

FUNGAL AND SUBSTRATE-ASSOCIATED FACTORS  
AFFECTING LIGNOCELLULOLYTIC MUSHROOM  
CULTIVATION ON WOOD SOURCES AVAILABLE  
IN SOUTH AFRICAN.

THESIS

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MARIA FATIMA DA SERRA

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## ABBREVIATIONS

acid swollen cellulose agar	ASCA
arbitrarily primed PCR	AP-PCR
aryl alcohol oxidase	AAO
basidiome size	BS
biological efficiency	BE
Blakeslee malt extract agar	B-MEA
deoxynucleotide triphosphate	dNTP
distilled water	dH <sub>2</sub> O
ethylene diaminetetra-acetic acid disodium salt	EDTA
figure	fig
<i>Flammulina velutipes</i> DSM 1684	F.V
glucose/malt/peptone/yeast medium	GMPY
kilobases	Kb
<i>Lentinus edodes</i> DSM 1899	1899
<i>Lentinus edodes</i> ATCC 48860	48860
<i>Lentinus edodes</i> ATCC 58762	58762
lignin agar	LA
lignin peroxidase	LiP
malt agar	MA
malt extract/peptone agar	MEPA
malt/yeast medium	M3
manganese peroxidase	MnP
N,N,N',N'-tetramethylethylene diamine	TEMED
Oak	O
<i>Pleurotus calyptratus</i> 36211	36211
<i>P. citrinopileatus</i> CCRC 36239	36239
<i>P.columbinus</i> CCRC 36215	36215



tris (hydroxymethyl)-aminomethane

Tris

unweighed pair group method with arithmetic average

UPGMA

veratryl alcohol oxidase

VAO

yeast/glucose medium

YG

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## ABSTRACT

Vast quantities of lignocellulosic materials, representing potential substrates for the cultivation of speciality mushrooms, are produced annually in South Africa. A number of these materials are derived as waste products of the timber and agricultural industries, e.g. Maranti (*Shorea spp.*) and Port Jackson Willow (*Acacia longifolia*), respectively. The screening of various wood-degrading fungi, which are cultivated worldwide for their production of speciality mushrooms, indicated that under the environmental conditions considered, certain species were adapted to cultivation on these lignocellulosic wastes (*Pleurotus* species) whereas others were not (*Lentinus edodes* and *Flammulina velutipes*). Furthermore, intra- and interspecies specific differences in the growth and production potential of the various lignocellulolytic fungi investigated on synthetic and natural medium were discovered. Biochemical and genetical investigations of these strains indicated differences between and within species which were often significant. Species varied qualitatively and quantitatively in the lignocellulolytic enzymes produced, which was loosely correlated with productivity on the different media investigated. Genetical studies, using RAPD fingerprinting, indicated that the *Pleurotus* genus is highly variable which supports the observed differences in growth, yield and enzymatic activity between different strains and species.

## CHAPTER 1

## GENERAL INTRODUCTION

## 1.1 INTRODUCTION

Lignocellulose is a complex polymer composed of cellulose, hemicellulose and lignin (Wood, 1985). Woody materials are the most commonly derived sources of lignocellulose, and vast quantities of this material is generated annually as waste by a large number of industries (Zadrazil, 1978; Janshekar and Fiechter, 1983; Buswell *et al.*, 1993). Lignocellulosic waste represents a valuable resource, if the appropriate bioconversion technology is applied (Kaneshiro, 1977; Zadrazil, 1978; Wood, 1985; Zadrazil and Peeraly, 1986; Buswell *et al.*, 1993; