QUANTIFICATION OF FUNGAL DEGRADATION OF *Pinus patula* AND *Eucalyptus grandis*.

VAHUNTH SINGH

A Thesis submitted to the Faculty of Science, University of Durban Westville, Durban, in partial fulfilment of the requirements for the degree of Master of Science.

Durban, 1992.

ABSTRACT

Previous studies of fungal decay have mainly examined long term effects of wood decay. In contrast, the present work, was designed to quantify fungal degradation of wood during incipient decay. Three facultatively anaerobic, dimorphic fungi were isolated from the rumen of sheep. These fungi were identified as *Mucor racemosus, Candida tropicalis* and *Geotrichum capitatum.* Scanning electron microscopy showed that these fungi colonised *Pinus patula* and *Eucalyptus grandis* extensively but did not appear to degrade the wood. The obligate anaerobe *Neocallimastix frontalis* colonised wood very sparsely, whereas the white rot basidiomycetes *Coriolus versicolor*, and *Phanaerochaete chrysosporium,* and the brown rotters *Coniophora puteana* and *Lentinus lepideus*, colonised wood under both aerobic and anaerobic conditions. The extents of colonisation were greater under aerobic conditions. The work then quantified the effects of the basidiomycetes *C.versicolor*, *P.chrysosporium*, *C.puteana* and *L.lepideus*, and the non-decay mould, *M.racemosus* in individual and coculture experiments. Wood colonisation was quantified by Kjeldahl nitrogen determinations converted to biomass assays, and degradation was quantified by weight losses, and Klason lignin determinations. Furthermore, the degraded wood samples were also analysed by HPLC analysis of hydrolysates and their sugar contents were determined to establish whether the glucose of cellulose and xylose + mannose of hemicellulose had been utilised by the respective fungi. The extent and nature of sugar utilisation by monocultures and cocultures in wood were then compared with the biomass and degradation data. Statistical analyses of these comparisons correlated the extents of colonisation, degradation, and the patterns of wood sugars predominantly utilised by each fungus. The results of the corresponding glucose, xylose and lignin analyses confirmed the brown rot physiological capacity of *C.puteana* in both woods. The white rot fungi behaved as simultaneous rotters and *M.racemosus* was shown to be ligninolytic in *P.patula*. The white rot physiological capacity of *C.versicolor* was confirmed in *E.grandis* and that of *P.chrysosporium* in *P.patula*. Antagonism and synergism in wood was detected between individuals within co-cultures during incipient decay. The significance of these findings becomes apparent when decayed wood of unknown history is analysed as described here. Such findings may be interpreted to provide valuable information describing the physiological nature of the responsible fungi, even if these are no longer viable or culturable.

DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Master of Science in the University of Durban Westville, Durban. It has not been submitted before for any degree or examination in any other University.

Singh

VAHUNTH SINGH

this 13th day of November, 1992.

DEDICATION

TO *RHONA, SHAHEEN, AND ASHLESHA*

PREFACE

Some of the material presented in this thesis has been published and presented elsewhere.

PUBLICATIONS

Singh, V., Shelver, G.D. and Baecker, A.A.W. (1990). Colonisation of wood elements by a facultatively anaerobic fungus isolated from the rumen. Proceedings of the Electron Microscopy Society of Southern Africa, 20 : 119 - 120.

Singh, V., Shelver, G.D. and Baecker, A.A.W. (1991). Reproductive structures produced by basidiomycetes in wood. Proceedings of the Electron Microscopy Society of Southern Africa, 21 : 147-148.

Singh, V., Tarin, M., Shelver, G.D. and Baecker, A.A.W. (1992). Quantification of wood decay effects by HPLC analysis. The International Research Group On Wood Preservation, Document No. IRG/WP/1576-92.

Singh, V., Tarin, M., Shelver, G. D. and Baecker, A.A.W. (1992). Quantification of fungal effects in wood. Microbios '92, Proceedings of the 7th Biennial Congress of the South African Society for Microbiology, 92.

PRESENTATIONS

Singh, V., Shelver, G.D. and Baecker, A.A.W. (1990). Colonisation of wood elements by a facultatively anaerobic fungus isolated from the rumen. Paper presented at EMSSA 90, 28th Annual Conference of the Electron Microscopy Society of Southern Africa, Grahamstown, RSA., 1990.

Singh, V., Shelver, G.D. and Baecker, A.A.W. (1991). Reproductive structures produced by basidiomycetes in wood. Paper presented at EMSSA 91, 29th Annual Conference of the Electron Microscopy Society of Southern Africa, Capetown, RSA., 1991.

Singh, V., Tarin, M., Shelver, G.D. and Baecker, A.A.W. (1992). Quantification of wood decay effects by HPLC analysis. Paper presented at IRG 23, 23rd Meeting of the International Research Group on Wood Preservation, Harrogate, UK, 1992.

Singh, V., Tarin, M., Shelver, G.D. and Baecker, A.A.W. (1992). Quantification of fungal effects in wood. Paper presented at Microbios 92, 7th Biennial Congress of the South African Society for Microbiology, Bloemfontein, RSA, 1992.

ACKNOWLEDGEMENTS

I wish to express my sincerest gratitude to Professor Albin A.W. Baecker, who initiated, designed, and supervised this work, guided its progress, whose interest, constant encouragement, invaluable critisms and suggestions, has made possible the successful completion of this research programme.

I also wish to record my appreciation to the following:

Mr G.D. Shelver, for all his assistance, advice, criticisms and suggestions, and especially for his invaluable assistance with the HPLC analyses and the analytical aspects of this work.

Professor K.C. Ryan, for his advice, suggestions and invaluable assistance with statistical analyses.

Mr V.Bandu and members of staff at the E.M. Unit, Pietermaritzburg, for their assistance and the many pleasant hours I spent working there.

Mr F.M. Swalaha, my colleague and friend, with whom I have spent long, memorable hours working in the laboratory, for being there when I needed him.

Miss M.Tarin, for all her assistance and support.

Teresa and Catherine Baecker for the hospitality they showed me during the numerous happy and pleasant hours I spent at their home while writing this dissertation.

Finally, I wish to thank my wife Rhona, for her patience, encouragement, constant understanding and support during the completion of this work.

LIST OF ABBREVIATIONS

C	-	degrees celcius
CA	-	Cellulose Agar
CB	-	Caldwell and Bryant's Medium No. 10
CM	-	Composite Medium
Eh	-	redox potential
HPLC	-	high performance liquid chromatography
l	-	liter
MM	-	Maintenance Medium
mm	-	millimeter
ml	-	milliliter
mg	-	milligram
$mgml^{-1}$	-	milligram per milliliter
$mgmg^{-1}$	-	milligram per milligram
Mr	-	relative molecular weight
MX	-	Malt Extract Agar
%	-	percent
SEM	-	Scanning Electron Microscopy
ul	-	microliter
um	-	micrometer
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight

glu	-	glucose
xyl	-	xylose
lig	-	lignin
XA	-	Xylan Agar

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1 INTRODUCTION

1.1 BACKGROUND

Forests comprise the natural reservoirs of the world's timber resources. The significance of forests in the global ecosystem has been reviewed by Spies (1988). They cover approximately one third of the world's land area and wood and forest products provide employment and income for millions of people world wide. On a global scale trade in forest products exceeds R2.08 x 10^{11} annually. Prior to large scale disturbances by man, the world's forests and woodlands covered approximately 6 x 10^{12} hectares. By 1954, however, the total had declined to approximately 4 x 10^{12} hectares. This 30% reduction reflects the increasing use of land for agriculture, pasture and dwellings. Due to the great variation of rainfall, temperature, land use, total land area and population density, forest resources are unevenly distributed among countries.

The Soviet Union has the largest area of forest (9.28 x 10^{8}hectares). Fifty two percent of the world's closed tropical forests occur in Brazil, Indonesia and Zaire. Together the twenty five most extensively forested countries, which contain

45% of the world's population, include 3.2 x 10^{12}hectares (74%) of the world's forest area. The Soviet Union, Canada and the United States together, have 1.7 x 10^{12} hectares or almost 54% of the world's total. In South Africa however, only 1% of the world's land area is under forests, whereas the figure for Africa as a whole is approximately 24%, therefore wood is in relatively short supply in this country.

Apart from the financial product contributions, forests also provide a wide range of services and non-quantifiable benefits that maintain or enhance productivity and both personal and national economic welfare, while sometimes having regional and international effects. These include :

i) soil stabilisation - in mountainous areas and regions of high rainfall;

ii) water flow moderation - resulting in a reduction of soil erosion and downstream flooding;

iii) amelioration - of physical and chemical structure of soil often resulting in the rehabilitation of degraded or abandoned agricultural land;

iv) climatic moderation - at the local, regional and possibly global scales. It has been shown that the loss of forests,

particularly the tropical rain forests can affect the global temperature rises that are being caused by the increase in atmospheric CO_2 (the greenhouse effect);

v) socio-economic benefits - to the individual and community in providing an income and employment, improved animal and human health through better drainage and sanitation, and

vi) conservation of genetic resources - of both the tree species themselves but also of plant and animal species (Rogers,1990).

More specifically, the timber from forests, i.e., the wood product itself is an extremely important commodity in the world today, as it has been in the past. It has been used for centuries for the construction of houses, implements and transport vehicles, and more recently as fence posts and transmission poles. In South Africa, the sale of wood and wood related products contributes approximately 10% of the Gross National Product (GNP) (Spies, 1988). The major consumers of wood in South Africa are the pulp and paper industry, the mining industry, the construction industry and agriculture.

The above figures clearly illustrate the very significant importance of wood in the South African context. Forming such a large proportion of the GNP, and being in relatively short supply

(above), it is important that the condition of the wood be carefully monitored both prior to and during service. This is essential not only to protect the economic value of wood but also for safety purposes and many other reasons. The losses associated with wood decay are therefore also of great significance to South Africa, and this thesis is based on the need to contribute to the fuller understanding of wood biodeterioration and its prevention. The specific objectives of the present work centre on the analysis of decayed wood and will be presented below (1.3) and reviewed in the appropriate Chapter Introductions. However it is necessary to place these objectives in the perspective of wood biodeterioration in general terms relevant to the content of this thesis.

1.2 Literature Review

1.2.1 The economic importance of wood

Throughout his history man has extensively used wood, initially depending on it for shelter, the manufacture of simple tools and as an energy source (Jane, 1957). Presently in most of the underdeveloped world, wood is an essential constructional material and is used extensively as a source of fuel. In the technically developed world, wood and wood products are used as raw materials supporting a wide variety of essential services including paper manufacture, building construction and the

production of furnishings. Even in highly technological societies where substitute materials may be synthesised for use as alternatives to wood, the timber trade still flourishes. In 1981 (King, 1981) Great Britain imported R15,000,000,000 (at 1992 exchang rates) worth of timber and wood products annually although she simultaneously exported forest thinnings to Scandinavia for pulping because recent market forces in the timber trade caused mature wood in many countries to have become too valuable to pulp.

Throughout the world nowadays an increasing awareness has therefore developed of the role of trees and forests in the supply of goods and services required or desired by man and his domestic animals. Many developed and developing countries are recognising the importance of wood for :

i) fuel - approximately half the wood used each year is consumed as fire wood or charcoal for heating and cooking, largely domestic and particularly in the tropical and sub-tropical areas. In some developed countries, the production of liquid and gaseous fuels from wood is technically feasible and may soon become economically feasible. This occurs in Brazil where timber is used for the production of wood alcohol;

ii) construction - for dwellings, furniture etc.;

iii) chemical extractives and derivatives- such as pharmaceutical products, beverages, pesticides, colourings and paintings; and,

iv) unprocessed wood - for building poles, fence posts and hedges.

Major reasons for the widespread use of wood today include its availability, its aesthetic appeal, its suitability in terms of strength (weight for weight it is stronger than steel), lightness, toughness, ease of conversion, and its adaptability for use as a constructional material. Increased demand for wood throughout the world has produced market trends raising the value of timber as mentioned above and many underdeveloped countries which had previously eradicated forests by fire to facilitate farming have consequently been stimulated to introduce practices which realise the economic value of their forests (King, 1981). Thus owing to reafforestation policies followed by many countries, wood has become the world's major renewable natural resource. This is especially the case in South Africa, where the high costs involved make it important to ensure that the durability of timber in service is maximised. This is necessary because incorrect usage or inadequate protection from its environment, can greatly reduce the service life of timber

through its deterioration as caused by various environmental influences acting either singly, or more usually in combination (Baecker, 1981).

The major causes of deterioration in timber include fire, mechanical wear, physical decomposition (e.g. hydrolyses by acids or alkalis) (Goldstein, 1973; Goldstein and Loos, 1973) and, principally, attack by living organisms (Cartwright and Findlay, 1958; Scheffer, 1973). Wood is a compacted source of a variety of nutritional carbon sources and depending on the availability of these nutrients, their uptake by living organisms may result in its biodeterioration. The term biodeterioration has been defined by Hueck (1968) as the process of biological interactions with materials resulting in a loss in the economic value of the material after such activity.

The organisms causing such damage to wood fall broadly into two broad groups - macroorganisms and microorganisms, the former including insects and their larvae, marine borers in salt water environments, woodpeckers and beetles. Microbial deterioration of wood is the most serious form in temperate climates (King, 1981). This may be initiated in the standing timber by parasites; however from the viewpoint of biodeterioration, the fungal saprophytes which colonise and attack wood as soon as the protective layer of bark is broken after felling are the most important. Two groups of fungi alone, the Brown Rot fungi and

the White Rot fungi, usually called the Decay Fungi produce the most serious destruction of wood. These fungi will be reviewed in more detail below (1.2.3.2). Scheffer (1973) stated that at least R300, 000,000 (at 1992 exchange rates) of damage to buildings alone was produced annually in the U.S.A. by decay. Such forms of biodeterioration may continue during timber conversion, seasoning, storage, construction and service, and although the microorganisms causing this are chiefly fungi and molds, bacteria and related groups are also involved (Baecker, 1981).

One of the most important uses of wood is in service in soil contact (Levy, 1968), in which it is used as transmission poles, fence posts, building supports and piles, railway sleepers and pit posts in mines. Therefore, effective methods of preserving such timber by protecting it from attack by numerous microbes in soil is essential to prevent its rapid deterioration and loss, particularly in warm climates such as that prevailing in South Africa.

Protection from biodeterioration by basidiomycetes may be achieved in wood by using various preservatives which are available commercially, but there is growing evidence of the failure of some preservative treated woods against microfungi causing soft rot in certain environments, e.g., in the tropics (Levy, 1965; Henningson and Nilsson, 1976). These failures

reflect the incomplete state of knowledge on the biodeterioration of wood by microorganisms. This has led several eminent workers in the field of wood biodeterioration (Levi, 1973; Scheffer, 1973; Wilcox, 1973; Kirk, 1973; Levi and Cowling, 1975; Liese and Greaves, 1975; Levy, 1982) to emphasise the need for further research with treated wood to provide the information required for the full understanding of the ecology of the colonisation and degradation of such wood by microorganisms. Such research preoccupies many groups around the world, however the present work examines important aspects of the biodeterioration of untreated wood as discussed in the appropriate Chapters of this thesis. Although the decay of preservative treated wood is therefore another very actively researched aspect of wood biodeterioration, this will not be considered further in the present Review.

1.2.2 The structure of wood

The structure of wood has been described in detail by Jane (1970) and broadly speaking wood consists of three or four basic structural elements which act as food storage, translocation and structural units in the living tree. The elements are arranged in characteristic patterns specific to each wood species. Timber of commercial value falls into two major classes : the soft woods derived from coniferous trees and the hard woods, from the broad leaved trees, which are of a more complex structure than the soft

woods. Anatomical differences between hardwoods and softwoods, based on the differences in the elements they contain are of great importance in determining decay susceptibility (King, 1981). Since Chapter 2 of this thesis considers the micromorphology of incipient decay, the microscopic structure of wood is reviewed below (1.2.2.1), and since Chapter 3 considers enzymatic effects of wood biodeteriogens, the chemical composition of wood is also reviewed here (1.2.2.2).

1.2.2.1 Microscopic structure

Coniferous or softwoods are simpler in structure than hardwoods. Tracheids and fibres are the major structural elements of softwoods and hardwoods respectively. Tracheids in softwoods provide physical support of the tree in addition to channelling of water and nutrients therein, whereas in hardwoods the fibres provide strength while specialised elements called vessels are used for liquid conduction. These are all vertical elements in the standing trees, but both hardwoods and softwoods also contain parenchyma tissue which runs in the horizontal plane in the medullary rays. Parenchymal cells have no structural function, but instead act as storage areas of synthesised nutrients for the living tree. These are thus nutrient rich zones in the tree when alive and remain as such after conversion of the timber unless removed by leaching. In living trees the phloem acts as the nutrient transport system. As such, the phloem and parenchymal

cells are rich in nutrients and when the tree is felled, converted and dried, these nutrient rich areas are the main avenues for microbial colonisation (Wilcox, 1973) during the early phases of decay, which will be examined in this thesis.

An individual wood cell is a multilayered structure in which the orientation of the cellulose strands in each cell wall layer is in a different direction (Jane, 1970). Each cell has a primary cell wall, characteristic of the phase of cell expansion which follows its origin from the cambium, and a secondary wall, laid down inside the primary wall during the latter stages of cellular differentiation. The "thin" wall between two young cambial derivatives has a tripartite organisation, consisting of a middle lamella, or intercellular layer, with a true primary wall (P) on each side of it (Bravery, 1975). In mature wood the corresponding thin line is often referred to as the middle lamella. The secondary wall to be found within this structure commonly consists of three layers, the outer, middle and inner layers, laid down in that order and usually termed S1, S2 and S3 respectively and helical thickening may be present on the inner surface of the S3 layer (Jane, 1970). The chemical compositions of these layers are also different and consequently only certain organisms are able to effect degradation (Thornber and Northcote, 1961). Selective colonisation of wood elements is considered in

Chapter 2 of this thesis. Therefore for ease of reference, a generalised cross section of a wood cell wall is presented below in Fig. 1.1.

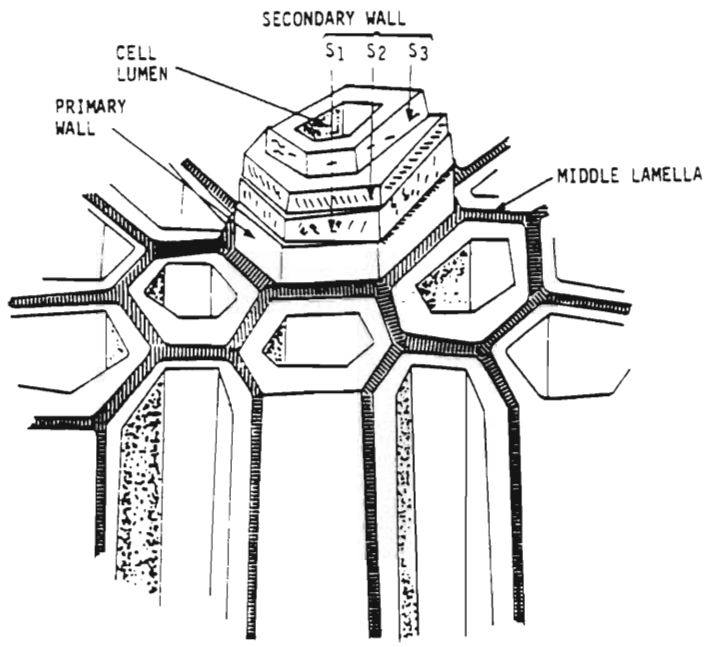


Fig. 1.1 Generalised cross section of wood illustrating compositional layers of a wood cell wall (Bravery, 1975).

1.2.2.2 Chemical constitution

The analysis of the selective degradation of wood constituents such as cellulose and lignin by given microorganisms comprises a major component of the present work (Chapter 3) therefore it was

felt appropriate to review the chemical constitution of wood in some detail here, for referral during the discussion (3.4) of those analyses.

The constituents of a wood cell wall are cellulose, hemicelluloses, lignin, extractives, minerals and water, the chemical constituents varying throughout the wall (Montgomery, 1982). Of the dry weight of wood, approximately 99% consists of cellulose and substances related to it, to which the general term "holocellulose" is applied, and lignin. Other substances such as pigments, silica, resins and gums make up the remaining 1% of the dry wood substance (Jane, 1970). Holocellulose accounts for approximately 70 - 80% of the wood cell wall and consists of a variety of carbohydrates together with certain related compounds. Forty to 50 % of the cell wall consists of cellulose. Cellulose is a homopolymer composed entirely of glucose (Fig. 1.2) whereas the hemicelluloses are heteropolymers composed of a mixture of sugars.

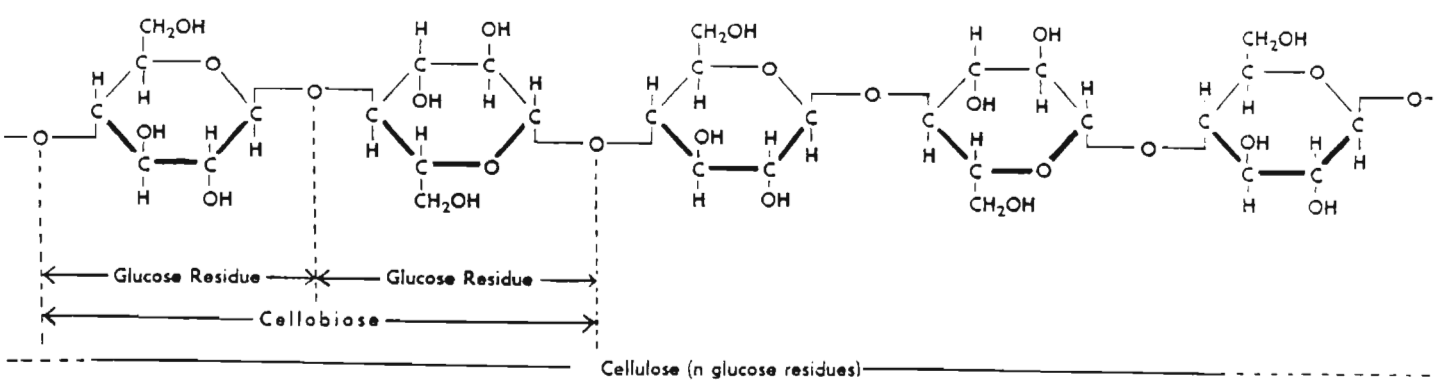


Fig. 1.2 Structure of cellulose molecule (Jane, 1970).

There are three main types of lignin differing in monomer units and linkage. Gymnosperm lignin is a polymer of coniferyl alcohol, angiosperm lignin is a polymer of coniferyl and sinapyl alcohols and grass lignin is a polymer of coniferyl, sinapyl and p - coumaryl alcohols, which involves some ester linkages (Knapp, 1985). In softwood , the lignin content is commonly between about 25 and 30 percent of the dry weight; in temperate hardwoods, it is rather less, ranging from 19 - 25%, while in tropical hardwoods it may be higher; 30% or more has been recorded (Jane, 1970). Compared with cellulose, much less is known of the structures of lignins, although it is known that they are polyaromatic dehydrogenation polymers with a basic repeating phenylpropanoid unit (Fig. 1.3). The monomers are not all linked in the same way since within one type of lignin there are C-C bonds and C-O bonds (and sometimes esters). Some monomers may be involved in several linkages and others only in one or two. The intermonomer bonds may link the monomers at different positions. The monomers have hydroxy and methoxy substituents, some of which are conserved in the polymer. It is emphasised that overall lignins are rather heterogeneous and much-branched polymers of undefined structure and size with estimates of molecular weight of lignin derivatives varying from a few thousand to over a million (Kirk and Fenn, 1982).

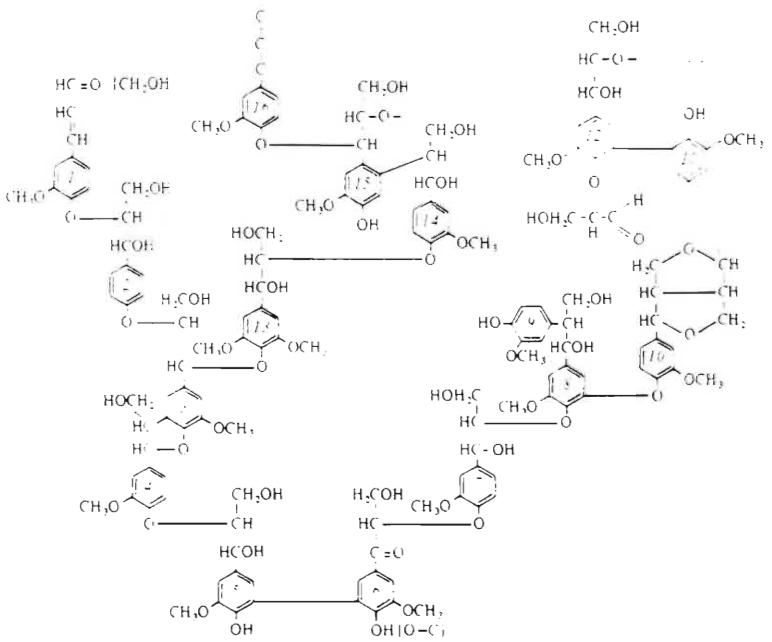


Fig. 1.3 Chemical structure of lignin (Kirk and Fenn, 1982).

Since the lignins of various species of wood have differing structures and chemical compositions, these in turn influence the decay rates of timbers because given microorganisms have differing enzymatic properties (1.2.3) which ultimately determine the particular mode of degradation of a given timber (Baecker, 1981) by given species.

1.2.3 Microbiological biodeterioration of wood

It was felt that the major component of the present review should place wood biodeterioration in perspective by focussing on the groups of microorganisms which decay wood, in order to permit the

use of Chapter Introductions to review the theory and technologies appropriate to the methods used to challenge the hypothesis defined below (1.3). Therefore the following review of wood biodeterioration attempts to outline the roles played by the major groups of microorganisms involved in this phenomenon.

The relationship between the composition of wood cells and the mode of degradation of the cells by microorganisms have been described by Wilcox (1973) and Kirk (1973) and excellently summarised by King (1981). Several major studies have elaborated on the variety of microorganisms which may colonise and attack wood (Garret, 1955, 1963; Cartwright and Findlay 1958; Findlay, 1966; Scheffer, 1973; Mowe *et al.*, 1983). Representatives of both prokaryotic and eukaryotic groups are involved in this process.

The diverse range of environments in which wood is attacked by microorganisms includes both marine and fresh water ecosystems eg. the seas, rivers, log ponds and water cooling towers, land environments such as buildings and wood constructions, timber yards, sawmills and pulpmills (including wood-chip piles, storage stacks and seasoning stacks), forest litter beds, and the soil (Baecker, 1981). Soil with a wide range of indigenous microorganisms provides a very variable environment in which the biodeterioration of wood occurs and the overall process is still not completely understood (Smith, 1980)

Differing soil types vary in moisture content, organic matter and mineral content, and hence pH, and temperature according to geographic location and climate. These factors tend to predetermine the dominant populations of microbes in a given soil sample. However, most soil samples contain high numbers of diverse groups of microorganisms per unit volume and hence wood in this environment is subject to colonisation and attack by a wide variety of microorganisms. Broadly speaking, however, the succession patterns of microorganisms occurring in wood in contact with differing soil types have been shown to have much in common by many researchers (Shigo, 1962; Corbett and Levy 1963a,b; Merril and French, 1966; Butcher, 1966, 1968a,b; Dowding, 1970; Bannerjee and Levy, 1971; Greaves, 1972) with similarities in the succession patterns displayed by the deteriorating wood samples.

Succession patterns of organisms in given ecosystems as they develop in complexity are well known to biologists and the succession of microbes in deteriorating wood has been studied in several countries throughout the world (Shigo, 1962, 1967; Corbett and Levy, 1963a,b; Levy, 1965; Butcher, 1966, 1968a,b, 1971, 1972; Merrill and French, 1966; Kaarik, 1967,1975; Gorshin and Krapivina, 1969; Butcher and Howard, 1968; Bannerjee and Levy, 1971; Greaves, 1972). The primary colonisers are the initial invaders of an ecosystem, and these are followed by a secondary group of colonisers. This process is repeated until

the final colonisers enter and gradually conquer the ecosystem. Often, representatives of each group of colonisers may thrive in the ecosystem simultaneously. It is as a result of the changes brought about in the ecosystem by primary colonisers that new ecological niches are formed in it, thus allowing new colonisers, the secondary colonisers to enter and develop a population (Garrett, 1963; Scheffer, 1973; Wilcox, 1973; Kaarik, 1975). In the case of wood as an ecosystem, the enzymology of specific microorganisms precludes their colonisation, and the subsequent biodeterioration of the wood. The make-up of the microbial population at any point is usually explained by theories of succession. The most popular of these appears to be the theory in which primary or pioneer invaders i.e., bacteria and staining fungi are thought to invade initially and live mainly saprophytically on cell contents. These organisms then alter the substrate, thereby providing a suitable niche for subsequent colonisers such as soft rot and basidiomycete fungi (Levy, 1975; Levy, 1982).

In summary, it has been stated that the succession patterns of colonising microorganisms in decaying wood may vary; the dominant microbial populations, and hence the precise forms of the patterns, are determined by a variety of environmental factors, the major influences being those of moisture presence, temperature, pH, geographic location, situation, aeration and of course wood type (Baecker, 1981). Specifically, it is during the

early stages of colonisation, i.e., during incipient decay, that these variables have most effect in producing lack of uniformity in the decay of the wood. In conclusion, these variables create the need for much research of an ecological nature, to clarify the roles played by individual groups of microorganisms during the successive decay of wood by mixed populations, and indeed, a great deal of such work is conducted by groups around the world. As will be shown below however, further review of overall succession patterns in wood is not considered necessary in this thesis, although the phenomena occurring during incipient decay are of primary importance in this work, as discussed below. Therefore the analytical nature of the present work was felt best conducted with defined monocultures and co-cultures of decay organisms. Therefore, this review will continue with the physiological groups of decay organisms in wood.

1.2.3.1 The action of bacteria on wood

Although fungi are traditionally the main agents of wood decay, bacteria have been shown to be among the earliest colonisers of wood (Levy, 1975). This early or primary colonisation may modify the environment so that the decay fungi may colonise the wood and proliferate. Recognition of the significant roles played by primary colonisers in any ecosystem therefore led many workers in wood biodeterioration to increase the effort dedicated to bacterial decay. Regardless of the type of wood, bacteria occur

mainly in the parenchymatous tissue, especially the rays (Ellwood and Eckland, 1959; Greaves, 1966; Courtois, 1966; Verall, 1969; Highley and Lutz, 1970). It has been shown that bacteria may travel via the pits (Duncan, 1965; Highley and Lutz, 1970) towards the parenchymatous ray cells where, like molds and staining fungi, they deplete storage material therein. According to Greaves (1970) the ray cells containing metabolites were the sites most heavily colonised. Bacterial attack is considered to occur in all wood elements although there are differences in lignification of wood (Greaves, 1969). Comparative and complimentary studies using scanning and transmission electron microscopy in recent years have helped define the various patterns of bacterial degradation more accurately. The mechanisms of action of cavitation, tunnelling and erosion bacteria producing these patterns have been reviewed by Singh and Butcher (1990) and are not considered further here.

Importantly, from the view point of the present work, when Greaves (1970) summarised contributions regarding the elucidation of bacterial isolation techniques in wood biodeterioration, the first reports (Karnop, 1967) of strict anaerobes in wood began to appear in the literature. *Clostridium omelianski* was isolated from wood and identified by Karnop (1967) and Liese and Karnop (1968). *Desulfovibrio desulfuricans* was isolated from pine by Knuth and McCoy (1962), but the role of this strict anaerobe in pine was not illucidated. Zeikus and Ward (1974) showed viable

methanogens to be present in the heartwood of living trees affected by the condition referred to as " wetwood". In view of the recent developments in the decay of wood by strict anaerobes, it is believed that wood probably contains certain zones where the oxygen levels are low as a result of the metabolism of facultatively anaerobic heterotrophic bacteria in such areas. This lowering of the oxygen levels could result in the formation of anaerobic microsites in the wood (Rogers and Baecker, 1988a), where strict anaerobes could begin to metabolise. This theory is supported by the isolation of a strictly anaerobic cellulolytic *Clostridium sp.* from a woody biomass digester (Yang, *et al.*, 1989), and confirmed by the isolation of *Clostridium xylanolyticum* from decayed *P.patula* wood chips (Rogers and Baecker, 1991). Similarly it has been shown (Singh, Nilsson and Daniel, 1990) that *Pinus sylvestris* sapwood can be degraded by a mixed bacterial population under near anaerobic conditions.

In summary, the effects of bacteria in wood have, to date, not appeared to be as serious as the degradation brought about by the rotting fungi, although Singh and Butcher (1990) have emphasised that there is still much to learn about the physiology and the full role of these bacteria in the biodeterioration of wood. With particular reference to the present thesis however, it was studies of bacterial degradation of wood which gave rise to the

concept that anaerobic decay of wood could occur. This concept will be referred to below when anaerobic fungi are discussed prior to their use in the present work.

1.2.3.2 The action of fungi on wood

Under normal conditions the main agents of wood decay are fungi. The wood rotting fungi are traditionally split up into different groups according to their mode of action and effects on the wood structure (Jurasek *et al.*, 1967; Norkrans, 1967; Wilcox, 1968 Liese, 1970). These groups include the sap stainers and moulds; the soft rotters and the decay fungi as discussed below.

1.2.3.2.1 Sap stainers and moulds

Those fungi causing abnormal discolourations in wood without appreciably decomposing it are commonly referred to as the sap - staining fungi, since their activity is almost exclusively confined to sapwood. These fungi fall into two classes : the moulds which grow and sporulate on the surface of the wood causing only superficial discolouration, and the true sap - staining fungi which penetrate into the sapwood and cause staining too deep to be easily removed. Stain fungi belong to the Ascomycetes and Fungi Imperfecti. These fungi use the wood cells as a habitat and do not utilise the chemical components (cellulose and lignin) of these elements (Butcher, 1966).

Instead, they penetrate the wood passively (Corbet, 1963) in the quest of nutrients within the parenchyma ray cells as indicated above (1.2.2). Thus staining fungi do not normally produce significant losses in the mechanical strength of wood (other than in impact resistance) but the spores are produced on the wood surface causing superficial discolouration. Because these fungi have highly pigmented hyphae (Butcher, 1968a; Liese, 1970) they stain the wood. Commonest is blue stain, but also occurring are red, pink, brown, yellow and green stains (Butcher, 1966; Scheffer, 1973).

Hyphae pass from cell to cell via pits, but many species of staining fungi have also been shown to produce bore holes through cell walls (Liese and Schmid, 1961; 1964; Sachs *et al.*, 1967; Levy, 1967; Wilcox, 1973; Scheffer, 1973). Under prolonged favourable conditions some stain fungi have also been observed to produce soft rot cavities (Duncan, 1960; Scheffer, 1973).

The mould fungi also belong to the Ascomycetes and Fungi Imperfecti, but are often colourless and consequently their presence is not always visually apparent. Most troublesome of the wood moulds are species of *Trichoderma, Gliocladium* and *Penicillium* all of which discolour surfaces with their green spores. Species of *Alternaria* and *Aspergillus* are responsible for much of the black mold (Scheffer, 1973). Mould fungi behave like most blue stainers in their invasion of wood, and may also

produce bore holes. Merril (1965) has shown that some mould fungi, although producing little effect on poplar wood cell walls, produced soft rot cavities in oak. One of the most striking effects of mould colonisation of wood is their capacity to tolerate or even degrade toxic chemicals (Scheffer, 1973; Nilsson, 1973; King and Eggins, 1973).

In the context of the present work, it is important to note from the above that sap stainers and moulds may colonise and decrease the value of the wood by discolouring it, without affecting its strength properties by extensive attack of cellulose and lignin. Therefore such colonisation cannot be monitored using conventional methods such as weight loss determinations (as reviewed in Chapter 3), and one of the questions raised during the present review centred on the development of any method which could be used to monitor such colonisation.

1.2.3.2.2 Soft rotters

The wood rotting, or decay fungi, possess the enzymes necessary for the breakdown of either the cellulose or the lignin of wood cell walls and consequently their actions seriously affect the strength of the wood.

Soft rot fungi produce a softening of the wood in its outer layers which led Savory (1954) to give them their name. They belong to the Ascomycetes and the Fungi Imperfecti and produce characteristic cavities within the cellulose layer of the cell walls by the action of cellulases (Courtois, 1963; Liese, 1963; Corbett, 1965; Schmid and Liese, 1965; Levy,1967). In a taxonomic study of soft rot fungi received at the U.S. Forest Products Laboratory, 69 species were identified. Although not uniformly destructive, Duncan (1960) found that most of them could cause substantial degradation of sweetgum sapwood. One of the best known of the more damaging species, *Chaetomium globosum* has been studied worldwide and used for testing resistance to soft rot. Duncan (1960; 1961) has stated that soft rot fungi are more prevalent in hardwoods than in softwoods, and that they are also more resistant to preservatives than Basidiomycetes. These findings were supported by other workers (Scheffer, 1973; Hulme and Butcher, 1977). However no wood is fully resistant to soft rot, and coniferous woods also are highly vulnerable to many of the fungi if they are in or on damp fertile soil or are wetted in conjunction with suitable nutrient minerals and vitamins. Duncan (1960; 1961) also showed that soft rot species can, as a group tolerate certain greater extremes of environment than Basidiomycetes; this hardiness was exhibited in tolerance of higher temperatures, higher pH and ability to grow with restricted oxygen.

Partial weakening of the lignin - carbohydrate complex, such as occurs in wood cooling towers wetted by water containing chlorine will increase susceptibility to soft rot. Many soft rotters isolated from cooling towers - but not from other sources - possessed a greater capacity than Basidiomycetes to gain weight in culture under severely restricted aeration. This finding supports the general assumption that the prevalence of soft rot but near absence of ordinary decay in the wet wood of cooling towers is attributable at least in part to a superior tolerance to oxygen defiency by the rotters involved (Scheffer, 1973). These assumptions are mentioned here because they prompted the initial theories of Rogers and Baecker (1987) which began to investigate the significance of anaerobes in wood decay, and this thesis includes further considerations (below) of anaerobic biodeterioration of wood.

1.2.3.2.3 Basidiomycetes

The decay fungi belong to the most advanced class of fungi in the evolutionary scale, the Basidiomycetes. This class name and that of a subdivision containing the decay - producing species, the Hymenomycetes are increasingly seen in semi technical literature on the subject of wood decay. Until Findlay and Savory (1950) first suggested the economic importance of soft rot, Basidiomycetes were regarded as the only fungi capable of causing severe economic degradation of timber in service. The two groups

of fungi which produce decay are the white rot fungi and the brown rot fungi and together these are called the decay fungi. Significant micromorphological differences between the white rots and the brown rots were not suspected until Bravery (1971, 1975, 1976) published a series of scanning electron photo-micrographs. His observations were confirmed by Nasroun (1971). Montgomery (1982), using a fluorescent stain to show up breakdown products of wood decay, has confirmed the micromorphological patterns for the three groups of wood rotting fungi by showing that the enzyme activity of the soft and white rots is restricted within the cavity or trough, whereas that of the brown rot appears to be diffused throughout the wall. The patterns of soft, white and brown rots are illustrated in Fig. 1.4. Since the basidiomycetes produce major, measurable effects in wood, such as weight losses, they were chosen as test organisms in the present work (Chapter 3) as outlined below (1.3). It is therefore necessary to consider their effects in wood.

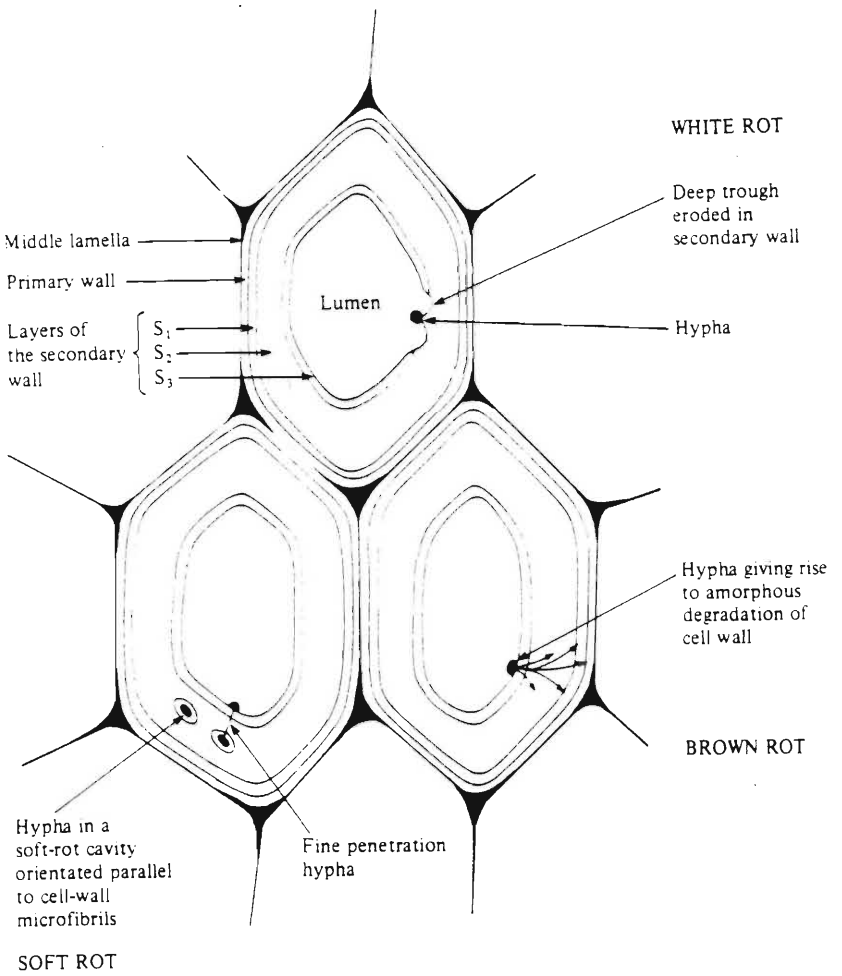


Fig. 1.4 Diagram of wood cells in transverse section showing patterns of degradation produced by three types of wood decay fungi (Montgomery, 1982).

White rot fungi are basidiomycetes which destroy both the holocellulose and lignin in the wood cell walls, giving the wood a bleached white colour (Kirk, 1975). The sap staining fungi invade and spread through the wood by growth within the cell lumina, and are capable of spreading to neighbouring cells by transverse penetration of pit membranes or by borehole formation.

Penetration by white rot hyphae appears to be chemical rather than mechanical, with enzymes capable of solubilising wood substances secreted in advance of penetration. The hyphae are initially greatly restricted where they pass through the wall, but may later increase the borehole size to many times the normal hyphal diameter by enzyme action. The hyphae thus established in cell lumina may cause, by cellulase, ligninase and other enzyme activity, the gradual erosion of all cell wall constituents from the lumen outward in the region of hyphal contact, forming a trough with a central ridge on which the hyphae rests; or more often, the formation of large cavities due to initial removal of lignin and later hydrolysis of cellulose a pocket rot (Levy, 1982; Blanchette, 1983). With continued branching of hyphae, the troughs or cavities formed coalese, eventually resulting in collapse of the woody tissue. Studies by Bravery (1971) and Levy (1982) using the white rot fungus *Coriolus versicolor* showed that where the trough was formed parallel to the cellulose microfibrils, its edges were smooth, but where it cut across their orientation the edges were ragged where the ends of the cellulose microfibrils projected. The formation of troughs or channels with well defined edges a relatively short distance from the hypha suggests that there could be some restrictions to free movement of extracellular enzymes involved in the lysis.

Brown rot fungi degrade and utilise only the cellulose and hemicellulose components of the wood, leaving the lignin of wood as a brown, brittle and friable residue (Levy, 1982). In 1971, Bravery showed that although the hyphae of brown rotters came to lie on the inner surface of the cell wall, no troughs or channels were formed, neither did the hypha penetrate into the walls and form chains or cavities inside. Instead the wall surface and the hyphae change very little but the inner layers of the wall are heavily degraded, becoming brown, friable and disorganised (Bravery, 1971; Nasroun, 1971; Levy, 1982). It gives the impression that in this type of fungus, the enzymes were capable of diffusing some distance away from the hyphae and penetrating into cell walls, where the cellulose and hemicellulose is destroyed, leaving the lignin largely unaltered as residue. The possible mechanisms of this are discussed below (1.2.4).

The kind of wood and whether it is above or below ground often determines whether decay may be by a brown rot or white rot fungus. In general, hardwoods in above ground service are much more succeptible to white rot than to brown rot and softwoods are more succeptible to brown rot (Knapp, 1985). There is no satisfactory explanation for this difference, but it does seem to exist and is further considered with relevant results of the present work in Chapter 3 of this dissertation.

Lignicolous marine fungi were first documented in 1944 and since that time an increasing number have been described (Mouzouras *et al.*, 1986). Although not of direct relevance to the present work, marine basidiomycetes are briefly mentioned here to illustrate another aspect of wood decay which leads to the conclusion that further research of anaerobic wood decay is justifiable.

There are six marine species belonging to the Basidiomycotina : *Digitatispora marina, Halocyphina villosa, Nia vibrissa, Melanotaenium ruppiae* (Jones, 1982), *Digitatispora lignicola* (Jones, 1986) and *Mycaureola dilsea* (Jones, 1986). Leightly and Eaton (1979) reported on the white rot characteristics of *N.vibrissa*. Since *D.marina* and *H.villosa* also cause white rot of wood, it appears that brown rot basidiomycetes are absent in the marine environment (Mouzouras *et al.*, 1986). The evidence available so far (Jones, 1982; Levy, 1982; Sutter *et al.*, 1984; Foisner *et al.*, 1985; Sutter and Jones, 1985; Mouzouras *et al.*, 1986) seems to indicate that fungal decay under waterlogged conditions will occur to a state where anaerobiosis ensues with subsequent decay by anaerobic bacteria. The type and extent of decay in waterlogged wood will depend on the conditions of the surrounding environment, hence a variation in results from different sites is inevitable. Further work in this field is

required (Mouzouras *et al.*, 1986), and this requirement again illustrates the need for a fuller understanding of the anaerobic wood decay process.

1.2.3.2.4 Rumen fungi

The anaerobic fungi represent a newly discovered (Orpin, 1975; Bauchop, 1979) group of organisms inhabiting the rumen ecosystems of sheep, cows and goats, and possess a life cycle alternating between a motile zoosporic stage and a non-motile zoosporangial stage (Bauchop, 1981; Lowe *et al.*, 1987; Citron *et al.*, 1987; Gold *et al.*, 1988). There have also been important advances in the understanding of the life cycle mechanisms and the controlling factors involved (Mountford, 1987), and these fungi are now known to more closely resemble Chytridiomycete fungi.

These organisms are *Neocallimastix frontalis* (Orpin, 1975); *Sphaeromonas communis* (Orpin, 1976) and *Piromonas communis* (Orpin, 1977). On morphological grounds anaerobic fungi were classified in the Class Chytridiomycetes (Heath *et al.*, 1983), but on the basis of the ultrastructural characteristics of the zoospores, they were assigned to the new family Neocallimasticaceae (Barr *et al.*, 1989). Gold *et al.*, (1988) suggested a subdivision of this family into the three genera containing monocentric species - *Neocallimastix, Piromyces* (previously *Piromonas*) and *Caecomyces* (previously *Sphaeromonas*)

which at that time were the only legitimately described genera of anaerobic fungi. Subsequently polycentric species have been described, e.g. *Orpinomyces bovis* (Barr *et al.*, 1989) and *Neocallimastix joyonni* (Breton *et al.*, 1989). Up to now, six anaerobic fungi isolated from foregut fermenters (sheep, cow) have been classified (Teunissen *et al.*, 1991) viz., *Neocallimastix patriciarum* (Orpin and Munn, 1986), *Neocallimastix frontalis* (Heath *et al.*, 1983), *Neocallimastix joyonni* (Breton *et al.*, 1989), *Piromyces communis* (Orpin, 1977), *Caecomyces communis* (Orpin, 1976) and *Orpinomyces bovis* (Barr *et al.*, 1989). The latter however does not conform to the proposed generic description. Another polycentric fungus which produces monoflagellate zoospores has recently formally been described and assigned to a new genus and species - *Ruminomyces elegans* (Ho *et al.*, 1990). Only one fungal species from a non-ruminant (horse) has been named, *Caecomyces equi* (Gold *et al.*, 1988).

The rumen is a compartment of the forestomach in ungulates termed ruminants. Here ingested plant material is retained and fermented by complex microbial populations. Volatile fatty acids, partially digested material and microorganisms are the carbon and energy sources of the ruminants. Until recently it was believed that metabolic transformations in the rumen were mediated by bacteria and protozoa and fungi were not considered as inhabitants of this ecosystem. However, there have been developments from *in vivo* and *in vitro* studies leaving little

doubt that these newly discovered anaerobic fungi make a significant contribution to rumen metabolism, particularly in the digestion and subsequent fermentation of plant structural materials (Mountford, 1987).

Microscopic observations of ruminal digesta have shown that in the vegetative stages, these fungi have a great affinity for plant particles and that they are rarely found in the liquid phase (Bauchop, 1979). *In vitro* experiments have demonstrated that the organisms are able to degrade and utilise components of plant fibre such as cellulose and xylan as energy sources (Orpin, 1977; Orpin and Letcher, 1979; Mountford and Asher, 1985; Lowe *et al.*, 1987). Their close associations with plant fragments suggested that these fungi are capable of degrading cell wall polysaccharides and may be important lignocellulose degraders (Bauchop,1979). Pure cultures of anaerobic fungi ferment cellulose to give lactate, acetate, CO_2, and H_2 as the major products. Ethanol and formate may also be produced. Anaerobic fungi also participate in hemicellulose and starch degradation (Mountford, 1987).

However, no quantitative information seems to be available on the ability of the ruminal fungi or bacteria to weaken plant fibres. Although there are a number of studies on the anaerobic degradation of stems and different grasses (Akin *et al.*, 1983;

Akin and Rigsby, 1987; Akin *et al.*, 1989; Theodorou *et. al.*, 1989) there is very little information on the degradation of wood by anaerobic fungi.

Since fungi are the major organisms responsible for wood degradation, it is thought that anaerobic fungi might show potential for wood degradation. Anaerobic fungi were first shown to colonise wood by Wiederhold *et al.*, (1989). Studies by Joblin and Naylor (1990) have indicated that anaerobic fungi were able to degrade wood to significant extents and investigations of anaerobic fungi will be continued in the present work.

1.2.4 Mechanisms of fungal decay

Since the main purpose of the work to be described in this thesis centres on method development for monitoring incipient decay in wood, it is appropriate to now review the mechanisms by which wood is decayed by the fungi discussed above.

Wood, although a high-calorie carbon source, suffers from the disadvantage of being highly polymeric and thus insoluble. Before it can be utilised by a wood degrading organism, such as a basidiomycete fungus, it must first be depolymerised. As wood is a very stable polymer, catalysts are needed and, since the wood is outside the organism, they must be extracellular enzymes. This implies that the enzymes must function in an uncontrolled

environment which, together with the nature of the substrate, puts several constraints on the enzymes and the way they act. They should be small to facilitate penetration of the substrate, although there must be a minimum functional size; they should remain active for as long as possible, although active turnover, i.e., absorption and production of the enzymes by the organisms, would reduce this requirement and effect a control mechanism; they should also be ionically suited to their environment, because if they were highly charged they would either be attracted by the wood and immobilised, or repelled and unable to function (Montgomery, 1982). These requirements should be considered in the context of the microstructure of the wood (1.2.2.1) and will be considered firstly in the context of cellulases (1.2.4.1) and then ligninases (1.2.4.2).

1.2.4.1 Action of polysaccharidase enzymes

According to Montgomery (1982), the first report on *in-vitro* cellulase activity appeared around 1906. Since then much work has been carried out, but the actual action of the polysaccharidase enzymes has still not been elucidated. This is mainly due to the great number of complex variables and the difficulties of studying enzyme activity on a solid phase substrate. Most of the work has been directed towards cellulase,

as cellulose is the major constituent of interest, but although less work has been done on hemicellulases, the mode of action appears to be similar (Montgomery, 1982).

In wood decayed by brown rot fungi the hemicellulose fraction is virtually absent and the degree of polymerisation of the cellulose is drastically reduced. Brown rot fungi rapidly depolymerise cellulose during early stages of wood decay but the decay mechanisms involved are not yet fully understood (Highley, 1987b). Attack of the cell wall by brown rot fungi is predominantly initiated by hyphae growing in the lumina in contact with the tertiary wall (S3) (Highley *et al.*, 1983).

Brown rot fungi apparently lack the synergistic endo-exo-glucanase cooperation to degrade crystalline cellulose but despite this they are effective wood decomposers (Highley, 1982; Lundborg, 1989c). Brown rotters produce endo-1,4 glucanases, but no report exists on the production of exoglucanases (Ritschkoff and Viikari, 1991). However no other enzyme systems are known to substitute these effects (Micales and Highley, 1988; Enoki *et al.*, 1990; Eriksson *et al.*, 1990). The nature of cellulose breakdown by brown rot fungi suggests that a small metabolic product may be involved in the decay. These fungi are known to produce extracellular hydrogen peroxide (Koenings, 1972a and 1974; Highley, 1987a; Enoki *et al.*, 1990; Ritschkoff and Viikari, 1991) which is believed to be at least one of these

small degradative agents. In the presence of Fe^{2+} or other transition state metals, the produced H_2O_2 is believed to form highly reactive radicals that initiate the oxidative degradation of wood carbohydrates (Highley, 1982 and 1987b; Illman *et al.*, 1988).

Eriksson *et al.*, (1990) suggested that when cultured on wood, brown rot fungi produce a factor able to diffuse into the pure cellulose and cause depolymerisation. Highly crystalline parts of the cellulose are more resistant to degradation than amorphous parts (Lundborg, 1989a). The action of reactive agents such as OH^- radical, O_2 or O_2^- radical is proposed to initiate degradation of crystalline cellulose and thus facilitate the enzymatic attacks (Lundborg, 1989b). Koenings (1972a,b) found that brown rot fungi are more powerful producers of H_2O_2 than white rot fungi and he therefore suggested that the initial attack on crystalline cellulose takes place via an oxidative H_2O_2/Fe^{2+} system. However the production of H_2O_2 is not observed in all brown rot fungi studied and recently it has been demonstrated that a small peptide or siderophore type of molecule might participate in the degradation of cellulose (Enoki *et al.*, 1990; Jellison *et al.*, 1991). These observations suggest that brown rotters could possess two different types of diffusible degradation agents which initiate the oxidative degradation of cellulose.

Several brown rot fungi are known to produce a pattern of extracellular wood carbohydrate hydrolysing enzymes including glycanases such as endo-B1-4 glucanase, endo-B1-4 xylanase and endo-B1-4 mannanase and glycosidases, such as a and B-D galactosidase, B-D-glucosidase and B-D-xylosidase (Micales and Highley, 1988). No reports exist on the production of exoglucanases by these organisms and no other enzyme systems are known to substitute this effect (Eriksson *et al.*, 1990). The initial hydrolysis of cellulose is believed to be a non enzymatic process since the molecular sizes of the enzymes are expected to be too large to penetrate the natural pores of the wood microstructure (Highley *et al.*, 1983; Strebotnik and Messner, 1990). However immunolabelling studies have shown that the penetration of fungal metabolites into the wood cell wall may take place. The cellulolytic enzymes or other metabolites may thus penetrate the wood structure at least to limited extents at early and moderate stages of decay (Goodal *et al.*, 1988; Kim *et al.*, 1990).

Brown rot fungi create a low pH while degrading wood, primarily by producing organic acids (Cowling, 1961). Oxalic acid produced by these fungi has been supposed to play a role in non enzymatic degradative pathways by reducing the ferric ions to ferrous ions (Schmidt *et al.*, 1981). Koenings (1974) suggested that the low pH maintained by brown rot fungi may play a role in solubilising and reducing the endogenous iron found in wood.

1.2.4.2 Formation and action of ligninolytic systems in basidiomycetes

The study of lignin biodegradation has until recently been a neglected area, but recent efforts have disclosed a fascinating and unusual process, even before many of the detailed features have been delineated.

Although the only microbes shown to metabolise lignin efficiently are basidiomycetes, other fungi are known to degrade it slowly (Lundstrom, 1973; Eslyn *et al.*, 1975; Haider and Trojanowski, 1980). Little is known about the chemical and physiological aspects of their activities. Many species - perhaps thousands - of wood and litter-degrading basidiomycetes metabolise lignin as they decompose lignocelluloses. Metabolism of lignin by the litter - degraders, unfortunately, has received little attention since the early work of Lindeberg (1944). The basidiomycetes that decompose wood have been studied extensively, and are known to cause either of two types of decay, brown rot or white rot, in which the lignin and polysaccharides (cellulose and hemicelluloses) are metabolised (Cowling, 1961).

The white rot fungi have been studied far more than other microbes from the standpoint of lignin biodegradation. Understanding of the specific reactions that compromise lignin biodegradation by white rot fungi is far from complete, but good

progress is now being made in several laboratories. Most of what is known has come from chemical and physical characterisation of partially biodegraded lignin isolated and purified from rotted wood.

Elemental and methoxy analyses and summative C_9 - unit formulae for sound and white rot lignins are shown in Table 1.1. Changes in other properties caused by the fungal degradation are summarised in Table 1.2. It is apparent from such investigations that biodegradation of the polymer consists primarily of an oxidative attack (Kirk and Fenn, 1982).

Table 1.1 Elemental and methoxy analyses (%) and C_9 - unit formulae for sound and white rotted spruce lignins.

Lignin sample[b]	C	H	O	OCH_3	C_9-formulae	Mol. wt of C_9-unit
Sound	62.85	6.08	31.07	15.11	$C_9H_{8.66}O_{2.75}(OCH_3)_{0.92}$	189.2
Fungus-degraded	57.97	4.70	37.23	11.33	$C_9H_{7.26}O_{3.95}(OCH_3)_{0.74}$	199.4

[a] From Kirk & Chang (1974).
[b] The sound sample was a milled wood lignin. The fungus-degraded sample was purified from wood which had been decayed to 50% weight loss by *Coriolus versicolor*.

Table 1.2 Changes in properties of lignin caused by white rot basidiomycete attack.

Property	Change[a] Increase	Decrease	Method of analysis[b]	Reference
Carboxyl content	+		C, S	Hata, 1966; Kirk & Chang, 1974, 1975
Carboxyl content	+		S	
Hydroxyl content				
Aliphatic		+	C, S	
Phenolic	+[c]	+	C, S	
Aromatic content		+	C, S	Kirk & Chang, 1975
Yield of low mol. wt aromatic compounds on oxidative chemical degradation		+	C	Higuchi, Kawamura & Kawamura, 1955; Hata, 1966; Kirk & Chang, 1975
Yield of low mol. wt aromatic compounds on hydrolytic degradation		+	C	Hata, 1966; Kirk & Chang, 1975

[a] Purified sound and fungus-degraded lignins were compared.
[b] C = various chemical procedures; S = spectroscopic methods (ultra-violet, infra-red and/or proton magnetic resonance).
[c] Hata (1966) reported an increase in phenolic hydroxyl content and variable results with aliphatic hydroxyl content.

Interpretation of the detailed chemical and physical comparisons of sound and white rot lignins have led to the conclusion that the oxidative attack by basidiomycetes occurs in both the aliphatic side chains, and in the aromatic nuclei still bound in the polymer (Kirk and Chang, 1975). Direct evidence for side chain oxidation is provided by the presence, in the degraded polymer, of aromatic acid moieties, and by the increase in alpha carbonyl content (Kirk and Chang, 1975). Evidence for the presence of residues of aromatic ring cleavage in the degraded lignin polymer is indirect :

a) the high carboxyl and oxygen contents which cannot be accomodated by side-chain oxidation alone;

b) the low methoxyl content and absence of (intact) demethylated aromatic nuclei;

c) the decrease in aromatic content;

d) the presence of a, B - unsaturated carboxyl groups which are not aryl-conjugated, and

e) the decrease in phenolic hydroxyl content

Other studies provide more direct support for ring cleavage in the polymer. Among the low molecular weight aromatics in extracts of wood partially decayed by *Phanaerochaete chrysosporium* were minute quantities of several compounds, each containing one intact aromatic ring attached to an aromatic carbon derived aliphatic moeity. The tentatively assigned structures of two of these compounds (I and II) are shown in Fig. 1.5.

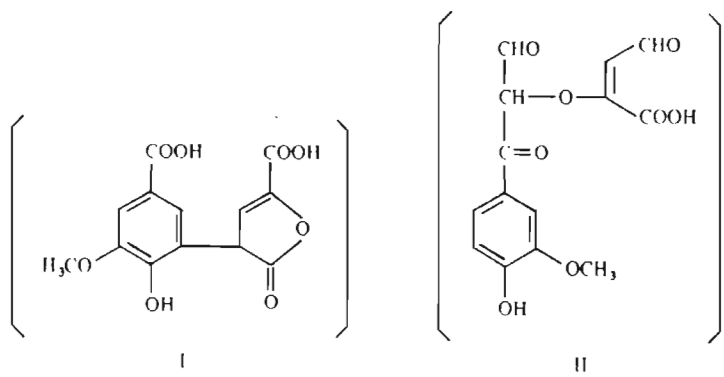


Fig. 1.5 Tentatively assigned structure of two lignin oxidation products in an extract of spruce wood partially decayed by *P.chrysosporium* (Kirk and Fenn, 1982).

With reference to the lignin structure in Fig. 1.3, it is evident that compound I could have arisen from units 5 - 6 and compound II from structures of the type illustrated by units 1 - 2. These results show clearly that some aromatic rings are oxidatively cleaved while still attached to a second ring (Kirk and Fenn, 1982). Side chain oxidation reactions can explain the origin of the aromatic acid moieties found in white rot lignin discussed above. However, it is not possible for these side chain oxidations alone, even with retention of the oxidised fragments in the polymer, to account for all the properties of the white rot lignin polymer (Kirk and Chang, 1975; Chang *et al.*, 1980). Oxidative cleavages of both side chains and aromatic nuclei are indicated (Kirk and Fenn, 1982).

The major extracellular proteins of white rot fungi thought to be responsible for lignin degradation are ligninase (Tien and Kirk, 1983) and manganese peroxidase (Huynh and Crawford, 1985). These are both peroxide requiring heme proteins of 42 000 and 45 000 molecular weights respectively (Flournoy, 1991). In addition white rot fungi possess the full complement of typical cellulolytic enzymes (Nilsson, 1974). The two extracellular heme peroxidases have been considered to play a major role in the degradation of the lignin component in wood because of their capability of the oxidative degradation of a range of lignin - related model compounds. However, the peroxidases have a phenol oxidase activity and partly polymerise lignin substrates rather

than depolymerise them (Haemmerli *et al.*, 1986; Odier *et al.*, 1988). Thus other systems to prevent or reverse these polymerisation reactions are also nessary for actual depolymerisation of the lignin polymer in wood. The one - electron oxidation activity of white rot fungi is also proportional to the degradation activity on wood (Tanaka *et al.*, 1986; Enoki *et al.*, 1988). White rot fungi generally produce a complete cellulase system of several hydrolytic enzymes able to hydrolyse natural cellulose to glucose and a ligninolytic system capable of degrading lignin. These fungi degrade hardwood much more rapidly than softwood and the lignin is preferentially degraded during wood degradation (Enoki *et al.*, 1988).

Several recent studies have examined the penetrability of wood decay enzymes into the cell wall by the use of transmission electron microscopy and immuno-gold labelling (Srebotnik *et al.*, 1988; Blanchette *et al.*, 1989; Daniel *et al.*,1989). These studies have concluded that lignin peroxidase (Mr - 42 000) is incapable of penetrating the walls of sound wood. The areas of the walls that have been heavily decayed by white rot fungi, which degrade lignin and hemicelluloses somewhat selectively, were penetrated by the enzyme (Blanchette *et al.*, 1989). This suggests that selective biological lignin removal, like alkaline delignification in pulping, opens up the cell walls.

It is hoped that the preceding review of cellulase and ligninase activity shows that the actions of these enzymes in wood produce significant effects on the substrate. Indeed, the advanced stages of these effects are quantifiable and such methods used are outlined below (1.2.5) to introduce the concept of examining alternative methods in the present work.

1.2.5 Quantification of fungal degradation

The main agents of wood decay are fungi and these organisms are separated into groups as outlined above (1.2.3.2).

Quantification cannot be ignored in any meaningful study of wood decay. Unfortunately, many disadvantages are associated with the quantitative methods currently available to the microbiologist. These are outlined here and reviewed in Chapter 3. The conventional methods of quantifying wood decay include the use of gravimetric weight losses, the Pressler Incremental Borer, the Pilodyn (Cown, 1978; Hedley and Naish, 1980; Leightley, 1981,1982), the Shigometer (Hedley and Naish, 1980; Leightley, 1981) and the Vibrante wood pole analyser (Vibrante Technologies, 1983-1987). It must be emphasised, however, that these methods have a number of limitations especially as far as incipient decay is concerned. A major drawback of these methods is that they do not provide any information on which components of the lignocellulosic matrix have been utilised. Importantly, from the

viewpoint of the present work, owing to the limitations of the conventional methods, there is a need to investigate other methods which would produce information which provides greater insight into the incipient decay process. For example, colonisation of wood is often visible, but can it be measured? Colonisation can be measured by biomass determinations (King *et al.*, 1981) and the most sensitive available means of measuring biomass is indirectly by nitrogen determination (Nkonge and Ballance, 1982) as discussed in Chapter 3. Degradation of wood can also be quantified by wood sugar analyses (Pettersen *et al.*, 1984) and Klason lignin determinations (Effland, 1977). These analytical techniques are considered superior to the conventional methods and their advantages are described in Chapter 3.

1.3 Scope of the Present Work

Drawbacks associated with gravimetric methods used to quantify microbial degradation of wood include lack of sensitivity, which produces large degrees of error. It was also felt that a disadvantage of such determinations was their inability to provide any information specifying those fractions of the lignocellulosic complex which may be selectively degraded by certain microorganisms. A further disadvantage associated with decay evaluations based soley on gravimetric determinations is that nil weight losses imply that no decay occurred, whereas the colonised wood may well contain microbial biomass in quantities greater than the mass of wood components which had been degraded.

The above review presents the significance of wood decay in broad terms, and justifies, inter alia, the need for further research of,

i) the significance of anaerobic and facultatively anaerobic fungi, including rumen isolates, in wood decay, and

ii) the quantification of degradation during incipient decay.

Occasions arise when microbiologists are required to establish whether colonised wood has been decayed during transit as untreated timber in the cargo holds of ships during passage

through tropical waters. As mentioned above (1.2.5) this is often difficult to establish in the early stages of decay when no significant decrease in wood density can be detected. It was therefore decided to establish whether analytical techniques are sensitive enough to confirm that wood degradation was measurable during incipient decay when colonisation may have occurred but may not have produced measurable weight losses. Specifically, it was decided to perform chromatographic analysis of degraded wood, in conjunction with gravimetric determinations, to establish that such analyses could ascertain the cellulolytic or ligninolytic physiology of the responsible decay fungi in the early stages of decay.

Having established in the above review that little information is available on anaerobic fungi in wood, it was decided to include these in the present studies to investigate their potential in wood decay. However, since previous work (Wiederhold *et al.*, 1989) had indicated that anaerobic fungi may colonise wood, but not degrade it significantly, it was felt that the primary advantage to be derived from the use of these fungi in the present work was that such isolates may provide suitable tools for the analysis of undecayed, but relatively well colonised, wood samples. Consequently, it was reasoned that such samples may be used as negative controls to provide biomass values

corresponding to low or nil weight losses, the positive control values would be obtained through the use of aggressive decay fungi, viz., basidiomycetes.

1.3.1 Hypothesis to be tested

On the basis of the above review, it was hypothesised that "degradation of wood by fungi can be analytically detected and quantified during the early stages of its colonisation".

1.3.2 Objectives

The following objectives were designed to challenge the above hypothesis :

i) to produce a culture collection of fungi appropriate for studies to assess the extent of colonisation of wood after inoculation and,

ii) to establish any correlations between colonisation and wood degradation during its incipient decay.

1.3.3 Aims

Aims pursued in order to realise the above (1.3.2) objectives are specified in the relevant Chapters of this dissertation, but within the scope of this work, the aims targeted :

A. the procurement of test cultures by :

i) isolation of local rumen fungi,

ii) examination of an obligately anaerobic fungus *N.frontalis* from Australia,

iii) examination of European standard cultures of wood decay fungi,

and the use of the above cultures to inoculate and colonise wood under both aerobic and anaerobic conditions and qualify the extent of colonisation using Scanning Electron Microscopy. The above work is described in Chapter 2 of this dissertation. The second major aim of the work was :

B. the quantification of incipient fungal degradation of wood by :

i) analytical techniques and,

ii) measurement of fungal biomass in wood,

and this work is described in Chapter 3 of this dissertation.

Chapter 4, the General Discussion, examines the significance of the results of the present work in the general context of wood biodeterioration.

CHAPTER 2

MICROSCOPICAL EVALUATION OF WOOD COLONISATION BY FUNGAL ISOLATES

2.1 INTRODUCTION

Chapter 1 has shown that the scope of this work encompassed the necessity to obtain suitable fungal isolates to conduct the analytical work described in this dissertation. Therefore, it was decided (Chapter 1) that part of the initial studies to be conducted should include the isolation of anaerobic fungi for possible subsequent tests. Since such work must take cognizance of environmental factors which affect the successful isolation of anaerobes, it is appropriate to consider the mechanisms of oxygen toxicity at this stage.

It is well known that the surface of the earth is in contact with the oxygenated atmosphere and all readily apparent life forms are oxygen dependent, but there are many ecosystems where oxygen is deficient. Organisms able to utilise the available nutrients in such an environment must be capable of an anaerobic way of life. Such life is almost entirely bacterial, and most prokaryotic species, unlike most eukaryotes, are capable of prolonged growth in the complete absence of oxygen (Holland, Knapp and Shoesmith, 1987).

Many anaerobic microorganisms are able to grow more in the presence of oxygen and are referred to as facultatively anaerobic organisms. There are those which prefer lower oxygen tensions for growth and these are referred to as microaerophiles. There are however, large numbers of organisms which readily grow anaerobically, but are unable to utilise oxygen productively, and in addition are inhibited or even killed by the presence of oxygen. These are the obligate anaerobes. The obligate anaerobe is best defined as an organism for which anaerobic cultivation methods give optimum growth and for which oxygen is inhibitory (Holland, Knapp and Shoesmith, 1987).

2.1.1 Sensitivity of anaerobes to oxygen

It must be noted that most of the work carried out in anaerobic microbiology have been conducted with bacteria. The bactericidal effects of oxygen have not been widely examined on a quantitative basis (Shoesmith and Worsely, 1984). The lethal effects of oxygen are more likely to be observed when handling fresh isolates from natural environments (Krieg and Hoffman, 1986). Some evidence suggests that bacterial oxygen sensitivity depends on the phase of growth. It has been shown that exposure of cultures to air, after one day's incubation and before slow growing colonies were visible, caused decreased recovery (Wren, 1980). This implies greater oxygen sensitivity of young cultures, and possibly interaction with the growth medium. The basis of the wide range of oxygen resistance in anaerobic bacteria is not clear. The variation applies to both

bactericidal effects , where tolerance extends to several hours in pure oxygen, and bacteriostatic effects where tolerance extends from the ability to grow slowly in air, to those anaerobes apparently inhibited by concentrations below the limits of measurement (Woods and Jones, 1986). These divergencies have led to the terms "oxygen tolerant anaerobes" and "extremely oxygen sensitive anaerobes" being adopted, but there are, unfortunately , no widely accepted definitions; the context of such usage usually needs to be examined to determine not only the tolerance level but also whether the tolerance refers to the practicality of handling an organism in air or to the level of oxygen in the growth environment (Loesche, 1969). This is especially the case with oxygen - tolerant anaerobes. No single factor can be defined as responsible for these variations in tolerance between different anaerobes or even between aerobes and anaerobes (Morris, 1979).

2.1.2 Eh and the growth of anaerobes

The oxidation - reduction (redox) potential (Eh) provides the most useful scale for measuring the degree of anaerobiosis (Hungate, 1969). Simply stated, the Eh is a measure of the tendency of a solution to give or take up electrons (i.e., to be oxidised or reduced). Measurements of Eh are expressed in units of electrical potential difference (i.e., volts), the more positive the number of volts, the higher the relative

concentration of oxidant to reductant in solution, and vice versa. The capacity of a medium to resist change in Eh is called poising, analogous to the buffering of pH.

In normal laboratory media, oxygen is primarily responsible for raising the Eh, and positive Eh values resulting from dissolved oxygen inhibit all anaerobic bacteria. However, positive Eh values created by the presence of other chemicals in a medium may not affect the growth of even stringent anaerobes (Onderdonk *et al.*, 1976; Walden and Hentges, 1975). Therefore, no specific tolerances for Eh can be set for various anaerobic bacteria. However, in standard media, most anaerobic bacteria are inhibited at Eh values higher than -100mV. Some will not initiate growth at potentials higher than -330mV; the theoretical concentration of oxygen at this Eh is 1.48×10^{-56} molecules per litre (Hungate, 1969). Even at a potential of -140mV, the calculated oxygen level is only 1.5×10^{11} molecules per litre, or less than 1 molecule of oxygen per cell in many cultures. This illustrates the importance of creating reducing conditions beyond those achieved by merely removing oxygen from media for the cultivation of the more stringent anaerobes.

The Eh of a solution is most accurately measured electrometrically. However, redox dyes are used much more widely to estimate the Eh of media and cultures. The useful dyes are reversibly oxidised and reduced and are coloured in the oxidised state and colourless in the reduced state. Each dye becomes reduced at a different Eh, and the Eh at which it is 50% oxidised

or reduced at pH 7.0 is the standard redox potential. The concept of Eh has long been used as an indication of the deoxygenation of growth media. Deoxygenation is shown by the use of indicator dyes, methylene blue and resasurin being the most commonly used (Holland, Knapp and Shoesmith, 1987). To obtain growth of an obligate anaerobe in any medium it is necessary to expel all dissolved oxygen (thereby lowering the Eh value) and to add some reducing agent (cysteine, thioglycollate, dithionite or ascorbate) to poise the culture Eh value at a still lower value (Morris, 1979). The indication of the extent of deoxygenation is then done by the use of the above dyes (Holland, Knapp and Shoesmith, 1987). Such agents must be non toxic at the concentration used and result in final potentials low enough for the particular organism under study.

2.1.3 Techniques for the isolation of anaerobes

Since air is the primary source of oxygen, air must be excluded from cultures of delicate anaerobes. Two techniques are currently being used in anaerobic microbiology : firstly, the method in which all precautions are taken to ensure that oxygen is excluded from every step of the handling procedure, including sampling, media preparation, transfer and incubation (Bryant, 1972) and, secondly, a technique where only the incubation step is maintained oxygen free. The second technique is convenient and has an unacceptably high failure rate in isolating strict anaerobes (Starr *et al.*, 1981). The first technique is slow,

time consuming and demanding on the operator, but when performed correctly is the only technique which will allow the successful handling of the strict oxygen sensitive anaerobes.

2.1.3.1 Anaerobic gas jars

The most widely used method for anaerobic incubation is the anaerobic jar, where the medium is prepared and inoculated aerobically. The inoculated medium is placed in the jar and the jar is sealed. The remaining oxygen in the jar is removed either by displacing it using an oxygen free gas, or by withdrawing the air by vacuum and replacing it with oxygen free gas. Sachets of palladinised aluminia are placed in the jar to remove any traces of oxygen remaining in the jar.
Modifications of the jar have been produced and these include bags or jars with H_2 + CO_2 generators. The system most commonly used is the Gas Pak system (Oxoid, Hamshire, England). No evacuation is required. After activation of the gas generating unit with water, the bag or jar is closed quickly. Methylene blue indicator strips may be sealed in the above transparent bags or jars to indicate the presence or absence of oxygen.

This technique is used for the culture of oxygen tolerant anaerobes that require anaerobic conditions for growth only. All the incubation steps are carried out anaerobically whereas all the other operations such as media preparation and subculturing are carried out in air using normal bacteriological techniques

(Holland, Knapp and Shoesmith, 1987). It was felt that the latter features precluded the use of anaerobic jars in the present work with stringently anaerobic fungi.

2.1.3.2 The Hungate Technique for stringent anaerobes

Many anaerobes found in the intestinal tract, rumen, and other natural anaerobic environments require very low redox potentials to initiate growth. Extraordinary precautions must be taken to protect media and cells from even brief exposure to oxygen. A roll tube technique was described by Hungate (1950) for the isolation and maintenance of pure cultures of stringently anaerobic bacteria, and many modifications of this technique have been developed (Hungate, 1966). Instead of petri dishes, roll tubes are used for the isolation of single colonies and for the estimation of viable populations of stringent anaerobes. Essential to this technique is the supply of oxygen free gas and the preparation and sterilisation of media under oxygen free conditions (Bryant, 1972). Medium prepared in such a manner is referred to as pre - reduced anaerobically sterilised medium (Hungate, 1950; Citron, 1984; Holland, Knapp and Shoesmith, 1987). Oxygen free gas can be purchased commercially. However, passing the gas through a column or oven containing copper heated to 350C removes any trace of oxygen. More recently, it has become more convenient to pass the gas through a column of palladinised aluminia pellets which removes any traces of oxygen from the gas by catalysing the reaction $2H_2 + O_2$ -----> $2H_2O$. This oxygen free gas is then used to displace oxygen from

bottles, syringes and needles, and media (Holland, Knapp and Shoesmith, 1987). Although reducing agents are often added to anaerobic media, oxidation of these agents may be inhibitory to some organisms (Fee, 1981). Therefore if these agents are to be added, the addition must be carried out after the medium has been deoxygenated. If the medium is to be used soon after autoclaving, the reducing agent may be added at this time provided it is heat stable; otherwise it should be added just before inoculation. The medium is dispensed into tubes or bottles equipped with butyl rubber stoppers while maintaining a constant flow of oxygen - free gas over the surface of the medium during the transfer, and each tube or bottle receiving medium is flushed with the gas before and during the transfer.

The roll tube method of Hungate (1969) can be used as shake or surface cultures. In this method the tube is inoculated through the butyl rubber stopper using a syringe and needle. The agar medium is held at 45C and after inoculation the tube is rolled under a cold water tap until the agar solidifies so that the walls are uniformly coated with agar. Mechanical spinners are available commercially and many tubes can be " rolled " simultaneously while ensuring uniform distribution of the agar on the walls of the tubes. Roll tubes may be streaked with a specimen by starting at the bottom of a prepared roll tube under a gassing canula and rotating the tube as the streaking loop is drawn straight up.

Even the most detailed descriptions of some modifications of the Hungate technique are frequently difficult to master without demonstration and it is often best to visit a laboratory where the technique is in use. The Hungate technique and variations thereof require patience and dextrous operators. It is probably these factors which have forced researchers working with anaerobes to turn their attention to using the anaerobic cabinet for the culture of anaerobes (Holland, Knapp and Shoesmith, 1987).

2.1.3.3 Anaerobic chambers

Costilow, (1982) described a flexible plastic anaerobic glove - box chamber which is as efficient as the roll tube method in isolating anaerobic microorganisms. This chamber has been tested and used in many laboratories. A number of glove box chambers are now commercially available.

The primary advantages of anaerobic chambers are that they permit use of standard bacteriological techniques including spread plates, replica plating, antibiotic sensitivity testing and they allow preparation of media in a convential manner. However, a sizable initial investment is required and a significant amount of laboratory space is occupied. Also there is some inconvenience in working with gloves, and it is necessary to anticipate media needs well in advance of use.

A simplified diagram (Fig. 2.1) showing the essential features of an anaerobic cabinet demonstrates the convenience of using such a cabinet over the other methods of cultivating anaerobes.

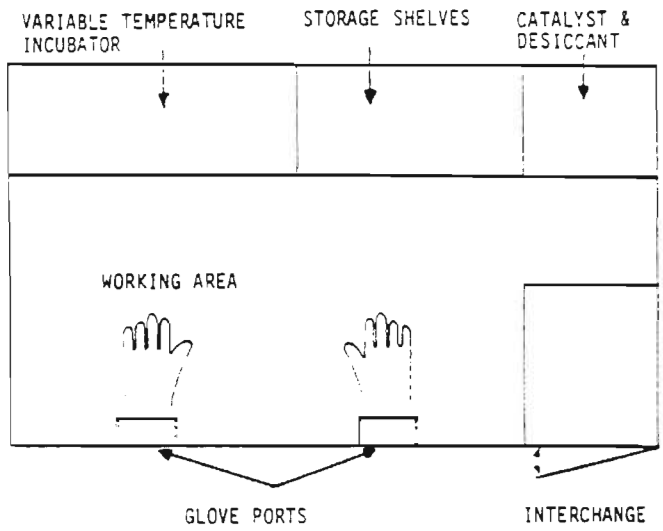


Fig. 2.1 Generalised diagram of an anaerobic cabinet illustrating the essential components (Rogers, 1990).

The interior of the cabinet has a large working area and incubator with variable temperature control. In addition to this there is a area for dessicant; palladinised aluminia catalyst helping to remove traces of oxygen; and activated charcoal to remove H_2S. The cabinet is maintained in an anaerobic state by an input gas mixture of oxygen free nitrogen, carbon dioxide and hydrogen. These systems operate at a slight positive pressure compared to atmospheric pressure. When carrying out manipulations of culturing and subculturing inside the cabinet, the operators hands are inserted through the glove ports.

Materials such as tubes, plates and flasks may be introduced or removed to or from the working area via an interchange - a double door interlock. The materials are placed in the interchange via the external door and three cycles of vacuum and addition of oxygen free gas are carried out automatically. Once the interchange and the interior working area have equalised, the inner door may be opened and the transfer of material into the working area may be carried out, and the outgoing material may be placed in the interchange ready for transport out (Rogers, 1990).

For the culture and isolation of strict anaerobes, pre - reduced media have to be used. This is prepared as previously described (Hungate, 1950; Citron, 1984; Holland, Knapp and Shoesmith, 1987). A modification to those techniques has been developed in order to use pre - reduced media in petri dishes. Schott bottles (Schott Glaswerke, West Germany) are used. A specially developed head (Fig. 2.2a) is inserted into the opening of the Schott bottle (Fig. 2.2b). The medium is prepared in the bottle and reduced by gassing with an oxygen free gas mixture and autoclaving for the appropriate time. Once sterilised, the medium is gassed again and the minerals and vitamins are added aseptically and anaerobically via the injection port. The redox poising agents are also added in this manner. Once the redox indicators show that the medium has been reduced, the medium is then dispensed via a repeating syringe into petri dishes inside the cabinet, or into McCartney bottles purged with oxygen free

gas. When petri dishes are used they are left in the cabinet overnight to ensure that the traces of oxygen are removed (Rogers, 1990).

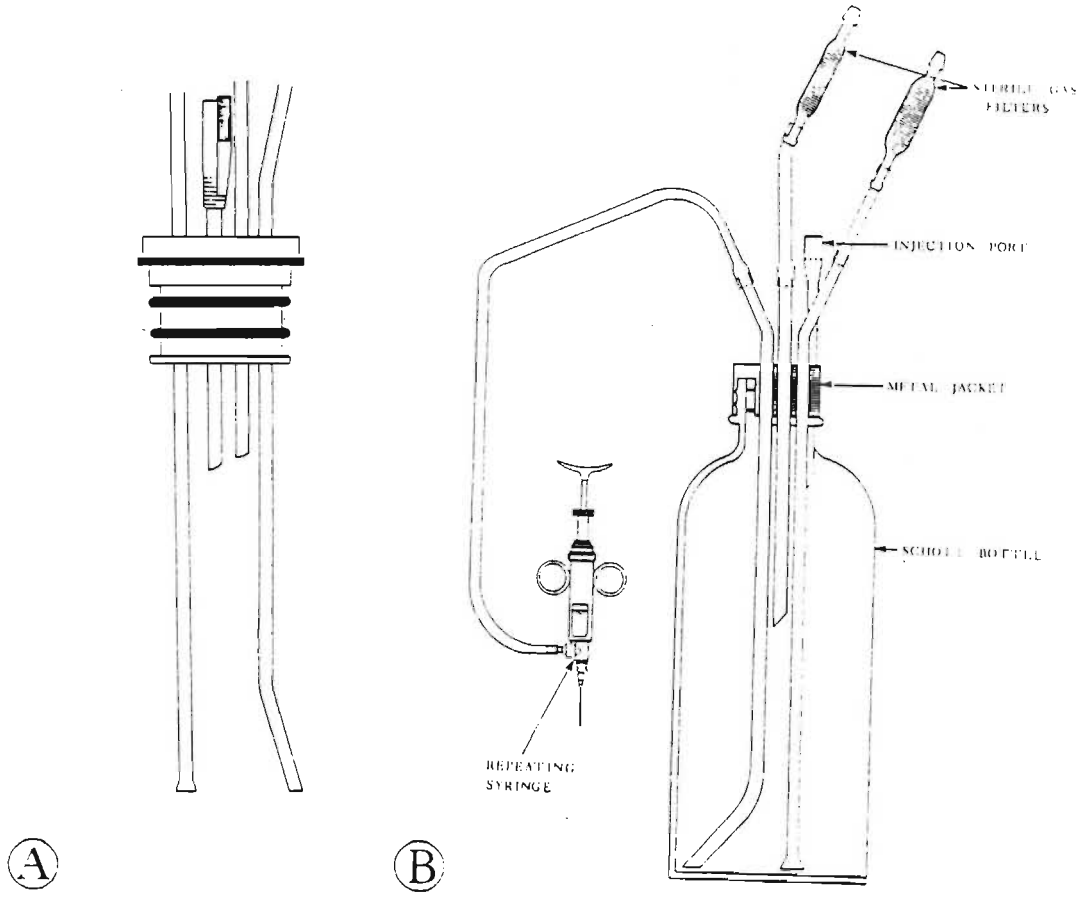


Fig. 2.2 Speciallly designed "head" apparatus (a) to fit into (b) a Schott bottle (Rogers, 1990).

2.1.4 Colonisation tests

Wood colonisation tests need to be conducted to determine whether the fungal test cultures colonise wood extensively or to limited extents. The wood samples (wood blocks or milled wood) need to be sterilised prior to being challenged with the test culture, but sterilisation by autoclaving is unacceptable in the present work since this process may have a destructive effect on wood, such as carbohydrate hydrolysis (Glassare, 1970; Savory and Bravery, 1970).

An alternative method of sterilising biological material is to irradiate it with penetrating electron radiation. All forms of radiation affect biological material by producing the extremely reactive H and OH radicals from the water which is always present in the tissue (Burns and MacDonald, 1970). It has been shown by Kenaga and Cowling (1959), and Lutomski and Lawniczak (1967), that gamma irradiation sterilised wood samples but most of these authors found that radiation dosages of 10^6 rads or more induced a tendency towards greater decay susceptibility in wood. The increased susceptibility of some timbers to decay after gamma irradiation may result because it has been shown that radiolysis (i.e., the effect of radiation on the degree of polymerisation) of holocellulose can occur (Lawton *et al.*, 1953; Charlesby, 1955; Glegg, 1957), although the effect of this phenomenon may be reduced in wood (Smith and Mixer, 1959) by naturally occurring aromatic compounds in lignin and other extractives. These compounds "protect" the aliphatic groups by effectively absorbing the heat of radiation in their own ring structures (Hansen,

1972). The decisive factor, with regard to sterilising dose, is the total received and for the sterilising effect it is of minor importance whether the irradiation is carried out over longer or shorter period (Rindorf and Christensen, 1969). The dose rate can, however, be of great importance when chemical changes in the wood material are considered. For example weak radiation over 1-2 days hardened a monomer, methylmethacrylate, whereas the same dosage administered in one second did not (Hansen, 1972). Hansen (1972) found that electron radiation of 5Mrad from a high energy linear accelerator (delivering 10^6 Mrad/min) effectively sterilised pine blocks without affecting their susceptibility to decay.

The sterilised wood blocks or wood slivers are placed onto media chosen for the colonisation tests and standard inocula of fungal cultures from actively growing mature lawns are placed adjacent to the wood on agar medium in petri dishes. The petri dishes are incubated for a designated period, depending on the nature of the investigation and wood samples are then removed and prepared for microscopical examination of the extent of colonisation.

Microscopic examination of samples is used for the assessment, at a cellular level, of physical effects produced in wood by the colonising microorganisms. The light microscope may conventionally be used when wood sections to be examined are cut sufficiently thin to allow light rays to penetrate them, but scanning electron microscopy may also be used on unsectioned wood (consequently undamaged by normal prepatory treatments e.g. microtoming) to obtain greater resolving power in these

observations. Such observations allow the worker to postulate the association of certain degradative effects produced in the wood with the microbes seen in the attacked cells. Care is required when making such correlations between degradation and causative agent because decayed wood usually supports a mixed population of different microbes and it is not always possible to establish "cause and effect" as the cause may be indistinguishable in the presence of many genera and species of microorganisms (Baecker, 1981).

An obvious advantage thus arises in this type of work when only one microorganism is involved, and even if atypical forms of this organism develop in the wood, the worker retains a firm basis to attribute the extent of colonisation and decay patterns observed to the only microorganism known to be present.

The use of the scanning electron microscope to monitor colonisation of wood by fungal isolates is further highlighted by studies conducted by Wiederhold *et al.*, (1989); Joblin and Naylor (1990) and Singh and Hedley (1990).

2.1.5 Objectives and Aims

On the basis of the above review, the objective of the work presented in this Chapter was to produce a screened culture collection of fungi appropriate for subsequent studies (Chapter 3) to quantify their colonisation of wood.

The primary aim of the work presented in this Chapter was therefore :

A) to isolate and obtain rumen fungi and standard wood decay cultures with the ability to extensively colonise wood.

The second aim of this work was :

B) to screen and select cultures suitable for the quantitative work of Chapter 3 and it was decided to do this qualitatively by using Scanning Electron Microscopy.

2.2 MATERIALS AND METHODS

Cultures were:

i) isolated from sheep (2.2.1), and

ii) obtained as stocks, viz.,

a) the obligate anaerobe - *Neocallimastix frontalis* (2.2.2) and,

b) four decay basidiomycetes (2.2.3).

2.2.1 Isolation of rumen fungi

Rumen fungi were isolated from fistulated sheep which had been fed a fibrous diet comprising lucerne hay supplemented with methionine.This diet was implemented 2 weeks prior to sampling,to promote the development of sporangia in the rumen (Gulati *et al.*, 1985 ; Philips and Gordon, 1989).

To include with media, rumen fluid from a rumen fistulated sheep was clarified by centrifugation at 10 000 x g for 30 min and stored at -20C. Caldwell and Bryant's Medium No.10 (CB) (Caldwell and Bryant, 1966) and a Composite Medium (CM) comprising malt extract agar (Oxoid, 2,5% w/w), potato dextrose agar (Biolab, 1,95% w/w) and clarified rumen fluid (40% w/v) in distilled water were each used to isolate fungi by the roll tube method. The complete recipes of these media are presented in Appendices 1 and 2 respectively, and the methods used for the preparation of pre - reduced media and the culture techniques used, eg., pre-gassing and the roll tube method, were as described by Hungate(1966).

As a source of fungal inocula, rumen fluid was freshly collected from a fistulated sheep and filtered through a layer of muslin. The rumen fluid was gassed with an anaerobic gas mixture (5,5%H_2, 31,8%CO_2 and 62,7%N_2) and used to inoculate the respective media above. For roll tubes, the CM or CB medium (4,0 ml) was placed in culture tubes and inocula of 0,5 ml rumen fluid were added to melted agar media in the tubes at 43C. Antibiotic solution (penicillin 0,04% w/v and streptomycin 0,01% w/v) was added to each tube before media solidified to inhibit bacterial growth. All cultures were incubated under a CO_2 atmosphere at 39C for 7 days.

Three fungal isolates were obtained in roll tubes under the strictly anaerobic conditions described above. These were purified and tested (below) for their ability to grow under aerobic conditions.

2.2.1.1 Colonisation tests under aerobic and anaerobic conditions

Pinus patula and *Eucalyptus grandis* sapwood was comminuted by hammer-milling (approximately 0,5 - 1,0 mm), sterilised by gamma irradiation (20 Kilorads) and sprinkled on the surfaces of CM in petri dishes which had been inoculated centrally with the three fungi isolated (2.2.1) from the rumen. The plates were incubated at 39C for 3 days when wood samples were removed and prepared as follows for examination by scanning electron microscopy using a Philips 500 SEM:

The wood samples were fixed for 14 hours with 3% gluteraldehyde in 0,1M sodium cacodylate buffer (Appendix 3) at room temperature. The samples were then washed in two 15 minute changes of 0,1M sodium cacodylate buffer and then dehydrated through progressively increasing concentrations of ethanol (10%, 30%, 50%, 70%, 80%, 90%, 100%), with 10 minutes in each solution and three changes in the 100% solution. The specimens were critical point dried in liquid carbon dioxide for 1h in a Hitachi Type HCP.2 Critical Point Dryer (Hitachi Co., Ltd., Tokyo, Japan), and then mounted on stubs using double sided sticky tape, followed by sputter coating (Polaron Equipment Ltd). The specimens were coated with approximately 10nm gold and examined using a Hitachi S-570 SEM (Hitachi, Tokyo, Japan).

Liquid media (CM Broth) were also supplemented (approximately 1% w/v) with *P.patula* and *E.grandis*. These broths were also inoculated with the three fungi and incubated for 7 days at 39C under both aerobic and anaerobic conditions. Wood samples were then removed and prepared as described above for examination by scanning electron microscopy.

2.2.1.2 Enzyme production

The cultures isolated were tested for the production of enzymes which could play a role in wood degradation.

2.2.1.2.1 *Cellulase activity*

The fungal isolates were inoculated onto 0,5% (w/v) Cellulose Agar(CA) to determine whether these fungi produce cellulases. The composition of this medium is recorded in Appendix 4. The medium was transferred to the anaerobic cabinet and was stirred to keep the insoluble cellulose substrate in suspension until plates were poured (9 ml per plate). After the medium had solidified, the plates were inoculated with the fungi and incubated at 39C under strictly anaerobic conditions in the anaerobic cabinet. The plates were monitored daily for growth and clear zones in the cellulose agar.

The above (CA) was also prepared without gassing and reducing agents, and inoculated petri dishes were incubated aerobically at 39C. These plates were also monitored daily for growth and zones of clearing in the agar.

2.2.1.2.2 *Xylanase Activity*

The fungi were used to inoculate Xylan Agar (XA) which comprised Caldwell and Bryant's Medium NO.10 (Appendix 1). The medium had been supplemented with 3% birch xylan (Sigma Chemical Company, St Louis, USA) prior to autoclaving. The plates were incubated aerobically and anaerobically at 39C and monitored daily for growth and zones of clearing.

2.2.2 Standard culture - obligately anaerobic *Neocallimastix frontalis*

As stated above (2.2.1.1), it was intended to compare the activities of rumen fungi isolated in South Africa (2.2.1) with those of the most well documented and widely described (Phillips and Gordon, 1988; Phillips and Gordon, 1989) anaerobic rumen fungus, *N.frontalis*, in the present work. No viable stock cultures of *N.frontalis* were obtainable in South Africa, therefore these cultures were kindly donated by Dr. M. W. Phillips (CSIRO, Division of Animal Production, Blackwell, Australia). The cultures were inoculated in Australia, and delivered to the author's laboratory 4 days later as :

(i) 2 agar roll tubes with CB overlays,

(ii) 2 agar roll tubes with CB + glycerol overlays, and also as the following broths,

(iii) 3 CB broths + sisal and grass as maintenance media.

(iv) 2 CB broths + sisal and grass + glycerol, as maintenance media.

In view of previous unsuccessful attempts (Singh, 1989) to regenerate such cultures after delayed transit from Australia to this laboratory, six subculture broths (Caldwell and Bryant's Medium No.10) were also sent together with the cultures to facilitate immediate subculture. On receipt of these cultures, they were immediately subcultured as follows:

(a) 0.5ml of CB broth from (i) above was taken from one tube and transferred to fresh medium, viz., Maintenance Medium (MM) (Bryant and Robinson, 1966) prepared in this laboratory (Appendix 5) and from the other to the supplied subculture broth. The tubes were incubated for 2-3 days and the subculture procedure repeated.

(b) 0.2ml of CB broth from (ii) above was taken from one tube and transferred to fresh MM and from the other tube to the supplied CB broth.

(c) Using a loop pieces of sisal/grass were removed from tubes specified in (iii) above and used to inoculate MM and the supplied CB broths.

(d) A loop was used to remove pieces of sisal and grass from tubes specified in (iv) above and inoculated into MM and the supplied subculture CB broths . These tubes were incubated for 2-3 days and the subculture procedure repeated.

All tubes were incubated at 39C and were subsequently subcultured every 3-4 days. The broths were examined microscopically (2.2.2.1) for evidence of growth.

2.2.2.1 Colonisation of sisal fibres in Maintenance Medium

Sisal fibres were removed from the MM inoculated above (2.2.2) and also from the cultures supplied from Australia. These were prepared for examination using SEM. The sisal fibres were fixed for 14 hours with 3% gluteraldehyde in 0.1M sodium cacodylate buffer (Appendix 3) at room temperature. The samples were then washed in two 15 minute changes of 0.1M sodium cacodylate buffer and then dehydrated through progressively increasing concentrations of ethanol (10%, 30%, 50%, 70%, 80%, 90%, 100%), with 10 minutes in each solution and three changes in the 100% solution.

The specimens were critical point dried in liquid carbon dioxide for 1h in a Hitachi Type HCP.2 Critical Point Dryer (Hitachi Co., Ltd, Tokyo, Japan), and then mounted on stubs using double sided sticky tape, followed by sputter coating (Polaron Equipment Ltd). The specimens were coated with approximately 10nm gold and examined using a Hitachi S-570 SEM (Hitachi, Tokyo, Japan), to search for zoospores which may have germinated on the substrate

i) during transit of stocks from Australia
ii) after subculture in this laboratory.

2.2.2.2 Wood colonisation tests

CB and MM were prepared anaerobically. The respective media (15ml) were dispensed into McCartney bottles which had been pregassed with the anaerobic gas mixture and sterilised. *P. patula* and *E. grandis* sapwood was hammer milled and sterilised by gamma irradiation (20 kilorads). The comminuted wood (0.5g) was then added to each bottle containing the respective medium.

Inocula were prepared from the CB roll tubes supplied from Australia (2.2.2) as follows:

i) the roll tubes were agitated vigorously to dislodge zoospores and sporangia from the agar into the CB overlay and,
ii) the agar in the roll tubes which had visible colonies of *N.frontalis* was also dislodged and a suspension prepared using the CB overlay.

The suspension was used (0.5ml aliquots) as *N.frontalis* inoculum which was added to each bottle and these bottles were incubated at 39C for 7 days. Wood samples were then removed, fixed and prepared for examination using SEM as described above (2.2.2.1).

2.2.3 Standard cultures - wood decay basidiomycetes

In conjunction with the above tests to compare the degradative potentials of South African rumen fungi with that of *N.frontalis* (2.2.2), it was decided to include similar tests using cultures of well documented wood degrading basidiomycetes, also for comparative purposes in the present work. Furthermore, it was felt that such investigations should comprise comparisons with basidiomycetes representing groups of decay fungi which produce the two well known decay patterns in wood, viz.,

i) white rot (ligninolytic) fungi and,
ii) brown rot (primarily cellulolytic) fungi.

Therefore, the following fungi for use in these tests were kindly supplied by Mrs L.M.Gibson, Dundee Institute of Technology, Scotland, UK;

Name	Decay Type
a) *Coriolus versicolor* FPRL 28A	white rot
b) *Phanaerochaete chrysosporium* BKMF 1767	white rot
c) *Coniophora puteana* FPRL 11E	brown rot
d) *Lentinus lepideus* FPRL 7B	brown rot

The cultures were supplied as slant cultures grown on 5% w/w Malt Extract (MX) Agar (Merck). These cultures were subcultured on fresh MX plates and incubated at 26C for 2 weeks. The cultures were examined for purity before commencing the colonisation tests below.

2.2.3.1 Colonisation tests

In keeping with the anaerobic investigations described above, these cultures of decay fungi were used to challenge wood under both aerobic and anaerobic conditions.

2.2.3.1.1 *Aerobic conditions*

Standard inocula of *Coriolus versicolor* FPRL 28A and *Phanaerochaete chrysosporium* (white rot), and *Coniophora puteana* FPRL 11E and *Lentinus lepideus* FPRL 7B (brown rot), grown on 5% w/w MX Agar for 2 weeks at 26C, were removed from mature lawn cultures using a sterile 5mm cork borer and separately plated on centres of fresh MX plates. *Pinus patula* and *Eucalyptus grandis* sapwood, was comminuted, sterilised by gamma irradiation (20 kilorads) and sprinkled on the surface of these plates as described previously (2.2.1.1). The plates were incubated at 26C for 2 weeks, and samples of wood and of mycelial mats of the respective inocula, were prepared as before (2.2.2.1) for examination using a Hitachi S-570 SEM.

2.2.3.1.2 *Anaerobic conditions*

A separate set of plates, prepared and inoculated as described above was incubated anaerobically at 26C for 2 weeks in the anaerobic cabinet. Samples of wood and mycelial mats of the respective inocula, were then removed, fixed and prepared for examination using SEM as described previously (2.2.2.1)

2.3 RESULTS

2.3.1 Identification

Various types of fungi were isolated (2.2.1) by the roll tube technique (Fig. 2.3). When subcultured (2.2.1.1) these fungi were found to be facultatively anaerobic in that they were able to grow both aerobically and anaerobically in CM broth. They were also shown to be dimorphic in that they were able to grow in both the yeast and mycelial form as described below.

Fig. 2.3 Colony growth in roll tubes used to isolate facultatively anaerobic fungi using Composite Medium (CM) and Caldwell and Bryant's Medium No. 10 (CB).

These fungi were identified (below) as :

i) *Mucor racemosus*

ii) *Candida tropicalis*

iii) *Geotrichum capitatum*

Some characteristic features used as taxonomic determinants in the identification of *M.racemosus*, *C.tropicalis* and *G.capitatum*, and illustrated in subsequent figures of this Chapter, are as follows:-

M.racemosus (Zygomycete)	*C.tropicalis* (Fungi Imperfecti)	*G.capitatum* (Fungi Imperfecti)
sporangia	yeasts	yeasts
sporangiospores	mycelium	mycelium
columellae	budding	arthrospores
yeast forms	chlamydospores	
	blastospores	

The above characters are discussed below but it is appropriate to mention them at this point as they formed part of the identification procedure. *M.racemosus* was conclusively identified by Dr C.J.Rabie (Division of Food Science and Technology, CSIR, Pretoria). *C.tropicalis* and *G.capitatum* were

conclusively identified by Mrs R.L.Steyn (Division of Food Science and Technology, CSIR, Pretoria) on the basis of the results presented in Appendix 6 and 7.

Fig. 2.4 depicts the gross morphology of these fungi when grown on CM agar plates. Since these grew aerobically, their capacities for facultatively anaerobic growth was confirmed.

Light microscopy revealed the microstructures of these fungi, and their modes of reproduction, as depicted in Figs. 2.5 and 2.6 (*M. racemosus*), Fig. 2.7 (*C. tropicalis*) and Fig. 2.8 (*G. capitatum*). The ultrastructures of the isolates were examined using SEM in the wood colonisation tests (2.2.1.1) and will be discussed during the description of those tests (2.3.1.2).

Fig. 2.5 indicates that the isolate identified as *M. racemosus* was a zygomycete because sporangia, sporangiospores and columellae were observed. Under anaerobic conditions, using medium that had been gassed with the anaerobic gas mixture and to which reducing agents had been added, this isolate grew predominantly in the yeast phase as depicted in Fig. 2.6. Such dimorphism is typical of *Mucor* species grown anaerobically (Gleason and Gordon,1988).

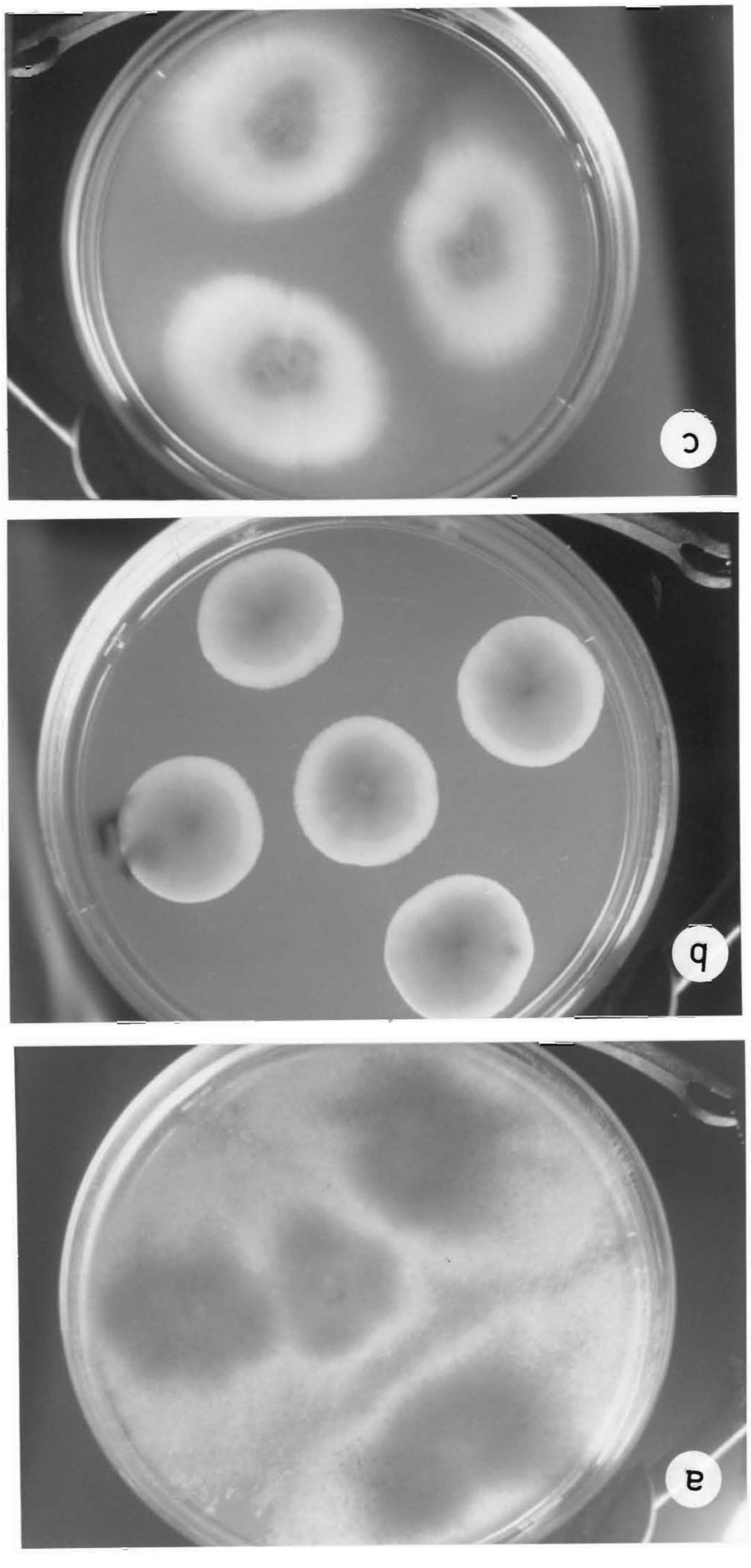


Fig. 2.4 Plate cultures of (a) *M.racemosus*,(b) *C. tropicalis* and (c) *G.capitatum*, each showing gross morphology and extensive mycelial growth under aerobic conditions.

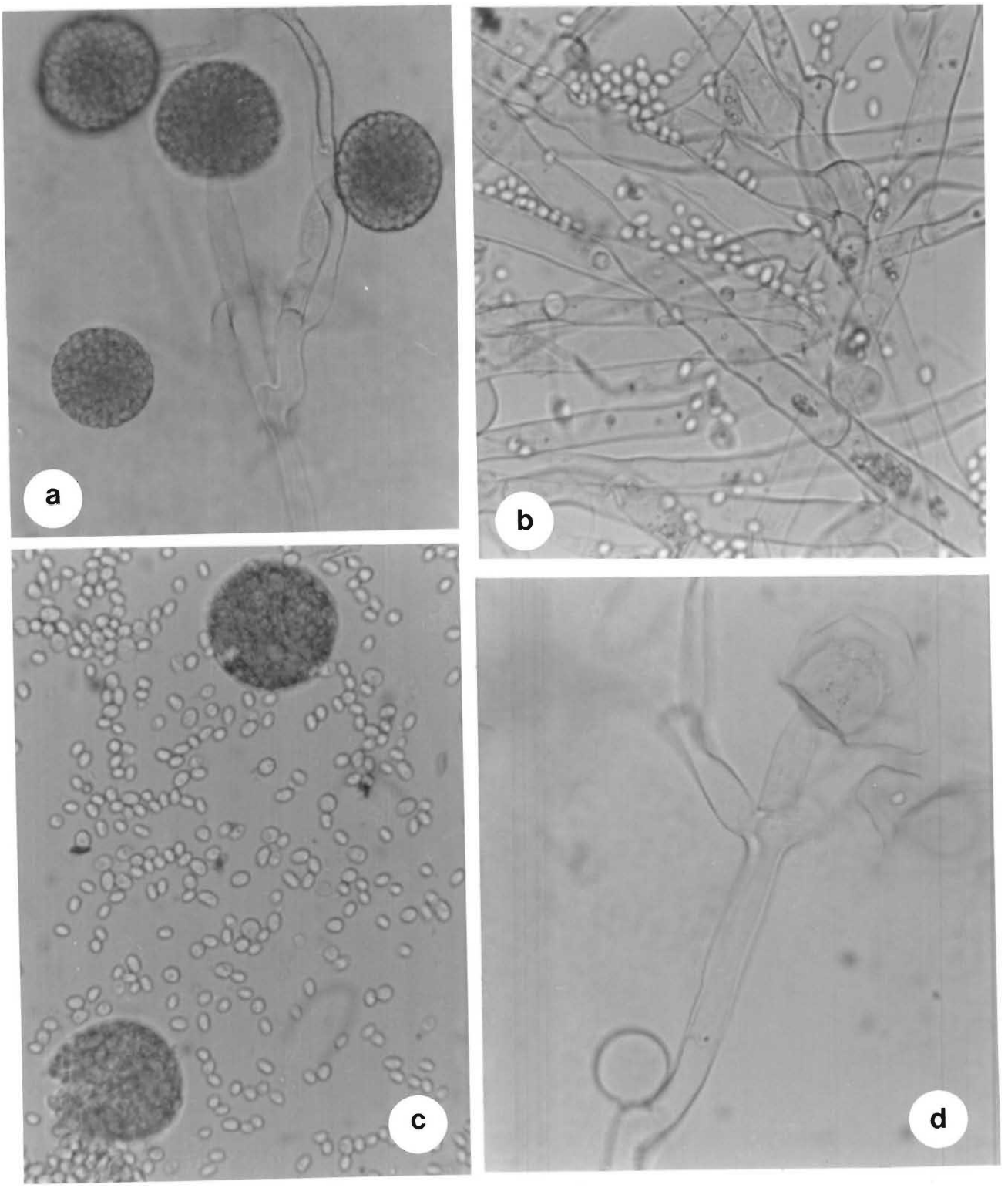


Fig. 2.5 Light micrographs (X 3000) of *M.racemosus* grown aerobically showing (a) intact sporangia containing sporangiospores, (b) thick branching hyphae, (c) disrupted sporangium showing the release of sporangiospores, and (d) columella with remnants of sporangial wall after release of sporangiospores.

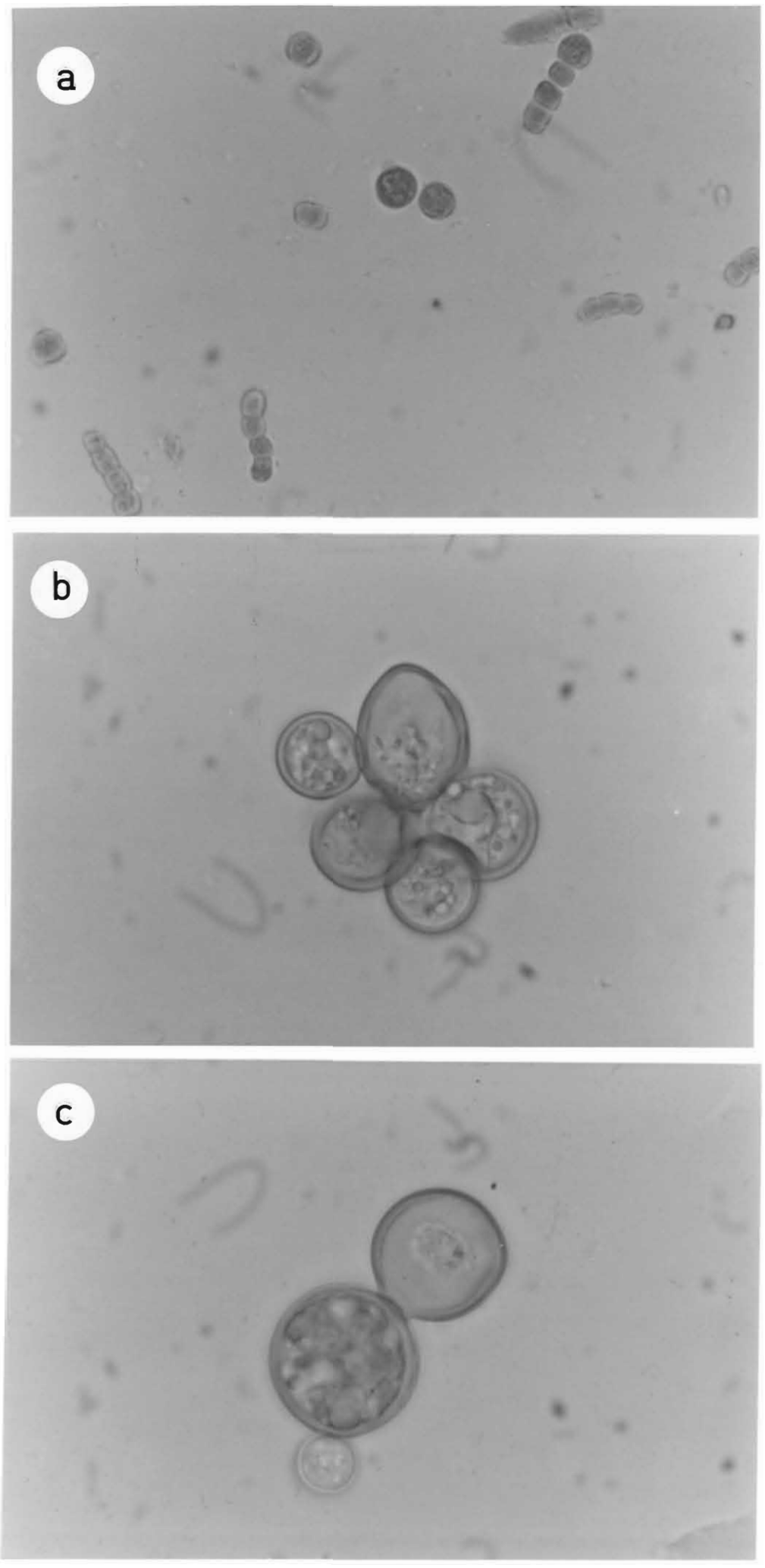


Fig 2.6 Light micrographs of *M.racemosus* grown under anaerobic conditions showing (a) yeast forms predominant (x 1000) (b) yeast forms budding (x3000), and (c) advanced budding (x 1000)

Fig. 2.7 shows that the second isolate also produced mycelial and elipsoid yeast forms, but these were more typical of *C.tropicalis* as demonstrated when their ultrastructures were observed using SEM during wood colonisation tests described above (2.2.1.1). These observations were consistent with the identity of this isolate confirmed as *C.tropicalis*.

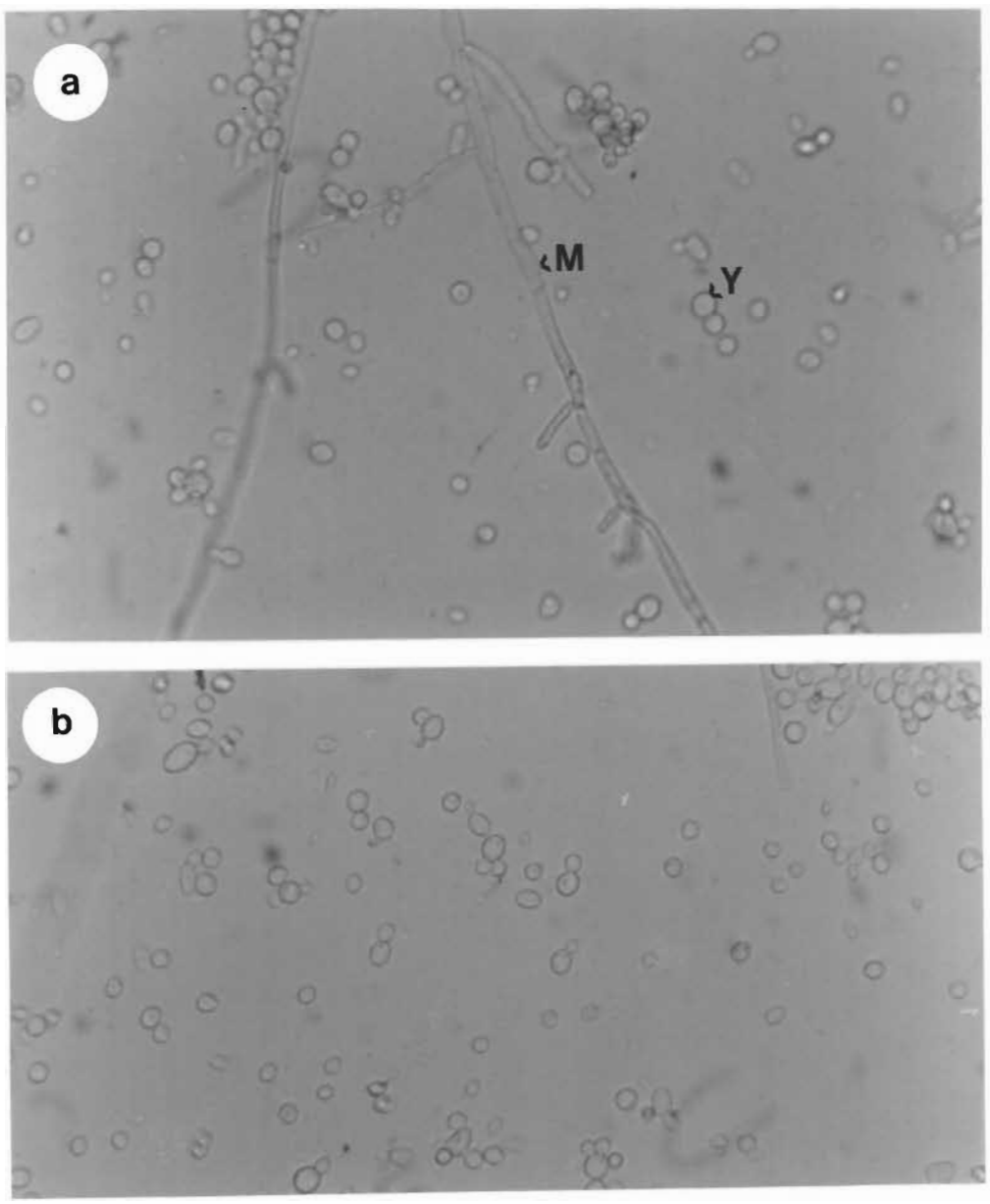


Fig. 2.7 Light micrograph (x1000) of *C.tropicalis* showing both (a) mycelial forms (M) and yeast forms (Y). Multiple buds clearly visible (b).

Fig. 2.8 shows that the third isolate was also dimorphic, but the microstructure of this isolate differed from that of the second in that the mycelium exhibited a greater degree of branching, while the yeast form also differed in microstructure. The ultrastructure observed during SEM studies of colonised wood (2.2.1.1), was consistent with that of the confirmed identity of *G.capitatum*, and is described below (2.3.2).

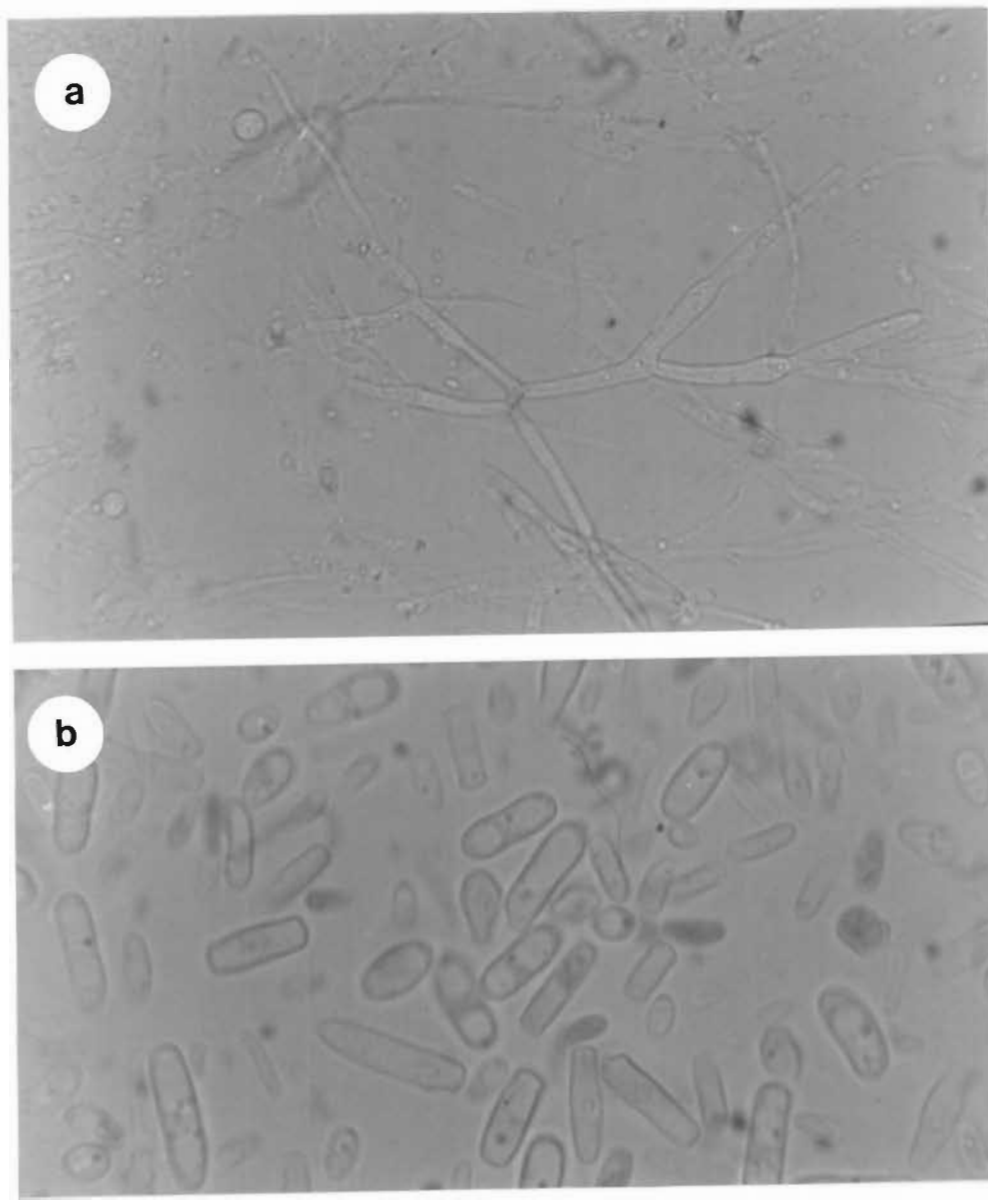


Fig. 2.8 Light micrograph of *G.capitatum* showing (a) mycelial forms (x1000) and (b) germinating yeast forms (x 3000).

2.3.1.1 Colonisation tests using ruminal isolates

Both *P.patula* (Fig. 2.9a) and *E.grandis* (Fig.2.9b) were extensively colonised by *M.racemosus* (Fig. 2.9c) under aerobic conditions. The life - cycle included the production of structures which were columellae (Fig. 2.9d), showing that the fungus was a sporangium - producing zygomycete. Linear hyphal growth orientated in the longitudinal direction of wood elements, a phenomenon not normally observed with wood decay fungi, occurred frequently. Such hyphae appeared to be in contact with middle lamellae (Fig. 2.9e). These morphological data all supported the identification of this isolate as *M. racemosus*.

Under anaerobic conditions, using liquid media, the above isolate again colonised both *P.patula* and *E.grandis*, but it grew predominantly in the yeast form with sparse mycelial growth on wood (Fig. 2.10a) and advanced budding was observed (Fig. 2.10b), again supporting the identification of the isolate as *M.racemosus*. While not constituting a character considered in identification, it was interesting to record the possible stages in the development of the yeast form as depicted in Fig. 2.11. This figure suggests that yeast forms initiate at the ends of hyphae and are released by detachment preceded by hyphal constriction at the sites of production.

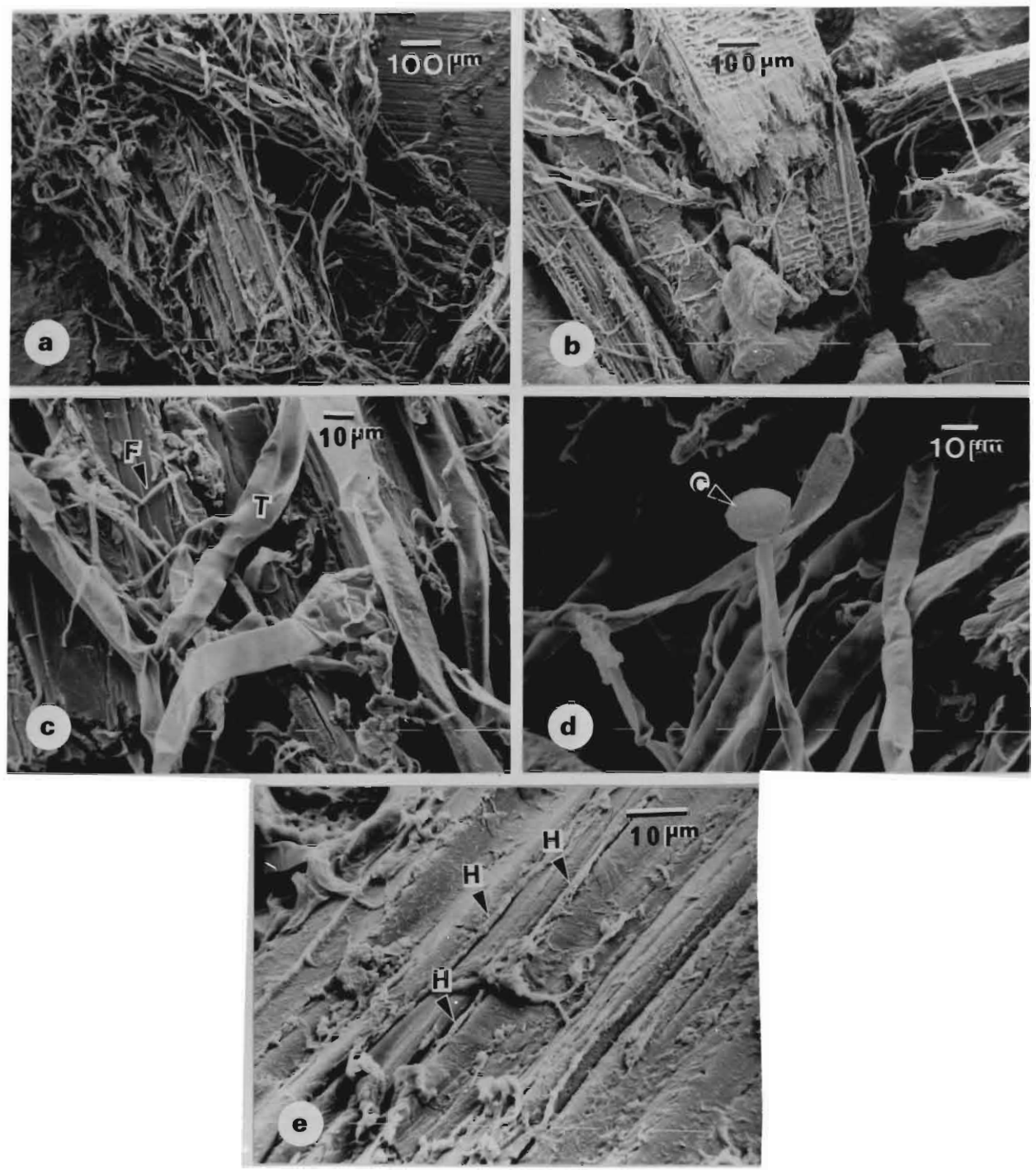


Fig. 2.9 Extensive aerobic colonisation of (a) *P.patula* and (b) *E.grandis* by *M.racemosus*.The dimorphic fungus produced (c) thick (T) and fine (F) hyphae, (d) columella - like structures as shown on *P.patula* (C), and hyphae (H) were often orientated on middle lamellae as shown on (e) *E.grandis*.

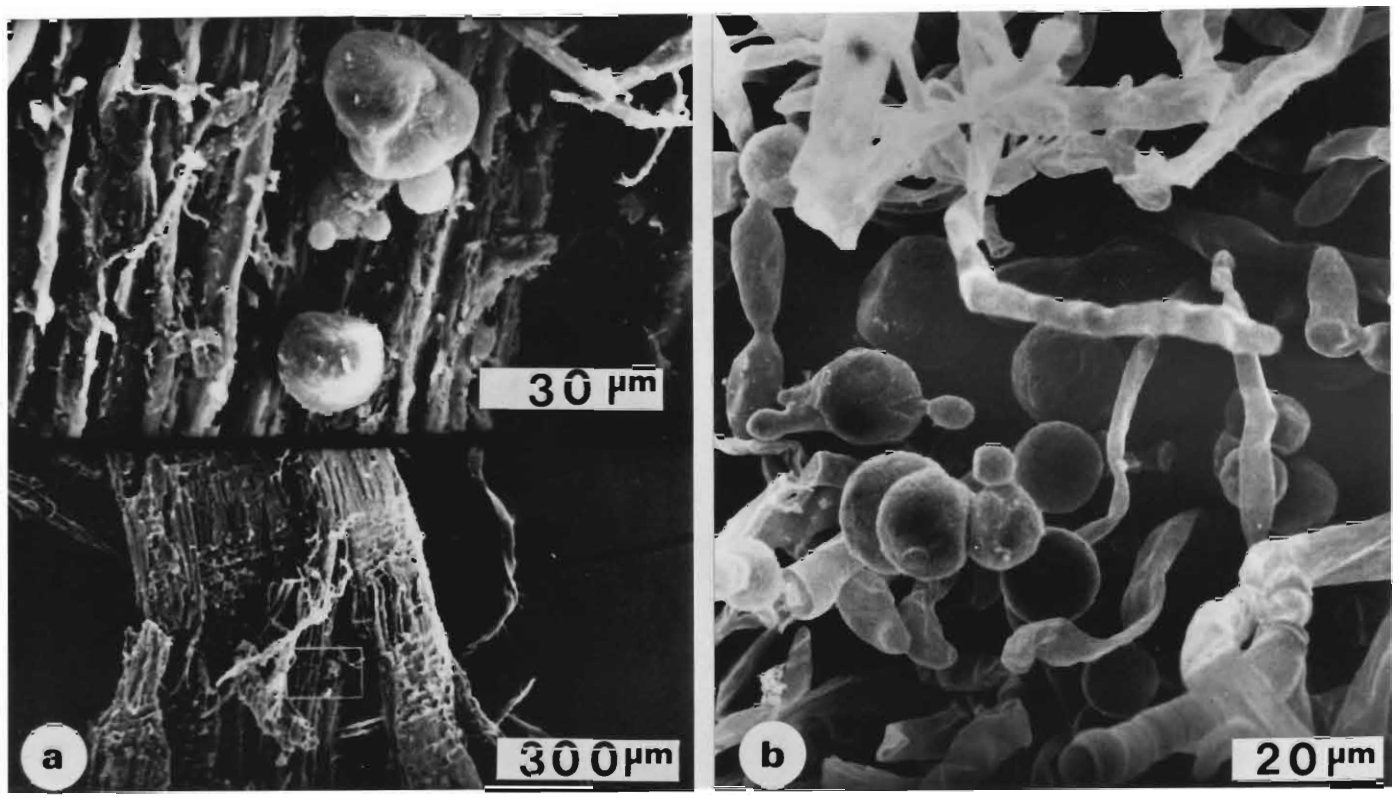


Fig. 2.10 Scanning electron micrograph of (a) *M.racemosus* on wood showing yeast form produced under anaerobic conditions, and (b) advanced budding of such yeast forms.

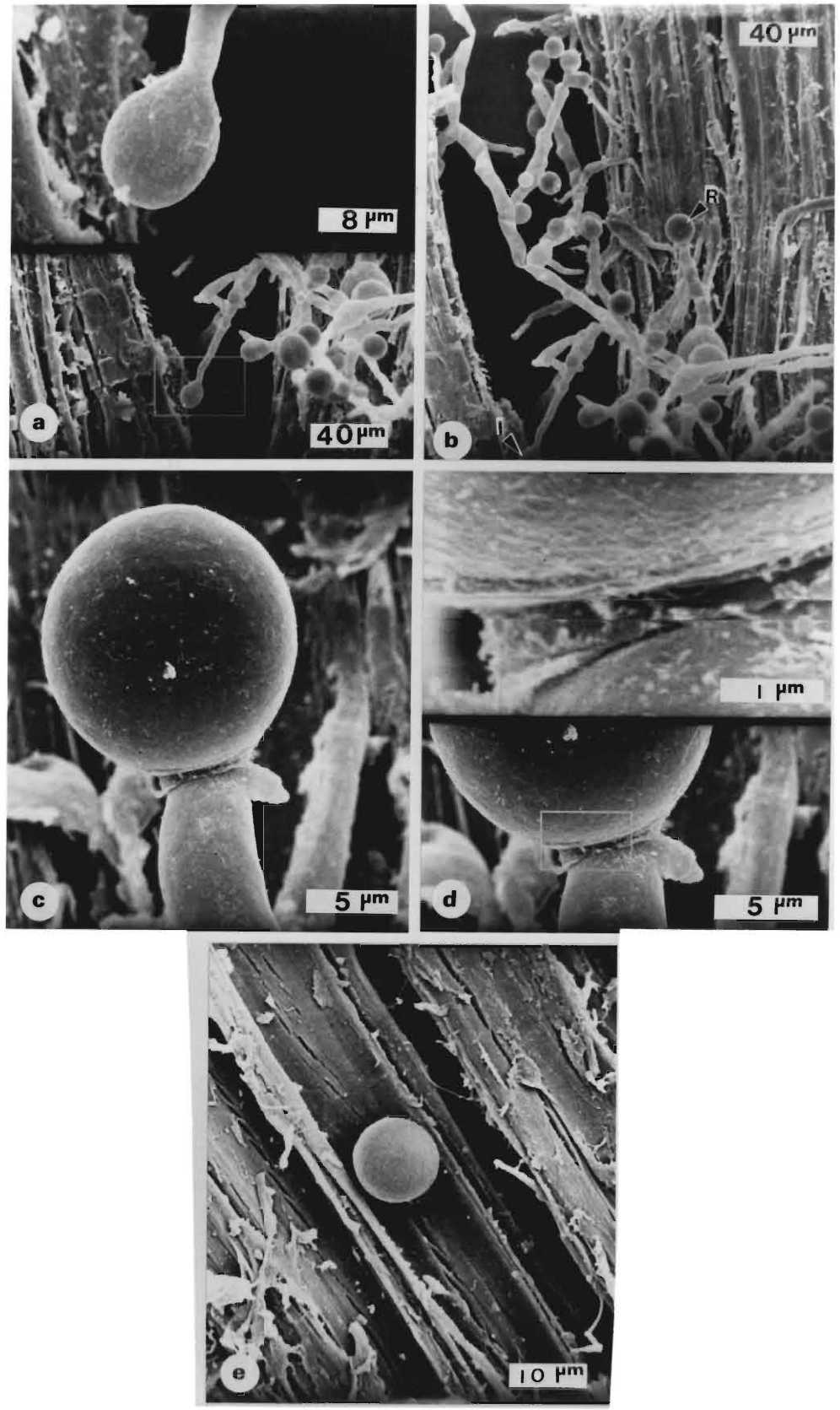


Fig. 2.11 (a) Initiation of the yeast form, (b) initiated yeast form(I) and fully rounded yeast forms(R) on terminae of hyphae, (c) fully developed yeast form detaching, (d) showing visible bud scar, and (e) completely detached yeast form.

The scar that remains after secession of the conidium is depicted in Fig. 2.12.

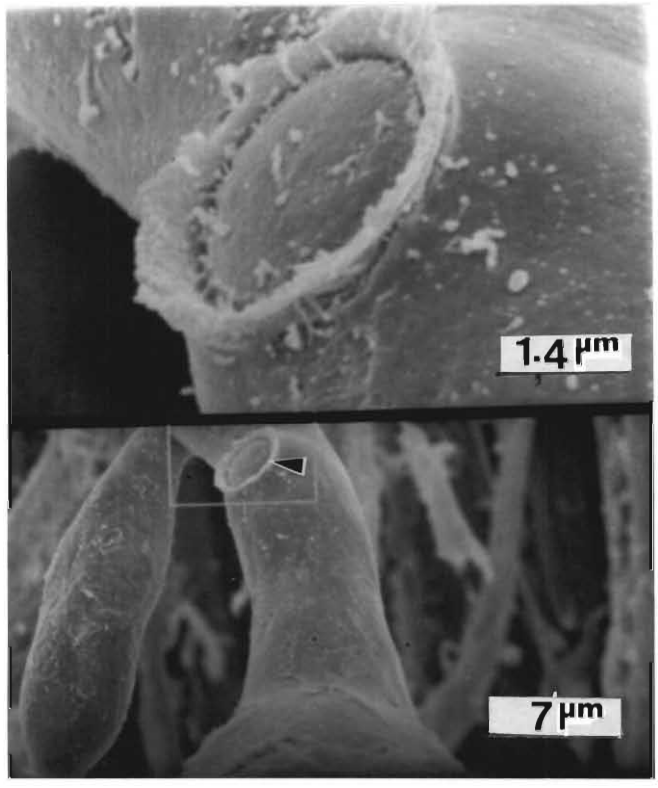


Fig. 2.12 Scar remaining after secession of a conidium.

On return to aerobic conditions, the yeast form appeared to germinate into the mycelial form as shown in Fig. 2.13.

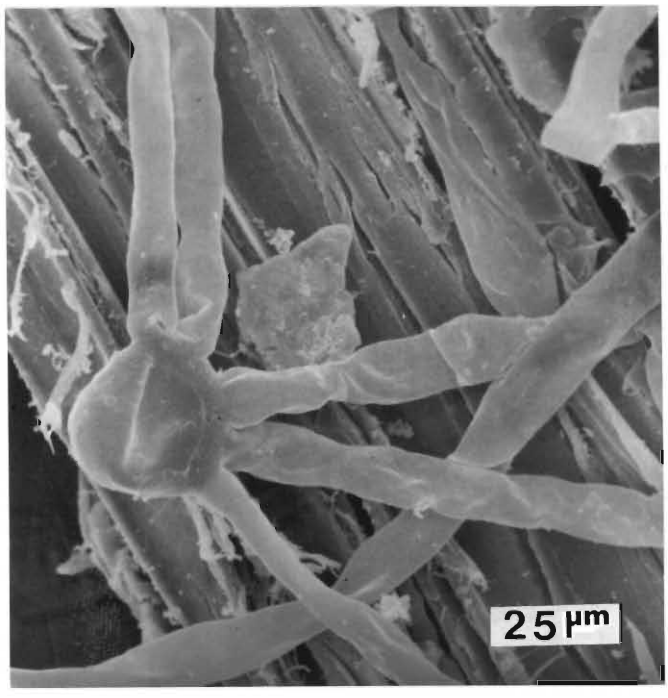


Fig. 2.13 Germination of yeast form to mycelial form under aerobic conditions.

Thus the life cycle between the two forms of the dimorphic fungus under aerobic and anaerobic conditions is depicted in the preceding micrographs, which all supported its identification as *M.racemosus*.

C.tropicalis colonised both *P.patula* and *E.grandis*. This fungus is also dimorphic as depicted in Fig. 2.14. On closer examination of the mycelium, it was noted that this was pseudomycelium as depicted in Fig. 2.15 and chlamydospores were visible as shown in Fig. 2.15b. The presence of blastospores were also evident as depicted in Fig. 2.16.

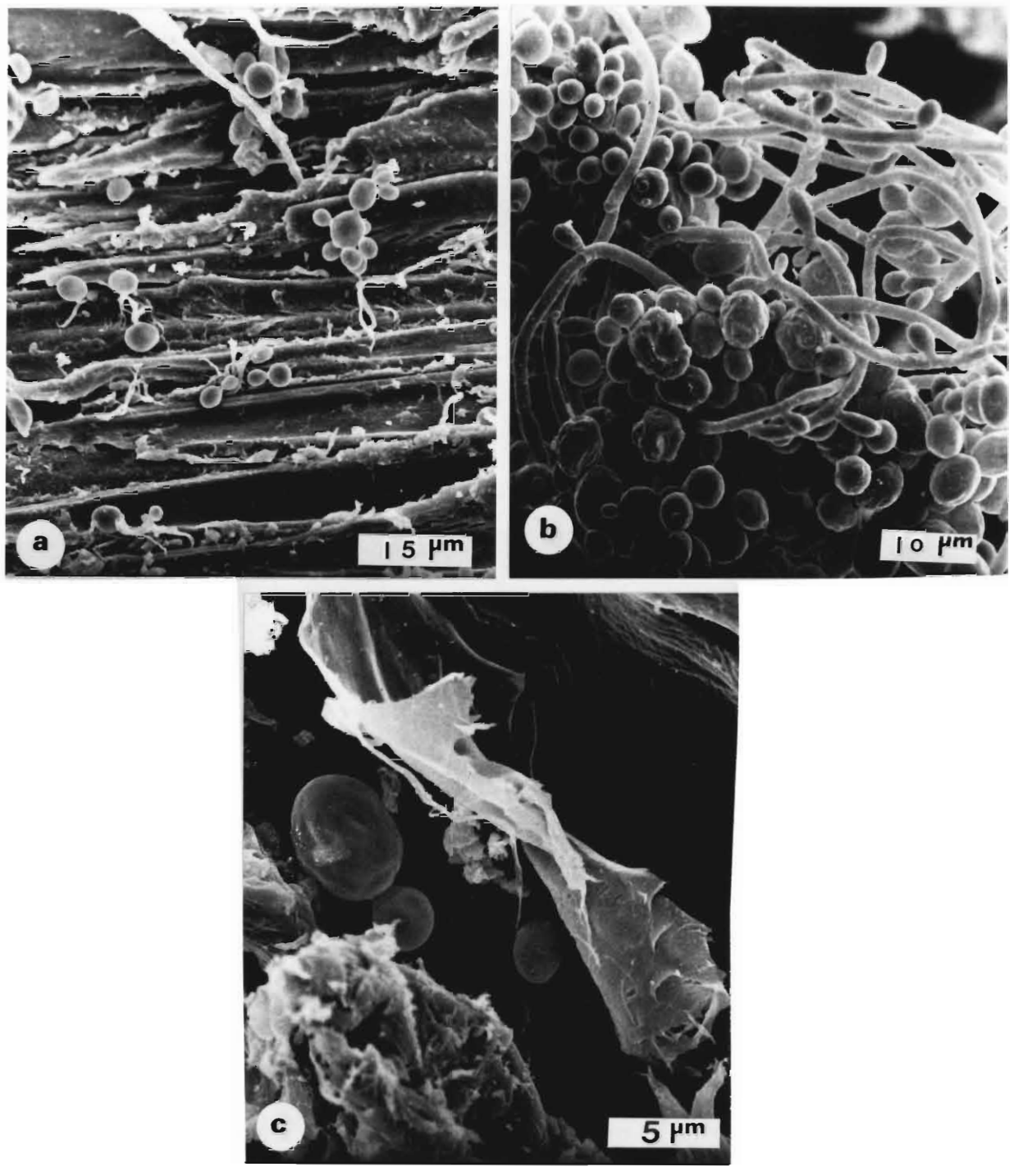
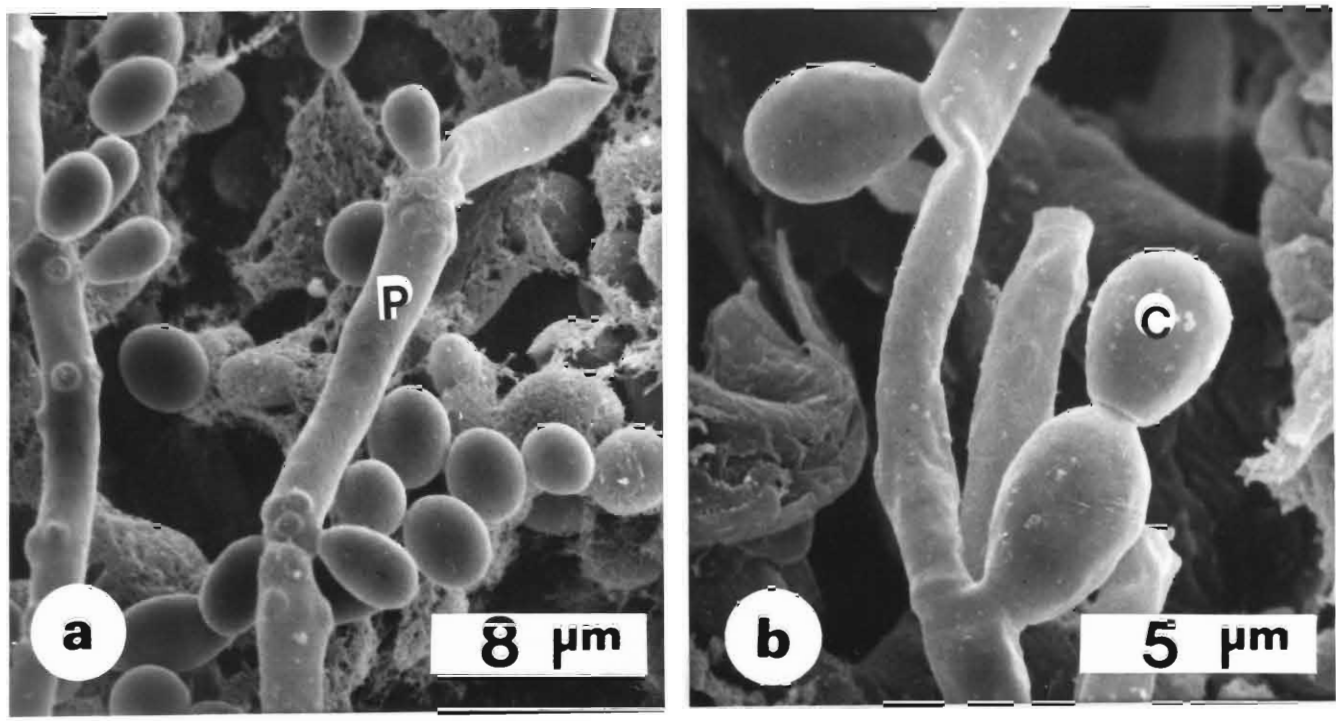


Fig. 2.14 *C.tropicalis* on *E.grandis* showing (a) predominant yeast forms with multiple budding evident, (b) mycelial form together with yeast form and (c) detached yeast form budding.



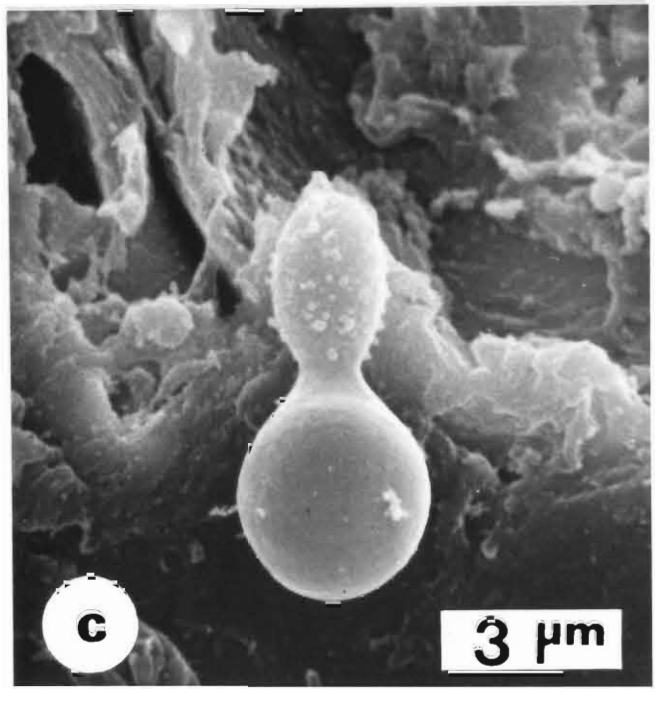


Fig. 2.15 *C.tropicalis* showing (a) pseudomycelium (P) and yeast forms, (b) pseudomycelium and chlamydospores (C) and (c) yeast form giving rise to more pseudomycelium.

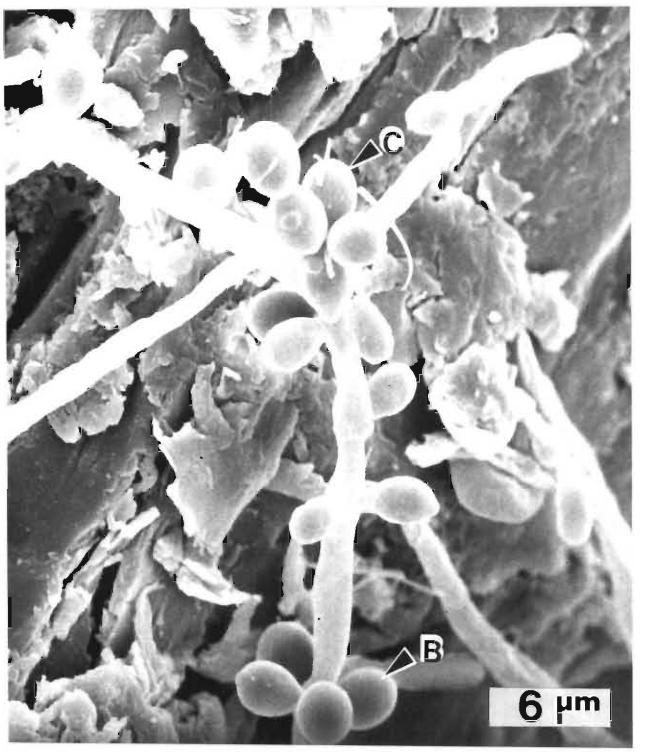


Fig. 2.16 *C.tropicalis* on *E.grandis* showing pseudomycelium, blastospores (B) and chlamydospores (C).

The third facultatively anaerobic fungus, *G.capitatum* also colonised both *E.grandis* and *P.patula*. This fungus was also dimorphic as shown in Fig. 2.17.

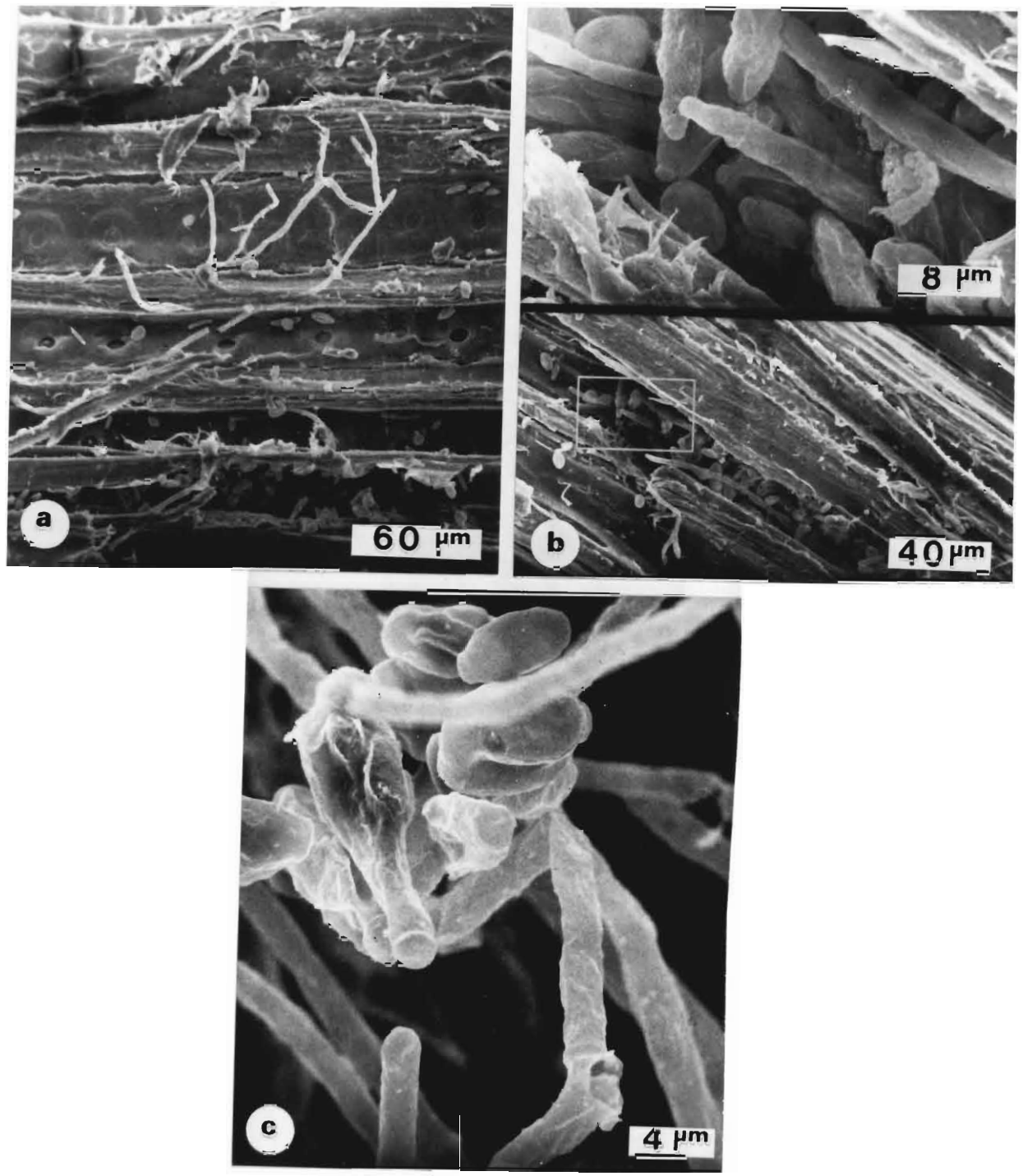


Fig. 2.17 *G.capitatum* colonising *P.patula* showing (a) mycelial form and yeast forms, (b) yeast forms in the fissures of *P.patula* and (c) germinating yeast form.

On closer examination of the mycelium, arthrospore formation is clearly evident as depicted in Fig. 2.18, fully consistent with the identification of this isolate as *G.capitatum*.

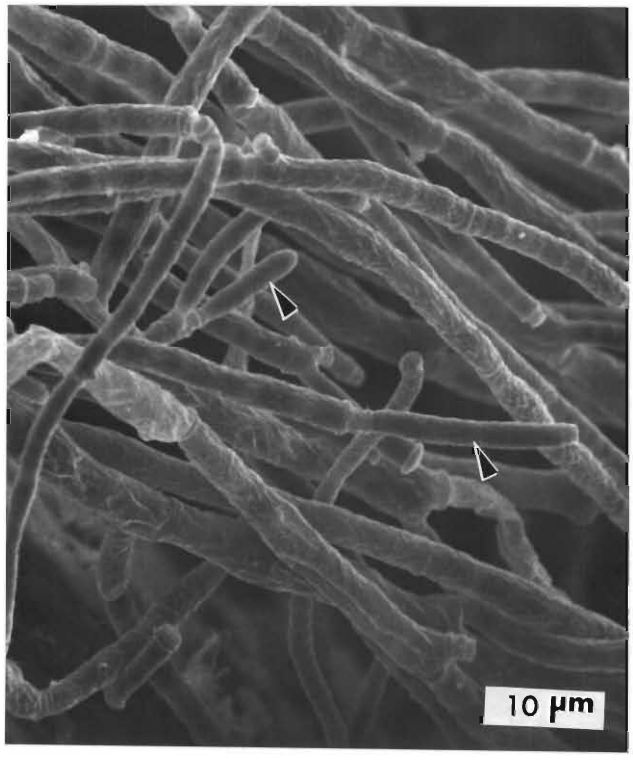


Fig. 2.18 *G.capitatum* on *P.patula* showing the formation of arthrospores.

2.3.1.2 Enzyme activity of ruminal isolates

2.3.1.2.1 *Cellulase*

All three facultatively anaerobic fungi grew on the 0,5% (w/v) Cellulose Agar medium under both aerobic and anaerobic conditions. However no clear zones were ever visible in the agar and it appeared that these fungi did not produce cellulases, even after prolonged incubation for 3 weeks. It was therefore concluded that they were non-cellulolytic, but this conclusion was revised as discussed in Chapter 3.

2.3.1.2.2 *Xylanase*

C.tropicalis and *G.capitatum* grew very well on the 3% Xylan Agar under both aerobic and anaerobic conditions, whereas *M.racemosus* grew very sparsely on this medium. After 2 weeks of incubation, there was no evidence of clear zones in the medium. The plates were incubated for a further 2 weeks, with no clearing visible. Hence it was concluded that these fungi were not xylanolytic, under the test conditions used.

2.3.2 Colonisation tests using *N.frontalis*

The results presented below show that *N.frontalis* had grown and extensively colonised the sisal fibres (2.3.2.1) during transit from Australia. When transferred to broths containing wood (2.2.2.2) the results shown below (2.3.2.2) suggested that *N.frontalis* failed to colonise the wood to the extent shown on the sisal fibres.

2.3.2.1 Sisal fibres

Examination of sisal fibres transferred from MM showed extensive mycelial colonisation of the supplied fibres by *N.frontalis* (Fig. 2.19a,b). Numerous sporangia were present on the sisal fibres (Figs. 2.20 and 2.22). The presence of young sporangia (Fig. 2.20) developing into bulbous (Fig. 2.20a) and extended forms (Fig. 2.21b) and finally to larger (size) mature sporangia (Fig. 2.22b,c) indicated clearly that *N.frontalis* had completed its life cycle on the sisal fibres. The mature sporangia were well distributed on sisal fibres as depicted in Fig. 2.23.

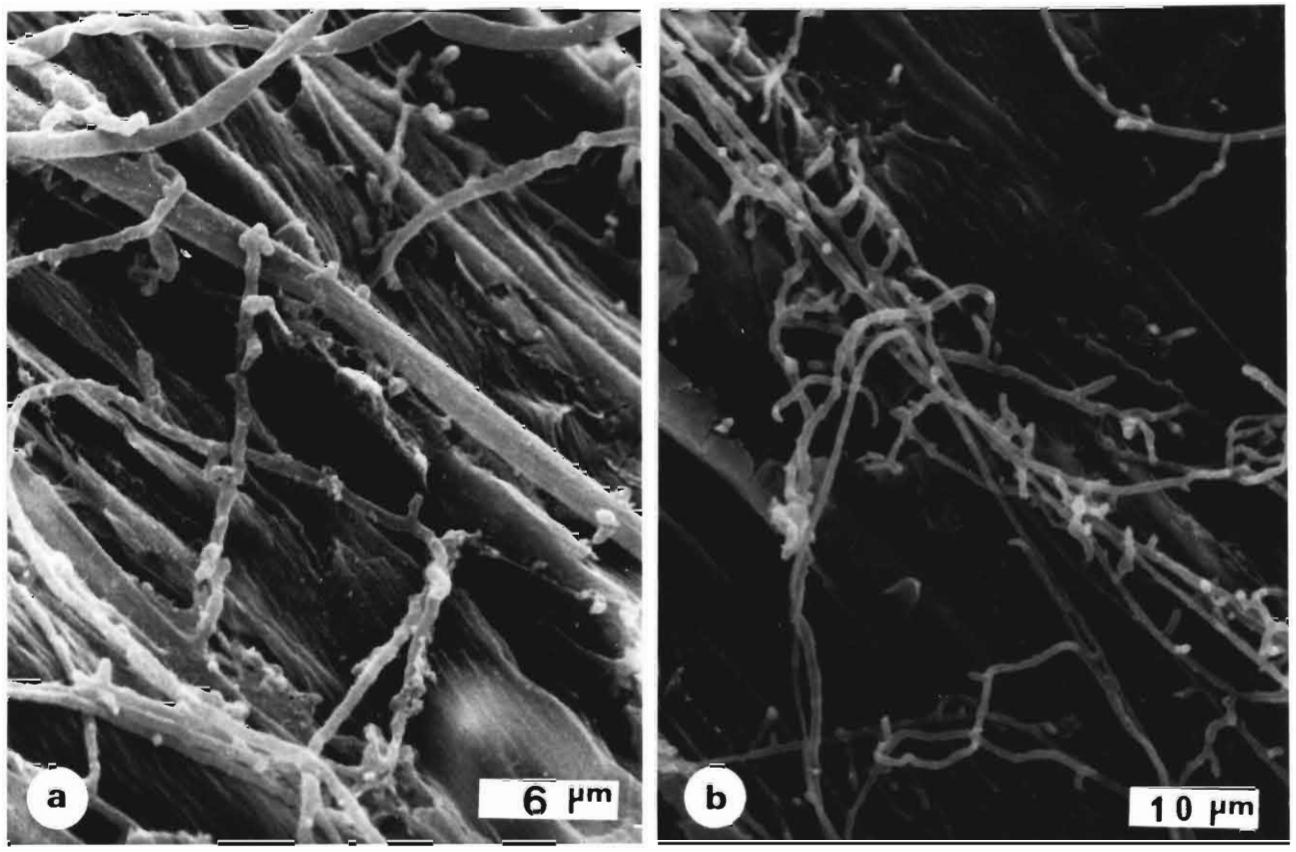


Fig. 2.19 *N.frontalis* mycelium on sisal fibres (a,b).

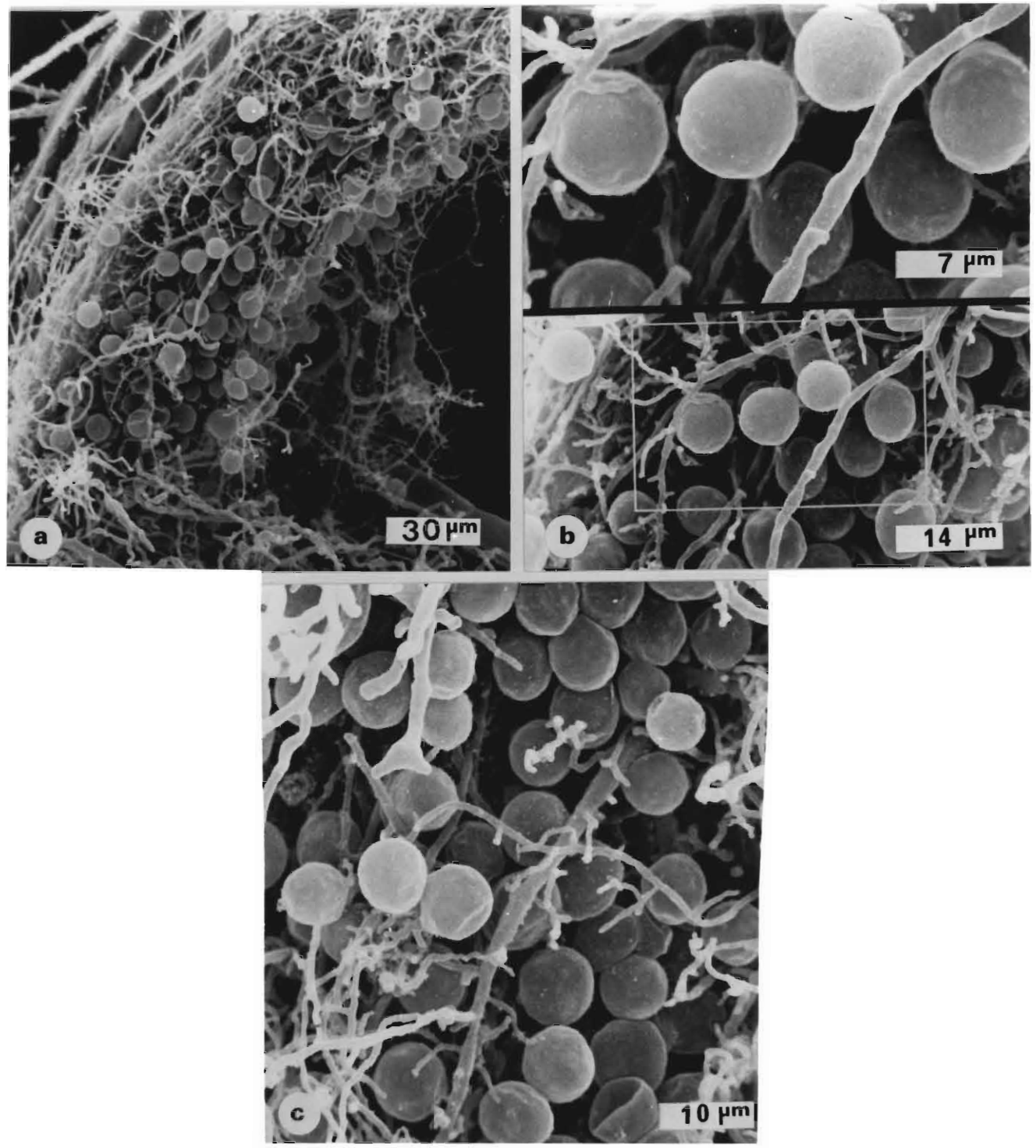


Fig. 2.20 Numerous young *N.frontalis* sporangia (a). These were spherical (b) and approximately 8um in diameter (c).

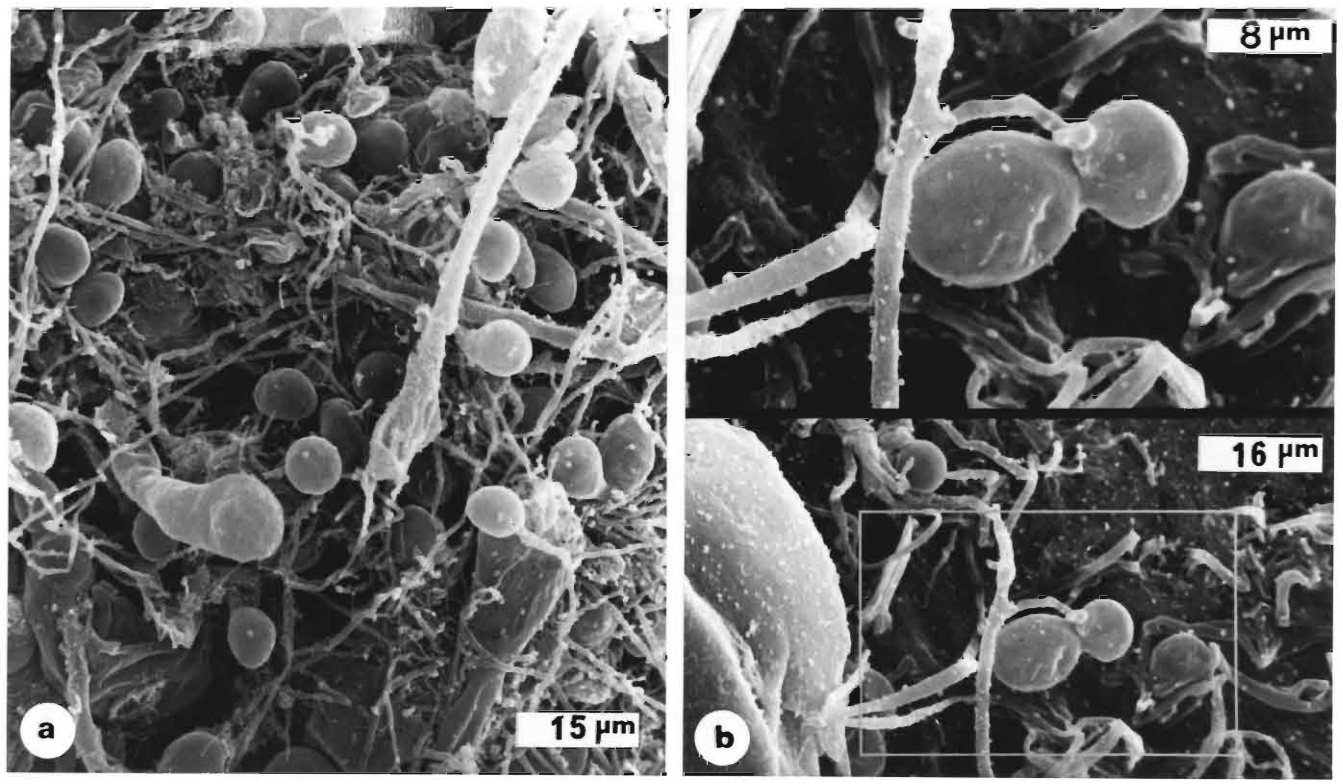


Fig. 2.21 Spherical young *N.frontalis* sporangia developing into bulbous (a) and also extended forms (b).

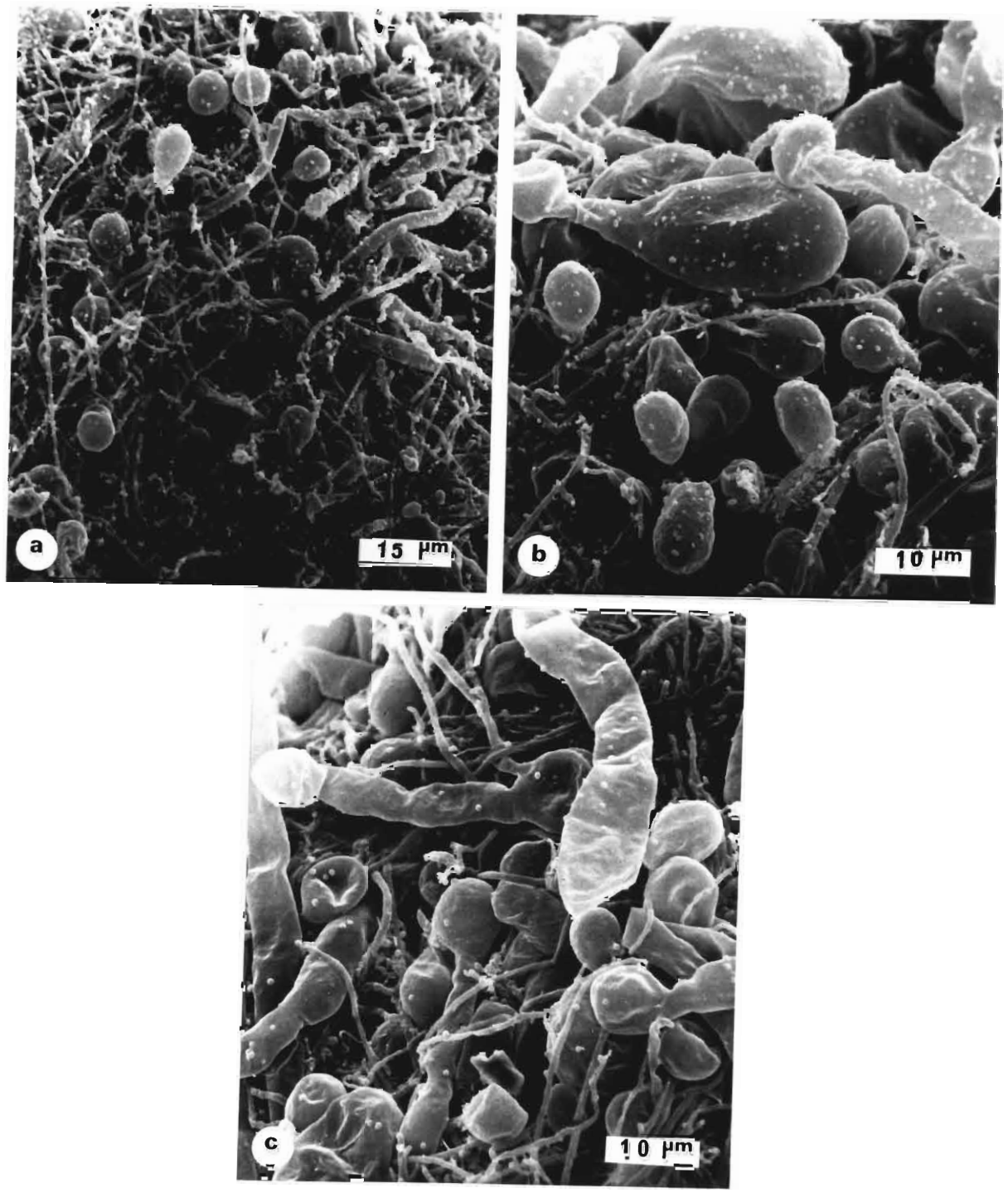


Fig. 2.22 Young *N.frontalis* sporangia (a) giving rise to larger, mature sporangia (b and c).

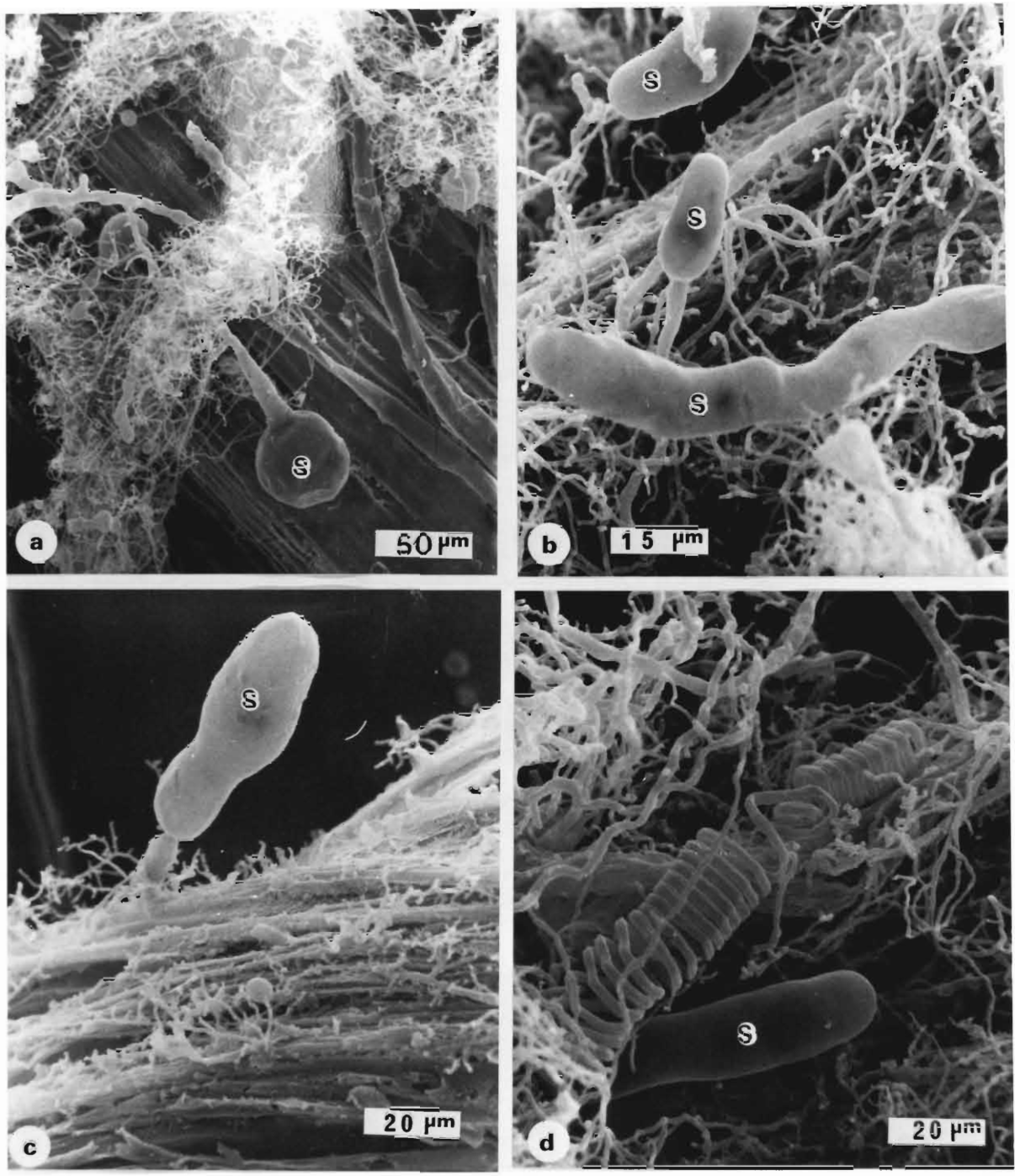


Fig. 2.23 Mature *N.frontalis* sporangia (S) well distributed on sisal fibres (a, b, c). Note the detached lignin which serves as a spiral thickening in sisal fibres (d).

The extensive mycelial colonisation of the sisal fibres and the presence of numerous young and well developed mature sporangia, showed clearly that *N.frontalis* had grown on the sisal fibres, and the fungus must have utilised the fibres as sole source of fermentable carbon in order to produce reproductive structures in completion of its life cycle.

2.3.2.2 Wood

In contrast to the above electronmicrographs showing extensive *N.frontalis* colonisation of supplied sisal fibres, wood samples inoculated from the supplied cultures never became extensively colonised. Fig. 2.24 presents the only evidence obtained showing mycelia on such wood. Such mycelia occurred only very sparsely on *E.grandis* and were not observed on *P.patula*.

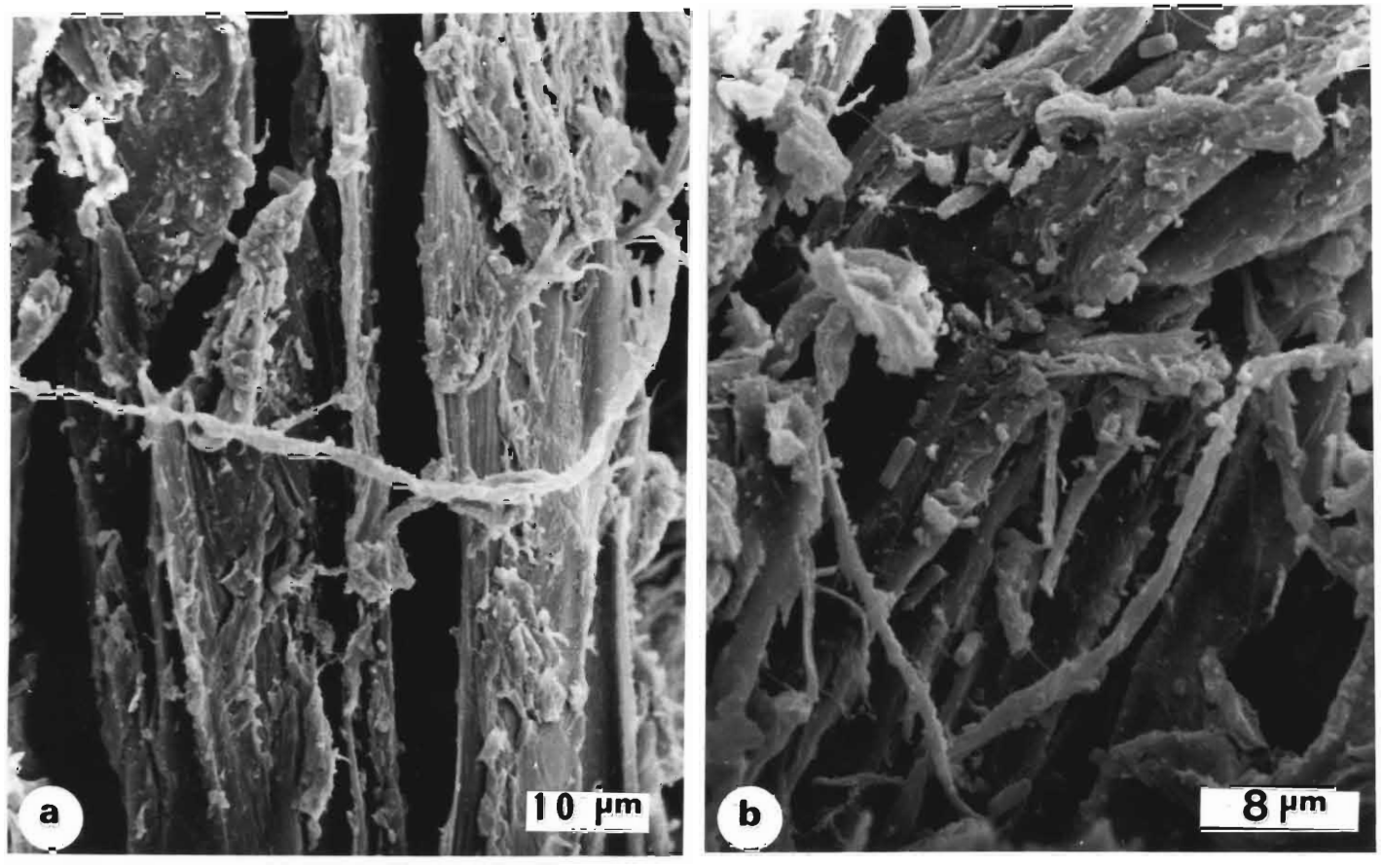


Fig. 2.24 Sparse mycelium on *E.grandis* (a, b) inoculated with *N.frontalis*.

2.3.3 Colonisation tests using basidiomycetes

The classification and characteristic features of the basidiomycetes used here are discussed by Webster (1978). In summary, the cultures were all representatives of the Class Hymenomycetes (Ainsworth, 1966). *Lentinus* falls within the Family Agaricaceae of the Order Agaricales, whereas *Coriolus* and *Coniophora* respectively fall within the Families Polyporaceae and Coniophoraceae of the Order Aphyllophorales. *Phanaerochaete* also falls within the Order Polyporales.

The points are summarised as:-

CLASS	**HYMENOMYCETES**			
ORDER	**Agaricales**		**Aphyllophorales**	
FAMILY	**Agaricaceae**	**Corticiaceae**	**Polyporaceae**	**Coniophoracea**
GENUS	*Lentinus*	*Phanaerochaete*	*Coriolus*	*Coniophora*
	monomitic		mono, di or trimitic	

Distinguishing features of fungi which this author considers to be of value when examining micrographs of fungi in wood include the nature of the hyphae. Agaricales produced only thin walled hyphae which become distended. Such construction is termed monomitic. In contrast, many Aphyllophorales are often more complex, being composed of thin walled generative hyphae, which may be accompanied by either thick - walled unbranched skeletal hyphae, or by thick - walled much - branched binding hyphae, or both. Such construction may thus be monomitic, dimitic or trimitic (Webster, 1978).

The basidiomycetes in the present work each produced mycelial mats on agar surfaces. The mycelium overgrew the wood particles and electron microscopy showed that the wood had been extensively colonised by each fungus. Initial mycelial development appeared to proceed at a faster rate under aerobic conditions, however there was no doubt that wood challenged under anaerobic conditions was also colonised. These findings are presented below.

2.3.3.1 Aerobic conditions

Scanning electron micrographs of wood colonised by the brown rotters *Coniophora puteana*, *Lentinus lepideus*, and by the white rotters *Coriolus versicolor* and *Phanaerochaete chrysosporium* are presented in Figs. 2.25, 2.26, 2.27 and 2.28 respectively.

The brown rotter *Coniophora puteana* produced mainly generative hyphae on agar (Fig. 2.25a) and in wood (Fig. 2.25b), although skeletal or binding hyphae were observed very occasionally (Fig. 2.25c) showing that this fungus could have been di - or trimitic (Webster, 1978). Although the wood samples were extensively colonised, clamps were never observed and basidia were rarely observed on this substrate (Fig. 2.25d). However, elongated, segmented epibasidia were observed (Fig. 2.25e) and these must have arisen from basidia concealed within the mycelial mat. Basidiospores of approximately 3um in diameter were produced on the epibasidia (Fig. 2.25f).

The white rotter *Coriolus versicolor* is, like *C.puteana* above, also a member of the Order Aphyllophorales and is therefore expected to be trimitic. In contrast to *C.puteana*, *C.versicolor* produced much binding, skeletal and generative hyphae on agar (Fig. 2.26a). In particular binding hyphae were produced much more frequently (Fig. 2.26b) than was the case with *C.puteana*. *C.versicolor* also displayed its undoubted trimitic construction in wood (Fig. 2.26c), and also unlike *C.puteana*, clamp connections were also produced by this fungus in wood (Fig. 2.26c,d), albeit occasionally. Similarly basidia were only occasionally produced in wood by *C.versicolor* (Fig. 2.24e), but in contrast to *C.puteana*, epibasidia were never observed. Basidiospores were rarely seen (Fig. 2.26f).

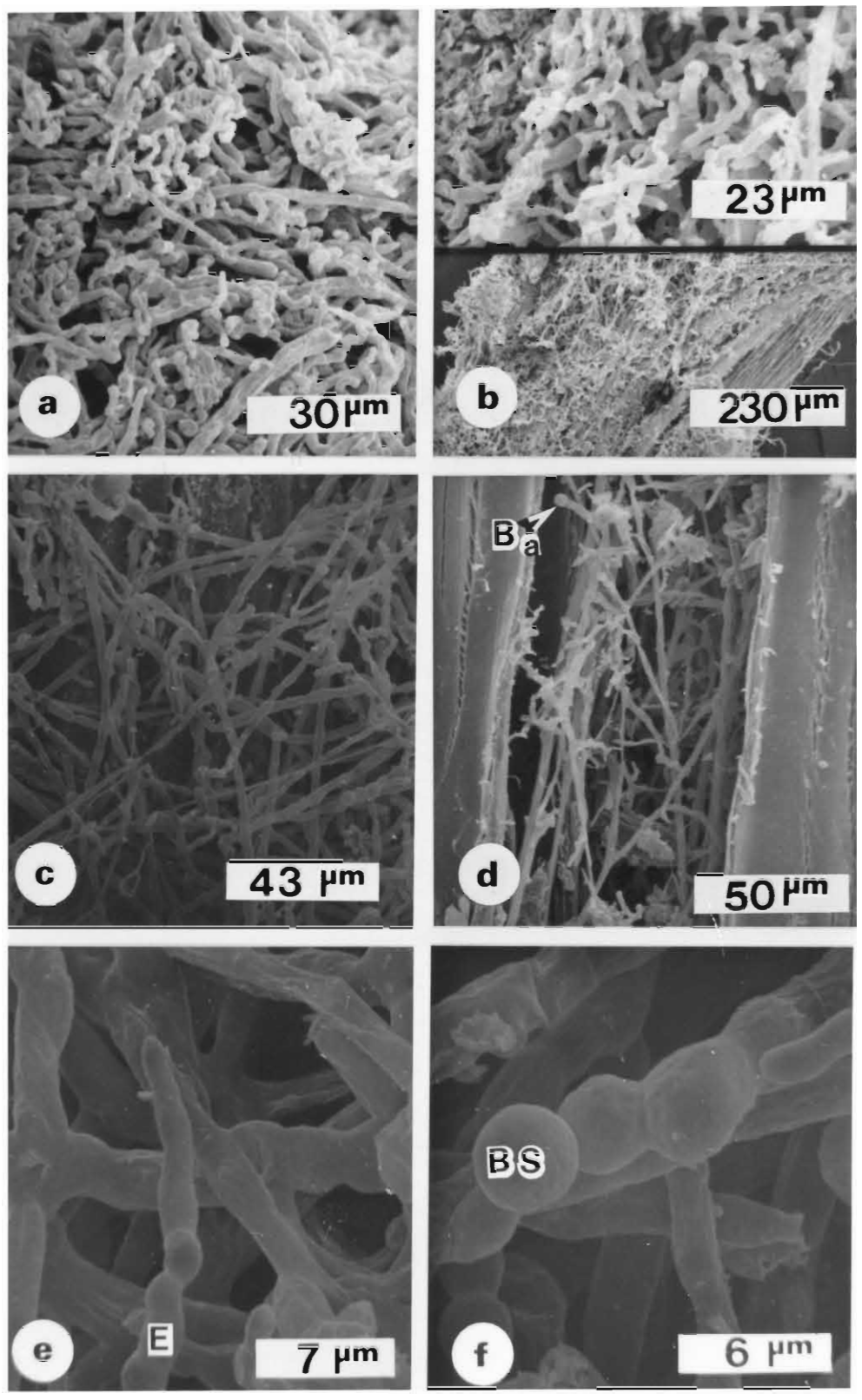


Fig. 2.25 Generative hyphae of *C.puteana* on Malt Extract Agar (a) and on (b) *P.patula*. Binding hyphae (c) were produced occasionally on *E.grandis* and (d) extensive colonisation of *P.patula* occurred, showing mycelium within wood and occasional basidia (B). Elongated segmented epibasidia (e) and (f) basidiospores (BS) were also produced, the latter on the segmented epibasidia.

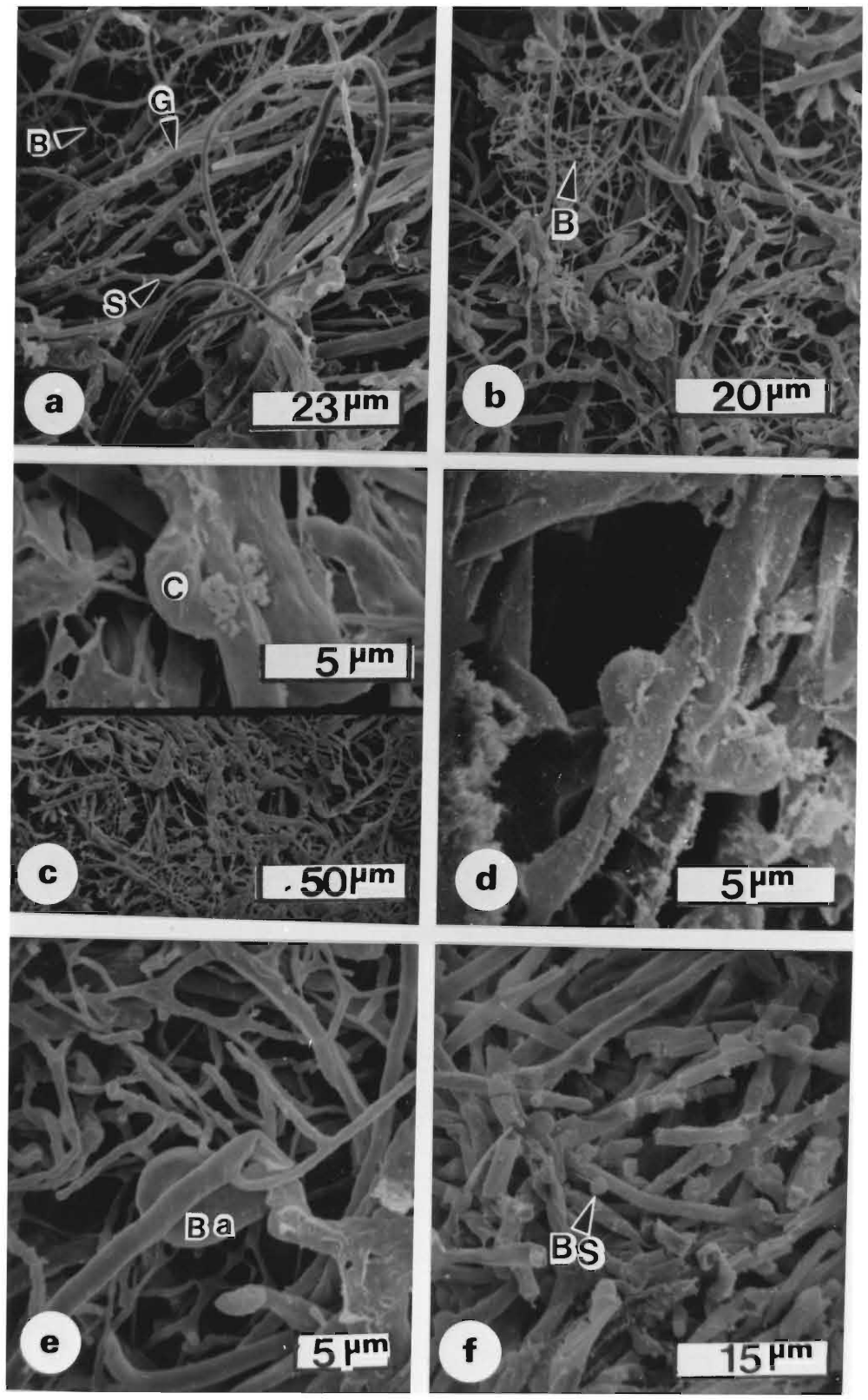


Fig. 2.26 Binding (B), skeletal (S) and generative (G) hyphae of *C.versicolor* on Malt Extract Agar (a). Frequent occurrence of binding hyphae (b) and trimitic hyphal construction with clamp connections were recorded on *P.patula* (c) and *E.grandis* (d). Occasional basidia (Ba) were produced on *P.patula* (e) and similarly, basidiospores were observed rarely (f).

Unlike the two fungi above, the brown rotter *Lentinus lepideus* falls within the order Agaricales whose representatives are all monomitic. In keeping with this definition, *L.lepideus* was observed to produce only generative hyphae in the present work, both in wood and on agar (Fig. 2.27a). In comparison with *C.versicolor,* clamps were more frequently produced (Fig. 2.27b) and basidia were also observed on agar (Fig. 2.27c). Wood was extensively colonised by *L.lepideus* (Fig. 2.27d) and the mycelium produced basidia on the wood surfaces (Fig. 2.27e). Basidiospores were also produced by *L.lepideus* on the wood (Fig. 2.27f).

Phanaerochaete chrysosporium produced few binding hyphae on agar (Fig. 2.28a) or in wood (Fig. 2.28b). Since no skeletal hyphae were observed it was felt that this basidiomycete was dimitic and most of the mycelium produced on these substrates comprised generative hyphae bearing basidia (Fig. 2.28c). Clamps were never observed but in contrast to the other fungi examined, the basidia produced by *P.chrysosporium* on wood were numerous (Fig. 2.28d). Although *P.chrysosporium* produced few binding hyphae (Fig. 2.28e) when compared with those produced by *C.versicolor*, the basidiospores produced by *P.chrysosporium* were numerous (Fig. 2.28f) whereas basidiospores produced by *C.versicolor* were rarely observed (Fig. 2.26f).

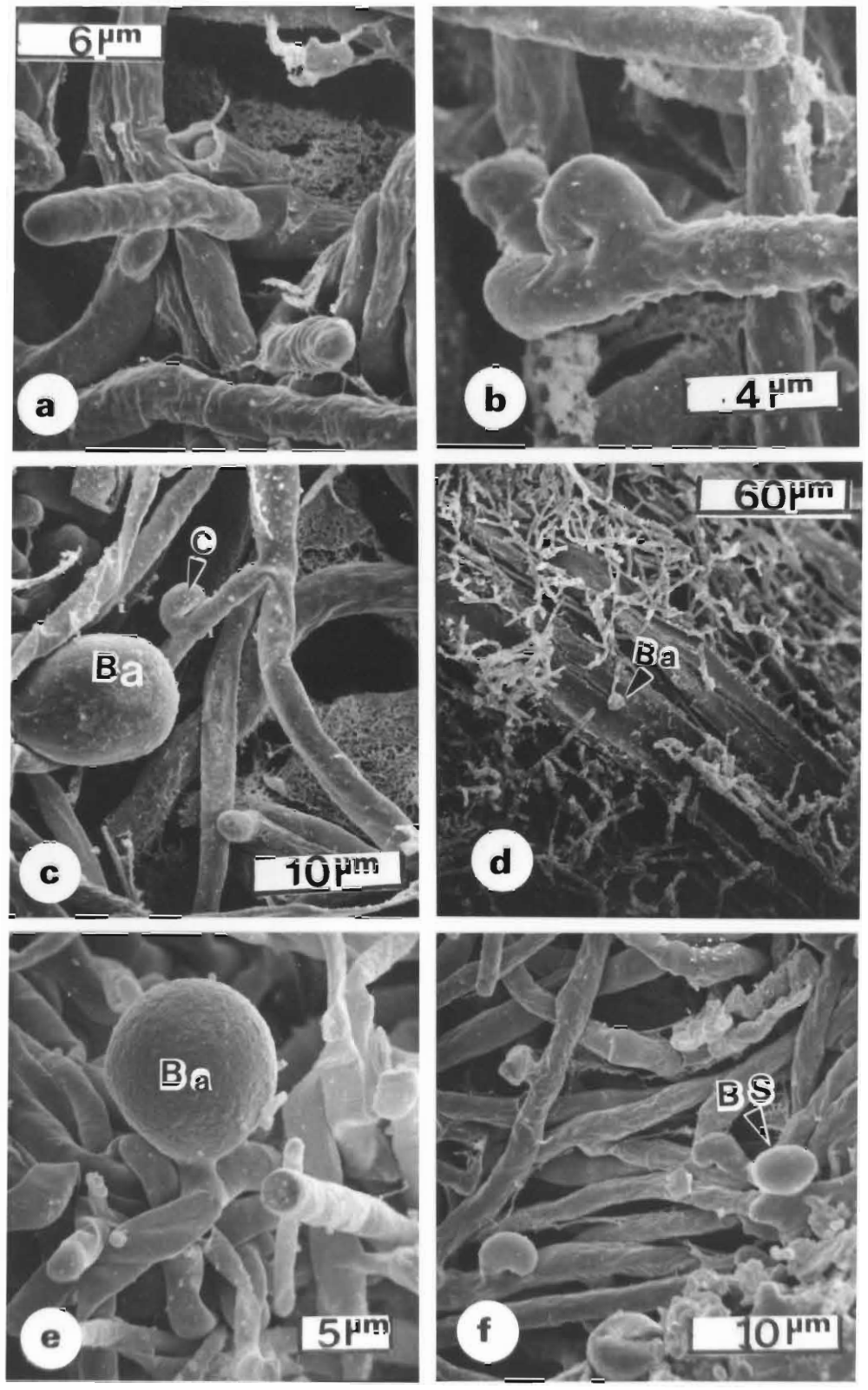


Fig. 2.27 *L.lepideus* on Malt Extract Agar showing generative hyphae (a), frequent production of clamp connections (b), and (c) a basidium. *P.patula* was extensively colonised (d), and basidia were produced on wood surfaces (d and e). Basidiospores observed on *E.grandis* (f).

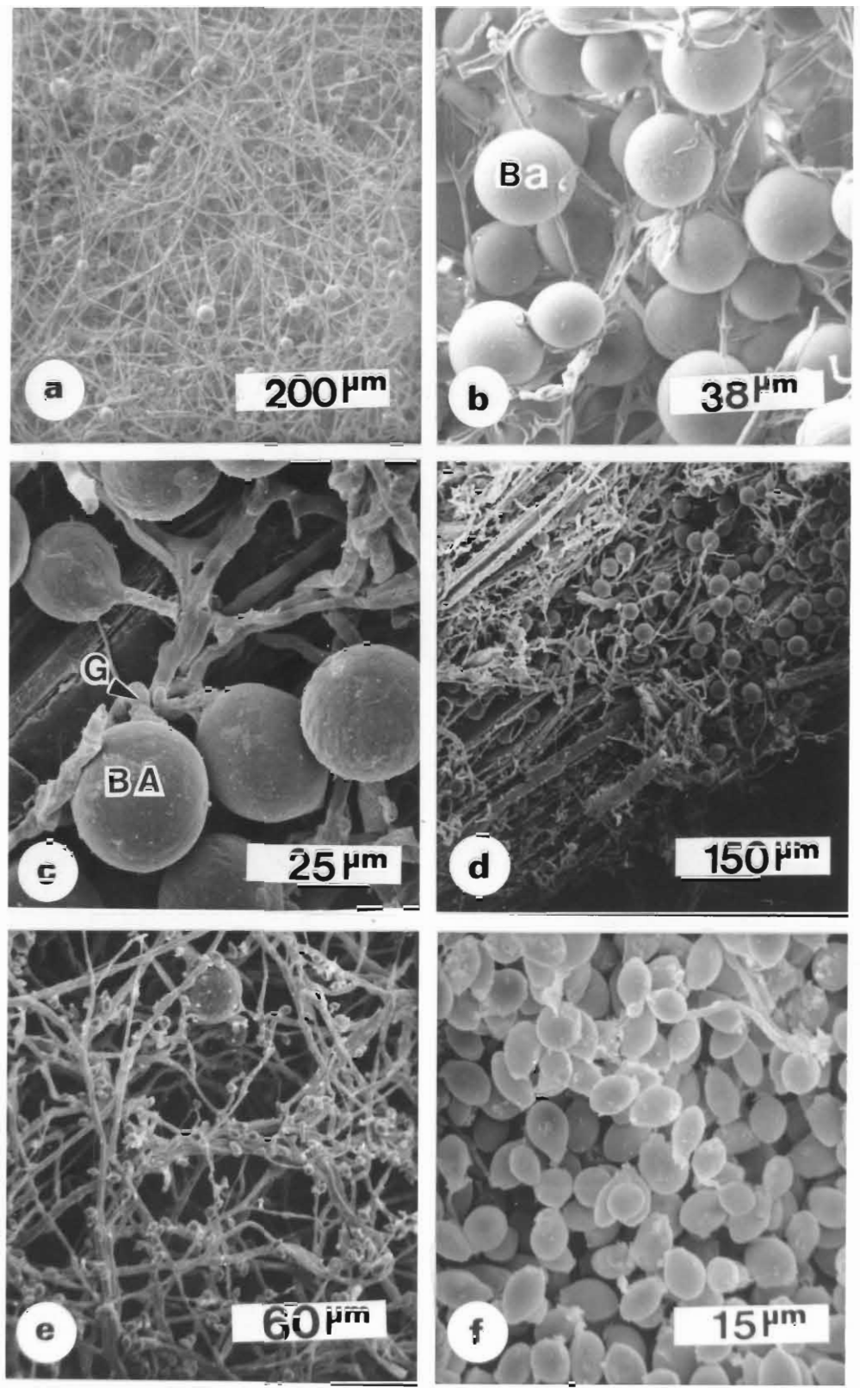


Fig. 2.28 Numerous *P.chrysosoporium* basidia produced on Malt Extract Agar (a) and on *P.patula* (b). Generative hyphae (G) bearing basidia on *P.patula* (c), and (d) extensive colonisation of *E.grandis* showing the presence of numerous basidia. Few binding hyphae were observed (c) and basidiospores (aleuriospores) produced were numerous (f).

On the basis of the above results, it was possible to construct Table 2.1, which summarises the distinguishing features of these basidiomycetes in wood.

Table 2.1 Distinguishing morphological characteristics of fungi observed in wood.

CHARACTER	*Coniophora puteana*	*Coriolus versicolor*	*Lentinus lepideus*	*Phanaerochaete chrysosporium*
mycelium	di-/trimitic	trimitic	monomitic	dimitic
skeletal hyphae	+/-	+	-	-
binding hyphae	+	+	-	+
generative hyphae	+	+	+	+
reproductive structures	MINIMAL			WIDESPREAD
clamp connections	-	occasional	frequent	-
basidia	rare	occasional	+	frequent
epibasidia	+	-	-	-
basidiospores	+	rare	+	frequent
decay patterns	brown	white	brown	white

2.3.3.2 Anaerobic conditions

C.versicolor, *C.puteana*, *L.lepideus* and *P.chrysosporium* were able to grow under anaerobic conditions, however their rate of growth was much slower when compared with that recorded under aerobic conditions. Scanning electron micrographs of wood colonised by these fungi are presented in Fig. 2.29.

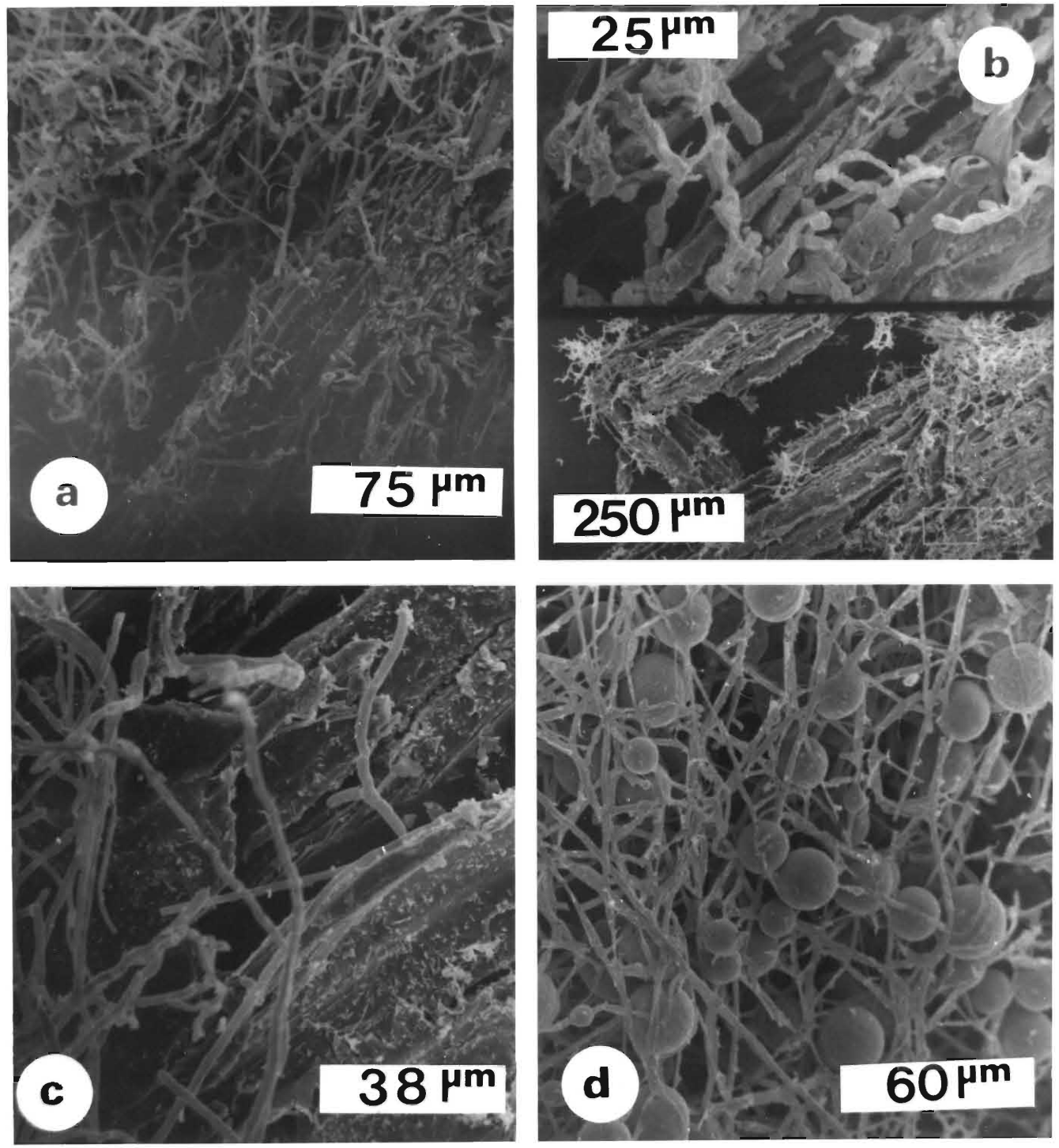


Fig. 2.29 Colonisation of wood elements under anaerobic conditions . *E.grandis* colonised by (a) *C.versicolor* and (b) *C.puteana*. *L.lepideus* (c) and *P.chrysosporium* (d) on *P.patula*.

The comparatively slow rate of growth of these fungi on wood under anaerobic conditions was not entirely unexpected, but neither was the observation of wood colonisation (Fig. 2.29) since these fungi are known to cause wood decay in the structural timbers of inadequately ventilated floors in buildings world wide (Bravery *et al.*, 1987).

It was tempting to attempt to compare the degree of colonisation of wood by each basidiomycete under aerobic (Figs. 2.25; 2.26; 2.27; 2.28) and the present anaerobic (Fig. 2.29) conditions. Clearly, such comparisons would be, at best, qualitative, but it was felt that the final extent of wood colonisation by the basidiomycetes under anaerobic conditions may have approached that which occurred aerobically (Figs. 2.25; 2.26; 2.27; 2.28). Unfortunately, as stated above, it must be borne in mind that SEM could not provide a reliably quantitative assessment of wood colonisation by these fungi.

2.4 DISCUSSION

The roll tube method used to isolate facultatively anaerobic fungi proved to be an efficient method for the isolation of rumen fungi. Using this method, three facultatively anaerobic dimorphic fungi were isolated.

The studies by Orpin (1975; 1976; 1977) showing the occurrence of Chytridiomycete - like fungi in the rumen established that these fungi are obligately anaerobic (Chapter 1). The obligate anaerobe *N.frontalis* (from Australia) used in this study has colonised the sisal fibres extensively with the production of numerous young as well as mature sporangia. However on wood, this fungus colonised wood very sparsely. No reproductive structures were visible and hence it was concluded that this fungus does not degrade wood and the very sparse colonisation of wood did not permit further study of wood degradation using this organism.

The fungi isolated in the present work were not obligately anaerobic, although they were able to grow under strictly anaerobic conditions. They were identified as *M.racemosus*; *C.tropicalis*; and *G.capitatum*. *M.racemosus* is a Zygomycete while *C.tropicalis* and *G.capitatum* belong to the Fungi Imperfecti. It was necessary to establish whether these fungi featured in lignocellulose degradation.

All three fungi were shown to colonise *P.patula* and *E.grandis* extensively but did not appear to degrade the wood. *M.racemosus* colonised wood to the greatest extent with extensive mycelial network and reproductive structures on the wood, indicating that the fungus completed its life cycle on wood. Previous studies of rumen fungi on wood in liquid culture (Wiederhold *et al.*, 1989) showed that colonisation did not occur as extensively as that observed when solid medium was used. The static environment in the present work may have favoured colonisation or alternatively *M.racemosus* may have had affinity for wood. Although distinct decay patterns were not observed in wood during this short term study, the possible delamination of wood elements by *M.racemosus* was directly comparable with the delamination of grass fibre elements by rumen fungi (Morrison and Mackie, 1989). This phenomenon may contribute to the biodeterioration of wood, particularly in conditions of reduced aeration, where many common wood decay fungi could not compete.

Obligately anaerobic fungi have been shown to produce cellulases and xylanases and it has been established that these fungi play an important role in fibre degradation. However the facultatively anaerobic fungi isolated in this study were neither cellulolytic nor were they xylanolytic, therefore their role in the rumen remains unclear. Furthermore under anaerobic conditions, using liquid media, *M.racemosus* grew predominantly in the yeast phase whereas under aerobic conditions, the mycelial form was predominant. Under anaerobic conditions however, the extent of colonisation was not as extensive as that observed

under aerobic conditions. Using scanning electron microscopy, it was possible to observe the different stages in the development of the yeast form. The occurrence of these dimorphic, facultatively anaerobic fungi in the rumen thus raised several important questions, viz.,

i) Does redox potential(Eh) affect dimorphism? In the case of *M.racemosus*, it has been found that redox potential definitely affects dimorphism in that under aerobic conditions this fungus exhibits mycelial growth only, whereas under anaerobic conditions using media where the redox potential has been lowered by the addition of reducing agents, the fungus exists predominantly in the yeast form. On return to aerobic conditions, the yeast form germinates into the mycelial form with multiple germ tubes emerging from the single yeast form. On the other hand *C.tropicalis* and *G.capitatum* display predominantly mycelial growth under anaerobic conditions whereas under aerobic conditions the yeast forms are dominant. The above facts are demonstrable, however the molecular mechanisms which operate at low redox potentials to control dimorphism are unknown.

ii) What role is played by these fungi in an anaerobic environment containing lignocellulose and other microorganisms? The role of obligately anaerobic fungi in the rumen has been partly clarified (Mountford, 1987), but the role of facultatively anaerobic fungi has not yet been established. For example zygomycetes have been isolated from environments with very low levels of dissolved oxygen such as from fermentation starters

(Hesseltine *et al.*, 1985) and from the rumen of sheep and cattle (Brewer *et al.*, 1972; Lund,1974), but the role of these fungi in these environments has not yet been established. It may well be the case that the major role of these fungi in the rumen is in synergistic or antagonistic relationships in cocultures of other anaerobic microorganisms which degrade lignocellulose directly. Future work in rumen microbiology should consider this aspect to determine whether there is synergism or antagonism in, for example, cocultures of anaerobic bacteria and these fungi.

In 1988, Gleason and Gordon worked with five different Zygomycetes and they found that only *Mucor genevensis* and *Benjaminiella poitrasii* were able to grow under obligately anaerobic conditions, however these cultures were not viable beyond the third transfer under anaerobic conditions. The cultures isolated in the present study could be maintained through successive subcultures and hence such work could continue to answer some of the questions raised regarding the role of facultatively anaerobic fungi in the rumen.

C.tropicalis and *G.capitatum* colonised both *P.patula* and *E.grandis* to lesser extents when compared with the colonisation patterns of *M.racemosus*. Although these fungi colonised wood extensively, there was no evidence of degradation. The incubation period (7 days) could have been too short and hence longer term studies may reveal evidence of wood degradation.

However, the three fungi used here appeared to be neither cellulolytic nor xylanolytic and it was decided that they were suitable for use as reference cultures of negative controls in further work (Chapter 3) to quantify the extent of wood colonisation by non-decay fungi and basidiomycetes during the early stages of such colonisation.

Fungal decay patterns produced in wood can be categorised mainly as white or brown rot (Chapter 1), but microscopical identification of the causal basidiomycetes relies upon characterisation of their reproductive structures. The four basidiomycetes used in this study, produced abundant mycelia and colonised wood extensively under aerobic conditions. *C.versicolor* produced much binding, skeletal and generative hyphae on agar and in wood and this fungus exhibited its undoubted trimitic hyphal construction on agar and in wood when compared with the other decay fungi used. *C.puteana*, *L.lepideus* and *P. chrysosporium* produced mainly generative hyphae on agar and in wood and the present study has shown that only *P.chrysosporium* produced numerous basidia in wood. These findings established that in spite of the information available in Table 2.1, it would be possible to microscopically identify only *P.chrysosporium* with certainty when present in mixed populations of the present fungi in decayed wood.

2.5 CONCLUSIONS

This Chapter has shown conclusively that extensive colonisation of wood by fungi can be assessed by scanning electron microscopy. It was further concluded that :

1. the obligate anaerobe *N.frontalis* did not appear to degrade wood under the conditions used in the present work, and,

2. the facultative anaerobes from the rumen of sheep did not appear to degrade wood in these short term tests but were shown to colonise wood quickly and hence could be used as reference cultures or negative controls, and,

3. the basidiomycetes used in this study which are known as active wood degraders, and scanning electron microscopic observations showed that these basidiomycetes colonised wood extensively and hence could be used as positive controls in subsequent studies.

Therefore on the basis of the results reported in this Chapter, it was decided to use analytical techniques to quantify the degradation of wood using those fungi that were able to colonise wood most extensively.

Under conditions of reduced aeration, the extent of wood colonisation by the four basidiomycetes was not as extensive as that observed under aerobic conditions. It was therefore decided to pursue the subsequent studies of the quantification of wood degradation under aerobic conditions, as described in Chapter 3.

CHAPTER 3: ANALYTICAL QUANTIFICATION OF FUNGAL DECAY IN WOOD

3.1 INTRODUCTION

Having concluded (Chapter 2) that *M.racemosus, C.tropicalis, G.capitatum, L.lepideus, C.puteana, P.chrysosporium* and *C.versicolor* all colonised the wood extensively, and that *M.racemosus, C.tropicalis* and *G.capitatum* did not produce visible (SEM) decay, whereas *L.lepideus, C.puteana, P.chrysosporium* and *C.versicolor* are known to do so, the establishment of the collection of cultures to examine the second objective was complete. The second objective (1.3.2) designed to establish correlations between colonisation and degradation, will be specified below (3.1.2), after the following review of methods available to quantify wood decay.

3.1.1 Review of decay test methods

3.1.1.1 Gravimetric methods

Traditionally, wood decay has been directly quantified using gravimetric determinations to measure weight losses produced in wood by decay agents. Wood blocks of a uniform size are used for most experiments and the assessment of attack is done by weighing the wood blocks before and after colonisation by fungi so that the loss of wood substance can be determined. The final dry weight subtracted from the initial dry weight gives the loss in

dry weight due to the fungal attack - this loss when calculated as a percentage of the original dry weight gives a useful measure of the amount of decay which has occurred under the standard conditions used.

This may be regarded as a special case of microbial growth in a closed environment. All the main nutrients are present in the form of water insoluable structural wood components i.e., cellulose and hemicellulose embedded in lignin. The latter substance is not considered to be utilisable as a nutrient, and on the contrary lignin will in most cases reduce the rate of polysaccharide consumption. It is not likely that the total amounts of cellulose and hemicellulose have any significant influence on the decay rate of a decay organism, at least not until any of the components become limiting in the later stages of decay. Thus it is rather the proportions of polysaccharides and lignins, the presence of extractives or other decay inhibiting substances and the anatomical structure that will influence the decay rate.

It is not problematical to convert weight loss figures to percent weight loss if wood blocks are of uniform size and density. Problems arise when comparisons are required for wood blocks of the same size but varying in density (differences in wood structure and extractives). Here it is obvious that the original dry weight of the wood block is determined by the density of the timber i.e., wood blocks from a high density timber weigh more than wood blocks from low density timbers (Nilsson and Daniel,

1992). Other drawbacks associated with gravimetric methods include the lack of sensitivity, which produces large degrees of error. Furthermore, in the present work, it was felt that another disadvantage of gravimetric determinations was their inability to provide any information specifying those fractions of the lignocellulosic complex which may be selectively degraded by certain microorganisms. A further disadvantage associated with decay evaluations based solely on gravimetric determinations is that nil weight losses imply that no decay occurred, whereas the colonised wood may well contain microbial biomass in quantities greater than the mass of wood components which had been degraded, particularly in incipient decay.

However, gravimetric determination is a standard method and, for reference purposes, should be included in any work designed to investigate alternative means of assessing wood decay.

3.1.1.2 Mechanical methods

Presler Increment Borer. This instrument is essentially an auger used to extract cores of wood from timber, and is used primarily to verify the penetration of preservatives in wood after treatment. It is however a destructive test method not considered suitable for use here.

The Shigometer. This instrument was developed as a mechanical means of quantifying the extent of decay in timber, eg., poles, in service. The electrical resistance of decayed wood is lower than that of sound wood and it is this that is measured by the Shigometer. The need for such an instrument arose because field stake tests traditionally relied on visual assessment of the condition of each stake. Depending on the extent of visible decay, stakes are allocated to one of five categories which numerically represent the percentage soundness, or residual strength, of each stake. Studies at the Forest Research Institute (Rotorua, New Zealand) have demonstrated that stakes treated with some preservative formulations, particularly those based on alkylammonium compounds, cannot always be readily assigned to the correct category on visual assessment alone (Leightley, 1981). Because of visibly extensive surface decay some stakes may be placed in the category representing 70% soundness, but subsequent dissection of such stakes has shown that decay may extend no more than 10 - 15 cells into the stake, corresponding to an estimated residual soundness greater than 95%. Although such a pessimistic visual assessment would have no effect on the ultimate time to failure of any stake, it can be grossly misleading in developmental work in which early appraisal of actual field performance is required. Consequently, a quantitative measure of stake decay, which eliminates the need for subjective assessment, is considered necessary (Hedley and Naish, 1980).

In a study by Leightley (1981), sixty poles of various eucalypt were inspected in North Queensland, for the presence of soft - rot decay using the Shigometer and Pilodyn (below). Examination of 30 poles for internal decay was made using the Shigometer. However, in 10 poles the Shigometer indicated that no or little decay was present, but microscopical examination revealed soft - rot decay. In some cases the decay was quite severe. In comparison the Shigometer indicated decay in four poles, but no soft rot could be found.

The low Shigometer readings were indicative of the presence of soft - rot. However, the depth of soft - rot ranged from 10 - 30 mm and was quite obvious on the surface of the pole. In the eucalypt soft - rot decay situation, the Shigometer seemed to offer little practical value, since a comparable visual examination could reveal the presence and extent of decay.

The Pilodyn. This instrument was originally developed to estimate the degree of soft rot in wooden poles by giving a quantitative measure of the extent or depth of decay in field test stakes. A sharp pin on the Pilodyn is driven into the wood at a given force, and the depth of penetration is proportional to the extent of decay in that area of wood. The instrument was used successfully in New Zealand to measure the wood density of standing *Pinus radiata* trees, although a disadvantage to its use was the relative insensitivity of the recording scale (Cown, 1978). In this work, a 6 - joule Pilodyn with a 2.5 diameter pin was used. The depth of pin penetration into the wood was over

the range 12.7 mm (basic density, 520 kg/m^3) to 20.6 mm (basic density, 320 kg/m^3). In a study by Hedley and Naish (1980), a 2-joule Pilodyn with 2.0 mm diameter pin was used in an attempt to limit depth of pin penetration (to 10 - 15 mm) without reducing scale insensitivity. The results demonstrated that by using the Pilodyn, it may be possible to quantify strength loss of test stakes caused by decay, thereby eliminating observer bias in visual assessments. Leightley (1981; 1982) examined a 12 - joule Pilodyn for performance on CCA treated eucalypt poles. Similarly, Hedley (1982) examined the limitations of the Pilodyn, and these authors found that before use of the Pilodyne could become a routine feature of stake inspections, further work is required - particularly in optimising Pilodyn spring strength and pin diameter, frequency of assessment, and loci on stakes where readings may be taken most advantageously. For example, a notable feature of the results obtained is the very close relationship between stake moisture content and Pilodyn readings at all three regions (60, 250 and 450mm from one end of each stake) from which data were collected. The influence of moisture content on Pilodyn pin penetration appeared to overide any effect decay might have had on readings. From week 8 to week 48, during which stake condition was reduced from 100% to 68% soundness, Pilodyn readings at groundline continued to rise and fall very similar to fluctuations in moisture content.

Vibrante wood pole analyser. North American Utilities own approximately 100 million wood poles representing an investment of more than R 150 billion at current exchange rates. Every year

up to 2 million poles are replaced, despite modern preservation treatments. Excessive decay - usually internal or below groundline rot - is the culprit causing the in - service life of a pole to range from 4 to 80 years. Assessing the condition of an individual pole at any given time presents a problem.

A structure's vibration properties are affected by structural defects. The principle of using Resonant Frequency Analysis as a measure of structural integrity has been applied in several industries, including inspection of oil rigs and nuclear power plant components.

The Vibrante wood pole analyser (Vibrante Technologies, Delta, Canada) incorporates this technique and applies the pole's natural resonant frequencies to a computer model. This computer model is used to calculate absolute stiffness (load vs deflection) since the way a pole vibrates depends on its stiffness. The Vibrante technique then compares absolute stiffness (how stiff the pole is) with how stiff it would be if it were in good condition. The result called relative stiffness, is a percentage of how much the pole has degraded. Expressed as a percentage, relative stiffness can be used to develop a Wood Pole Degradation Scale.

Through extensive testing (Vibrante Technologies summary of internal reports, 1983 -7) involving more than 1000 poles, it was determined that any pole with relative stiffness of less than 60% falls into the fail category. Those poles with a relative

stiffness rating of greater than 80% pass and are in "like new" condition. The "suspect range" lies between 60% and 80%. These poles may be monitored more closely.

This wood pole degradation scale was used in a field test to evaluate fully configured poles owned by Alcan Smelters in Canada. The test conducted by an independent contractor, indicated that 75% of the poles met the pass criterion, 4% failed and 21% fell into the suspect category. The 4% which fell into the fail category of the degradation scale seemed fine and in rather exceptional condition for the age of the poles. However, additional below - ground inspection of the failed poles revealed that over 40% of the poles had rotted away.

The limitations of the wood pole analyser include:

i) will only work with poles,

ii) does not indicate the type of rot or defect in a pole,

iii) incremental boring (holes made by pin of wood pole analyser at different regions on the pole) could introduce decay in the pole, and,

iv) provides no information as to which components of the lignocellulosic matrix are degraded especially in the case of brown rot or white rot.

On the basis of the above limitations, it was decided not to use mechanical methods to assess wood decay in the present work.

3.1.1.3 Analytical Methods

It was felt that analytical methods would provide the sensitivity desired in the present work to investigate incipient decay. Fundamental chemical research concerning wood and wood derived - products depends on a knowledge of the material's nitrogen, lignin and carbohydrate composition.

Wood Nitrogen and Biomass Determinations. The Kjeldahl method has been widely used for estimation of protein in cereals and the protein can be estimated from the nitrogen value (Nkonge and Ballance, 1982). The method has also been used to measure nitrogen in wood (King, Henderson and Murphy, 1980).

The classical Kjeldahl procedure involves two steps :

i) digestion of the sample in concentrated acid, and

ii) distillation and titration of the liberated nitrogen as ammonia.

The second step requires special equipment for distillation, and where large numbers of samples are to be analysed, this step is relatively slow and time consuming thereby limiting the number of samples that can be processed per day. Thus, alternative methods for the estimation of liberated ammonia directly from the digest have been examined. These include spectrophotometric estimation of volatilised ammonia, colorimetric methods and quantitation using an ammonia specific electrode (Nkonge and Ballance, 1982).

A colorimetric method based on the Barthelot reaction (Nkonge and Ballance, 1982) seems useful because of its sensitivity, the availability of equipment, and the potential to automate the method at a later date. The need for sensitivity in the nitrogen assay lies :

i) in the desire to use the minimum amount of sample for digestion, and
ii) the requirement that the ammonia can be accurately quantitated after the digest has been diluted sufficiently to avoid interference by the Kjeldahl catalyst ions.

Various combinations of different phenol reagents and sources of available chlorine have been tested in the Barthelot reaction of ammonia estimation. Because of conflicting reports in the literature regarding optimum conditions, Nkonge and Ballance (1982) have reexamined many of the parameters not only to obtain the optimised conditions but also to determine how a change in any one parameter affects the overall sensitivity of the reaction. The nitrogen assay procedure developed by Nkonge and Ballance (1982) could be used in conjunction with two other assays so that half a kernel of cereal would provide adequate sample for all three analyses.

It was therefore concluded that the sensitivity of this method would be suitable to monitor biomass in wood in work of the nature envisaged here.

Wood Sugar Determination - Colorimetric Methods. In trying to determine the enzymatic hydrolysis of cellulose numerous authors have recognised that most of the methods used to measure cellulolytic activity are influenced by the nature of the substrate, the composition of the cellulase complex and the methods used for analysing hydrolysis products (Rivers *et al.*, 1984; Breuil and Saddler, 1985a;).

The dinitrosalicylic acid (DNS) method (Miller, 1959) and the Nelson and Somogyi (N&S) method (Nelson, 1944; Somogyi, 1952), are fast and convenient procedures that measure the amount of reducing sugars released from the cellulosic substrate. However, these methods are not able to to determine the composition or degree of polymerisation of the sugars arising during the course of the enzymatic reaction. Usually the reaction products are estimated as glucose equivalents, although the absorption coefficients of glucose, cellobiose and dextrins with a higher degree of polymerisation are not identical when the values are determined on the basis of the same weight (Breuil and Saddler, 1985a; Ghose, 1987).

In some of the assays structural analogues of cellulose intermediates are used as substrates, resulting in non representative values. This has been shown to be the case for p-nitrophenyl-B-D-glucopyranoside or salicin, when they are used as standard substrate for assaying the B-glucosidase activity (Andreotti *et al.*, 1977; Kubicek, 1982). Breuil *et al.*, (1986) recommended using cellobiose as the substrate for the

determination of B-glucosidase activity and that the released glucose be measured by the glucose peroxidase method (Keston, 1956). Unfortunately the accuracy of this method, which is based on an enzymatic system, can be influenced by interference with various compounds (Breuil and Saddler, 1985b; Ghose, 1987).

Wood Sugar Determination - HPLC. During the last 8 years, high performance liquid chromatography (HPLC) has advanced to the stage where this instrument has become a standard piece of laboratory equipment available to most groups working on the enzymatic modification of cellulose. Although colorimetric assays still predominate in the literature, more groups are using HPLC to characterise products present in enzymatic hydrolysates of cellulose (Grohmann *et al.*, 1985; Sudo *et al.*, 1986).

Although the HPLC method has been clearly demonstrated (Wentz *et al.*, 1982; Paice *et al.*, 1982) there are presently few quantitative data available concerning the analysis of wood and pulp samples. Pettersen *et al.*, (1984) have provided quantitative results from a variety of woods and wood pulps using both the HPLC method and the older, established method, paper chromatography (Saeman *et al.*, 1954) and the HPLC method was shown to be superior showing better precision overall. Schawald *et al.* (1988) have compared HPLC with colorimetric methods for measuring cellulolytic activity and a comparison of methods for quantifying B-glucosidase activity showed that the enzyme based

methods could be influenced by inhibitory products present in pretreated wood substrates. HPLC analysis was not influenced by wood decomposition products present in culture filtrates.

In summary HPLC has been shown to be a fast and efficient means of separating wood sugar residues in neutral, aqueous solutions. Separation is achieved with a commercial column packed with cation exchange resin in the lead (II) form. A differential refractive index detector and an electronic integrator provide quantitative measurements for each sugar. An automatic sample injector and a system controller provide continuous sample and data flow 24 hours per day without operator attention. Separation and quantitation for one sample requires 60 - 70 minutes.

The advantages of HPLC include :

i) precision
ii) high efficiency
iii) fast i.e. rapid elution of peaks
iv) qualitative and quantitative analysis of individual sugars
v) nanogram sample amounts can be used,

and this method was therefore selected to perform wood sugar analyses in the present work.

Lignin Determination - Hydrolysis and Gravimetric Measurement. If wood is treated with a strong acid, carbohydrates are hydrolysed and solubilised. The insoluble residue is by definition Klason lignin (Effland, 1977) and can be measured gravimetrically. The standard method of analysis requires samples of 1 to 2 grams of wood or pulp. In some laboratories, these amounts of samples are often not available for analytical determinations. Effland (1977) has thus developed a modification of the standard procedure of Saeman *et al.* (1954), suitable for much smaller sample amounts. Effland (1977) showed excellent results obtained for three hard woods and five soft woods when comparing this procedure with the standard procedure. Repeated analysis of a sample of aspen wood on 12 days showed a standard deviation of 0.3% and a standard error of mean of 0.09. This procedure has been used extensively with very satisfactory results on samples as small as 25 mg. It should be noted that the lignin determined in this manner (known as Klason lignin) does not take into account the lignin that dissolves in the sulphuric acid during hydrolysis. Although this is a small amount in soft woods, the soluble lignin in hard woods may be higher. The advantage of this procedure is that small amounts of sample can be used for analysis and more importantly, two types of analyses can be performed from the same sample i.e. the insoluble residue is by definition Klason lignin and can be measured gravimetrically and the filtrate contains the hydrolysed carbohydrates as their constituent monosaccharides, which can

then be assayed with techniques such as HPLC. It was therefore decided to use the modified procedure of Effland (1977) to determine acid - insoluble lignin in wood in the present work.

3.1.2 Objectives and Aims

In the scope of the work of this dissertation (1.3.2) the second objective of the work was, in general terms, to establish whether correlations between colonisation and decay could be demonstrated during the incipient stages of decay.

On the basis of the above review (3.1), the specific objective of the work presented here was to correlate the extent of wood colonisation with the degree of cellulose and lignin degradation by the given fungi. It was decided to monitor colonisation by biomass determination, to monitor cellulose degradation by wood sugar analyses and to monitor lignin degradation by analysis of Klason lignin, all during a six week colonisation period when incipient decay should occur.

The aims of the work were therefore :

a) to set up an experiment to colonise *P.patula* and *E.grandis* with *L.lepideus, C.puteana, P.chrysosporium, C.versicolor* and *M.racemosus*, as monocultures and cocultures;

b) to monitor, by Kjeldahl nitrogen determinations, biomass produced in the colonised wood;

c) to monitor, by HPLC, any changes in wood sugar ratios in colonised wood;

d) to monitor residual lignin in colonised wood by gravimetric determination of Klason lignin.

To ensure that the findings could be interpreted in terms of established knowledge and standards in this field, a corresponding aim was to monitor wood degradation in the conventional manner by weight loss determinations.

3.2 **MATERIALS AND METHODS**

3.2.1 Preparation of wood samples

Mature trees of *Pinus patula* and *Eucalyptus grandis* were selected in Swaziland and felled in 1988. Four one metre bolts were removed from each tree, from just above the butt-swelling. The bolt at breast height was taken in the case of each timber and converted to 5cm thick planks (by quarter sawing "through and through"). The plank containing the pith was selected in each case for test material.

The planks were taken to the National Timber Research Institute, CSIR, Pretoria and the bark was removed from one edge of each. They were shortened to 750mm in length and oven dried at 50C until dry (14 days drying was required to bring the planks to constant weight) when the heartwood and evaporative surfaces (to a depth of 10mm) were removed and discarded. The heartwood was not used for tests because it contained high concentrations of tannins and phenols (Jane, 1970) which are generally toxic to microorganisms. The evaporative surfaces containing redistributed soluble nutrients (King, Oxley and Long, 1976) were likewise discarded because of their atypically high nitrogenous content present as redistributed soluble nutrients which has been shown to enhance wood decay (King *et al.*, 1981).

The remaining sapwood was cut longitudinally into 750mm sticks of cross-section 1 square centimetre. These sticks were then cross-cut to prepare blocks of dimensions 10mm x 10mm x 5mm with the 5mm edge running in the longitudinal direction. 164 blocks were prepared from each timber and those of each species were considered to have similar nitrogen [growth limiting factor of microorganisms in wood (Merrill and Cowling, 1966)] contents for the following reasons:-

1. all blocks were of the same size,
2. trees with minimum tension wood (Jane, 1970), and therefore timber of constant density, was used for block preparation,
3. all blocks originated at the same heights in the tree trunks,
4. all blocks were from the same annual ring groups, counting the pith as 0, in their respective trees.
5. all blocks contained little, if any, redistributable soluble nutrients.

As stated above, 164 blocks of *P.patula* and 164 blocks of *E.grandis* were cut and these blocks were sanded to ensure that the edges were smooth. The blocks were then labelled using India ink according to codes developed (Appendix 8a), dried overnight at 103C and quickly transferred to dessicators to cool (it had previously been found that hot wood was hygroscopic and such blocks absorbed 0.5% of their dry weight in atmospheric moisture between removal from the oven and weighing on the balance in the

laboratory). After they had cooled the blocks were acurately weighed to four places of decimals on a standardised balance and these weights were recorded.

It was necessary to then sterilise the wood blocks prior to their exposure to colonisation by the fungal cultures, but sterilisation by autoclaving was unacceptable in the present work, since this process may have a destructive effect on wood, such as carbohydrate hydrolysis (Glassare, 1970; Savory and Bravery, 1970). The blocks were packed into petri dishes which were then sealed with parafilm. The blocks were sterilised by gamma irradiation (20 kilorads) as had been done with the comminuted wood (2.2.3.1).

3.2.2 Cultures and inoculation

Plastic coated wire was cut into pieces and made into Z-shaped forms to support wood blocks on agar surfaces during the tests to be described. These supports were autoclaved and allowed to cool. Malt extract agar plates were freshly prepared and the supports were placed at 5 points in each petri dish. The preweighed blocks which had been labelled and sterilised (3.2.1) were then placed on the supports so that the blocks did not touch the agar (Fig. 3.1). There were 5 blocks of *P.patula* per plate and similarly, 5 blocks of *E.grandis* per plate. Ten blocks of each wood were challenged by the separate fungi listed below.

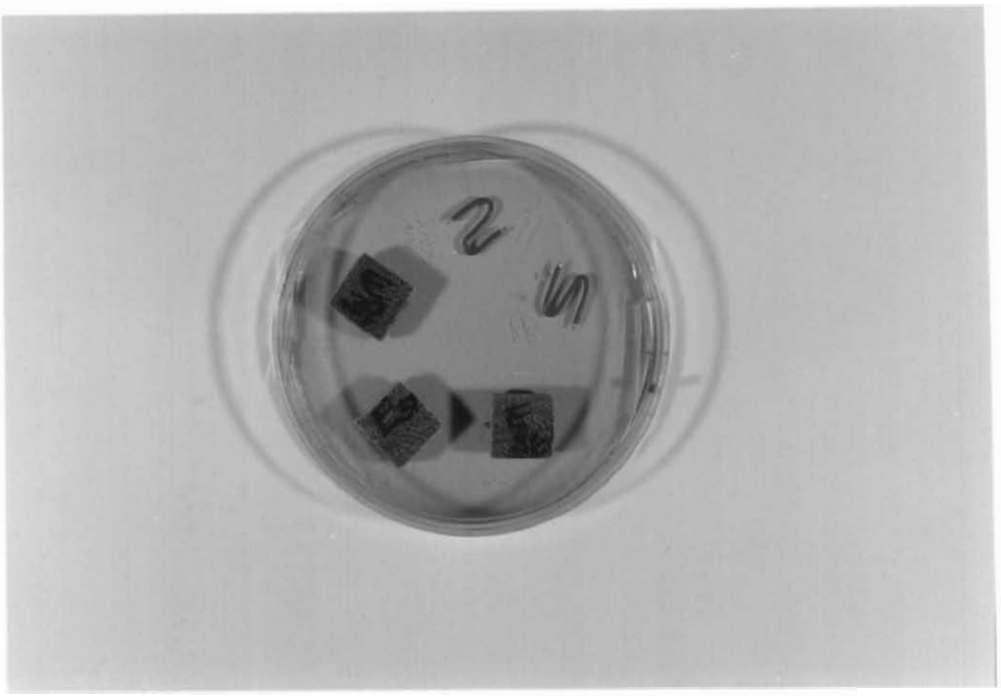


Fig. 3.1 Wire supports used to separate test blocks from direct contact with surfaces of Malt Extract Agar in petri dishes.

The monocultures used in these decay tests were :

i) *Coriolus versicolor* FPRL 28A

ii) *Coniophora puteana* FPRL 11E

iii) *Lentinus lepideus* FPRL 75

iv) *Phanaerochaete chrysosporium* BKM-F 1767

v) *Mucor racemosus* (isolated by the author - Chapter 2)

The cocultures of the above were :

i) *M.racemosus* and *C.puteana*

ii) *P.chrysosporium* and *C.puteana*

These combinations were used to determine whether there existed a synergistic or antagonistic relationship between i) a sugar fungus and a brown rotter and ii) a white rotter and a brown rotter.

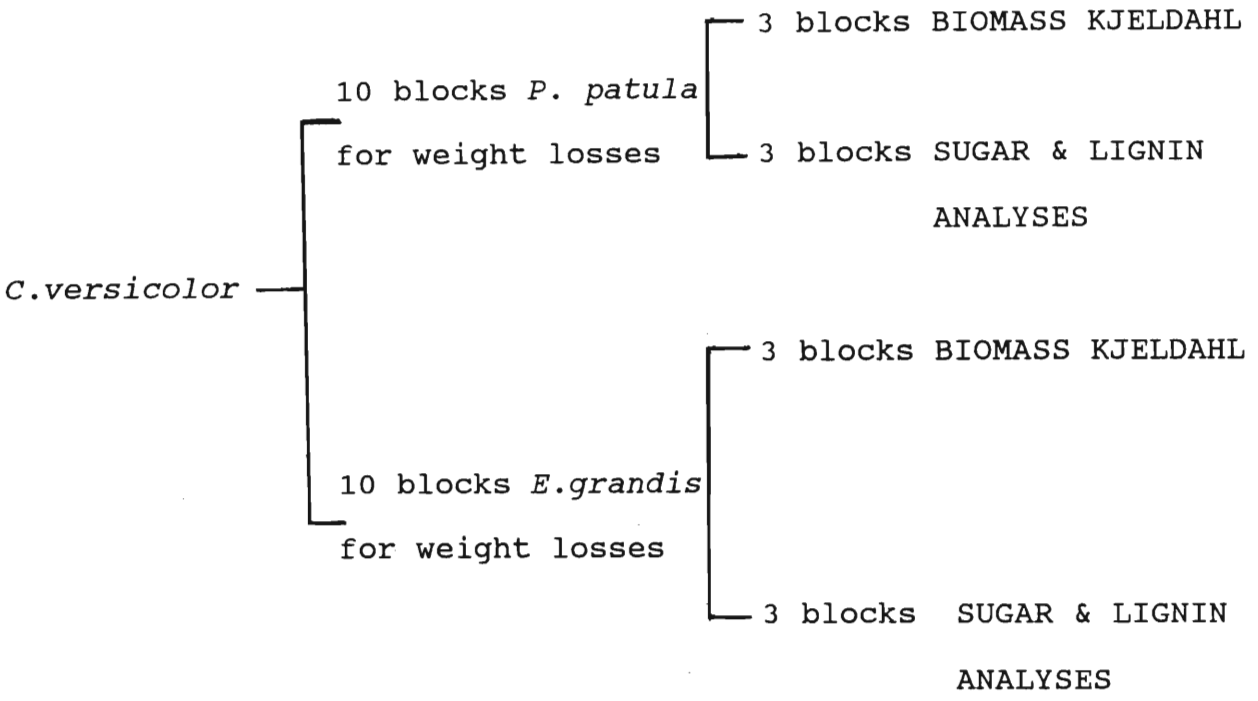
Standard inocula were removed from mature lawns of these cultures using a sterile 5mm cork borer and the plates were inoculated adjacent to each wood block as depicted in Fig. 3.3. Similarly when cocultures were used, standard inocula were removed and the plates were inoculated with both fungi adjacent to each block. The inoculated petri dishes were then placed in glass jars to prevent dehydration of the media. Replicate jars containing the test samples were incubated at 26C for 3 weeks and 6 weeks respectively. The jars were opened every 3 days to check for the presence of contamination and also to allow sufficient oxygen to be present in the jar.

3.2.3 Sampling and analyses

The incubation periods used in this experiment were 0, 3 and 6 weeks (Fig. 3.2). As far as the 0 week samples were concerned, the initial dry weight was recorded and the blocks were then used in biomass, lignin and sugar determinations.

As far as the 3 week and 6 week samples were concerned, the final dry weight was recorded, before the wood samples were used for biomass and sugar determinations. Of the 10 blocks of each wood challenged by the respective cultures, 3 challenged by each test culture were used for biomass determinations, and a further 3 replicates of each wood challenged by each test culture were used for sugar determinations. The protocol is summarised below :

Six week incubation period :



and similarly, the blocks challenged by *C.puteana, L.lepideus, P.chrysosporium, M.racemosus* and cocultures *M.racemosus* and *C.puteana; P.chrysosporium* and *C.puteana* were also analysed as shown above.

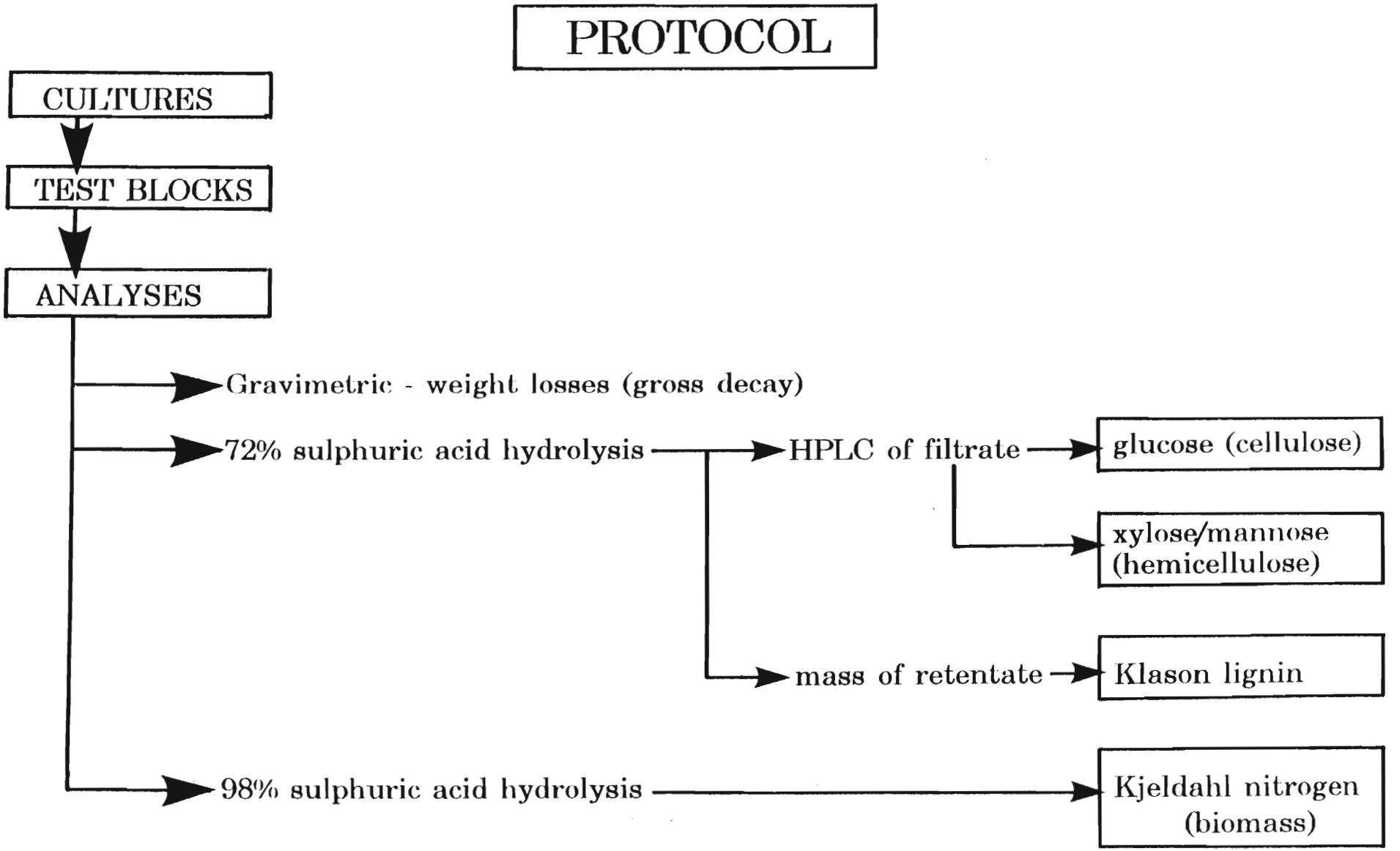


Fig. 3.2 Protocol.

3.2.3.1 Gravimetric determinations - weight losses

For gravimetric analyses at 3 and six weeks of incubation, the test blocks were removed from petri dishes and mycelium colonising the wood was brushed off each block. Each block was then placed in a separate labelled McCartney bottle and dried at 103C for 2 days. The bottles containing the blocks were removed from the oven and placed in a dessicator and allowed to cool because hot wood is hygroscopic and the final dry weight of each wood block was determined using a Sartorius Model electronic balance. The final dry weight was subtracted from the initial dry weight of each block, and hence weight losses or weight gains were recorded. These were then expressed as percentage weight losses or percentage weight gains as outlined below :

$$\% \text{ WL} = \frac{W1 - W2}{W1} \cdot 100$$

WL = weight loss

W1 = original dry weight of the wood block

W2 = dry weight after decay of the same wood block

It is obvious that W1 will be determined by the size of the wood block and the density of the timber used in the experiments.
W1 - W2 represents the weight of the wood substance that has been metabolised by the decay organism.

3.2.3.2 Biomass determinations

In the present work, the nitroprusside colorimetric method of Nkonge and Ballance (1982) was used to estimate ammonia as follows :

Three blocks of *P.patula* and 3 blocks of *E.grandis* from the zero week samples were digested in separate Micro - Kjeldahl flasks. Two glass beads were added to each flask and 10ml of concentrated H_2SO_4 (Merck, AR grade) and 0.5g $CuSO_4$ (Merck, AR grade) were added to each flask. The flasks were then placed in the Micro - Kjeldahl digestion apparatus and the mixture was heated under slow heat for 20 min, and subsequently the heat was increased. The digestions were carried out in a fume cupboard and samples were digested for approximately 8h, or until the digests had reached a clear colour.

The digests were allowed to cool and then transferred to 100ml volumetric flasks. The digestion flasks were rinsed with distilled water and the mixture was transferred to the volumetric flasks. Distilled water was used to bring the contents in the flask up to volume i.e., 100ml. The diluted digests were transferred to labelled glass medical - flat bottles, and these were stored in the refrigerator (4C) for subsequent analyses.
The blocks used from the 6 week incubation periods were also digested as outlined above and therefore a total of 42 digestions were carried out on these test samples.

Standard nitrogen solutions to be used for establishing optimum assay conditions (Nkonge and Ballance, 1982) were prepared from using oven dried (3h at 103C) ammonium sulphate. The ammonium sulphate (47.16mg) was substituted for the sample in the above digest conditions. This digest, after dilution to 100ml, yielded a solution of 100ug of N per ml. Blank digests were prepared in an identical manner by using only catalyst ($CuSO_4$) and acid; blank digests were used for dilution of the 100ug of N per ml standard solution to suitable volumes.

The reagents used for ammonia estimations were as follows :
(1) the working buffer was prepared from two stock solutions. Solution A was composed of Na_2HPO_4 (0.2M), NaOH (0.2M), and sodium potassium tartrate - tetrahydrate (0.36M) prepared in distilled deionised water. Solution B was 2.5M NaOH. The working buffer was prepared by mixing A and B on a 1:1 volume basis,
(2) salicylate - nitroprusside reagent was prepared by dissolving sodium salicylate (20.0g) and sodium nitroprusside (30.0mg) in distilled deionised water and diluting to 100ml.
(3) hypochlorite solution of the appropriate concentration was prepared just prior to use by dilution of commercial bleach [5.2% w/v; available chlorine as determined by Method 6.111 (AOAC, 1975)] with distilled deionised water.

The procedure used for the ammonia assay was as follows :
To 0.5ml of diluted digest, 1.5ml of working buffer was added. After addition of 0.40ml of salicylate - nitroprusside reagent, the solution was mixed and placed in a water bath and allowed to

equilibrate to the incubation temperature (25C) before 0.2ml of the hypochlorite solution was added. The reagent proportions used were based on those of Nkonge and Ballance (1982). The complete reaction mixture was mixed and incubated for a further 30min in a shaking bath to allow the colour to develop. The sample was then diluted with 10ml of distilled water and the absorbance at 660nm was measured.

This method did not prove successful in nitrogen analysis because of the variability of the results obtained for replicate readings of the same sample and values obtained were over estimates when compared to the values determined by Schippenkoetter *et al.*, (1988).

Hence it was decided to use an automated colorimetric total nitrogen analysis method based on the Barthelot reaction - the Technicon Auto Analyser II (Industrial Method No 334 - 74A, 1976). The principle of this analysis is that initially all the organic nitrogen in the sample would be converted to ammonium sulphate after digestion in sulphuric acid. During the automatic analysis each sample flows alongside a diffusion membrane and the ammonium ions present in the sample pass through to an acidic carrier solution. This solution is then reacted with a strong alkali to release the nitrogen as ammonia. This free ammonia is then mixed with an alkaline salicylate solution which contains nitroprusside as a catalyst. A hypochlorite solution is added

and this reacts with ammonia and salicylate in the presence of nitroprusside to form indophenol blue, the absorbance of which is measured at 660nm (Shelver, 1992).

5 ml of each hydrolysate (obtained after digestion as described above) was filtered (0.22um filters) prior to analysis of nitrogen content using the Technicon Auto Analyser II Method. Nitrogen contents were quantified and converted by a factor of 6.25 to dry protein contents, and by subtraction of control values, subsequently to viable fungal biomass, expressed as fungal protein, in each block, although nitrogen contents are also tabulated as % (w/w) dry wood in this thesis.

3.2.3.3 Klason lignin determinations

From the zero week samples, 3 blocks of *P.patula* and 3 blocks of *E.grandis*, and of the 6 week samples, 21 blocks of each wood (3 from each test) were selected for Klason lignin and subsequent sugar determinations.

Each block was oven dried as described previously (3.2.3.1), cooled in a desiccator, weighed and comminuted to wood flour and transferred to a shell vial. For each 50 mg of sample, 0.5 ml of 72% sulphuric acid (pour 65 ml of concentrated H_2SO_4 into 300 ml of water, cool, and make up to 1000 ml; standardise to a strength of 72% by titration with a standard alkali or by measuring specific gravity) was added. The mixture was placed in a 30 C water bath, stirring frequently to assure complete solution.

After exactly one hour, the sample was diluted to 14.5 ml in distilled water and secondary steam hydrolysis was carried out in an autoclave. A steam autoclave with distilled water preboiler was used to ensure constant take up and take down times and freedom from volatile water-treatment chemicals. The autoclave was held at 120 C for 1 hr.

While keeping the final hydrolysate solutions hot, the lignin was filtered off through preweighed glass fibre filters and the filtrates were used for the sugar analyses described below. The Klason lignin residue was thoroughly washed with hot water to completely remove the acid and the filters and the retentates were oven dried, cooled in a dessicator and reweighed to determine the mass of Klason lignin in each sample (Effland, 1977).

3.2.3.4 Sugar determinations - HPLC

The above filtrates (3.2.3.3) were analysed after neutralisation with Ba_2CO_3 (2g). The Ba_2CO_3 was added slowly with mixing to neutralise the contents. When all the CO_2 evolution had ceased, the samples were left stationary, so that the precipitates would settle to the bottom of the tube. The supernatants were carefully removed and filtered (0.22um with 1um prefilter) and the filtrates were stored in septum capped bottles at 4C until analysed.

The filtrates were analysed using a High Pressure Liquid Chromatography System (Waters, Milford USA) consisting of a 510 solvent delivery system, WISP autosampler, 401 refractive index detector and 740 data module.

The mobile phase was deionised water at a flow rate of 0.5ml min^{-1}. The column used was a Sugar - Pak I at 85C and the sample injection volume was 20ul. Standards in the range of 1.0 to 0.25 $mgml^{-1}$ were used to calibrate the system and the glucose and xylose plus mannose fractions of the samples were quantified.

3.2.3.5 Statistical methods

All data were analysed by GENSTAT 5.2 (Rothamstead Experimental Station,U.K) using the analysis of variance (ANOVA) technique. The weight losses, nitrogen values, cellulose, hemicellulose and lignin values were analysed separately. For weight losses three factors were considered, viz.,

i) period - 3 and 6 weeks
ii) fungi - 7 cultures (5 monocultures and 2 cocultures)
iii) wood - *P.patula* and *E.grandis*

For the remaining values, period was constant at 6 weeks, therefore only factors ii) and iii) above were considered.

3.3 RESULTS

It was decided to present the results of this Chapter in three parts, i.e. the first part (3.3.1) describes the results of weight loss determinations, while the biomass developed by fungi which had produced these weight losses in the wood follow in 3.3.2. Similarly, residual sugars and Klason lignin in these samples are presented in 3.3.3. All raw data and statistical analyses of variance are included in Appendices (itemised below), however mean values of these data are presented as tables and figures in the relevant sections of the text.

3.3.1 Weight losses in colonised wood

The raw data from *C.versicolor*, *C.puteana*, *L.lepideus*, *P.chrysosporium* and *M.racemosus* as monocultures in *P.patula* and *E.grandis* and as cocultures of *M.racemosus* + *C.puteana* and *P.chrysosporium* + *C.puteana* are recorded in Appendix 8a. The mean values of all these data are presented in Tables 3.1 and 3.2 respectively below. The analysis of variance of the three - factor interaction, viz., wood, incubation period and fungus (Appendix 8b) showed that all effects apart from the incubation period x wood interaction were statistically significant ($P < 0.05$). This can be interpreted as confirming that the weight loss effects produced by the fungi in the wood were statistically significant. Tables 3.1 and 3.2 constitute tables of means of this three - factor interaction.

Table 3.1 Mean % weight losses[1] produced in *P.patula* blocks by fungal monocultures and cocultures.

Culture	Incubation Period (weeks)		
	0	3	6
C.versicolor	ND	10.34	24.89
C.puteana	ND	-0.93	0.14
L.lepideus	ND	0.51	0.38
P.chrysosporium	ND	0.12	0.60
M.racemosus	ND	-0.32	1.91
M.racemosus + *C.puteana*	ND	-0.53	1.21
P.chrysosporium + *C.puteana*	ND	-0.93	0.35

1 All values are means of 10 determinations, and the statistical analysis of these data is presented in Appendix 8b. The standard error of the difference between any 2 means is 1.460.

ND : not determined.

Table 3.2 Mean % weight losses[1] produced in *E.grandis* blocks by fungal monocultures and cocultures.

Culture	Incubation Period (weeks)		
	0	3	6
C.versicolor	ND	20.60	28.11
C.puteana	ND	-1.29	-0.28
L.lepideus	ND	-1.80	-0.98
P.chrysosporium	ND	4.86	9.39
M.racemosus	ND	0.28	1.94
M.racemosus + *C.puteana*	ND	1.49	1.84
P.chrysosporium + *C.puteana*	ND	2.14	0.62

1 All values are means of 10 determinations, and the statistical analysis of these data is presented in Appendix 8b. The standard error of the difference between any 2 means is 1.460.
ND : not determined.

The data show (Tables 3.1 and 3.2) that with some monocultures weight losses were incurred, whereas with others weight gains were incurred. All blocks appeared to be extensively colonised, and some blocks, eg., those challenged by *C.versicolor* and *P.chrysosporium* appeared more colonised than others, eg., those

challenged by *M.racemosus* (Fig. 3.3). The apparently anomalous production of weight gains in colonised wood is discussed below in conjunction with the biomass values produced in these samples.

Considering the standard error of 1.460 and since no weight losses occurred in unchallenged wood, only *C.versicolor* and *P.chrysosporium* appeared to produce significant degradation of *E.grandis* (Table 3.2) as monocultures. However, the difference between *P.chrysosporium* monoculture and *P.chrysporium* + *C.puteana* coculture was also statistically significant, therefore *C.puteana* exerted a significantly antagonistic effect on *P.chrysosporium* in *E.grandis* after 6 weeks. Furthermore, these trends were repeated when *P.patula* was examined (Table 3.1), although *P.chrysosporium* produced no apparent weight loss in *P.patula*. However this apparent anomaly will also be discussed below, in conjunction with biomass produced (3.3.2) and the ligninolytic potential of *P.chrysosporium*.

Since most weight losses at the three week period were biologically insignificant (< 5%), it was decided to analyse only 6 week samples below (3.3.2 and 3.3.3).

3.3.2 Biomass produced in wood

The nitrogen and protein contents of *P.patula* and *E.grandis* (sound wood) and also those of these woods colonised by the monocultures and cocultures are recorded in Appendix 9a.

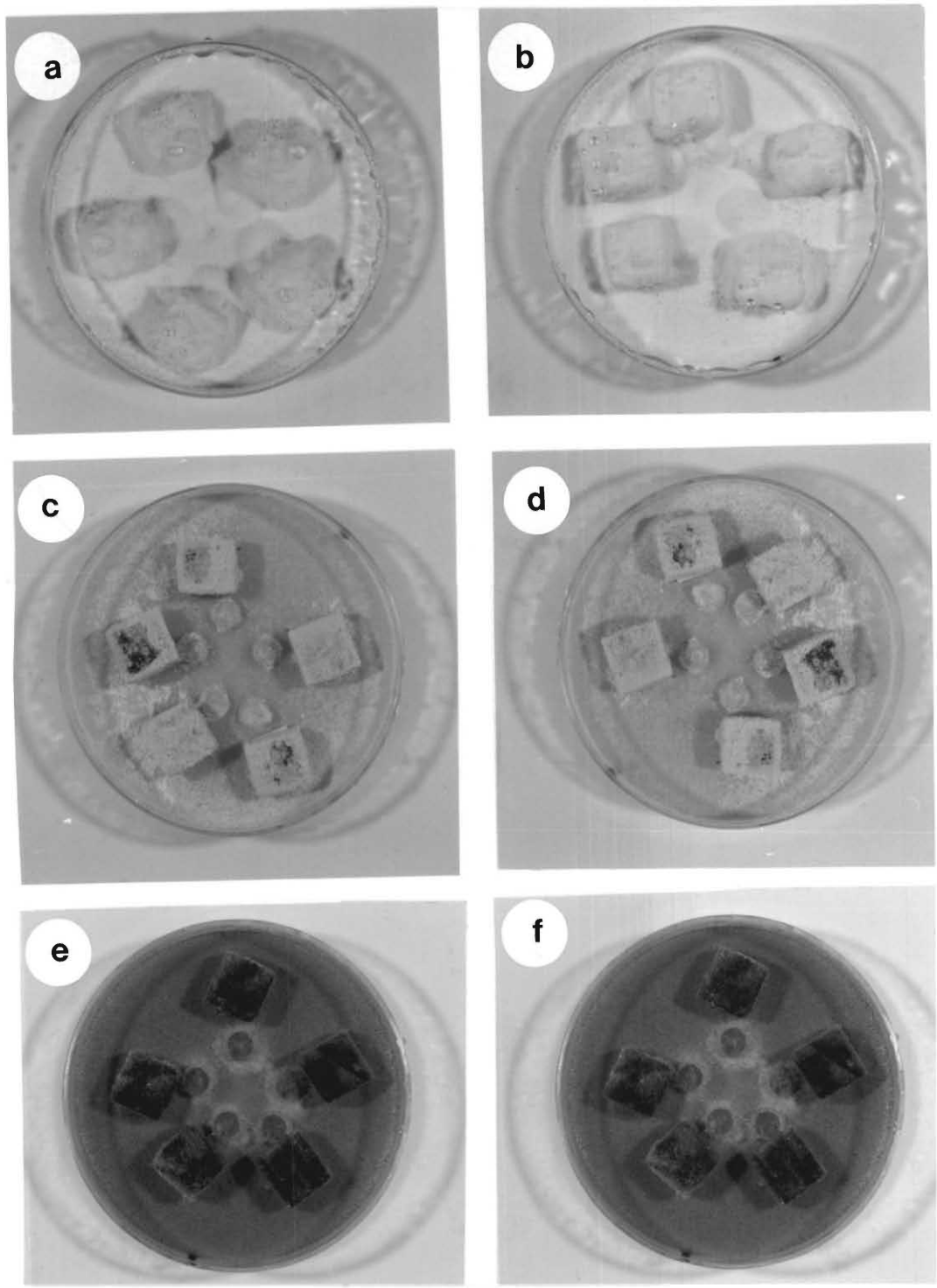


Fig. 3.3 Colonisation produced by (a) *C.versicolor* on *P.patula*, (b) *C.versicolor* on *E.grandis*, (c) *P.chrysosporium* on *P.patula*, (d) *P.chrysosporium* on *E.grandis*, (e) *M.racemosus* on *P.patula*, and (f) *M.racemosus* on *E.grandis*.

The corresponding analysis of variance of nitrogen contents is recorded in Appendix 9b and the mean values of the raw data are presented in Tables 3.3 and Table 3.4, respectively.

Table 3.3 Mean percentage nitrogen and protein contents[1] of *P.patula* blocks colonised by fungal monocultures and cocultures after 6 weeks' incubation.

Culture	Nitrogen[2] (% w/w)	Protein (% w/w)	Biomass[3] (% w/w)
Control	0.029	0.178	___
C.versicolor	0.341	2.129	1.951
C.puteana	0.222	1.390	1.212
L.lepideus	0.231	1.443	1.265
P.chrysosporium	0.217	1.356	1.178
M.racemosus	0.165	1.030	0.852
M.racemosus + *C.puteana*	0.162	1.015	0.837
P.chrysosporium + *C.puteana*	0.122	0.763	0.585

[1] All values are means of three determinations and the statistical analysis of these data is presented in Appendix 9b.
[2] The standard error of the difference between any 2 means in this column is 0.0796 based on statistical analysis.
[3] Expressed as dry weight fungal protein.

The statistical analyses of variance (Appendix 9b) confirmed that the significant effects were for fungi and the interaction wood x

fungi, i.e., the various fungi each produced significantly different amounts of biomass in a given wood species. This correlation was consistent with that shown above (3.3.1) in conjunction with the weight losses. There was no significant overall difference between the two woods (Appendix 9b), i.e., in general, any given fungus produced similar amounts of biomass in each wood species. However, on inspecting the wood x fungi tables of means, significant differences between *P.patula* (Table 3.3) and *E.grandis* (Table 3.4) in terms of biomass production by *P.chrysosporium* were noted, i.e., more biomass was produced in *E.grandis*. This statistically significant observation supported the weight loss data which showed (3.3.1) that *P.chrysosporium* produced significant weight losses only in the hardwood *E.grandis*.

Table 3.4 Mean percentage nitrogen and protein contents[1] of *E.grandis* blocks colonised by fungal monocultures and cocultures after 6 weeks' incubation.

Cultures	Nitrogen[2] (% w/w)	Protein (% w/w)	Biomass[3] (% w/w)
Control	0.045	0.278	-----
C.versicolor	0.224	1.397	1.119
C.puteana	0.145	0.907	0.629
L.lepideus	0.141	0.880	0.602
P.chrysosporium	0.517	3.230	2.952
M.racemosus	0.109	0.676	0.398
M.racemosus + *C.puteana*	0.178	1.110	0.832
P.chrysosporium + *C.puteana*	0.177	1.107	0.829

[1] All values are the means of three determinations and the statistical analysis of these data is presented in Appendix 9b.
[2] The standard error of the difference between any 2 means is 0.0796.
[3] Expressed as dry weight fungal protein.

Importantly, the biomass values explained why nil weight losses had been recorded (3.3.1) in conjunction with wood which appeared to be extensively colonised. It can be seen that blocks from which nil weight losses or weight gains were recorded, viz., *C.puteana* and *L.lepideus* (Table 3.1 and Table 3.2), usually contained statistically significant amounts of fungal protein.

This phenomenon suggests that real weight losses could have been produced in these samples, but were nullified by biomass production. In particular, *P.chrysosporium* had produced a significant weight loss of 9.39% in *E.grandis*, but no apparent weight loss in *P.patula* (Table 3.1). Further, Fig. 3.3 and the scanning electron micrographs in Chapter 2 show that *P.chrysosporium* colonised *P.patula* extensively. It is proposed that Table 3.3 helps interpret this anomaly when it is noted that *P.chrysosporium* produced 1.178% dry protein in *P.patula*, i.e., at least 5 or 6% biomass, which must therefore have nullified its contribution to the reduction of wood substrate normally observed as a weight loss in the wood. This is further discussed below (3.3) in conjunction with the sugar and lignin determinations.

It was also interesting to note that the other cultures which produced significant weight losses in wood (Tables 3.1 and 3.2), viz., *C.versicolor*, also produced significant amounts of biomass (Fig. 3.3) in wood (Tables 3.3 and 3.4).

3.3.3 Residual cellulose, hemicellulose and Klason lignin

The raw data of the residual cellulose, expressed as glucose, and hemicellulose, expressed as xylose + mannose values obtained after HPLC analysis of hydrolysed wood samples are recorded in Appendix 10. The analyses of variances are recorded in Appendix 11a and 11b respectively. The corresponding data of Klason lignin determinations are recorded in Appendix 10 and the analysis of variance is recorded in Appendix 11c. The mean

percentages of residual sugars, i.e., cellulose (glucose), hemicellulose (xylose plus mannose) and Klason lignin in *P.patula* and *E.grandis* colonised by the fungal monocultures and cocultures for six weeks are presented in Tables 3.5 and 3.6 respectively. As was the case with biomass values above, only the statistically significant findings will be considered here.

It is very important to examine the glucose and xylose + mannose data holistically in conjunction with the corresponding lignin values. The reason for this is as follows :- a given mass of decayed wood which had lost lignin but not cellulose would appear to contain a higher percentage of cellulose than a mass of wood which had lost both cellulose and lignin, or which had lost neither of these components. Therefore, percentage residual values of each component would be confusing if interpreted separately. Furthermore, the corresponding weight losses and biomass values require to be included in such examination, in order to interpret the data meaningfully. For ease of comparison, the ratios of all these values are presented in histograms (Figs. 3.4 and 3.5) which summarise the data tabulated in this section.

Table 3.5 Mean percentage residual sugar and lignin contents[1] of *P.patula* blocks colonised by fungal monocultures and cocultures for six weeks.

Culture	Glucose[2] (% w/w)	Xylose + Mannose[3] (% w/w)	Klason lignin[4] (% w/w)
Control	40.6	17.2	37.0
C.versicolor	39.4	16.5	38.3
C.puteana	39.9	14.6	38.3
L.lepideus	43.5	16.5	38.2
P.chrysosporium	43.3	15.4	35.9
M.racemosus	40.9	17.9	31.5
M.racemosus + *C.puteana*	40.3	16.1	40.5
P.chrysosporium + *C.puteana*	40.5	16.0	34.9

1 All values are the means of 3 determinations.
2 HPLC analysis of hydrolysed cellulose and the standard error of the difference between any 2 means in this column is 1.409.
3 HPLC analysis of hydrolysed hemicellulose and the standard error of the difference between any 2 means in this column is 0.838.
4 Klason lignin determined by gravimetric analysis and the standard error of the difference between any 2 means is 4.219.

Table 3.6 Mean percentage residual sugar and lignin contents[1] of *E.grandis* blocks colonised by fungal monocultures and cocultures for six weeks.

Cultures	Glucose[2] (% w/w)	Xylose + Mannose[3] (% w/w)	Klason lignin[4] (%w/w)
Control	47.1	10.1	26.7
C.versicolor	47.6	10.5	37.9
C.puteana	47.1	9.2	32.1
L.lepideus	48.8	11.6	27.9
P.chrysosporium	45.1	8.1	30.3
M.racemosus	44.3	10.1	29.8
M.racemosus + *C.puteana*	45.1	9.7	30.3
P.chrysosporium + *C.puteana*	46.4	9.8	26.8

1 All values are the means of 3 determinations.
2 HPLC analysis of hydrolysed cellulose and the standard error of the difference between any 2 means in this column is 1.409.
3 HPLC analysis of hydrolysed hemicellulose and the standard error of the difference between any 2 means in this column is 0.838.
4 Klason lignin determined by gravimetric analysis and the standard error of the difference between any 2 means is 4.219.

With regard to glucose, the analysis of variance (Appendix 11a) showed that all effects were significant, except the interaction term wood x fungi, although the wood effects were much higher (F probability < 0,001) than the other 2 effects. This meant that the analyses confirmed that *E.grandis* had a significantly higher

level of cellulose viz., 47.1% (w/w) than did *P.patula* which contained 40.6% cellulose. If the different fungi showed no differences in their respective interactions with each wood, the above difference, i.e., the interaction effect between the cell contents of *E.grandis* and *P.patula* would be reproduced after colonisation by each fungus. This was not the case when *P.chrysosporium* colonised *P.patula* (Table 3.5) and *E.grandis* (Table 3.6), and in effect, *P.chrysosporium* appeared to raise *P.patula*'s glucose content to that of *E.grandis*. This was clearly not possible, and in reality, reflected the reduction of lignin in *P.patula* producing an apparent increase in cellulose in that wood as explained above. Therefore *P.chrysosporium* showed a greater ligninolytic potential than the other fungi, illustrating the well known (Tien and Kirk, 1983; 1984) ligninolytic potential of this white rot fungus. However, this effect was demonstrated to a greater extent in *P.patula* than in *E.grandis* where its ligninolytic potential was not apparent in *E.grandis*. Therefore the main points shown by the glucose values were that *P.chrysosporium* and to a lesser extent, *M.racemosus*, were ligninolytic in *P.patula*.

In contrast Tables 3.5 and 3.6 indicate that *M.racemosus* may appear to depress the glucose content of *E.grandis*, which in turn may be interpreted as showing that *M.racemosus* is cellulolytic in this wood, which in turn caused this writer to amend the conclusion in Chapter 2.

With regard to hemicellulose utilisation, the analysis of variance (Appendix 11b) showed that only the wood and fungi main effects were significant but the interaction term wood x fungi was not significant. This meant that the hemicellulose content of *P.patula*, at 15.76%, was significantly higher than that of *E.grandis* at 9.54%, but, unlike the case of cellulose above , no fungus affected this difference. Therefore, there was no notable effect of a given fungus on a particular wood, showing that the fungi demonstrated no preference for the source of xylose in a particular wood. However, two fungi did demonstrate significant hemicellulolytic potentials, viz., *C.puteana* in *P.patula* and *P.chrysosporium* in *E.grandis*.

Interestingly, the data show that the average glucose content was 43.74% (grand mean) and the residual value was 2.976. However when the % lignin is considered, the average was 33.52%, but the variance was 26.7% which is almost a 10 fold increase with respect to the glucose figures. These results show that although the blocks received the same treatment, there was greater variation within lignin analyses than with glucose analyses. The reason for this variation is that the lignin determination was a gravimetric determination which produced large degrees of error. Therefore, since the residual variation was high, the ligninolytic effect of *P.chrysosporium* was not strikingly different from the others. In spite of this variation it was still possible to examine possible trends in the lignin values. The analysis of variance in lignin values (Appendix 11c) showed that only the wood main effect was significant, i.e., it was

confirmed that the lignin content of *P.patula* (36.98%) was significantly different from that of *E.grandis* at 26.65%. On average there was a difference in lignin content of approximately 6% between the uncolonised wood species. Further, the interaction term wood x fungi was not significant, meaning that no fungus significantly affected the difference in lignin between the two woods. It therefore appeared that no fungus demonstrated marked ligninolytic potential, however *C.versicolor* did significantly increase the lignin content of *E.grandis*, showing its cellulolytic potential, and *M.racemosus* reduced the lignin contents in *E.grandis* to some extent. However, the major ligninolytic effect observed was the above-mentioned potential of *P.chrysosporium* to degrade lignin in *P.patula*, although this effect was not demonstrated in *E.grandis*.

The range of data presented above (Tables 3.5 and 3.6) should theoretically account for 99% of the wood mass. It was of interest to ascertain the efficiency of recovery of these constituents, viz., cellulose, hemicellulose and lignin.
Therefore, the results presented above, i.e., the sugar and lignin contents of all test blocks are summarised in Table 3.7 with the wood weight losses and corresponding biomass production and the percentage recovery of wood constituents. Interestingly, all percentage recoveries were less than 100%, reflecting consistency of recovery rates. However, yields varied between 83.2% and 98.4%. The discrepancies between these values and 100% recovery were attributed to lignin solubility during hydrolysis, and this will be further discussed below.

Table 3.7 Sugar, lignin and nitrogen ratios and weight - losses[4] of wood analysed after six weeks colonisation by fungi.

WOOD	FUNGI	WEIGHT LOSS (% w/w)	GLUCOSE[1] (% w/w)	XYLOSE[2] + MANNOSE (% w/w)	LIGNIN[3] (% w/w)	NITROGEN[4] (% w/w)	% RECOVERY[5]
PINE							
PINE	CONTROL	--	40.6	17.2	37.0	0.029	94.8
PINE	*P.chrysosporium* & *C.puteana*	0.4	40.5	16.0	34.9	0.122	91.5
PINE	*M.racemosus* & *C.puteana*	1.2	40.3	16.1	40.5	0.162	97.1
PINE	*M.racemosus*	1.9	40.9	17.9	31.5	0.165	90.5
PINE	*L. lepideus*	0.4	43.5	16.5	38.2	0.231	98.4
PINE	*C.versicolor*	24.9	39.4	16.5	38.3	0.341	94.5
PINE	*C.puteana*	0.1	39.9	14.6	38.3	0.222	93.0
PINE	*P.chrysosporium*	0.6	43.3	15.4	35.9	0.217	94.8
EUCALYPT							
EUCALYPT	CONTROL	--	47.1	10.1	26.7	0.045	83.9
EUCALYPT	*P.chrysosporium* & *C.puteana*	0.6	46.4	9.8	26.8	0.177	83.2
EUCALYPT	*M.racemosus* & *C.puteana*	1.8	45.1	9.7	30.3	0.178	85.3
EUCALYPT	*M.racemosus*	1.9	44.3	10.1	29.8	0.109	84.3
EUCALYPT	*L. lepideus*	-1.0	48.8	11.6	27.9	0.141	88.4
EUCALYPT	*C.versicolor*	28.0	47.6	10.5	37.9	0.224	96.2
EUCALYPT	*C.puteana*	-0.3	47.1	9.2	32.1	0.145	88.5
EUCALYPT	*P.chrysosporium*	9.4	45.1	8.1	30.3	0.517	84.0

[1]HPLC analysis of hydrolysed cellulose
[2]HPLC analysis of hydrolysed hemicellulose
[3]Klason lignin
[4]Technicon auto-analysis of Kjeldahl nitrogen
[5]Total recovery of wood sugars, lignin and nitrogen
[4]Weight losses are the means of 10 replicates and all other values are the means of 3 replicates

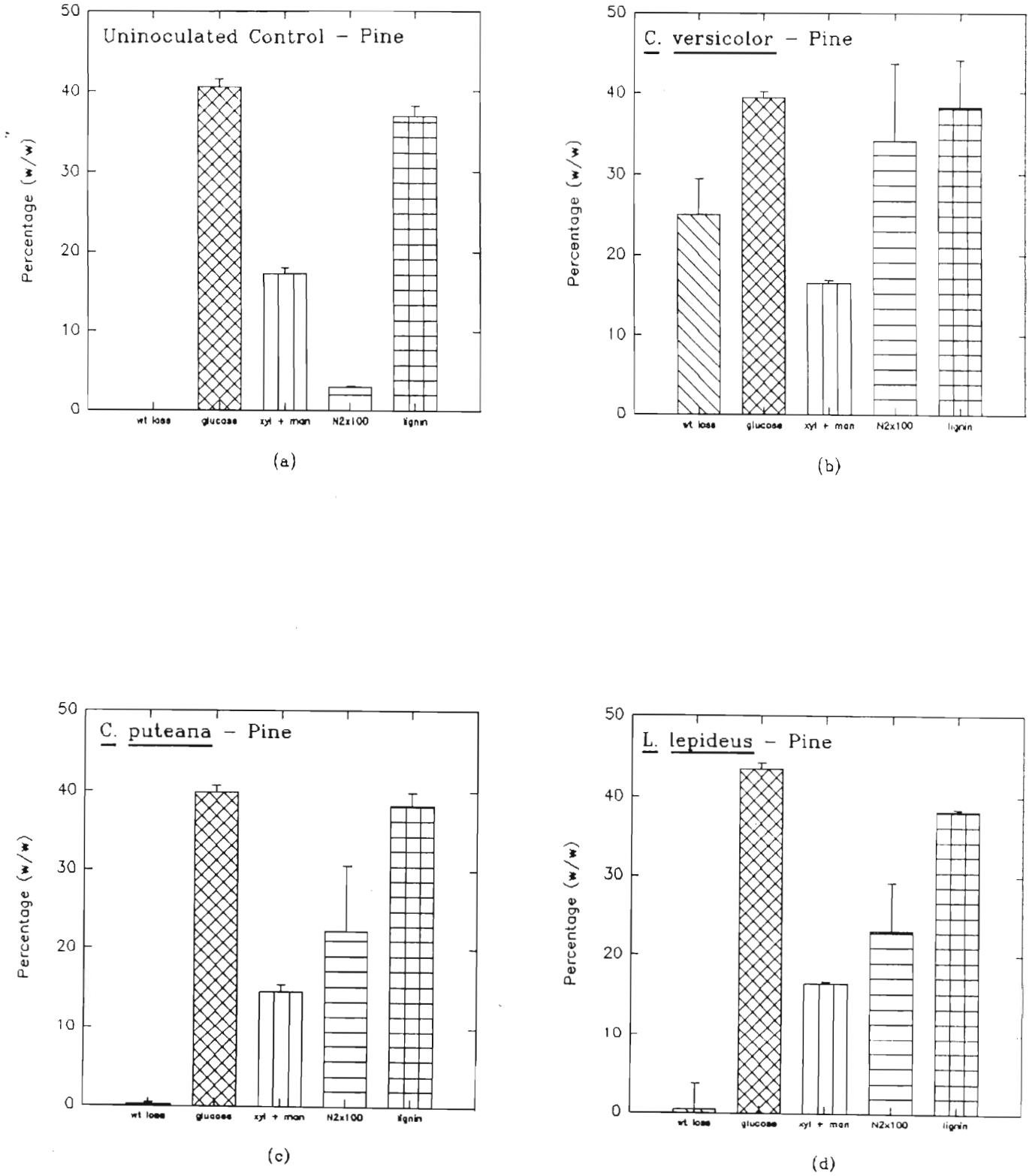
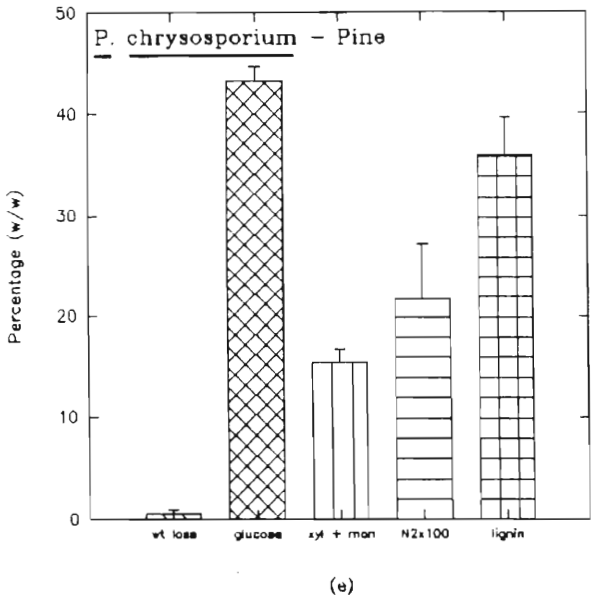
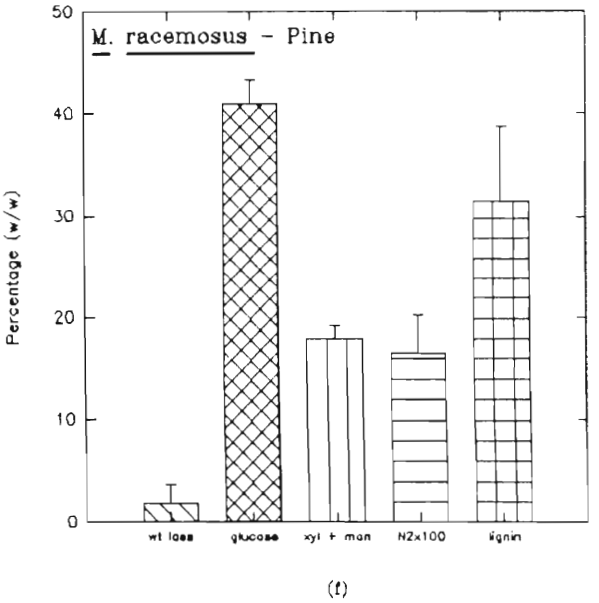


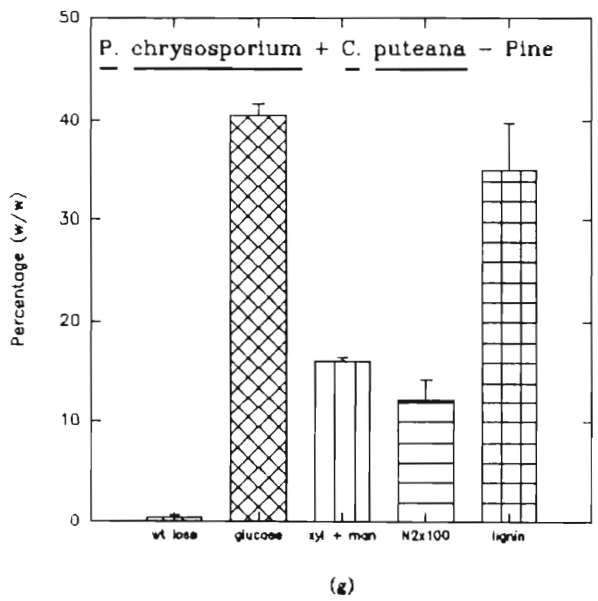
Fig. 3.4 Weight losses produced in *P.patula*, compared with the corresponding cellulose, hemicellulose, nitrogen and Klason lignin contents of *P.patula* after its colonisation by fungi for 6 weeks.



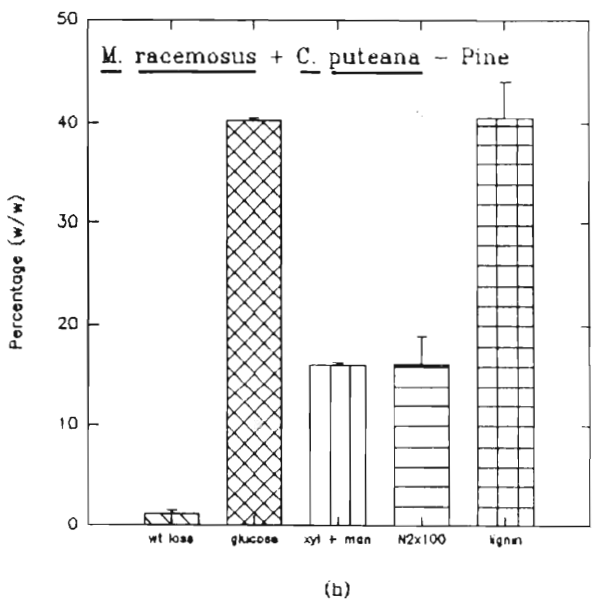
(e)



(f)



(g)



(h)

Fig 3.4 (cont'd)

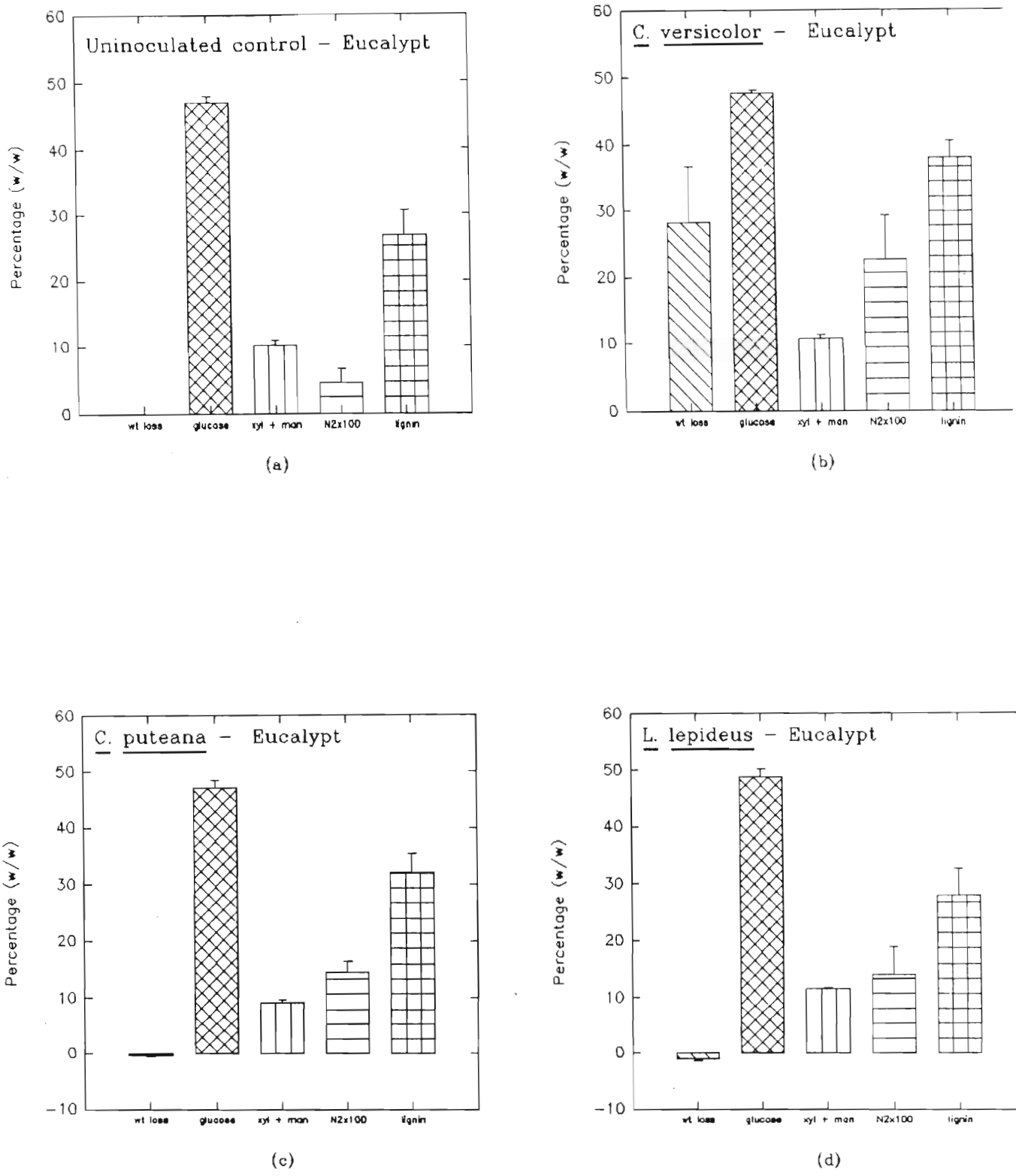
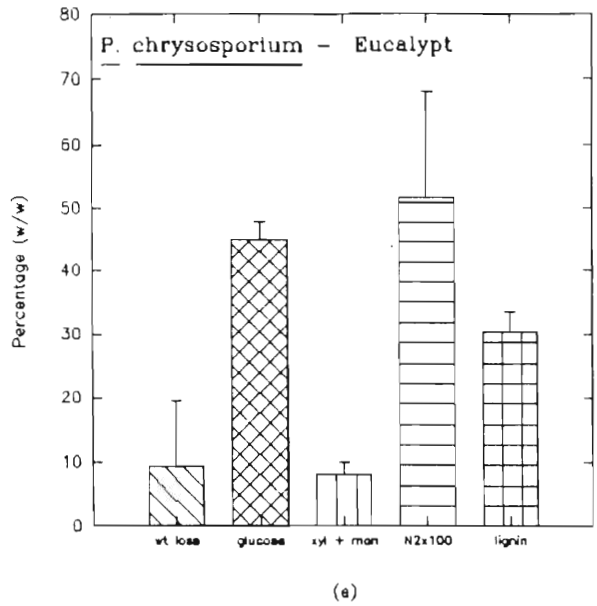
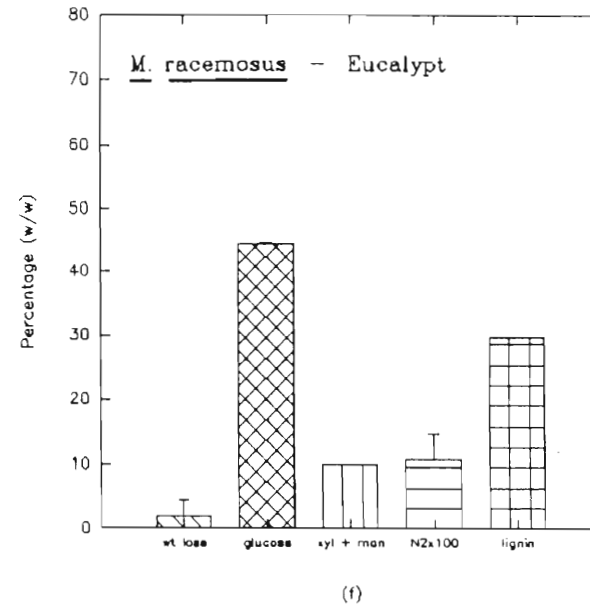


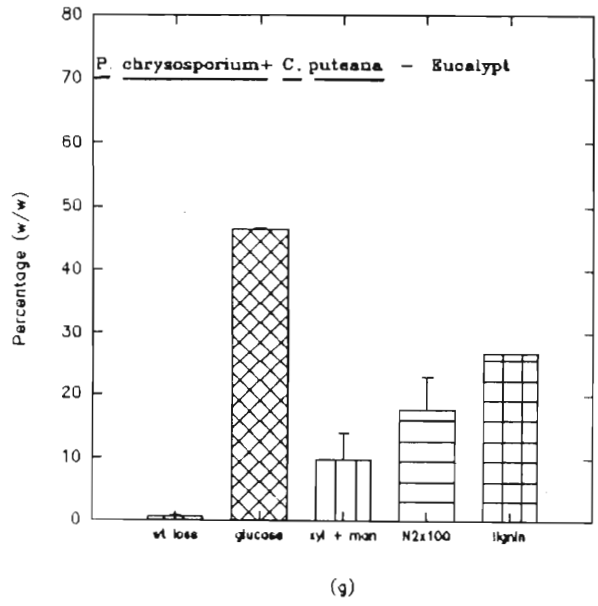
Fig. 3.5 Weight losses produced in *E.grandis* compared with the corresponding cellulose, hemicellulose, nitrogen and Klason lignin contents of *E.grandis* after its colonisation by fungi for 6 weeks.



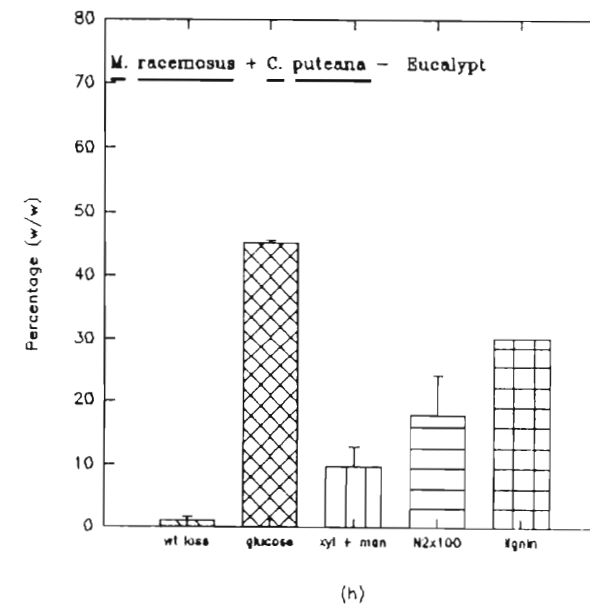
(e)



(f)



(g)



(h)

Fig 3.5 (cont'd)

3.4 DISCUSSION

As described in Chapter 1, and summarised here for the purpose of the following discussion, the constituents of a wood cell wall are cellulose, hemicellulose, lignin, extractives, minerals and water, the chemical constituents varying throughout the wall (Montgomery, 1982). Of the dry weight of wood, approximately 99% consists of cellulose and substances related to it , to which the general term " holocellulose" is applied, and lignin. Other substances such as pigments, silica, resins and gums make up the remaining 1% or so of the dry wood substance (Jane, 1970). Holocellulose accounts for approximately 70-80% of the wood cell wall and consists of a variety of carbohydrates together with certain related compounds. Approximately 40-50% of the cell wall consists of cellulose. In soft wood, the lignin content is commonly between 25 and 30 % of the dry weight; in temperate hard woods it is rather less, ranging from 19-25 %, while in tropical hardwoods it may be higher; 30 % or more has been recorded (Jane, 1970).

Limitations of the present work should be considered before detailed discussion of the present findings. From Table 3.7, it was seen that the percentage recovery of wood constituents i.e. cellulose, hemicellulose and lignin ranged between 83.2 - 98.4%. Specifically, *P.patula* recovery rates were higher and ranged between 90.5 and 98.4%, whereas the *E.grandis* rates were consistently lower than 90%, with only one value of 96.2%. Primarily, the recovery rates indicated that the analytical

methods used had been effective in measuring the proportions of cellulose, hemicellulose and lignin. An explanation of lower yields in *E.grandis* has been provided by Effland (1977) who showed that the lignins of hardwoods are more soluble during hydrolysis than those of softwoods. The results presented in Table 3.7 are therefore consistent with those of Effland (1977). Furthermore, the present findings consequently suggest that the lignin values may thus have been affected by a) gravimetric variability, and also by b) solubility, therefore this possibility should be considered when interpreting the relevant data below, particularly with regard to *E.grandis* values. Indeed, the consistently low lignin recoveries from decayed samples are quite evident as trends illustrated in the histograms presented in Fig. 3.5.

As stated above (3.3), it became clear that many of the effects produced by the cultures were slight during the incipient decay process studied here, therefore to avoid over-speculative argument, the present discussion seeks to draw conclusions from statistically significant measurements obtained. In particular, the detailed reviews of the complexity of basidiomycete enzymology in wood (Chapter 1), in conjunction with the many statistically insignificant effects shown by basidiomycetes in the present Chapter, strongly suggests that attempts to analyse these slight effects would be outside the scope of the present work. However such effects are considered in Chapter 4 with respect to possible future work of the present nature.

The work showed that after 3 weeks' incubation (Table 3.1), *C.versicolor, L.lepideus, P.chrysosporium,* and *M.racemosus* all produced weight losses in *P.patula*, with the white rotter *C.versicolor* showing the highest weight loss (10.3%). In contrast the brown rotter *C.puteana* and the cocultures *M.racemosus* + *C.puteana* and *P.chrysosporium* + *C.puteana* produced weight gains. However, all cultures produced weight losses after six weeks'colonisation, with *C.versicolor* values increasing to 24.9%. When compared with the weight losses produced in *E.grandis* (Table 3.2), *C.versicolor, P.chrysosporium* and the cocultures again produced significant weight losses after three weeks' incubation and these weight losses were higher than those recorded in *P.patula*. It is also evident that the brown rotters, viz., *C.puteana* and *L.lepideus* produced weight gains after 6 weeks' colonisation whereas these cultures had ultimately produced weight losses in *P.patula* after 6 weeks. In general therefore, the brown rotters appeared to produce less decay than the other cultures whereas the white rotters *C.versicolor* and *P.chrysosporium* consistently produced significantly higher weight losses (28.1% and 9.4% respectively) in *E.grandis*. It was therefore clear that during incipient decay, the brown rotters used in this study produced higher weight losses in *P.patula* than in *E.grandis*, whereas the white rotters produced higher weight losses in *E.grandis*. Thus, without yet considering any of the other parameters analysed, the weight losses conclusively showed that the brown rotters utilised soft wood components more extensively than those of hard woods whereas the white rotters degraded hard woods to a greater extent. Such findings are

entirely consistent with those described by Knapp (1985) and confirmed the abilities of the fungi to degrade the wood as expected of them as standard test cultures in work of the present nature.

Similarly, examination of the biomass values (Tables 3.3 and 3.4) showed that amounts of biomass produced varied according to the cultures and wood species challenged. These values were consistent with the findings of other workers (King *et al.*, 1980) who impregnated wood blocks with bacterial suspensions and therefore confirmed that all blocks in the present work had been successfully and extensively colonised by the fungi used.

The above weight gains recorded by *C.puteana* and *L.lepideus* in *E.grandis* corresponded with lag phases during incipient decay (Bravery, 1976) and are consistent with recent findings by other workers (Schippenkoetter, *et al.*, 1988) using these cultures. It would over-simplify the findings if it was concluded that nil weight losses or weight gains implied that the fungi had simply colonised but not decayed wood. While it was felt that biomass accumulation in the absence of decay would produce weight gains, it was strongly felt that weight gains should not be interpreted as showing solely that no decay had occurred. Indeed, it was considered essential that the weight loss or weight gain values should be interpreted statistically in conjunction with all other parameters measured before conclusive statements regarding the action of the various fungi on wood could be made.

Interpretation of biomass values in conjunction with the weight loss data (Tables 3.1 and 3.2) indeed provided greater insight of the colonisation process occurring during the incipient decay process. Using the above example, the white rotter *C.versicolor* produced weight lossses of 24.9% in *P.patula* and the fungal biomass produced was considered to be biologically significant at 1.95%. Therefore, when compared with the other cultures used, *C.versicolor* produced the highest weight loss and the highest level of fungal biomass in *P.patula*. In contrast however, although *C.versicolor* produced a higher weight loss in *E.grandis* (28.1%) than in *P.patula* (24.9%), the fungal biomass produced in *E.grandis* (1.1%) was significantly lower than that produced in *P.patula*, showing that even comparatively low amounts of this white rotter could extensively degrade the hard wood. This trend is clearly illustrated in Figs. 3.4 and 3.5. A similar readily apparent trend was noted when the other white rotter, *P.chrysosporium* produced weight losses of 9.4% in *E.grandis* but only 0.6% in *P.patula*. Further, when the biomass values were examined (Figs. 3.4 and 3.5) the relatively aggressive *P.chrysosporium* produced 2.95% in *E.grandis* and 1.18% in *P.patula*. Thus it is evident that this white rotter also degraded the hardwood but weight loss levels in *P.patula* were negligible in spite of having produced considerable biomass in this wood. Such findings allow one to confirm that the lack of wood degradation in *P.patula* by *P.chrysosporium* was not attributable to failure to colonise the wood. Instead, it was concluded that the fungus did not degrade *P.patula* during the incipient decay process.

When the brown rotters *C.puteana* and *L.lepideus* were considered, the value or importance of biomass determinations became very clear. In *E.grandis*, these fungi produced weight gains and the biomass levels recorded were substantial. Similarly, in *P.patula*, these fungi produced biologically insignificant weight losses, while their biomass levels were 1.21% and 1.26% respectively. The above results obtained from monocultures collectively show that during the early stages of decay, when zero or nil weight losses occur, the biomass levels play a very important role in the interpretation of what actually occurs during the early stages of decay.

In summary, all cultures produced significant biomass as reflected by nitrogen increases in colonised blocks (Figs. 3.4 and 3.5). However during these early stages of decay, only *C.versicolor* and *P.chrysosporium* produced biologically significant weight losses in the wood, in conjunction with the highest biomass levels recorded. This study has therefore shown conclusively that although weight gains were recorded by these brown rotters, thereby implying that no decay occurred, both woods were extensively colonised by these fungi. Although decay had not progressed to a significant extent, the measurable and biologically significant extent of colonisation (biomass) indicated that the onset of wood decay was imminent. It was therefore concluded that the biomass determinations had provided important additional information on the likelihood of the onset of wood decay, particularly in those cases where weight loss determinations alone indicated that decay had not occurred.

However, biomass analyses alone did not identify the ratios in which each member of a coculture were present in wood, i.e., it was difficult to assess with certainty, whether one fungus had inhibited another. However, on the basis of weight loss and biomass data it was possible to make limited interpretation of certain events occurring in wood colonised by cocultures of basidiomycetes. For example it was stated above that *P.chrysosporium* had produced high weight losses in *E.grandis* whereas *C.puteana* had not (Table 3.2). Cocultures of these fungi produced lower weight losses than did *P.chrysosporium* monocultures, indicating that *C.puteana* had colonised the wood to an extent which inhibited *P.chrysosporium*. Similar trends were observed in *P.patula*. Furthermore, *C.puteana* also appeared to inhibit the effects of *M.racemosus*, particularly in *P.patula* (Table 3.1) where weight losses produced by the latter were lower in coculture than in monoculture. It could again be speculated that the initial weight gain produced by *C.puteana* in *P.patula* showed that this fungus had produced much biomass in the wood, which could in turn possibly explain its ability to outcompete and inhibit *M.racemosus* in coculture. Clearly, it was impossible to make conclusive comments of this nature based solely on weight loss data of the cocultures, however, biomass values, with the sugar and lignin analysis discussed below, confirm many such speculations.

The example used above showed that *C.puteana* colonised *P.patula* extensively (Table 3.3) without weight loss (Table 3.1) and it is therefore possible to conclude on the basis of weight loss and

biomass data that *C.puteana* had inhibited any decay potential of *M.racemosus* in this wood (Table 3.1). A similar trend occurred in *E.grandis* (Table 3.2) and with the basidiomycete cocultures.

It was argued in the Introduction of this Chapter that sugar and lignin analyses would provide the additional information required to show which fungus in a given coculture exerted the greater effect on the wood, or inhibited the other fungus, because, by ascertaining how the proportions of the ratios of cellulose : hemicellulose : lignin change during degradation, it should be possible to establish :

i) whether the wood has been degraded or not,

ii) which fractions of the lignocellulosic matrix have been utilised, and,

iii) the physiological nature of the decay fungus.

However, as mentioned above (3.3.3) and discussed below, it should be noted that depletion of each fraction equally will result in no change to the above ratio and in the absence of any weight loss values, may thus appear to suggest that no decay had occurred. Before considering cocultures, these concepts should be explored in examination of the results obtained with monocultures.

The Introduction (Chapter 1) reviewed white rot in wood by basidiomycetes which deplete lignin but which also have the capacity to remove other cell wall components. However, for the purposes of the present discussion, it should now be noted that

some species are selective and preferentially attack lignin without extensively removing cellulose and hemicellulose, although other species remove all cell wall components concurrently (Cowling,1961). A white rot fungus which previously has received considerable study, *Trametes versicolor*, is not selective for lignin removal and degrades all cell wall components simultaneously (Wilcox, 1968). The decay caused by this fungus has been referred to as a simultaneous rot (Liese, 1970). From Fig. 3.4, it is evident that *C.versicolor* in *P.patula* utilised cellulose and hemicellulose without changing the amount of lignin significantly. Thus this fungus behaved primarily as a brown rotter in Pine during the early stages of decay. In Eucalypt, however, the ratios of cellulose : hemicellulose : lignin did not change significantly from that of the control. However, biomass was produced and weight losses were generated, therefore wood constituents must have been degraded uniformly. This is consistent with the above description of this fungus as a simultaneous rotter. These findings are also consistent with those of Blanchette *et al.* (1988) where lignin and sugar analyses of wood decayed for 3 months by *T.versicolor* indicated that lignin, cellulose and hemicellulose were removed in approximately the same proportions in which these compounds are found in the wood.

Interestingly,the results obtained in the case of *M.racemosus* were unexpected. Although the results of Chapter 2 suggested that this fungus was not a wood degrader, *M.racemosus* in *P.patula* produced a significant decrease in the proportion of

lignin, thereby suggesting that it is ligninolytic which in turn would imply that it degraded only lignin, as the proportions of cellulose and hemicellulose increased (Table 3.5). This fungus was isolated from the rumen of sheep and the role of the recently discovered facultatively anaerobic Zygomycetes in the rumen has not yet been established (Chapter 2). In *E.grandis*, however, a decrease in the proportion of cellulose occurred while the proportion of hemicellulose remained unchanged and the proportion of lignin increased (Table 3.6). These findings suggest that contrary to the findings of the short term studies of Chapter 2, *M.racemosus* is cellulolytic in *E.grandis* after a lag phase has elapsed.

When *C.puteana* colonised *P.patula*, the proportion of hemicellulose decreased with a slight decrease in cellulose while the proportion of lignin correspondingly increased. Similarly, in *E.grandis*, the proportion of hemicellulose decreased with no change in cellulose and the proportion of lignin again increased, representing more of the wood residue when other components were depleted preferentially. The present findings are therefore consistent with the description of *C.puteana* as a brown rotter (Knapp, 1985) and have demonstrated its brown rot physiological capacity in both a hardwood and a softwood. In the same context *P.chrysosporium* acted predominantly as a brown rotter in *E.grandis* and the proportions of cellulose and hemicelluloses decreased while lignin increased. In contrast however, this fungus behaved as a simultaneous rotter, i.e., as a non selective wood degrader in that biomass produced by this fungus in *P.patula*

corresponded with simultaneous removal of all cell wall components. It will be recalled that the biomass production also correlated with nil weight losses in *P.patula* (Fig. 3.4) which underscores a major point argued in this thesis, viz., the importance of considering all analytical data holistically in order to meaningfully interpret the events taking place during incipient decay. In the present case, the nil weight losses actually reflected *P.chrysosporium* biomass development in conjunction with simultaneous rot of *P.patula* components, but this finding could not have been made without the analytical determinations described.

As far as the cocultures are concerned, *M.racemosus* + *C.puteana* in *P.patula* produced a decrease in the proportion of hemicellulose, slight decrease in cellulose and an increase in lignin proportions. Since *M.racemosus* was firstly, shown to be ligninolytic and secondly, when grown as a coculture with *C.puteana*, to produce no evidence of ligninolysis, it was clear that *C.puteana* in *P.patula* was antagonistic towards *M.racemosus*. Furthermore,the relevant biomass and weight loss data (Fig. 3.4) supported this finding, and cocultured *C.puteana* behaved in *P.patula* as it had done as a monoculture. In *E.grandis*, however, a degree of synergism was implied as the proportions of cellulose and hemicellulose decreased and the proportion of lignin was increased by the coculture (Fig. 3.5). However, *C.puteana* in monoculture did not change the proportion of cellulose while the proportion of hemicellulose decreased. In contrast, *M.racemosus* in monoculture reduced the proportion of cellulose with no change

in hemicellulose proportions. As a coculture, however, these fungi together, were able to reduce the proportions of both cellulose and hemicellulose which they could not achieve as monocultures. These findings again illustrate the value of analytical information when examining events taking place during wood degradation, especially when more than one fungus is involved, and emphasise the danger of interpreting weight loss and biomass data in isolation.

P.chrysosporium and *C.puteana* as cocultures in *P.patula* significantly decreased the proportion of lignin and hemicellulose and slightly decreased cellulose content. However the decrease in the proportion of lignin produced by the coculture was greater than that produced when *P.chrysosporium* colonised *P.patula* as a monoculture. *C.puteana* as a monoculture did not degrade lignin at all and hence, when cocultured in *P.patula*, these fungi selectively degraded lignin to a greater extent than *P.chrysosporium* as a monoculture. Hence there exists a degree of synergism between *P.chrysosporium* and *C.puteana* in *P.patula*. These findings again illustrate the degree of caution which must be exercised when interpreting biomass data in isolation. For example, on the basis of weight loss and biomass values it was reasoned above that *C.puteana* had inhibited *P.chrysosporium*, however the present sugar and lignin analyses confirmed that in fact, the ligninolytic effects of the latter were enhanced in coculture with *C.puteana*, showing that synergistic degradation had occurred.

In the hard wood, viz., *E.grandis*, the proportions of cellulose, hemicellulose and lignin were not significantly altered by this coculture in comparison with that of the control, although weight losses confirmed that decay had occurred, *P.chrysosporium* behaved as a brown rotter in *E.grandis* (Fig. 3.5e) whereas *C.puteana* did not change the proportion of cellulose (Fig. 3.5c) although hemicelluloses decreased and the proportion of lignin increased. However as a coculture in *E.grandis* (Fig.3.5g), a synergistic relationship clearly existed since this coculture behaved as a simultaneous rotter.

3.5 CONCLUSIONS

This study has shown that for the different fungi used, the changes in the proportions of cellulose, hemicellulose and lignin values, in conjunction with weight loss and biomass values, provided important insight into the incipient decay process. The ratios of cellulose, hemicellulose and lignin residues determined by analysis of decayed wood can be interpreted to determine the physiological nature of the responsible fungi as follows :

i) in the case of aggressive fungi which produce large weight losses in wood, cellulose : hemicellulose : lignin ratios readily illustrated the capacity of given fungi to exclusively produce brown or white rot, or a combination of these detectable as simultaneous rot;

ii) when low weight losses occurred in conjunction with biomass development during the early stages of decay, the determination of the type of decay fungi present was not predictable, although the occurrence of decay could be established on analysis;

iii) it became possible to identify antagonistic or synergistic relationships between fungi during incipient decay.

On the basis of the above conclusions, and those drawn in Chapter 2, it was decided that the theme of the General Discussion presented in Chapter 4 should firstly review these experimentally derived conclusions, before considering the applications of this work.

CHAPTER 4 GENERAL DISCUSSION AND CONCLUSIONS

The diverse range of environments in which wood is attacked by microorganisms includes both marine and fresh-water ecosystems, e.g., the seas, rivers, log ponds and water cooling towers, land environments such as buildings and wooden constructions, timber yards, sawmills and pulpmills (including wood-chip piles, storage stacks and seasoning stacks), soil and forest litter beds. All these environments, exhibit to varying extents, fluctuations in aeration, moisture presence, temperature, pH, geographic location, situation and wood type. Thus it is evident that the environment in which wood biodeterioration occurs is very variable, with each environment having a wide range of indigenous microorganisms.

Owing to its economic importance, the decay of wood has been extensively studied in the past as reviewed in Chapter 1. However the overall process of the biodeterioration of wood is still not completely understood (Smith, 1980; King *et al.*, 1981). Under normal conditions the main agents of wood decay are fungi, bacteria only being significant in waterlogged timber. However recent studies have shown that although bacteria do not degrade wood at rates comparable with those of fungi, they are considered as being the primary colonisers of wood, and may therefore create environments that favour the growth and proliferation of the wood decay fungi (Rogers and Baecker, 1988a,b,c; Singh *et al.*, 1990). Wood rotting fungi are traditionally separated into different groups according to their mode of action and effects on the wood

structure. These groups include the blue stain fungi; the soft rot fungi, the brown rot fungi and the white rot fungi. Since fungi are regarded as the principal degraders of wood, most enzymological studies of wood decay have concentrated on the enzymes, mainly on cellulases and hemicellulases, produced by these wood degraders (Jurasek and Paice, 1988) and more recently (Srebotnik and Messner, 1988; Daniel *et al.*, 1989; Srebotnik and Messner, 1990) on the lignin peroxidase enzymes.

Studies of bacterial degradation of wood under anaerobic conditions (Rogers and Baecker, 1988a,b; Singh *et al.*, 1990) led to the concept of degradation of wood by anaerobic fungi (Wiederhold *et al.*, 1989). As stated in Chapter 1, anaerobic fungi represent a new group of organisms inhabiting the rumen ecosystem and possess a life cycle alternating between a motile flagellated form (zoospore) and a non-motile vegetative reproductive form (thallus). Developments from *in vivo* and *in vitro* studies leave little doubt that anaerobic fungi make a significant contribution to rumen metabolism, particularly in the digestion and subsequent fermentation of plant structural materials (Mountford, 1987). The precise quantification of the contribution of the anaerobic fungi to overall rumen metabolism still needs to be assessed. *In vivo* determination of fungal biomass and associated enzymatic activities would be required and this would not be without difficulties particularly in situations where extensive penetration of plant tissues is produced by fungal rhizoid.

The nature of the life cycle of anaerobic fungi would pose additional problems. Whether these fungi are truely primary invaders of plant tissue facilitating secondary attack by fermentative bacteria is open to question. It has been noted, however, that at least in the case of *N.frontalis* the pattern of cellulase production is similar to that observed for several rumen cellulolytic bacteria (Bauchop and Mountford, 1981). The role of anaerobic fungi in the degradation of hemicelluloses has received meagre attention and much more information is required in the elucidation of the fermentation patterns and the enzymes involved. In this regard, it was unfortunate that anaerobic fungi could not be cultured on the wood tested in the present work, since the analyses of such wood could have provided important information on hemicellulolysis.

Although the obligately anaerobic fungus, *N.frontalis*, showed very sparse colonisation of wood, and hence did not permit further study of wood degradation using this fungus, the facultatively anaerobic fungi produced extensive colonisation of wood elements. The three facultatively anaerobic, dimorphic fungi, viz., *M.racemosus, C.tropicalis* and *G.capitatum*, were isolated from the rumen of sheep (Chapter 2) and each colonised the softwood *P.patula* and the hardwood *E.grandis* extensively. However, the scanning electron micrographs (Chapter 2) showed no evidence of decay. *M.racemosus* colonised both *P.patula* and *E.grandis* to a greater extent than the other two isolates and the delamination of wood elements by *M.racemosus* was similar to the delamination of grass fibre elements by rumen fungi (Morrison and

Mackie, 1989). This phenomenon could increase surface to volume ratios of fibrous material and contribute to the biodeterioration of wood, especially under conditions of reduced aeration where many common wood decay fungi could not compete. The three facultative anaerobes were shown to be neither cellulolytic nor xylanolytic and hence several questions were considered regarding the role of these fungi in the rumen (Chapter 2).

The basidiomycetes (*C.versicolor, C.puteana, L.lepideus* and *P.chrysosporium*) used in this study (Chapter 2) colonised wood extensively under aerobic conditions. Under anaerobic conditions, however, the extent of colonisation was not as extensive as that observed under aerobic conditions. Importantly, the scanning electron micrographs showed clearly (Chapter 2) that it is difficult to identify these fungi readily using microscopy, especially when present as mixed fungal populations. Normally the presence of clamp connections, basidia and basidiospores would enable one to establish that the fungus is a basidiomycete. The present work has shown that not all basidiomycetes produced clamp connections in the wood which made it difficult to identify these fungi with certainty. *P.chrysosporium* produced numerous basidia and basidiospores and hence it was possible to microscopically identify only this fungus with certainty when present in decayed wood.

Unfortunately, although the scanning electron micrographs of these fungi in wood (Chapter 2) provided valuable information, such as colonisation patterns and the degree or extent of colonisation, the work was qualitative.

Because of the various disadvantages associated with the conventional methods used to quantify wood degradation (3.1.1), it was decided to examine the use of analytical methods, to permit more informed interpretation to be made of microbiological effects in wood during incipient decay. Thus the parameters examined included weight loss determinations (for reference purposes), biomass determinations, wood sugar determinations (cellulose and hemicellulose) and Klason lignin determination.

Fungal biomass determined analytically (Chapter 3) confirmed that statistically significant amounts of biomass were produced by the monocultures and cocultures of *M.racemosus*, *C.versicolor*, *C.puteana*, *L.lepideus* and *P.chrysosporium* which had colonised wood extensively in Chapter 2.

One of the most important findings of the present work was that this study clearly showed the dangers of interpreting weight loss data in isolation, especially during incipient decay, where low weight losses or weight gains were recorded. For example the biologically insignificant weight losses in *P.patula* and weight gains in *E.grandis* after 6 weeks' colonisation by *C.puteana* implied that no decay had occurred. However, the substantial amounts of biomass produced showed that the fungus had colonised

both woods extensively. The weight gains recorded corresponded with lag phases during incipient decay (Bravery, 1976) and are consistent with recent findings by other workers (Schippenkoetter *et al.*, 1988) and the cellulose, hemicellulose and lignin values provided some insight of what was actually occurring during the incipient decay process. Therefore, in spite of undetectable weight losses, the analyses conducted allowed this writer to confirm that *C.puteana* had demonstrated its brown rot physiological capacity (Knapp, 1985) in both a hardwood and a softwood. Similarly *P.chrysosporium* was shown by analysis to behave as a brown rotter in *E.grandis* and a simultaneous rotter in *P.patula* while *C.versicolor* behaved as a simultaneous rotter in *E.grandis*.

Interestingly, *M.racemosus* was shown to be cellulolytic in *E.grandis* (Chapter 3) although previous enzymatic investigations (Chapter 2) suggested that it was neither cellulolytic nor xylanolytic. A possible explanation of this anomaly is that ball milled filter paper was used in the Cellulose Agar to determine whether this fungus was cellulotytic. This fungus possibly degrades the native cellulose of *E.grandis* thereby accounting for the decrease in the proportion of cellulose in *E.grandis*. However, the reason for its ability to degrade native cellulose while unable to degrade ball milled filter paper cellulose is presently unknown. In *P.patula*, on the other hand, *M.racemosus* was shown to be ligninolytic.

In summary therefore, it is proposed that the importance of analysing and interpreting weight loss data in conjunction with biomass, cellulose and hemicellulose, and lignin data cannot be overemphasised. As illustrated by the above examples, the analytical methods used provided knowledge of which fractions of the lignocellulosic matrix were utilised, and thus greater insight of what occurs during incipient decay. Such data undoubtedly provided information which superseded that of the conventional methods of quantifying wood decay. Moreover, analyses such as those performed here would allow a scientist to confirm that unknown wood samples had been decayed, or colonised by decay fungi.

Similarly, the information generated using the analytical techniques permitted the examination and interpretation of events which occurred during incipient decay by cocultures. The microbiological effects of cocultures in wood have not previously been examined as shown in this work. This study has shown that the analytical methods used were sufficiently sensitive to detect changes in the proportions of wood components, which in turn permitted identification of the synergistic and antagonistic relationships between the fungi. It is believed that longer term tests using cocultures could markedly demonstrate synergistic or antagonistic phenomena more clearly. As far as the monocultures are concerned, this study has shown that for the different fungi used, the changes in the proportions of cellulose, hemicellulose and lignin in conjunction with biomass and weight loss data provide important insight into the incipient decay process.

Although no other study has researched incipient decay using the analytical methods described here, Blanchette *et al.* (1988) has used longer term (12 weeks) decay tests which produced higher weight losses than those recorded here, and the changes noted in the relative proportions of cellulose : hemicellulose : lignin readily illustrated the capacity of given fungi as either white or brown rot. Unfortunately that study did not quantify biomass, whereas the present study has shown that biomass values are crucial in order to make conclusive statements regarding the degree of colonisation of samples, especially during incipient decay where low weight losses or weight gains are often recorded.

Current studies on wood decay by microorganisms have focussed primarily on fungi. Most studies have been conducted using monocultures. Since bacteria are the primary invaders of wood, and the succession of microbes in wood have been established to some extent, future work should examine the use of consortia of microbes rather than monocultures, as wood decay under normal conditions involves numerous microbes, with each organism having its own role to play. With regard to the applications of the work described in this thesis, the present investigations of fungal degradation of wood, has provided information of physiological differences among decay types. Fungi that selectively degrade wood are of particular interest. The biotechnological potential of fungi that delignify wood is enormous. One use currently being tested includes the pretreatment of wood chips with fungi that selectively remove lignin for pulp and paper production. For example, pretreated

chips with selected strains of *P.chrysosporium* and *Ceriporiopsis subvermispora* improved paper strength properties while requiring up to 68% less energy than standard mechanical pulping procedures (Blanchette *et al.*, 1991). Work of the present nature would be of value in screening suitable organisms for use in chip pretreatment.

Another aspect of white rot fungi which may be of significant use is their ability to "bleach" wood. The effluents produced during chemical bleaching of kraft pulping are major contributors to water pollution, and new methods of bleaching wood pulp with fungi or their enzymes may provide a viable substitute for some of the chemicals now used (Paice *et al.*, 1989; Shelver *et al.*, 1991). Delignification processes may also be used to modify wood or agricultural products to increase the nutritional value for ruminant animal feed. The digestibility of straw, bagasse and wood can be dramatically increased when treated with various white rot fungi, and have shown no problems of pallatibility or toxicity (Farrell, 1987). Investigations showing that fungi with high ligninolytic activity are able to degrade environmental pollutants such as PCB, DDT, dioxins, industrial dyes and chlorinated phenols (Bumpus *et al.*, 1985) are also of major significance. Again, it is proposed that work of the present nature would be of use in screening procedures to isolate ligninolytic fungi.

In summary, there seems no doubt that current trends in quantification of fungal degradation of wood follow the analytical route, and that many applications of such work exist. The ability of decay fungi to modify their ionic environment is of potential relevance to their ability to colonise and degrade wood. In a recent study, Jellison *et al.* (1992) used plasma emmission and atomic absorption spectrophotometry to examine changes in the concentration and composition of cations within wood colonised by brown and white rot fungi. That study showed that in Spruce degraded by *P.chrysosporium* to 58% weight loss, calcium, magnesium and manganese contents were significantly higher in degraded wood. Furthermore, McCormack *et al.* (1992) examined a Nuclear Magnetic Resonance Spectroscopic technique of measuring Ti spin-lattice relaxation times for its potential in quantification of the microbial decay of wood. Their results indicated that NMR technology may provide a useful tool for the non-destructive evaluation of the molecular changes in wood resulting from fungal decay. Essner and Tas (1992) have examined the detection of dry rot by air analysis using gas chromatography mass spectrometry. Their findings show that each fungus has its own specific "blue print" of volatile metabolic compounds. Thus air analysis could have application as another method for detecting decay caused by fungi. It is proposed that future work should compare the efficacies of these techniques with the present methods in order to select the most practical method for rapid, on-site, assessment of the decay status of stockpiled wood or other forest products.

In conclusion, the present study has shown that the fungal degradation of wood can be quantified using the analytical techniques described above. Statistical analyses of the results obtained have enabled the writer to identify the patterns of wood sugars predominantly utilised by certain fungi.

Two objectives were set in this work to challenge the working hypothesis that "degradation of wood by fungi can be analytically detected and quantified during the early stages of its colonisation". These objectives were achieved and having produced the culture collection necessary to study incipient decay, the major conclusions drawn from the results were :

i) the extent of wood colonisation by vigorous basidiomycetes was quantifiable and correlated in some cases with the degree of decay measured gravimetrically and by analysis of ligninolytic or cellulolytic activity, and,

ii) when lag phases preceeding the onset of decay had not been completed, the progress of the incipient decay process was analytically quantifiable by biomass assays, and also in some cases, also by cellulose, hemicellulose and lignin assays.

The lack of definitive correlations between the measured wood decay activities of the less vigorous fungi, and cocultures of these, clearly illustrated the complexity of the physiological events which take place in wood during its incipient decay.

In view of the above findings, it was therefore concluded that analysis of cellulose : hemicellulose : lignin ratios of decayed wood could be used to establish the predominant physiological types of fungi which had degraded fresh or ancient specimens even if the responsible fungi are no longer viable or culturable. Modern molecular biological techniques such as PCR and DNA probes will resolve the problem of identifying the fungi at the genus or species level.

A secondary major benefit of these investigations should be the increased comprehension of microbial degradation mechanisms which would undoubtedly improve the understanding of pathological relationships between decay fungi and wood.

APPENDIX 1

Caldwell and Bryant's Medium No.10 (Caldwell and Bryant, 1966).

I. Mineral solution 1 (40x final concentration)

K_2HPO_4	11.8442 g/l

II. Mineral solution 2 (40x final concentration)

KH_2PO_4	7.0766 g/l
$(NH_4)_2SO_4$	17.9710 g/l
NaCl	1.7768 g/l
$MgSO_4$	3.7466 g/l
$CaCl_2.2H_2O$	2.4111 g/l

The latter two constituents were individually predissolved in distilled water and added dropwise with stirring. Both mineral solutions were stored at room temperature in the undiluted state, as they were sufficiently concentrated to prevent microbial growth.

III. Haemin solution

Haemin solution was prepared by adding iomg of haemin to 100ml of solution containing 25% ethanol and 0.05M KOH. Storage was at 4C.

IV. Indigocarmine solution

Indigocarmine	0.5 g/100ml

Stored at 4C.

V. Volatile fatty acid salts solution

Acetic acid	178.33 g
Propionic acid	59.60 g
Butyric acid	38.36 g
Isobutyric acid	9.50 g
n-valeric acid	9.39 g
Isovaleric acid	9.31 g
DL-a-methylbutyric acid	9.41 g

Sodium hydroxide (10M) (460ml)was measured out and approximately 250ml of this was transferred to a stirred beaker surrounded with crushed ice. The weighed out acids were slowly poured along a glass rod into the chilled alkali while stirring. The remaining alkali was then added and the entire contents were transferred to a 1l volumetric flask and made up to volume using distilled water.

VI. Vitamin solution

Vitamin stock solution contained 400mg of each of the following:

Pyridoxine.HCl
Pyridoxamine.2HCl
Thiamine.HCl
Nicotinamide
Ca-D-pantothenate

20mg of each of p-aminobenzoate and biotin
2mg of folic acid and 0.1mg of cyanocobalamin (Vitamin B_{12})

Cyanocobalamin, folic acid and biotin were dissolved individually in minimal amounts of 0.1M NH_4HCO_3, before being added to the mixture. The solution was made up to 2l total volume with distilled water. This stock solution was filtered through Whatman No.1 filter paper, filter sterilised (0.22um) and 100ml aliquots were aseptically added to sterile 200ml glass infusion bottles. All bottles were stored at 4C.

VII. Sulphide solution

12.5g of sodium sulphide was weighed off into a stoppered 100mm measuring cylinder, being purged with N_2 gas. The volume was made upto 100ml using boiled distilled water. This solution was then added to a N_2 purged 250ml glass ampoule with a screw cap containing abutyl rubber septum. The ampoule was sterilised at 121C for 25 minutes.

VIII. Cysteine solution

This solution was prepared as described in VII above using 12.5g of Cysteine.HCl.

IX. Sodium carbonate solution

39.6g of Na_2CO_3 was added to a 1l volumetric flask and brought up to volume with distilled water. This solution (400ml) was combined with 100ml VFA solution in a 1l glass bottle.

The above components (I - IX) were used in the preparation of Medium No.10.

Medium No. 10. (Caldwell and Bryant, 1966)

	Concentration/l
Peptone	2 g
Mineral Solution 1[I]	25 ml
Mineral solution 2[II]	25 ml
Haemin solution[III]	10 ml
Indigocarmine[IV]	1 ml
VFA solution[V]	10 ml
Vitamin solution[VI]	10 ml
Sulphide solution[VII]	2 ml
Cysteine solution[VIII]	2 ml
Na_2CO_3 solution[IX]	40 ml

Bring up to volume with distilled water.

The above medium may be supplemented with one of the following :

Cellobiose	0.3%
Ball milled wood	1.00%
Ball milled cellulose	1.00%
Glucose	0.3%
Xylan	3.00%
Avicel	1.00%

The bulk medium is purged with anaerobic gas mixture before and directly after sterilisation.

APPENDIX 2

Composite Medium

	Concentration/500 ml
Malt Extract Agar	12.5 g
Potato Dextrose Agar	9.75 g
Clarified Rumen Fluid	200.00 ml

Bring up to volume with distilled water.

Sterilise at 121C for 15 minutes. pH after autoclaving (6.8). Gas the medium with the anaerobic gas mixture and add reducing agents aseptically.

Na_2S	1 ml
Cysteine HCl	1 ml

Transfer medium to anaerobic cabinet and allow to cool to 50C before pouring into plates.

APPENDIX 3

3% Glutaraldehyde in 0.1M Sodium Cacodylate buffer.

3ml of a 25% solution of glutaraldehyde
12.5ml of 0.2M cacodylate buffer
Make up to 25ml with distilled water

Store at 4C and use within 24h.

APPENDIX 4

0.5% Cellulose Agar

In to a 2l Schott bottle containing a teflon-coated magnetic stirrer bar measure, while stirring on a hotplate/magnetic stirrer :

Distilled water	255.0ml
Mineral Solution 1	25.0ml
Mineral Solution 2	25.0ml
Clarified rumen fluid	400.0ml
Whatman cellulose (2.0% m/v ball milled suspension)	250.0ml
Indigocarmine (0.5% m/v solution)	1.0ml
Agar	5.0ml

Bring the contents of the bottle to the boil while stirring vigorously in order to expel dissolved oxygen and to melt the agar. Fit the bottle with a multiple inlet/outlet head (Fig.2.2). Clamp off tubing between gas inlet filter and the head, but leave the connection to the gas outlet filter open. Sterilise the medium at 121C for 30 minutes. After sterilisation attach the inlet gas filter to a supply of anaerobic gas mixture, unscrew the clamp and gas at a rate of about 5l/h, while allowing the medium to cool to about 50C. Inject through the inlet port, with aseptic precautions :

Na_2CO_3	40.0ml
Cysteine.HCl	2.0ml
$Na_2S.9H_2O$	2.0ml

Gas for a further 10 minutes, while stirring, then clamp off inlets and outlets and disconnect from gas supply. Transfer bottle to anaerobic cabinet, and remove all clamps. Allow temperature of medium to drop to 50C while swirling to keep the insoluble substrate in homogenous suspension and pour medium into Petri dishes (10ml per plate), and mix well to ensure even distribution of cellulose in medium.

APPENDIX 5

Maintenance Medium (Bryant and Robinson, 1961)

I. Component	Concentration/500ml
Agar	1 0.0 g
Mineral solution 1	118.75 ml
Mineral solution 2	218.75 ml
D-glucose	0.165 g
Cellobiose	0.165 g
Xylan	0.33 g
Soluble starch	0.33 g
Clarified rumen fluid	200.0 ml
Indigocarmine solution	5.0 ml
Deionised water	257.5 ml

II. Mineral solution 1

3g K_2HPO_4 was dissolved and made up to 500ml with deionised water.

III. Mineral Solution 2

6g NaCl, 6 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.6 g$CaCl_2$ and 1.23 g $MgSO_4.7H_20$ were dissolved and made up to 500ml with deionised water.

APPENDIX 6

Identification of *C.tropicalis*.

Name Keys to Candida tropicalis ref.: F6

Fermentation	
F 1 glucose	+
F 2 galactose	+
F 3 maltose	+
F 4 methyl α-glucoside	
F 5 sucrose	+
F 6 trehalose	
F 7 melibiose	
F 8 lactose	
F 9 cellobiose	
F10 melezitose	
F11 raffinose	
F12 inulin	
F13 soluble starch	
F14 xylose	

colonies: red/pink/orange/yellow/buff/cream/white; fluid/mucoid/butyrous/membranous;
budding/stalks/fission; apiculate cells;
filaments: none/primitive/well developed
pseudohyphae/septate hyphae

arthroconidia/endospores/chlamydospores
ballistoconidia: none/symmetric/asymmetric

asci: none/evanescent/persistent
ascospores: round/oval/conical/reniform/cap-/hat-/saturn-/walnut-/needle-shaped/whip- like;
rough/smooth; ____ per ascus
teliospores/basidia: none/____
conjugation: cells/cell-bud/clamps ____

Growth 25 °C				date: 26/10/90 conditions Potator			
C 1 D-glucose	+			C34 5-keto-D-gluconate	+		
C 2 D-galactose	+			C35 D-gluconate	+		
C 3 L-sorbose	+			C36 D-glucuronate	−		
C 4 D-glucosamine	D			C37 D-galacturonate	−		
C 5 D-ribose	D	W		C38 DL-lactate	D		
C 6 D-xylose	+			C39 succinate	+		
C 7 L-arabinose	−			C40 citrate	+		
C 8 D-arabinose	−			C41 methanol	−		
C 9 L-rhamnose	−			C42 ethanol	−		
C10 sucrose	+			C43 propane 1, 2 diol	D	W	
C11 maltose	+			C44 butane 2, 3 diol	−		
C12 α, α trehalose	+			C45			
C13 methyl α-glucoside	+						
C14 cellobiose	D			N1 nitrate −ve			
C15 salicin	D			N2 nitrite −ve			
C16 arbutin				N3 ethylamine +ve			
C17 melibiose	−			N4 L-lysine			
C18 lactose	−			N5 cadaverine			
C19 raffinose	−			N6 creatine			
C20 melezitose	+			N7 creatinine			
C21 inulin	−			N8 glucosamine			
C22 soluble starch	+			N9 imidazole			
C23 glycerol	D						
C24 *meso* erythritol	−			V1 w/o vitamins +			
C25 ribitol	+			V2 w/o inositol			
C26 xylitol	D			V3 w/o pantothenate			
C27 L-arabinitol	−			V4 w/o biotin			
C28 D-glucitol	+			V5 w/o thiamin			
C29 D-mannitol	+			V6 w/o bio + thia			
C30 galactitol	−			V7 w/o pyridoxine			
C31 *myo*-inositol	−			V8 w/o pyr + thia			
C32 glucono δ-lactone	+			V9 w/o niacin			
C33 2-keto-D-gluconate	+			V10 w/o PABA			

T 25°C ____ T 30°C ____ T 35°C ____
T 37°C ____ T 42°C +ve

01 0.01% cycloheximide +
02 0.1% cycloheximide +
03 1% acetic acid ____
04 50% glucose ____
05 60% glucose ____

M1 starch production ____
M2 acetic acid production ____
M3 urea test ____
M4 DBB reaction −ve

APPENDIX 7

Identification of *G.capitatum*.

Name Keys to Geotrichum capitatum. ref.: F 1

Fermentation	negative		
F 1 glucose	−ve	creeping pellicle	
F 2 galactose	−ve		
F 3 maltose	−ve		
F 4 methyl α-glucoside			
F 5 sucrose	−ve		
F 6 trehalose			
F 7 melibiose	−ve		
F 8 lactose			
F 9 cellobiose			
F10 melezitose			
F11 raffinose			
F12 inulin			
F13 soluble starch			
F14 xylose			

colonies: red/pink/orange/yellow/buff/cream/white; fluid/mucoid/butyrous/membranous;
budding/stalks/fission; apiculate cells;
filaments: none/primitive/well developed
pseudohyphae/septate hyphae

arthroconidia/endospores/chlamydospores
ballistoconidia: none/symmetric/asymmetric

asci: none/evanescent/persistent
ascospores: round/oval/conical/reniform/cap-/hat-/saturn-/walnut-/needle-shaped/whip- like;
rough/smooth; _____ per ascus
teliospores/basidia: none/_____
conjugation: cells/cell-bud/clamps _____

Growth 25 °C			
C 1 D-glucose	+ve		
C 2 D-galactose	+ve		
C 3 L-sorbose	D		
C 4 D-glucosamine	−		
C 5 D-ribose	−		
C 6 D-xylose	−		
C 7 L-arabinose	−		
C 8 D-arabinose	−		
C 9 L-rhamnose	−		
C10 sucrose	−		
C11 maltose	−		
C12 α, α trehalose	−		
C13 methyl α-glucoside	−		
C14 cellobiose	−		
C15 salicin	−		
C16 arbutin	/		
C17 melibiose	−		
C18 lactose	−		
C19 raffinose	−		
C20 melezitose	−		
C21 inulin	−		
C22 soluble starch	−		
C23 glycerol	+		
C24 *meso* erythritol	−		
C25 ribitol	−		
C26 xylitol	−		
C27 L-arabinitol	−		
C28 D-glucitol	−		
C29 D-mannitol	−		
C30 galactitol	−		
C31 *myo*-inositol	−		
C32 glucono δ-lactone	?		
C33 2-keto-D-gluconate	−		

date: 13:11:90 conditions	Rotator		
C34 5-keto-D-gluconate	−		
C35 D-gluconate	−		
C36 D-glucuronate			
C37 D-galacturonate			
C38 DL-lactate	D		
C39 succinate	+		
C40 citrate	−		
C41 methanol	−		
C42 ethanol	+		
C43 propane 1, 2 diol	+		
C44 butane 2, 3 diol	+		
C45			

N1 nitrate	−ve
N2 nitrite	−ve
N3 ethylamine	+
N4 L-lysine	
N5 cadaverine	
N6 creatine	
N7 creatinine	
N8 glucosamine	
N9 imidazole	
V1 w/o vitamins	
V2 w/o inositol	
V3 w/o pantothenate	
V4 w/o biotin	
V5 w/o thiamin	
V6 w/o bio + thia	
V7 w/o pyridoxine	
V8 w/o pyr + thia	
V9 w/o niacin	
V10 w/o PABA	

T 25°C	T 30°C T 35°C
T 37°C	T 42°C +ve (strong)
01 0.01% cycloheximide	+
02 0.1% cycloheximide	+
03 1% acetic acid	
04 50% glucose	
05 60% glucose	
M1 starch production	
M2 acetic acid production	
M3 urea test	
M4 DBB reaction	−ve

APPENDIX 8a

Raw data : Weight losses in *P.patula* and *E.grandis*.

Codes used :

Fungus	Block No.	Wood

Fungus

C - *C.versicolor*
Cn- *C.puteana*
L - *L.lepideus*
P - *P.chrysosporium*
M - *M.racemosus*
MC - *M. racemosus* + *C.puteana*
PC - *P.chrysosporium* + *C.puteana*

Wood

P = *P.patula*
E = *E.grandis*

Zero hour control = Uninoculated wood blocks
POh = Pine
EOh = Eucalypt

The above codes are also used in Appendices 9a and 10.

Uninoculated wood blocks - Zero hour Controls

Pine and Eucalypt

PINE	
BLOCK NUMB.	INITIAL DRY WT
P1	0.3238
P2	0.3304
P3	0.2658
P4	0.3073
P5	0.3516
P6	0.3428
P7	0.2375
P8	0.2928
P9	0.2685
P10	0.3169
P11	0.2613
P12	0.2307
P13	0.2620
P14	0.3494
P15	0.3254
P16	0.2748
P17	0.2740
P18	0.3249
P19	0.2454
P20	0.2313
P21	0.2631
P22	0.2418
P23	0.3005
P24	0.3451
P25	0.2730
P26	0.2537
P27	0.2216
P28	0.3101
P29	0.2371
P30	0.2378

EUCALYPT	
BLOCK NUMB.	INITIAL DRY WT
E1	0.3659
E2	0.3043
E3	0.3602
E4	0.4434
E5	0.3588
E6	0.3782
E7	0.3003
E8	0.3161
E9	0.3776
E10	0.3114
E11	0.4522
E12	0.2944
E13	0.3237
E14	0.4009
E15	0.3020
E16	0.3204
E17	0.3052
E18	0.3484
E19	0.3143
E20	0.2821
E21	0.4529
E22	0.3432
E23	0.4005
E24	0.4166
E25	0.4366
E26	0.4015
E27	0.3390
E28	0.4513
E29	0.3106
E30	0.3592

THREE WEEK INCUBATION PERIOD

FUNGUS : *Coriolus versicolor*

Wood: PINE

Block	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
C1P	0.3550	0.3358	0.0192	5.4085
C2P	0.2835	0.2484	0.0351	12.3810
C3P	0.2345	0.2069	0.0276	11.7697
C4P	0.3237	0.2909	0.0328	10.1328
C5P	0.3004	0.2675	0.0329	10.9521
C6P	0.2938	0.2644	0.0294	10.0068
C7P	0.3634	0.3361	0.0273	7.5124
C8P	0.3351	0.3016	0.0335	9.9970
C9P	0.2379	0.2022	0.0357	15.0063
C10P	0.2893	0.2598	0.0295	10.1970
		MEAN	0.0303	10.3364
	STD DEVIATION		0.0047	2.4691

Wood: EUCALYPT

C1E	0.2972	0.2055	0.0917	30.8546
C2E	0.4186	0.3611	0.0575	13.7363
C3E	0.3757	0.3064	0.0693	18.4456
C4E	0.3720	0.2900	0.0820	22.0430
C5E	0.3681	0.2852	0.0829	22.5211
C6E	0.3904	0.3443	0.0461	11.8084
C7E	0.3756	0.3009	0.0747	19.8882
C8E	0.3226	0.2466	0.0760	23.5586
C9E	0.3570	0.2786	0.0784	21.9608
C10E	0.3602	0.2839	0.0763	21.1827
		MEAN	0.0735	20.5999
		STD DEVIATION	0.0125	5.0142

FUNGUS : *Coniophora puteana*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
Cn11P	0.3044	0.3075	-0.0031	-1.0184
Cn12P	0.2598	0.2636	-0.0038	-1.4627
Cn13P	0.2835	0.2856	-0.0021	-0.7407
Cn14P	0.2663	0.2685	-0.0022	-0.8261
Cn15P	0.2281	0.2310	-0.0029	-1.2714
Cn16P	0.2798	0.2824	-0.0026	-0.9292
Cn17P	0.3217	0.3255	-0.0038	-1.1812
Cn18P	0.2477	0.2494	-0.0017	-0.6863
Cn19P	0.2442	0.2456	-0.0014	-0.5733
Cn20P	0.2626	0.2643	-0.0017	-0.6474
		MEAN	-0.0025	-0.9337
	STD DEVIATION		0.0008	0.2800

Wood: EUCALYPT

Cn11E	0.3103	0.3155	-0.0052	-1.6758
Cn12E	0.3229	0.3271	-0.0042	-1.3007
Cn13E	0.3159	0.3213	-0.0054	-1.7094
Cn14E	0.2740	0.2783	-0.0043	-1.5693
Cn15E	0.3493	0.3541	-0.0048	-1.3742
Cn16E	0.3598	0.3647	-0.0049	-1.3619
Cn17E	0.3471	0.3520	-0.0049	-1.4117
Cn18E	0.4183	0.4208	-0.0025	-0.5977
Cn19E	0.2991	0.3026	-0.0035	-1.1702
Cn20E	0.3484	0.3511	-0.0027	-0.7750
		MEAN	-0.0042	-1.2946
	STD DEVIATION		0.0010	0.3448

FUNGUS : *Phanaerochaete chrysosporium*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
P21P	0.2483	0.2480	0.0003	0.1208
P22P	0.2761	0.2790	-0.0029	-1.0503
P23P	0.3624	0.3635	-0.0011	-0.3035
P24P	0.2859	0.2867	-0.0008	-0.2798
P25P	0.2875	0.2897	-0.0022	-0.7652
P26P	0.3324	0.3289	0.0035	1.0529
P27P	0.2911	0.2879	0.0032	1.0993
P28P	0.2716	0.2727	-0.0011	-0.4050
P29P	0.3554	0.3507	0.0047	1.3225
P30P	0.3425	0.3412	0.0013	0.3796
		MEAN	0.0005	0.1171
	STD DEVIATION		0.0025	0.7817

Wood: EUCALYPT

P21E	0.3678	0.3576	0.0102	2.7732
P22E	0.3142	0.3157	-0.0015	-0.4774
P23E	0.4041	0.3731	0.0310	7.6714
P24E	0.3869	0.3778	0.0091	2.3520
P25E	0.4024	0.3747	0.0277	6.8837
P26E	0.4018	0.3760	0.0258	6.4211
P27E	0.3850	0.3581	0.0269	6.9870
P28E	0.4021	0.3662	0.0359	8.9281
P29E	0.3557	0.3465	0.0092	2.5864
P30E	0.3876	0.3702	0.0174	4.4892
		MEAN	0.0192	4.8615
	STD DEVIATION		0.0114	2.8275

FUNGUS : *Lentinus lepideus*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
L31P	0.3180	0.3182	-0.0002	-0.0629
L32P	0.2566	0.2538	0.0028	1.0912
L33P	0.2607	0.2553	0.0054	2.0713
L34P	0.2422	0.2426	-0.0004	-0.1652
L35P	0.2456	0.2451	0.0005	0.2036
L36P	0.2452	0.2439	0.0013	0.5302
L37P	0.2701	0.2690	0.0011	0.4073
L38P	0.2969	0.2968	0.0001	0.0337
L39P	0.3026	0.3016	0.0010	0.3305
L40P	0.2206	0.2191	0.0015	0.6800
		MEAN	0.0013	0.5120
		STD DEVIATION	0.0016	0.6287

Wood: EUCALYPT

L31E	0.3474	0.3548	-0.0074	-2.1301
L32E	0.3306	0.3358	-0.0052	-1.5729
L33E	0.2646	0.2710	-0.0064	-2.4187
L34E	0.3893	0.3959	-0.0066	-1.6954
L35E	0.3811	0.3878	-0.0067	-1.7581
L36E	0.3618	0.3681	-0.0063	-1.7413
L37E	0.3489	0.3561	-0.0072	-2.0636
L38E	0.2975	0.3021	-0.0046	-1.5462
L39E	0.3049	0.3099	-0.0050	-1.6399
L40E	0.3535	0.3587	-0.0052	-1.4710
		MEAN	-0.0061	-1.8037
		STD DEVIATION	0.0009	0.2876

FUNGUS : *Mucor racemosus*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
M41P	0.2547	0.2535	0.0012	0.4711
M42P	0.2767	0.2767	0.0000	0.0000
M43P	0.3052	0.3051	0.0001	0.0328
M44P	0.2950	0.2950	0.0000	0.0000
M45P	0.2584	0.2577	0.0007	0.2709
M46P	0.2150	0.2164	-0.0014	-0.6512
M47P	0.2259	0.2268	-0.0009	-0.3984
M48P	0.2183	0.2204	-0.0021	-0.9620
M49P	0.2708	0.2742	-0.0034	-1.2555
M50P	0.2916	0.2936	-0.0020	-0.6859
		MEAN	-0.0008	-0.3178
		STD DEVIATION	0.0014	0.5330

Wood: EUCALYPT

M41E	0.3528	0.3509	0.0019	0.5385
M42E	0.3876	0.3858	0.0018	0.4644
M43E	0.3286	0.3258	0.0028	0.8521
M44E	0.3482	0.3459	0.0023	0.6605
M45E	0.4253	0.4220	0.0033	0.7759
M46E	0.4098	0.4102	-0.0004	-0.0976
M47E	0.3456	0.3464	-0.0008	-0.2315
M48E	0.3897	0.3898	-0.0001	-0.0257
M49E	0.3592	0.3601	-0.0009	-0.2506
M50E	0.3394	0.3391	0.0003	0.0884
		MEAN	0.0010	0.2775
		STD DEVIATION	0.0015	0.4043

COCULTURE : *Mucor racemosus* and *Coniophora puteana*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
MC51	0.2956	0.2955	0.0001	0.0338
MC52	0.2963	0.2978	-0.0015	-0.5062
MC53	0.2533	0.2531	0.0002	0.0790
MC54	0.2989	0.3020	-0.0031	-1.0371
MC55	0.2835	0.2840	-0.0005	-0.1764
MC56	0.2807	0.2820	-0.0013	-0.4631
MC57	0.2875	0.2901	-0.0026	-0.9043
MC58	0.2569	0.2591	-0.0022	-0.8564
MC59	0.2856	0.2884	-0.0028	-0.9804
MC60	0.2678	0.2692	-0.0014	-0.5228
		MEAN	-0.0015	-0.5334
		STD DEVIATION	0.0011	0.3906

Wood: EUCALYPT

MC51	0.3915	0.3880	0.0035	0.8940
MC52	0.3176	0.3162	0.0014	0.4408
MC53	0.3947	0.3873	0.0074	1.8748
MC54	0.4024	0.3949	0.0075	1.8638
MC55	0.3957	0.3939	0.0018	0.4549
MC56	0.3993	0.3928	0.0065	1.6278
MC57	0.3564	0.3537	0.0027	0.7576
MC58	0.3421	0.3401	0.0020	0.5846
MC59	0.4390	0.4286	0.0104	2.3690
MC60	0.4193	0.4025	0.0168	4.0067
		MEAN	0.0060	1.4874
		STD DEVIATION	0.0046	1.0629

COCULTURE : *Phanaerochaete chrysosporium* and *Coniophora puteana*

Wood: PINE Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
PC61	0.3009	0.3046	-0.0037	-1.2296
PC62	0.2528	0.2568	-0.0040	-1.5823
PC63	0.2463	0.2496	-0.0033	-1.3398
PC64	0.3169	0.3199	-0.0030	-0.9467
PC65	0.3014	0.3048	-0.0034	-1.1281
PC66	0.2702	0.2724	-0.0022	-0.8142
PC67	0.2399	0.2421	-0.0022	-0.9170
PC68	0.2570	0.2597	-0.0027	-1.0506
PC69	0.2634	0.2653	-0.0019	-0.7213
PC70	0.2368	0.2359	0.0009	0.3801
		MEAN	-0.0025	-0.9350
	STD DEVIATION		0.0013	0.5010
Wood: EUCALYPT				
PC61	0.4468	0.4184	0.0284	6.3563
PC62	0.3607	0.3376	0.0231	6.4042
PC63	0.3085	0.2929	0.0156	5.0567
PC64	0.4003	0.3817	0.0186	4.6465
PC65	0.3698	0.3674	0.0024	0.6490
PC66	0.4236	0.4246	-0.0010	-0.2361
PC67	0.2872	0.2895	-0.0023	-0.8008
PC68	0.3244	0.3263	-0.0019	-0.5857
PC69	0.3488	0.3495	-0.0007	-0.2007
PC70	0.2852	0.2849	0.0003	0.1052
		MEAN	0.0083	2.1395
	STD DEVIATION		0.0112	2.9037

SIX WEEK INCUBATION PERIOD

FUNGUS : *Coriolus versicolor*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
C71P	0.2449	0.1792	0.0657	26.8273
C72P	0.2926	0.2052	0.0874	29.8701
C73P	0.2889	0.2275	0.0614	21.2530
C74P	0.2480	0.1757	0.0723	29.1532
C75P	0.2652	0.1933	0.0719	27.1116
C76P	0.2661	0.2154	0.0507	19.0530
C77P	0.2397	0.1734	0.0663	27.6596
C78P	0.2438	0.1802	0.0636	26.0870
C79P	0.2884	0.2125	0.0759	26.3176
C80P	0.3103	0.2621	0.0482	15.5334
		MEAN	0.0663	24.8866
		STD DEVIATION	0.0110	4.4458

Wood: EUCALYPT

C71E	0.3367	0.2074	0.1293	38.4021
C72E	0.3693	0.2348	0.1345	36.4203
C73E	0.4155	0.2996	0.1159	27.8941
C74E	0.4305	0.3185	0.1120	26.0163
C75E	0.3501	0.3279	0.0222	6.3410
C76E	0.4111	0.2782	0.1329	32.3279
C77E	0.3636	0.2478	0.1158	31.8482
C78E	0.3478	0.2300	0.1178	33.8700
C79E	0.4634	0.3589	0.1045	22.5507
C80E	0.4275	0.3186	0.1089	25.4737
		MEAN	0.1094	28.1144
		STD DEVIATION	0.0306	8.6954

FUNGUS : *Coniophora puteana*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
Cn81P	0.2614	0.2623	-0.0009	-0.3443
Cn82P	0.3633	0.3632	0.0001	0.0275
Cn83P	0.3060	0.3067	-0.0007	-0.2288
Cn84P	0.2378	0.2372	0.0006	0.2523
Cn85P	0.2557	0.2558	-0.0001	-0.0391
Cn86P	0.2445	0.2438	0.0007	0.2863
Cn87P	0.2612	0.2616	-0.0004	-0.1531
Cn88P	0.3598	0.3587	0.0011	0.3057
Cn89P	0.2391	0.2381	0.0010	0.4182
Cn90P	0.2413	0.2393	0.0020	0.8288
		MEAN	0.0003	0.1354
		STD DEVIATION	0.0009	0.3338

Wood: EUCALYPT

Cn81E	0.3396	0.3412	-0.0016	-0.4711
Cn82E	0.3102	0.3129	-0.0027	-0.8704
Cn83E	0.3566	0.3572	-0.0006	-0.1683
Cn84E	0.3407	0.3417	-0.0010	-0.2935
Cn85E	0.3586	0.3601	-0.0015	-0.4183
Cn86E	0.3612	0.3614	-0.0002	-0.0554
Cn87E	0.2831	0.2850	-0.0019	-0.6711
Cn88E	0.3521	0.3526	-0.0005	-0.1420
Cn89E	0.3791	0.3787	0.0004	0.1055
Cn90E	0.3707	0.3702	0.0005	0.1349
		MEAN	-0.0009	-0.2850
		STD DEVIATION	0.0010	0.3098

FUNGUS : *Phanaerochaete chrysosporium*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
P91P	0.2497	0.2476	0.0021	0.8410
P92P	0.2245	0.2225	0.0020	0.8909
P93P	0.2524	0.2526	-0.0002	-0.0792
P94P	0.3346	0.3323	0.0023	0.6874
P95P	0.2906	0.2880	0.0026	0.8947
P96P	0.2586	0.2573	0.0013	0.5027
P97P	0.2950	0.2941	0.0009	0.3051
P98P	0.3681	0.3662	0.0019	0.5162
P99P	0.3468	0.3434	0.0034	0.9804
P100P	0.2919	0.2905	0.0014	0.4796
		MEAN	0.0018	0.6019
		STD DEVIATION	0.0009	0.3100

Wood: EUCALYPT

P91E	0.3984	0.3552	0.0432	10.8434
P92E	0.4010	0.3950	0.0060	1.4963
P93E	0.3404	0.3394	0.0010	0.2938
P94E	0.3004	0.2478	0.0526	17.5100
P95E	0.3651	0.3675	-0.0024	-0.6574
P96E	0.3380	0.2669	0.0711	21.0355
P97E	0.3590	0.2554	0.1036	28.8579
P98E	0.3113	0.3125	-0.0012	-0.3855
P99E	0.2986	0.2552	0.0434	14.5345
P100E	0.2816	0.2806	0.0010	0.3551
		MEAN	0.0318	9.3884
		STD DEVIATION	0.0350	10.1603

FUNGUS : *Lentinus lepideus*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
L101P	0.2536	0.2510	0.0026	1.0252
L102P	0.2664	0.2683	-0.0019	-0.7132
L103P	0.3034	0.3042	-0.0008	-0.2637
L104P	0.2638	0.2850	-0.0212	-8.0364
L105P	0.3180	0.3119	0.0061	1.9182
L106P	0.3228	0.3212	0.0016	0.4957
L107P	0.2737	0.2715	0.0022	0.8038
L108P	0.3564	0.3547	0.0017	0.4770
L109P	0.2802	0.2622	0.0180	6.4240
L110P	0.2448	0.2407	0.0041	1.6748
		MEAN	0.0012	0.3805
	STD DEVIATION		0.0091	3.3710

Wood: EUCALYPT

L101E	0.3086	0.3126	-0.0040	-1.2962
L102E	0.3773	0.3814	-0.0041	-1.0867
L103E	0.3086	0.3129	-0.0043	-1.3934
L104E	0.4158	0.4180	-0.0022	-0.5291
L105E	0.3671	0.3698	-0.0027	-0.7355
L106E	0.3316	0.3367	-0.0051	-1.5380
L107E	0.3160	0.3197	-0.0037	-1.1709
L108E	0.3267	0.3298	-0.0031	-0.9489
L109E	0.3525	0.3548	-0.0023	-0.6525
L110E	0.3682	0.3698	-0.0016	-0.4345
		MEAN	-0.0033	-0.9786
	STD DEVIATION		0.0011	0.3604

FUNGUS : *Mucor racemosus*

Wood: PINE

	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
M111	0.2746	0.2739	0.0007	0.2549
M112	0.2530	0.2520	0.0010	0.3953
M113	0.2932	0.2945	-0.0013	-0.4434
M114	0.3530	0.3512	0.0018	0.5099
M115	0.2601	0.2588	0.0013	0.4998
M116	0.2539	0.2448	0.0091	3.5841
M117	0.2401	0.2288	0.0113	4.7064
M118	0.3420	0.3334	0.0086	2.5146
M119	0.2778	0.2660	0.0118	4.2477
M120	0.3544	0.3445	0.0099	2.7935
		MEAN	0.0054	1.9063
		STD DEVIATION	0.0049	1.7820

Wood: EUCALYPT

M111	0.3555	0.3357	0.0198	5.5696
M112	0.3813	0.3713	0.0100	2.6226
M113	0.3547	0.3520	0.0027	0.7612
M114	0.3300	0.3277	0.0023	0.6970
M115	0.3398	0.3228	0.0170	5.0029
M116	0.3289	0.3251	0.0038	1.1554
M117	0.3606	0.3577	0.0029	0.8042
M118	0.4532	0.4486	0.0046	1.0150
M119	0.3009	0.2981	0.0028	0.9305
M120	0.3341	0.3314	0.0027	0.8081
		MEAN	0.0069	1.9367
		STD DEVIATION	0.0062	1.7612

COCULTURE : *Mucor racemosus* and *Coniophora puteana*

Wood: PINE

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
MC12	0.2449	0.2416	0.0033	1.3475
MC12	0.3553	0.3518	0.0035	0.9851
MC12	0.2787	0.2753	0.0034	1.2199
MC12	0.2633	0.2608	0.0025	0.9495
MC12	0.3379	0.3344	0.0035	1.0358
MC12	0.3311	0.3264	0.0047	1.4195
MC12	0.3031	0.2995	0.0036	1.1877
MC12	0.2661	0.2640	0.0021	0.7892
MC12	0.3429	0.3393	0.0036	1.0499
MC13	0.2520	0.2466	0.0054	2.1429
		MEAN	0.0036	1.2127
		STD DEVIATION	0.0009	0.3585

Wood: EUCALYPT

MC12	0.3090	0.3061	0.0029	0.9385
MC12	0.2931	0.2887	0.0044	1.5012
MC12	0.2961	0.2931	0.0030	1.0132
MC12	0.3991	0.3886	0.0105	2.6309
MC12	0.3680	0.3596	0.0084	2.2826
MC12	0.3116	0.3076	0.0040	1.2837
MC12	0.3564	0.3451	0.0113	3.1706
MC12	0.3898	0.3819	0.0079	2.0267
MC12	0.3110	0.3087	0.0023	0.7395
MC13	0.3940	0.3831	0.0109	2.7665
		MEAN	0.0066	1.8353
		STD DEVIATION	0.0034	0.8134

COCULTURE : *Phanaerochaete chrysosporium* and *Coniophora puteana*

Blk.No	Init Dry wt	Final Dry wt	Wt Loss/ Gain	%Wt Loss Gain
PINE				
PC131	0.2365	0.2359	0.0006	0.2537
PC132	0.2512	0.2506	0.0006	0.2389
PC133	0.2479	0.2482	-0.0003	-0.1210
PC134	0.2542	0.2539	0.0003	0.1180
PC135	0.2751	0.2733	0.0018	0.6543
PC136	0.3500	0.3483	0.0017	0.4857
PC137	0.2762	0.2753	0.0009	0.3259
PC138	0.2716	0.2713	0.0003	0.1105
PC139	0.3493	0.3478	0.0015	0.4294
PC140	0.3431	0.3396	0.0035	1.0201
		MEAN	0.0011	0.3515
		STD DEVIATION	0.0010	0.3036
EUCALYPT				
PC131	0.4048	0.4013	0.0035	0.8646
PC132	0.3786	0.3771	0.0015	0.3962
PC133	0.3982	0.3950	0.0032	0.8036
PC134	0.3311	0.3289	0.0022	0.6645
PC135	0.3450	0.3427	0.0023	0.6667
PC136	0.3211	0.3185	0.0026	0.8097
PC137	0.2988	0.2967	0.0021	0.7028
PC138	0.3781	0.3755	0.0026	0.6876
PC139	0.3142	0.3129	0.0013	0.4137
PC140	0.3138	0.3133	0.0005	0.1593
		MEAN	0.0022	0.6169
		STD DEVIATION	0.0008	0.2121

APPENDIX 8b : Analysis of variance of % weight losses for all blocks.

Variate : % weight loss

Source of variation	d.f[1]	s.s[2].	m.s[3].	v.r[4].	F.pr[5].
period	1	452.37	452.37	42.45	<.001
fungi	6	14376.14	2396.02	224.84	<.001
wood	1	304.00	304.00	28.53	<.001
period.fungi	6	888.36	148.06	13.89	<.001
period.wood	1	16.85	16.85	1.58	0.210
fungi.wood	6	690.38	115.06	10.80	<.001
period.fungi.wood	6	175.46	29.24	2.74	0.013
residual	252	2685.42	10.66		
Total	279	19588.97			

[1] degrees of freedom
[2] sum of squares
[3] mean square
[4] variance ratio
[5] F probability

APPENDIX 9a : Raw data : Nitrogen and protein contents of wood

NITROGEN ANALYSIS

				PERCENTAGE NITROGEN			PERCENTAGE PROTEIN		
Sa	Code	Mass	Dilut	% NIT	MEAN	SD	% PROT	MEAN	SD
1	P50h	0.3516	100	0.027	0.029	0.002	0.166	0.178	0.015
2	P60h	0.3428	100	0.027			0.17		
3	P80h	0.2928	100	0.032			0.199		
4	E80h	0.3161	100	0.03	0.045	0.021	0.185	0.278	0.131
5	E90h	0.3776	100	0.074			0.463		
6	E100h	0.3114	100	0.03			0.187		
7	PC135P	0.2733	100	0.137	0.122	0.020	0.854	0.763	0.125
8	PC136P	0.3483	100	0.094			0.586		
9	PC137P	0.2753	100	0.136			0.848		
10	PC131E	0.4013	100	0.14	0.177	0.041	0.872	1.107	0.257
11	PC136E	0.3185	100	0.234			1.465		
12	PC137E	0.2967	100	0.157			0.983		
13	MC130E	0.3831	100	0.219	0.178	0.031	1.37	1.110	0.197
14	MC125E	0.3596	100	0.143			0.892		
15	MC128E	0.3819	100	0.171			1.069		
16	MC126P	0.3264	100	0.2	0.162	0.027	1.251	1.015	0.172
17	MC123P	0.2753	100	0.136			0.848		
18	MC130P	0.2466	100	0.151			0.946		
19	M112E	0.3713	100	0.101	0.248	0.198	0.628	1.551	1.239
20	M111E	0.3357	100	0.528			3.302		
21	M115E	0.3228	100	0.116			0.723		
22	M119P	0.2660	100	0.193	0.165	0.037	1.206	1.030	0.234
23	M120P	0.3445	100	0.19			1.185		
24	M118P	0.3334	100	0.112			0.7		
25	L106E	0.3367	100	0.208	0.141	0.048	1.299	0.880	0.300
26	L107E	0.3197	100	0.117			0.73		
27	L102E	0.3814	100	0.098			0.612		
28	L103P	0.3042	100	0.245	0.231	0.061	1.534	1.443	0.383
29	L101P	0.2510	100	0.297			1.859		
30	L105P	0.3119	100	0.15			0.935		
31	C72P	0.2052	100	0.364	0.341	0.095	2.274	2.129	0.589
32	C71P	0.1792	100	0.443			2.767		
33	C77P	0.1734	100	0.215			1.346		
34	C76E	0.2782	100	0.168	0.224	0.068	1.048	1.397	0.429
35	C78E	0.2300	100	0.183			1.141		
36	C77E	0.2478	100	0.32			2.001		
37	Cn82E	0.3129	100	0.119	0.145	0.020	0.746	0.907	0.126
38	Cn87E	0.2850	100	0.147			0.921		
39	Cn85E	0.3601	100	0.168			1.053		
40	Cn84P	0.2372	100	0.295	0.222	0.084	1.844	1.390	0.528
41	Cn88P	0.3587	100	0.104			0.65		
42	Cn86P	0.2438	100	0.268			1.675		
43	P99E	0.2552	100	0.658	0.517	0.163	4.114	3.230	1.016
44	P91E	0.3552	100	0.289			1.806		
45	P97E	0.2554	100	0.603			3.769		
46	P95P	0.2880	100	0.194	0.217	0.056	1.215	1.356	0.348
47	P92P	0.2225	100	0.294			1.835		
48	P99P	0.3434	100	0.163			1.019		

APPENDIX 9b : Analysis of variance of % nitrogen.

Variate : % N

Source of variation	d.f[1].	s.s[2].	m.s[3].	v.r[4].	F.pr[5].
wood	1	0.006394	0.006394	0.67	0.418
fungi	7	0.391019	0.055860	5.87	<.001
wood.fungi	7	0.185584	0.026512	2.79	0.022
Residual	32	0.304456	0.009514		
Total	47	0.887453			

[1] degrees of freedom
[2] sum of squares
[3] mean square
[4] variance ratio
[5] F probability

APPENDIX 10

Raw data: Cellulose (glucose), hemicellulose (xylose + mannose) and Klason lignin.

Sugar Analysis

Sa	Code	Mass	Glu Pk Area	Xyl Pk Area	Glu mg/ml	Xyl Mg/ml	Mg wood /ml	Glu Mg/Mg	Xyl Mg/Mg
1	Pine0h	0.0500	1599631	673876	1.44	0.63	3.448	0.419	0.183
2	Pine0h	0.0502	1521679	614163	1.37	0.57	3.462	0.396	0.165
3	Pine0h	0.0501	1542999	621946	1.39	0.58	3.455	0.403	0.167
4	Euc0h	0.0500	1836321	418139	1.66	0.38	3.448	0.482	0.109
5	Euc0h	0.0501	1781531	389587	1.61	0.35	3.455	0.466	0.101
6	Euc0h	0.0501	1776731	359830	1.61	0.32	3.455	0.465	0.092
7	PC131P	0.0500	1601765	608158	1.45	0.56	3.448	0.419	0.164
8	PC132P	0.0500	1489375	601376	1.34	0.56	3.448	0.389	0.162
9	PC134P	0.0501	1561289	578234	1.41	0.54	3.455	0.408	0.155
10	PC132E	0.0500	1775942	363551	1.61	0.32	3.448	0.466	0.093
11	PC135E	0.0502	1762824	388762	1.59	0.35	3.462	0.460	0.100
12	PC139E	0.0503	1787592	390929	1.62	0.35	3.469	0.466	0.101
13	MC122E	0.0503	1759237	355090	1.59	0.31	3.469	0.458	0.090
14	MC123E	0.0500	1713048	378308	1.55	0.34	3.448	0.449	0.098
15	MC126E	0.0503	1711654	397544	1.55	0.36	3.469	0.446	0.103
16	MC122P	0.0502	1552495	598428	1.40	0.56	3.462	0.405	0.160
17	MC125P	0.0500	1528557	607543	1.38	0.56	3.448	0.400	0.164
18	MC129P	0.0500	1549639	594454	1.40	0.55	3.448	0.405	0.160
19	M118E	0.0502	1557252	407605	1.40	0.37	3.462	0.406	0.106
20	M119E	0.0501	1769857	385775	1.60	0.34	3.455	0.463	0.100
21	M120E	0.0501	1757089	374964	1.59	0.33	3.455	0.460	0.096
22	M112P	0.0502	1466687	615698	1.32	0.57	3.462	0.382	0.165
23	M114P	0.0500	1548252	650166	1.40	0.61	3.448	0.405	0.176
24	M115P	0.0500	1679145	720914	1.52	0.68	3.448	0.440	0.196
25	L105E	0.0503	1800769	445918	1.63	0.40	3.469	0.469	0.116
26	L108E	0.0500	1904742	450953	1.72	0.41	3.448	0.500	0.119
27	L109E	0.0502	1888379	432232	1.71	0.39	3.462	0.493	0.113
28	L106P	0.0502	1629671	614513	1.47	0.57	3.462	0.425	0.165
29	L107P	0.0500	1674534	603801	1.51	0.56	3.448	0.439	0.163
30	L108P	0.0501	1689643	620138	1.53	0.58	3.455	0.442	0.167
31	C73P	0.0502	1534436	610982	1.38	0.57	3.462	0.400	0.164
32	C75P	0.0501	1527993	634674	1.38	0.59	3.455	0.399	0.171
33	C78P	0.0501	1467916	599532	1.32	0.56	3.455	0.383	0.161
34	C73E	0.0503	1844661	378700	1.67	0.34	3.469	0.481	0.097
35	C74E	0.0500	1802674	427721	1.63	0.39	3.448	0.473	0.112
36	C80E	0.0501	1810873	403740	1.64	0.36	3.455	0.474	0.105
37	Cn83E	0.0500	1856130	370725	1.68	0.33	3.448	0.487	0.095
38	Cn84E	0.0503	1819030	373688	1.64	0.33	3.469	0.474	0.096
39	Cn88E	0.0500	1724734	332270	1.56	0.29	3.448	0.452	0.084
40	Cn81P	0.0500	1485398	591492	1.34	0.55	3.448	0.388	0.159
41	Cn83P	0.0501	1554491	527290	1.40	0.48	3.455	0.406	0.140
42	Cn87P	0.0503	1547504	525022	1.40	0.48	3.469	0.402	0.139
43	P92E	0.0501	1623682	276077	1.47	0.24	3.455	0.424	0.068
44	P95E	0.0500	1677987	264097	1.52	0.22	3.448	0.440	0.065
45	P100E	0.0500	1867273	419130	1.69	0.38	3.448	0.490	0.109
46	P96P	0.0500	1655669	548768	1.50	0.51	3.448	0.420	0.140
47	P98P	0.0504	1740614	644165	1.57	0.60	3.476	0.453	0.173
48	P100P	0.0503	1633604	565322	1.47	0.52	3.469	0.425	0.151

MEAN LIG	LIG	SD	GLU	SD	XYL	SD
0.380						
0.354						
0.376	0.370	0.012	0.406	0.009	0.172	0.008
0.218						
0.314						
0.268	0.267	0.039	0.471	0.008	0.101	0.007
ERR						
0.282						
0.382						
0.384	0.349	0.048	0.405	0.012	0.16	0.004
0.257						
0.335						
0.211	0.268	0.051	0.464	0.003	0.098	0.003
0.244						
0.274						
0.390	0.303	0.063	0.451	0.005	0.097	0.005
0.412						
0.446						
0.357	0.405	0.037	0.403	0.002	0.161	0.002
0.352						
0.266						
0.276	0.298	0.038	0.443	0.026	0.101	0.004
0.277						
0.251						
0.417	0.315	0.073	0.409	0.024	0.179	0.013
0.240						
0.346						
0.252	0.279	0.047	0.488	0.013	0.116	0.002
0.385						
0.382						
0.378	0.382	0.003	0.435	0.007	0.165	0.002
0.458						
0.380						
0.312	0.383	0.059	0.394	0.008	0.165	0.004
0.411						
0.378						
0.347	0.379	0.026	0.476	0.004	0.105	0.006
0.359						
0.324						
0.278	0.321	0.033	0.471	0.014	0.092	0.005
0.396						
0.360						
0.394	0.383	0.016	0.399	0.008	0.146	0.009
0.287						
0.276						
0.346	0.303	0.031	0.451	0.028	0.081	0.02
0.379						
0.307						
0.392	0.359	0.037	0.433	0.014	0.154	0.014

%Lig	SD	CV	%Glu	SD	CV	%XYL	SD	CV
37.0	1.2	3.1	40.6	0.9	2.2	17.2	0.8	4.7
26.7	3.9	14.7	47.1	0.8	1.7	10.1	0.7	6.9
34.9	4.8	13.6	40.5	1.2	3.0	16.0	0.4	2.5
26.8	5.1	19.1	46.4	0.3	0.6	9.8	0.3	3.1
30.3	6.3	20.8	45.1	0.5	1.1	9.7	0.5	5.2
40.5	3.7	9.0	40.3	0.2	0.5	16.1	0.2	1.2
29.8	3.8	12.9	44.3	2.6	5.9	10.1	0.4	4.0
31.5	7.3	23.2	40.9	2.4	5.9	17.9	1.3	7.3
27.9	4.7	16.9	48.8	1.3	2.7	11.6	0.2	1.7
38.2	0.3	0.8	43.5	0.7	1.6	16.5	0.2	1.2
38.3	5.9	15.5	39.4	0.8	2.0	16.5	0.4	2.4
37.9	2.6	6.9	47.6	0.4	0.8	10.5	0.6	5.7
32.1	3.3	10.4	47.1	1.4	3.0	9.2	0.5	5.4
38.3	1.6	4.2	39.9	0.8	2.0	14.6	0.9	6.2
30.3	3.1	10.1	45.1	2.8	6.2	8.1	2.0	24.7
35.9	3.7	10.4	43.3	1.4	3.2	15.4	1.4	9.1

APPENDIX 11a : Analysis of variance of % glucose.

Variate : % glucose

Source of variation	d.f[1].	s.s[2].	m.s[3].	v.r[4].	F.pr[5].
wood	1	347.225	347.225	116.66	<.001
fungi	7	50.980	7.283	2.45	0.040
wood.fungi	7	44.326	6.322	2.13	0.069
Residual	32	95.247	2.976		
Total	47	537.778			

[1] degrees of freedom
[2] sum of squares
[3] mean square
[4] variance ratio
[5] F probability

APPENDIX 11b : Analysis of variance of % xylose + mannose.

Variate : % xylose

Source of variation	d.f[1].	s.s[2].	m.s[3].	v.r[4].	F.pr[5].
wood	1	495.367	495.367	470.29	<.001
fungi	7	32.589	4.656	4.42	0.002
wood.fungi	7	10.296	1.471	1.40	0.241
Residual	32	33.707	1.053		
Total	47	571.959			

---

[1] degrees of freedom
[2] sum of squares
[3] mean square
[4] variance ratio
[5] F probability

APPENDIX 11c : Analysis of variance of % Klason lignin.

Variate : % lignin

Source of variation	d.f[1].	s.s[2].	m.s[3].	v.r[4].	F.pr[5].
wood	1	528.25	528.25	19.78	<.001
fungi	7	275.50	39.36	1.47	0.212
wood.fungi	7	159.73	22.82	0.85	0.552
Residual	32	854.41	26.70		
Total	47	1817.89			

---

[1] degrees of freedom
[2] sum of squares
[3] mean square
[4] variance ratio
[5] F probability

REFERENCES

Ainsworth, G. C. 1966. A general purpose classification of fungi. Bibliography of Systematic Mycology Part 1, 1-4.

Akin, D. E., and L. L. Rigsby. 1987. Mixed fungal populations and lignocellulosic tissue degradation in the bovine rumen. Applied and Environmental Microbiology **53:**1987-1995.

Akin, D. E., G. L. Gordon, and J. P. Hogan. 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulphur. Applied and Environmental Microbiology **55:**611-616.

Akin, D. E., C. E. Lyon, W. R. Windham, and L. L. Rigsby. 1989. Physical degradation of lignified stem tissues by ruminal fungi. Applied and Environmental Microbiology **55:**611-616.

Andreotti, R. E., M. Mandels, and C. Roche. 1977. Effect of fermentation variables on growth and cellulase production by *Trichoderma* QM 9414, p. 249-267. *In*: T.K.Ghose (ed.), Proceedings of the Bioconversion Symposium. Raj Bandu Industrial Company, Dehli.

AOAC "Official Methods of Analysis", 12th ed.; W. Horwitz (ed.), AOAC, Washington DC.

Baecker, A. A. W. 1981. The role of actinomycetes in the biodeterioration of wood. Ph.D. thesis. Dundee Institute of Technology, Scotland, U.K.

Bannerjee, A. K., and J. F. Levy. 1971. Fungal succession in wooden fence posts. Material und Organismen **6:**1-25.

Barr, D. J. S., H. Kudo, K. D. Jakober, and K. G. Cheng. 1989. Morphology and development of rumen fungi: *Neocallimastix sp; Piromonas communis* and *Orpinomyces bovis* gen. nov., sp.nov. Canadian Journal of Botany **67:**2815-2824.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. Applied and Environmental Microbiology **38:**148-148.

Bauchop, T. 1981. The anaerobic fungi in rumen fibre digestion. Agriculture and Environment **6:**339-348.

Bauchop, T., and D. O. Mountford. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Applied and Environmental Microbiology **42:**1103-1110.

Blanchette, R. A. 1983. An unusual decay pattern in brown rotted wood. Mycologia **75:**552-556.

Blanchette, R. A., T. A. Burns, G. F. Leatham, and M. J. Effland. 1988. Selection of white rot fungi for biopulping. Biomass **15:**93-101.

Blanchette, R. A., A. R. Abad, R. L. Farrel, and T. D. Leathers. 1989. Detection of lignin peroxidase and xylanase by immunocytochemical labelling in wood decayed by basidiomycetes. Applied and Environmental Microbiology **55:**1457-1465.

Blanchette, R. A., G. F. Leatham, M. C. Attridge, M. Akthar, and G. C. Myers. 1991. Biomechanical pulping with *C.subvermispora*. U.S. Patent No. 5,055, 159.

Bravery, A. F. 1971. the application of scanning electron microscopy in the study of timber decay. Journal of the Institute of Wood Science **5:**13-19.

Bravery, A. F. 1975. Micromorphology of decay in preservative treated wood. *In* W. Liese (ed.), Biological Transformation of Wood by Microorganisms. Springer-verlag, Berlin.

Bravery, A. F. 1976. Interactions between solvent preservatives, wood and decay fungi. Material und Organismen **3:**331-334.

Bravery, A. F., R. W. Berry, J. K. Carey, and D. E. Cooper. 1987. Recognising wood rot and insect damage in buildings. Building Research Establishment Report, 120pp.

Breton, A., A. Bernalier, F. Bonnemoy, G. Fonty, B. Gaillard, and P. Gouet. 1989. Morphological and metabolic characterisation of a new species of strictly anaerobic rumen fungus : *Neocallimastix joyonni*. FEMS Microbiology Letters **58:**309-314.

Breuil, C., and J. N. Saddler. 1985a. Comparison of the 3,5-dinitrosalicylic acid and Nelson-Somogyi method of assaying for reducing sugars and determining cellulase activity. Enzyme and Microbial Technology **7:**327-332.

Breuil, C., and J.N. Saddler. 1985b. Limitations of using the D-glucose oxidase peroxidase method for measuring glucose derived from lignocellulosic substrates. Biotechnology Letters **7:**191-196.

Breuil, C., P. Mayers, and J. N. Saddler. 1986. Substrate conditions that influence the assays used for determining the B-glucosidase activity of cellulolytic microorganisms. Biotechnology and Bioengineering **28:**1653-1656.

Brewer, D., J. M. Duncan, S. Safe, and A. Taylor. 1972. The survival at low oxygen partial pressure of fungi isolated from the contents of the ovine rumen. Canadian Journal of Microbiology **18:**1119-1128.

Bryant, M. P. 1972. Commentry on the Hungate technique for culture of anaerobic bacteria. American Journal of Clinical Nutrition **25:**1324-1328.

Bryant, M. P., and I. M. Robinson. 1961. An improved non - selective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. Journal of Dairy science **44:**1446-1456.

Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust. 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science **228:**1434-1436.

Burns, D. M., and S. G. G. MacDonald. 1970. Physics for biology and pre-medical students. Addison Wesley Publishing Company, London, California, Ontario, Sydney, Manila, 614 pp.

Butcher, J. A. 1966. Fungal infection of ground produce during seasoning. Proceedings of the New Zealand Wood Preservers Association 22-30.

Butcher, J. A. 1968a. Fungi and preservative treated timber. Proceedings of the New Zealand Wood Preservers Association 38-47.

Butcher, J. A. 1968b. The ecology of fungi infecting untreated sapwood of *Pinus radiata*. Canadian Journal of Botany **46:**1557-1589.

Butcher, J. A. 1971. Techniques for the analysis of fungal flora in wood. Material und Organismen **6:**209-232.

Butcher, J. A. 1972. Analysis of the fungal population of wood, p. 319-325. *In* A. H. Walters and E. H. Hueck-van der Plas (ed.), Biodeterioration of Materials. Applied Science Publishers, London.

Butcher, J. A., and M. Howard. 1968. Outside storage of *Pinus radiata* wood chips in New Zealand. *Tappi* **51:**117-122.

Caldwell, D. R., and M. P. Bryant. 1966. Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. Applied Microbiology **14:**794-801.

Cartwright, K. St. G., and W. P. K. Findlay. 1958. Decay of timber and its prevention. Her Majesty's Stationery Office, London, 332p.

Chang, H.-m., C. L. Chen, and T. K. Kirk. 1980. the chemistry of lignin degradation by white rot fungi. *In* T. K Kirk, T. Higuchi and H.-m. Chang (ed.), p. 215-230. Lignin Biodegradation: Microbiology Chemistry and Potential Applications. Boca Raton, Florida: CRC Press.

Charlesby, A. 1955. The degradation of cellulose by ionising radiation. Journal of Polymer Science. **15:**263-270.

Citron, D. M. 1984. Specimen collection and transport, anaerobic culture techniques and identification techniques. Reviews of Infectious Diseases **6:**551-558.

Citron, A., A. Brenton, and G. Fonty. 1987. Rumen anaerobic fungi. Bulletin de Institut Pasteur **85:**329-343.

Corbett, N. H. 1963. Anatomical, ecological and physiological studies on microfungi associated with decaying wood. Ph.D. thesis. University of London.

Corbett, N. H. 1965. Micromorphological studies on the degradation of lignified cell walls by ascomycetes and fungi imperfecti. Journal of the Institute of Wood Science **14:**18-29.

Corbett, N. H., and J. F. Levy. 1963a. Ecological studies on fungi associated with wooden fence posts. British Wood Preservers Association News Sheet 27:1-3.

Corbett, N. H., and J. F. Levy. 1963b. Ecological studies on fungi associated with wooden fence posts. British Wood Preservers Association News Sheet **28:**1-10.

Costilow, R. N. 1982. Biophysical factors in growth. *In* : P. Gerhardt, R. G. E. Murray and R. N. Costilow (ed.), p. 66-78. Manual of Methods for general Bacteriology. American Society fr Microbiology.

Courtois, H. 1963. Mikromorphologische Befallsymptome biem Holzabbau durch Moderfaulepilze. Holzforschung und Holzververtung 15:88-101.

Courtois, H. 1966. On the decomposition of cell wall by bacteria in coniferous wood. Holzforschung **20:**148-154.

Cowling, E.B. 1961. Comparative biochemistry of the decay of sweet gum by white rot fungi. United States Department of Agriculture Technical Bulletin No. 1258, 79pp.

Cown, D. J. 1978. Comparison of the Pilodyn and torsiometer methods for the rapid assessment of wood density in living trees. New Zealand Journal of Forest Science **8:**384-391.

Daniel, G., T. Nilsson, and B. Petterson. 1989. Intra and extracellular localisation of lignin peroxidase during the degradation of solid wood and wood fragments by *Phanaerochaete chrysosporium* by using transmission electron microscopy and immunogold labelling. Applied and Environmental Microbiology **55:**871-888.

Dowding, P. 1970. Colonisation of freshly bored pine sapwood surfaces by staining fungi. Transactions of the British Mycological Society **55:**399-412.

Duncan, C. G. 1960. Wood attacking capacities and physiology of soft rot fungi. United States Forest Service, Forest Products Laboratory Report No. 2173.

Duncan, C. G. 1961. Relative aeration requirements by soft rot and basidiomycete wood destroying fungi. United States Forest Service, Forest Products Laboratory Report No. 2218

Duncan,C. G. 1965. Determining resistance to soft rot fungi. United States Forest Service, Forest Products Laboratory Report No.48.

Effland, M. J. 1977. Modified procedure to determine acid-insoluable lignin in wood and pulp. *Tappi*, vol.60, No.10.

Ellwood, E. L., and Eckland, B. A. 1959. Pine logs in pond storage. Forests Products Journal **9:**283.

Enoki, A. H., H. Tanaka, and G. Fuse. 1988. Detection of lignin related compounds, pure cellulose and wood components by white rot and brown rot fungi. Holzforschung **42:**85-93.

Enoki, A., S. Yoshioka, H. Tanaka, and G. Fuse. 1990. Extracellular H_2O_2 producing and one -electron oxidation system of brown rot fungi. The International Research Group on Wood Preservation, Document No. IRG/WP/1445.

Eriksson, K. L., R. Blanchette, and P. Ander. 1990. Microbial and enzymatic decomposition of wood and wood components. Springer-Verlag, Berlin, 397p.

Eslyn, W. E., T. K. Kirk, and M. J. Effland. 1975. Changes in the chemical composition of wood caused by six soft rot fungi. Phytopathology **65:**473-476.

Essner, P. M., and A. C. Tas. 1992. Detection of dry rot by air analysis. The International Research Group on Wood Preservation, Document No. IRG/WP/2399.

Farrell, R. L. 1987. Industrial applications of lignin in transforming enzymes. Philosophical Transactions of the Royal Society of London on Biological Sciences **321:**549-553.

Fee, J. A. 1981. A comment on the hypothesis that oxygen toxicity is mediated by superoxide. In : Oxygen and Life, Royal Society of Chemistry, London.

Findlay, W. P. K. 1966. Ecology of wood-destroying and wood-inhabiting fungi, p. 191-211. *In* G. Becker and W. Liese (ed.), Holz und Organismen, Internationales Symp., Berlin-Dahlem-1965. Dunker and Humblot, Berlin.

Findlay, W. P. K. and Savory, J. G. 1950. Proceedings of the 7th International Congress, Stockholm, p. 316.

Flournoy, D. S. 1991. Changes in pore structure and cell wall volume in wood decayed by brown and white rot fungi. The International Research Group on Wood Preservation, Document No. IRG/WP/1501.

Foisner, R., K. Messner, H. Stachelberger, and M. Rohr. 1985. Wood decay by basidiomycetes : extracellular tripartite membranous structures. Transactions of the British Mycological Society **85:**257-266.

Garrett, S. D. 1955. Microbial ecology of the soil. Transactions of the British Mycolological Society **38:**1-9.

Garrett, S. D. 1963. Soil Fungi and Soil Fertility. Pergammon Press, London, 100pp.

Ghose, T. K. 1987. Measurement of cellulase activities. Pure and Applied Chemistry **59:**257-268.

Glassare, P. 1970. Volatile compounds from *Pinus sylvestris* stimulating the growth of wood rotting fungi. Archives of Microbiology **72:**333-343.

Gleason, F. H., and G. L. R. Gordon. 1988. Techniques for anaerobic growth of Zygomycetes. Mycologia **80:**249-252.

Glegg, R. E. 1957. The influence of oxygen and water on the after effect in cellulose degradation by gamma rays. Radiation Research **6:**467-473

Gold, J. J., I. B. Heath, and T. Bauchop. 1988. Ultrastructural description of a new chytrid genus of a caecum anaerobe *Caecomyces equi* gen. nov., sp. nov., assigned to the Neocallimastiaceae. Biosystems **21:**403-415.

Goldstein, I. S. 1973. Degradation and protection of wood from thermal attack, p. 307-340. *In* D. H. Nicholas (ed.), Wood Deterioration and its Prevention by Preservative Treatments. Syracuse University Press, New York.

Goldstein, I. S., and W. E. Loos. 1973. Special treatments, p. 341-371. *In* D. H. Nicholas (ed.), Wood Deterioration and its Prevention by Preservative Treatments. Syracuse University Press, New York.

Goodell, B., J. Jellison, and J. P. Hoesli. 1988. serological detection of wood decay fungi. Forests Products Journal **38:**59-62.

Gorshin, S. N., and I. G. Krapivinia. 1969. The role of ascomycetes and imperfect fungi in effecting the degradation of wood. Mikolgia I Fitopatologiya **3:**477-480.

Greaves, H. 1966. Effect of bacterial action on some wood cubes in shake culture, p.61-67. Holz und Organismen, International Symposium, Berlin.

Greaves, H. 1969. Micromorphology of the bacterial attack of wood. Wood Science and Technology **3:**150-160.

Greaves, H. 1970. The effect of selected bacteria and actinomycetes on the decay capacity of some wood-rotting fungi. Material und Organismen **5:**265-279.

Greaves, H. 1972. Microbial ecology of untreated and CCA treated stakes exposed in a tropical soil. Canadian Journal of Microbiology **18:**1923-1931.

Grohmann, K., R. Torgett, and M. Himmel. 1985. Optimisation of dilute acetic acid pretreatment of biomass. Biotechnology and Bioengineering Symposium **15:**59-80

Gulati, S. K., J. R. Ashes, G. L. R. Gordon, and M. W. Phillips. 1985. Possible contribution of rumen fungi to fibre digestion in sheep. Proceedings of the Nutrition Society of Australia **10:**96.

Haemmerli, S. D., M. S. A. Leisola, and A. Fiechter. 1986. Polymerisation of lignins by ligninases from *Phanaerochaete chrysosporium*. FEMS Microbiology Letters **35:**33-36.

Haider, K., and J. Trojanowski. 1980. A comparison of the degradation of ^{14}C-labelled DHP and corn stalk lignins by micro- and macrofungi and by bacteria, p. 111-134. *In* T. K. Kirk, T. Higuchi and H. Chang (ed.), Lignin Biodegradation : Microbiology, Chemistry and Potential Applications. CRC Press, Boca Raton, Florida.

Hansen, J. 1972. Sterilisation of test objects for mycological testing using electron radiation. Material und Organismen **7:**73-80.

Hata, K. 1966. Investigations on lignin and lignification. XXXIII. Studies on lignins isolated from spruce wood decayed by *Poria subacida*. Holzforschung **20:**142-147.

Heath, I. B., T. Bauchop, and R. A. Skipp. 1983. Assignment of the rumen anaerobe *Neocallimastix frontalis* to the Spizellomycetales on the basis of its polyflagellate zoospore ultrastructure. Canadian Journal of Botany **61:**295-307.

Hedley, M. E. 1982. Limitations of the Pilodyn when assessing decay in graveyard stakes. The International Research Group on Wood Preservation, Document No. IRG/WP/2168.

Hedley, M. E., and R. W. Naish. 1980. Field stake test assessment with the Pilodyn. The International Research Group on Wood Preservation, Document No. IRG/WP/2136.

Henningson, B., and T. Nilsson. 1976. Some aspects on microflora and the decomposition of preservative treated wood in ground contact, p. 307-318. *In* G. Becker and W. Liese (ed.), Organismen und Holz: Internationales Symposium Berlin-Dahlem-1975. Dunker and Humblot, Berlin.

Hesseltine, C. W., C. L. Featherson, G. L. Lombard, and V. R. Dowell. 1985. Anaerobic growth of molds isolated from fermentation starters used for foods in Asian countries. Mycologia **77:**390-400.

Highley, T. 1982. Is extracellular hydrogen peroxide involved in cellulose degradation by brown rot fungi? Material und Organismen **17:**205-214.

Highley, T. 1987a. Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay fungi in solid medium. FEMS Microbiology Letters **48:**373-377.

Highley, T. 1987b. Biochemical aspects of white and brown rot decay. The International Research Group on Wood Preservation, Document No. IRG/WP/1319.

Highley, T., and J. F. Lutz. 1970. Bacterial attack on wood stored bolts. Forests Products Journal **20:**43-44.

Highley, T., L. Murmanis, and J. Palmer. 1983. Electron microscopy of cellulose decomposition by brown rot fungi. Holzforschung **37:**271-277.

Higuchi, T., I. Kawamura, and H. Kawamura. 1955. Properties of the lignin in decayed wood. Journal of the Japan Forestry Society **37:**298-302.

Ho, Y. W., N. Abdullah, and S. Jalaludin. 1990. *Ruminomyces elegans* gen. et. sp. nov., a polycentric anaerobic rumen fungus from cattle. Mycotaxon **38:**397-405.

Holland, K. T., J. S. Knapp, and J. G. Shoesmith. 1987. Anaerobic Bacteria. Chapman and Hall, New York.

Hueck, H. J. 1968. The biodeterioration of materials - an appraisal, p. 6-12. *In* A. H. Walters and J. J. Elphick (ed.), Biodeterioration of Materials. Elsiever, London.

Hulme, M. A., and J. A. Butcher. 1977. Soft rot control in hardwoods treated with chromated copper arsenate preservatives. Material und Organismen **12:**81-95.

Hungate, R.E. 1950. An anaerobic mesophilic cellulolytic bacterium. Bacteriological reviews **14:**1-49.

Hungate, R. E. 1966. The Rumen and its Microbes. Academic Press Incorporated, New York.

Hungate, R. E. 1969. A roll tube method for the cultivation of strict anaerobes, p 117 -132. In (J. R. Norris and D. W. Ribbons eds.), Methods in Microbiology Volume 3B. Academic Press, New York.

Huynh, V. B., and R. L. Crawford. 1985. Novel extracellular enzymes (ligninases) of *Phanaerochaete chrysosporium*. FEMS Microbiology Letters **28:**119-123.

Illman, B. L., D. C. Meinholtz, and T. Highley. 1988. Generation of hydroxyl radical by the brown rot fungus, *Postia placenta*. The International Research Group on wood Preservation, Document No. IRG/WP/1360.

Jane, F. W. 1957. Wood science - its past, present and future. Journal of the Institute of Wood Science **1:**1-11.

Jane, F. W. 1970. The Structure of Wood, 2nd ed. A. and C. Black, London, 478pp.

Jellison, J., K. C. Smith, and W. T. Shortle. 1992. Cation analysis of wood degraded by white and brown rot fungi. The International Research Group on Wood Preservation, Document No. IRG/WP/1552.

Jellison, J., B. Goodell, F. Fekete, and V. Chandhoke. 1991. Fungal siderophores and their role in wood biodegradation. The International Research Group on Wood Preservation, Document No. IRG/WP/1442.

Joblin, K. N., and G. E. Naylor. 1990. Degradation of woods by obligately anaerobic fungi. The International Research Group on Wood Preservation, Document No. IRG/WP/1452.

Jones, E. B. G. 1982. Decomposition by basidiomycetes in aquatic environments, p. 191-192. *In* J. C. Frankland, J. N. Hedger and M. J. Swift (ed.), Decomposer Basidiomycetes, their Biology and Etiology. Cambridge University Press, London.

Jones, E. B. G. 1986. *Digitatispora lignicola* sp. nov. a new marine lignicolous Basidiomycotina. Mycotaxon **27:**55-159.

Jurasek, L., R. J. Calvin, and D. R. Whitaker. 1967. Microbial aspects of the formation and degradation of cellulosic fibres. Advances in Applied Microbiology **9:**131-170.

Jurasek, L., and M. G. Paice. 1988. Biological treatment of pulp. Biomass **15:**103-108.

Kaarik, A. 1967. Colonisation of pine and spruce poles by fungi after six months. Material und Organismen **2:**97-108.

Kaarik, A. 1975. Succession of microorganisms during wood decay, p. 39-51. *In* W. Liese (ed.), Biological Transformation of Wood by Microorganisms. Springer- verlag, Berlin, Heidelberg, New York.

Karnop, G. 1967. Der Betall von Wassergelagertan Nadelholz durch Bakterian mit besondererBeruchsichtigung des anaeroben Cellulose Abbans durens *Bacillus omelianski*. Ph.D.thesis. University of Hamburg.

Kenaga, D. L., and E. B. Cowling. 1959. Effect of gamma radiation on ponderosa pine. Forest Products Journal **9:**112-116.

Keston, S. A. 1956. Specific colorimetric enzymatic analytical reagents for glucose. Abstracts of the 129th ACS Meeting Dallas, April 1956, p31c.

Kim, Y. S., B. Goodell, and J. Jellison. 1990. Immuno-electron microscopic localisation of extracellular metabolites in spruce wood decayed by brown rot fungus *Postia placenta*. The International Research Group on Wood Preservation, Document No. IRG/WP/1441.

King, B. 1981. The durability of timber and timber products. Bulletin of the Institute of Conventional Science and Technology **19:**5-11.

King, B., and H. O. W. Eggins. 1973. Decay mechanisms of microfungi which might produce an enhanced permeability in wood. International Biodeterioration Bulletin **9:**35-43.

King, B., T. A. Oxley, and K. D. Long. 1976. Some biological effects of redistribution of soluble nutrients during decomposition of wood. Material und Organismen **3:**263-276.

King, B., W. J. Henderson, and M. E. Murphy. 1980. A bacterial contribution to wood nitrogen. International Biodeterioration Bulletin **16:**79-84.

King, B., G. M. Smith, A. A. W. Baecker, and A. Bruce. 1981. Wood nitrogen control of toxicity of copper-chrome-arsenic preservatives. Material und Organismen **16:**105-118.

Kirk, K. T. 1973. The chemistry and biochemistry of decay, p. 149-182. *In* D. H. Nicholas (ed.) Wood Deterioration and its Prevention by Preservative Treatments. Syracuse University Press, New York.

Kirk, K. T. 1975. Chemistry of lignin degradation by wood destroying fungi, p. 161-178. *In* W. Liese (ed.), Biological Transformation of Wood by Microorganisms. Springer-verlag, Berlin.

Kirk, T. K., and H. Chang. 1974. Decomposition of lignin by white rot fungi. I. Isolation of heavily degraded lignins from decayed spruce. Holzforschung **28:**217-222.

Kirk, T. K., and H. Chang. 1975. Decomposition of lignin by white rot fungi. II. Characterisation of heavily degraded lignins from decayed spruce. Holzforschung **29:**56-64.

Kirk, T. K., and P. Fenn. 1982. Formation and action of ligninolytic systems in Basidiomycetes. *In* : J. C. Frankland, J. N. Hedger and M. J. Swift (ed.), p. 67-91. Decomposer Basidiomycetes - their Biology and Ecology. Cambridge University Press, New york.

Knapp, J. S. 1985. Biodegradation of celluloses and lignins. *In* M. Moo-Young (ed.), p.835-846. Comprehensive Biotechnology: The Principles, Applications and Regulation of Biotechnology in Industry, Agriculture and Medicine, Vol. 4. Pergamon Press.

Knuth, D. T., and E. McCoy. 1962. Bacterial degradation of pine logs in pond storage. Forest Products Journal **12:**437-442.

Koenings, J. 1972a. Production of extracellular hydrogen peroxide and peroxidase by brown rot fungi. Phytopathology **62:**100-110.

Koenings, J. 1972b. *Poria weirii* as a possible commercial source of peroxidase. Applied Microbiology **23:**835-836.

Koenings, J. 1974. Production of hydrogen peroxide by wood-decaying fungi in wood and its correlation with weight loss, depolymerisation and pH changes. Archives of Microbiology **99:**129-145.

Kubicek, C. P. 1982. B-glucosidase excretion by *Trichoderma pseudokoningi*: correlation with cell wall bound B-1,3-glucanase activities. Archives of Microbiology **132:**349-354.

Krieg, N. R., and P. S. Hoffman. 1986. Microaerophily and oxygen toxicity. Annual Review of Microbiology **40:**107-130.

Lawton, E., A. Buechie, and J. Balwitt. 1953. Irradiation of polymers by high-energy electrons. Nature **172:**76-77

Leightley. L. E. 1981. The use of the Shigometer and Pilodyn as non-destructive test methods for detecting decay in CCA treated eucalypt poles. The International Research Group on Wood Preservation, Document No. IRG/WP/2153.

Leightley, L. E. 1982. Examination of the Pilodyn as a non destructive test method for detecting decay in CCA treated eucalypt poles. The International Research Group on Wood Preservation, Document No. IRG/WP/2177.

Leightley, L. E., and R. A. Eaton. 1979. *Nia vibrissa* - a marine white rot fungus. Transactions of the British Mycological Society **73:**35-40.

Levi, M. P. 1973. Control Methods, p. 183-216. *In* D. H. Nicholas (ed.), Wood Deterioration and its Prevention by Preservative Treatments. Syracuse University Press, New York.

Levi, M. P., and E. B. Cowling. 1975. Extension : an obligation of all wood-products pathologists to society, p. 182-192. *In* W. Liese (ed.), Biological Transformation of Wood by Microorganisms. Springer-verlag, Berlin, Heidelberg, New York.

Levy, J. F. 1965. The soft rot fungi: their mode of action and their significance in the degradation of wood. Advances in Botanical Research **2:**323-357.

Levy, J. F. 1967. Decay and degradation of wood by soft rot fungi and other organisms. Records of the Annual Convention of the British Wood Preservers Association **7:**147-175.

Levy, J. F. 1968. Studies on the ecology of fungi in wooden fence-posts, p. 424-428. *In* A. H. Walters and J. J . Elphick (ed.), Biodeterioration of Materials. Elsiever, London.

Levy, J. F. 1975. Bacteria associated with wood in ground contact, p. 64-73. *In* W. Liese (ed.), Biological Transformation of wood by Microorganisms. Springer-verlag, Berlin.

Levy, J. F. 1982. The place of basidiomycetes in the decay of wood in contact with the ground, p. 161-178. *In* J. C. Frankland, J. N. Hedger and M. J. S. Swift (ed.), Decomposer Basidiomycetes - their Biology and Ecology.Cambridge University Press, London.

Liese, W. 1963. Tertiary wall and warty layer in wood cells. Journal of Polymer Science **2:**213-219.

Liese, W. 1970. Ultrastructural aspects of woody tissue disintegration. Annual Review of Phytopathology **8:**231-257.

Liese, W., and H. Greaves. 1975. Micromorphology of bacterial attack, p. 64-73. *In* W. Liese (ed.), Biological Transformation of Wood By Microorganisms. Springer-verlag, Berlin.

Liese, W., and G. Karnop. 1968. On the attack of coniferous wood by bacteria. Holz Als Roh-u-Werkstof 26(6):202-208.

Liese, W., and R. Schmid. 1961. Licht-und Elektromikroskopische utersuchungen uber das Waschtum von Blauepilzen in Kiefern-und Fichtenholz. Holz Als Roh-und Werkstof 19:329-337.

Liese, W., and R. Schmid. 1964. Uber das Wachstum von Blauepilzen durch verholzte Zellwande. Phytopathologische Zeitschrift 51:385-393.

Lindeberg, G. 1944. Uber die Physiologie lignin-abbauender Bodenhymenomyzeten. Symbolae Botanicae Upsalienses **8:**1-183.

Loesche, W. J. 1969. Oxygen sensitivity of various anaerobic bacteria. Applied Microbiology **18:**723-727.

Lowe, S. E., M. K. Theodoru, and A. P. J. Trinci. 1987. Cellulases and xylanases of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose and xylan. Applied and Environmental Microbiology **53:**1216-1223.

Lund, A. 1974. Yeasts and molds in the bovine rumen. Journal of General Microbiology **81:**453-462.

Lundborg, A. 1989a. Interactions between fungi affect their ability to degrade crystalline cellulose. Material und Organismen **23:**171-189.

Lundborg, A. 1989b. A simple non-destructive method for estimating degradation of crystalline cellulose in agar cultures. Material und Organismen **23:**191-196.

Lundborg, A. 1989c. Deformation of agar by wood decaying fungi- a possible indication of the occurrence of radicals. Material und Organismen **23:**259-269.

Lundstrom, H. 1973. Studies of the wood decay capacity of the soft rot fungi *Allecheria terrestris, Phialophora luteo viridis* and *Phialophora richardsiae*. Royal College of Forestry, Department of Forest Products, Research Notes, No.R87, Stockholm.

Lutomski, K., and M. Lawniczak. 1967. Uber den Holzeigenschaften durch x - strahlung. Materialpruf **4:**416- 426

McCormack, P. W., A. E. G. Cass, and R. J. Murphy. 1992. NMR TI relaxation time as a non-destructive method for the study of decay in wood. The International Research Group on Wood Preservation, Document No. IRG/WP/2406.

Merril, W. 1965. Decay of wood and wood fibre boards by common fungi imperfecti. Material und Organismen **1:**69-76.

Merril, W., and E. B. Cowling. 1966. Nitrogen in wood and its role in wood deterioration. Canadian Journal of Botany **44:**1539-1554.

Merril, W., and D. W. French. 1966. Colonisation of wood by soil fungi. Phytopathology **56:**301-303.

Micales, J., and T. Highley. 1988. The effect of tunicamycin on the production and secretion of extracellular carbohydrate degrading enzymes by *Postia placenta*. The International Research Group on Wood Preservation, Document No. IRG/WP/1342.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugars. Analytical Chemistry **31:** 426-428.

Montgomery, R. A. P. 1982. The role of polysaccharidase enzymes in the decay of wood by basidiomycetes, p.51-65. *In* J. C. Frankland, J. N. Hedger, and M. J. Swift (ed.), Decomposer Basidiomycetes - their Biology and Ecology. Cambridge University Press, London, New york.

Morris, J. G. 1979. Nature of oxygen toxicity in anaerobic microorganisms, p. 149-162. *In* M. Shilo (ed.), Strategies of Microbial Life in Extreme Environments. Dahlem, Konferenzen, Berlin.

Morrison, M., and R. Mackie. 1989. Fungi in the grazing stakes. Nuclear active, International Journal of the Atomic Energy Corporation of South Africa, No.40, p 17-24.

Mountford, D. O. 1987. The rumen anaerobic fungi. FEMS Microbial Reviews **46:**401.

Mountford, D. O., and R. A. Asher. 1985. Production and regulation of cellulase by two strains of the rumen anaerobic fungus *Neocallimastix frontalis*. Applied and Environmental Microbiology **49:**1314-1322.

Mouzouras, R., E. B. G. Jones, R. Venkatasamy, and S. T. Moss. 1986. Decay of wood by microorganisms in marine environments. British Wood Preservers Association Annual Convention, 18p.

Mowe, G., B. King, and S. J. Senn. 1983. Tropic responses of fungi to wood volatiles. Journal of General Microbiology **129:**779-784.

Nasroun, T. A. 1971. Mode of changes in the ultrastructure and microstructure of wood attacked by white rots and brown rots. Diploma thesis, Imperial college, University of London.

Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry **153:**373-380.

Nilsson, T. 1973. Studies on wood degradation and cellulolytic activity of microfungi. Studia Forestialia Suecica, No.104, 40pp.

Nilsson, T. 1974. Comparative study on the cellulolytic activity of white rot and brown rot fungi. Material und Organismen **9:**173-198.

Nilsson, T., and G. Daniel. 1992. On the use of % weight loss as a measure for expressing results of laboratory decay experiments. The International Research Group on Wood Preservation, Document No. IRG/WP/2394.

Nkonge, C., and G. M. Ballance. 1982. A sensitive colorimetric procedure for Nitrogen Determination in micro-kjeldahl digests. Journal of Agricultural and Food Chemistry **30:**416-420.

Norkrans, B. 1967. Cellulose and cellulolysis. Advances in Applied Microbiology **9:**91-129.

Odier, E., M. D. Mozuch, B. Kalayanaramen, and T. K. Kirk. 1988. Ligninase-mediated phenoxy radical formation and polymerisation unaffected by cellobiose : quinone oxidoreductase. Biochimie **70:**847-857.

Onderdonk, A. B., J. Johnston, J. W. Mayhew, and S. L. Gorbach. 1976. Effect of dissolved oxygen and Eh on *Bacteroides fragilis* during continous culture. Applied and environmental Microbiology **31:**168-172.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. Journal of General Microbiology **91:**249-262.

Orpin, C. G. 1976. Studies on the rumen flagellate *Sphaeromonas communis*. Journal of General Microbiology **94:**270-280.

Orpin, C. G. 1977. The rumen flagellate *Piromonas communis* : its life history and invasion of plant material in the rumen. Journal of General Microbiology **99:**107-117.

Orpin, C. G., and A. J. Letcher. 1979. Utilisation of cellulose, starch, xylan and other hemicelluloses for growth by the rumen phycomycete *Neocallimastix frontalis*. Current Microbiology **3 :** 121 - 124.

Orpin, C. G., and E. A. Munn. 1986. *Neocallimastix patriciarum* sp.nov., a new member of the Neocallimasticaceae inhabiting the rumen of sheep. Transactions of the British Mycological Society **86:**178-181.

Paice, M. G., L. Jurasek, and M. Desrochers. 1982. Simplified analysis of wood sugars. *Tappi* **65:**103-106.

Paice, M. G., L. Jurasek, C. Ho, R. Bourbonnais, and F. Archibald. 1989. Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*. *Tappi* **5:**217-221.

Pettersen, R. C., V. H. Schwandt, and M. J. Effland. 1984. An analysis of the wood sugar assay using HPLC : a comparison with paper chromatography. Journal of Chromatographic Science **22:**478-484.

Phillips, M. W., and G. L. R. Gordon. 1988. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. Biosystems **21:**377-383.

Phillips, M. W., and G. L. R. Gordon. 1989. Growth characteristics on cellobiose of three different anaerobic fungi isolated from the ovine rumen. Applied and Environmental Microbiology **55:**1695-1702.

Rindorf, H., and A. Christensen. 1969. A plant for bacteria reduction according to requirements (Danish). Farmaceutisk Tidende **30:**705-714.

Ritschkoff, A. C., and L. Viikari. 1991. The influence of crystalline and amorphous cellulose on extracellular hydrogen peroxide production by brown rot fungi. The International Research Group on Wood Preservation, Document No. IRG/WP/1482.

Rivers, D. B., S. J. Gracheck, L. C. Woodford, and G. H. Emmert. 1984. Limitations of the DNS assay for reducing sugars from saccharified lignocellulosics. Biotechnology and Bioengineering **26:**800-802.

Rogers, G. M. 1990. Immunolocalisation of xylanases produced by *Clostridium xylanolyticum* during anaerobic degradation of *Pinus Patula*. Ph.D. Thesis, University of Witwatersrand, Johannesburg.

Rogers, G. M., and A. A. W. Baecker. 1987. Theories on the degradation in wood associated with glycocalyx producing bacteria. Journal of the Institute of Wood Science **12:**78-85.

Rogers, G. M., and A. A. W. Baecker. 1988a. A new method for the study of microbiological decay of wood in a strictly anaerobic environment. The International Research Group on Wood Preservation, Document No. IRG/WP/2319.

Rogers, G. M., and A. A. W. Baecker. 1988b. The study of anaerobic bacteria in wood. Microbios 1988. Proceedings of the 5th Biennial Congress of the South African Society for Microbiology, Pretoria, 1988. ISBN No.0620126183. CSIR Pub.No. S.483, 4.6.

Rogers, G. M., and A. A. W. Baecker. 1988c. The micromorphology of wood degradation by anaerobic bacteria. Proceedings of the Electron Microscopy Society of Southern Africa **18:**127-128.

Rogers, G. M., and A. A. W. Baecker. 1991. *Clostridium xylanolyticum* sp. nov., an anaerobic xylanolytic bacterium from decayed *Pinus patula* wood chips. International Journal of Systematic Bacteriology **41:**140-143.

Sachs, I. B., V. M. G. Mair, and J. R. Kuntz. 1967. Penetration and degradation of cell walls in oak infected with *Ceratocystis fagacearum*. Phytopathology **60:**1399-1404.

Saeman, J. F., W. E. Moore, R. L. Mitchell, and M. A. Millet. 1954. Techniques for the determination of pulp constituents by quantitative paper chromatography. *Tappi* **37:**336-343.

Savory, J. G. 1954. Breakdown of timber by ascomycetes and fungi imperfecti. Annals of Applied Biology **41:**336-247.

Savory, J. G., and A. F. Bravery. 1970. Collaborative experiments in testing the toxicity of wood preservatives to soft rot fungi. Material und Organismen **5:**59-80.

Schawald, W., M. Chan, C. Breuil, and J. N. Saddler. 1988. Comparison of HPLC and colorimetric methods for measuring cellulolytic activity. Applied Microbiology and Biotechnology **28:**398-403.

Scheffer, T. C. 1973. Microbial degradation and the causal organisms, p. 31-106. *In* D. H. Nicholas (ed), Wood Deterioration and its Prevention by Preservative Treatments. Syracuse University Press, New york.

Schippenkoetter, W. H., L. D. Abraham, and A. A. W. Baecker. 1988. Analysis of relative performances of DNBP and CCA wood preservatives in accelerated decay tests. Material und Organismen **23:**301-319.

Schmid, R., and W. Liese. 1965. Zur Aussenstruktur der Hyphen von Blauepilzen. Phytopathology **54:**275-284.

Schmidt, C. J., B. K. Whitten, and D. D. Nicholas. 1981. A proposed role for oxalic acid in non-enzymatic wood decay by brown rot fungi. Proceedings of the American wood Preservers Association **77:**157-164.

Shelver, G. D. 1992. Ph.D. thesis. Interactions between continously cocultured anaerobic rumen bacteria during the degradation of maize cell walls. University of Witwatersrand, Johannesburg.

Shelver, G. D., U. Matai, W. van Wyk, and A. A. W. Baecker. 1991. Effects of the anaerobic wood decay bacterium *Clostridium xylanolyticum* on untreated Pinus and Eucalyptus pulp. The International Research Group on Wood Preservation, Document No. IRG/WP/1506.

Shigo, A. L. 1962. Observations on the succession of fungi on hardwood pulpwood bolts. Plant Disease Reporter **46:**379-380.

Shigo, A. L. 1967. Succession of organisms in discoloration and decay of wood. Internal Review of Forest Research No.2.

Shoesmith, J.G., and B. W. Worsely. 1984. Anaerobes and exposure to oxygen, p. 127-146. *In* : M.H.E. Andrew and A.D. Russel(ed.), The revival of Injured Microbes. Academic Press, New York.

Singh, V. 1989. Isolation of anaerobic fungi from sheep rumen fluid. Mycology Practical Report, University of Durban Westville, 22p.

Singh, A. P., and J. A. Butcher. 1990. Bacterial degradation of wood cell wall : a review of degradation patterns. The International Research Group on Wood Preservation, Document No. IRG/WP/1460.

Singh, A. P., and M. E. Hedley. 1990. Ultrastructure of degraded, CCA-treated *Pinus radiata* wood from a marine pile. The International Research Group on Wood Preservation, Document No. IRG/WP/1461.

Singh, A. P., T. Nilsson, and G. Daniel. 1990. Bacterial attack of *Pinus sylvestris* wood under near anaerobic conditions. Journal of the Institute of Wood Science **11:**237-249.

Smith, D. N. R. 1980. Study of decay of preservative-treated wood in soil. Journal of the Institute of Wood Science **8:**194-200.

Smith, D. M., and R. Y. Mixer. 1959. The effect of lignin on the degradation of wood by gamma irradiation. Radiation Research **11:** 776-780.

Somogyi, M. 1952. Notes on sugar determination. Journal of Biological Chemistry **195:**19-23.

Spies, P. H. 1988. Global and regional trends in demography, markets and human needs in relation to development to South African Forestry. Wood Southern Africa **14:**24-25.

Srebotnik, E., and K. Messner. 1988. Spatial arrangement of lignin peroxidase in pine decayed by *Phanaerochaete chrysosporium* and *Fomitopsis pinicola*. The International Research Group on Wood Preservation, Document No. IRG/WP/1343.

Srebotnik, E., and K. Messner. 1990. Immunogold labelling of size marker proteins in brown rot degraded pine wood. The International Research Group on Wood Preservation, Document No. IRG/WP/1428.

Srebotnik, E., K. Messner, and R. Foisner. 1988. Penetrability of white rot wood by the lignin peroxidase of *Phanaerochaete chrysosporium*. Applied and Environmental Microbiology **54:**2608-2614.

Starr, M. P., H. Stolp, H. G. Truper, A. Ballows, and H. Schlegal. 1981. The Prokaryotes. A Handbook on Habitats, Isolation and Identification of Bacteria, Volumes I & II. Springer Verlag, New York.

Sudo, K., K. Shimizu, T. Ishii, T. Fugii, and S. Nagasawa. 1986. Enzymatic hydrolysis of wood. Part IX. Catalysed steam explosion of soft wood. Holzforschung **40:**339-345.

Sutter, H. P., and E. B. G. Jones. 1985. Interactions between copper and wood degrading fungi. Record of the 1985 Annual Convention of the British Wood Preservers Association.

Sutter, H. P., E. B. G. Jones, and O. Walchi. 1984. Occurrence of crystalline sheaths in *Poria placenta*. Journal of the Institute of Wood Science **10:**19-23.

Tanaka, H., A. Enoki, and G. Fuse. 1986. Correlation between ethylene production from <-oxo-x-methylthiobutyric acid and degradation of lignin dimeric model compounds by wood-inhabiting fungi. Mokuzai Gakkaishi **32:**125-135.

Technikon Auto Analyser II Industrial Method No.334-74A, 1976.

Teunissen, M. J., H. J. M. Op Den Camp, C. G. Orpin, J. H. J Huis in 'T Veld, and G. D. Vogels. 1991. Comparison of growth characteristics of anaerobic fungi isolated from ruminant and non-ruminant herbivores during cultivation in a defined medium. Journal of General Microbiology **137:**1401-1408.

Theodorou, M. K., A. C. Longland, M. S. Dhanoa, S. E. Lowe, and A. P. J. Trinci. 1989. Growth of *Neocallimastix* sp. strain R1 on Italian ryegrass hay : removal of neutral sugars from plant cell walls. Applied and Environmental Microbiology **55:**1365-1367.

Thornber, J. P., and D. H. Northcote. 1961. Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissues in trees. Biochemistry Journal **81:**449-455.

Tien, M., and T. K. Kirk. 1983. Lignin-degrading enzyme from the hymenomycete *Phanaerochaete chrysosporium* Burds. Science **221:**661-663.

Tien, M., and T. K. 1984. Lignin-degrading enzyme from *Phanaerochaete chrysosporium*: purification, characterisation and catalytic propertiesof a unique H_2O_2-requiring oxygenase. Proceedings of the National Academy of Science, USA **81:**2280-2284.

Verall, A. F. 1969. Attack by plant organisms on southern pine wood. Forest Products Journal **19:**40-46.

Vibrante Technologies Incorporated. 1983-1987. Wood pole inspection simplified. Summary of Internal Reports, 1983-1984, 4pp.

Walden, W. C., and D. J. Hengtes. 1975. Differential effects of oxygen and oxidation-reduction potential on the multiplication of 3 species of anaerobic bacteria. Applied Microbiology **30 :** 781 - 785.

Webster, J. 1978. Introduction to Fungi. Cambridge University Press, New York, 424pp.

Wentz, F. E., A. D. Marcy, and M. J. Gray. 1982. Analysis of wood sugars in pulp and paper industry samples by HPLC. Journal of Chromatographic Science **20:**349-352.

Wiederhold, A. H., M. Morrison, and A. A. W. Baecker. 1989. The colonisation pattern of wood by anaerobic fungi. The International Research Group on Wood Preservation, Document No. IRG/WP/1403.

Wilcox, W. W. 1968. Changes in wood microstructure through progressive stages of decay. United States Forest Services Research Paper FPL-70, 46pp.
Wilcox, W. W. 1973. Degradation in relation to wood structure, p. 107-148. *In* D. H. Nicholas (ed.), Wood Deterioration and its Prevention by Preservative Treatments. Syracuse University Press, New York.

Woods, D. R., and D. T. Jones. 1986. Physiological responses of *Bacteroides* and *Clostridium* strains to environmental stress factors. Advances in Microbial Physiology **23:**1-64.

Wren, M. W. D. 1980. Prolonged incubation in the isolation of anaerobic bacteria from clinical specimens. Journal of Medical Microbiology **13:**257-263.

Yang, J. C., D. Williams, R. Brigmon, and D. P. Chynowth. 1989. Repetitive isolation of a new cellulolytic *Clostridium* species from a woody-fermenting anaerobic digester, Abstract No. 1-110, p. 235. Annual Meeting of the American Society of Microbiology, 1989.

Zeikus, J. G., and J. C. Ward. 1974. Methane formation in living trees: a microbial origin. Science **184:**1181-1183.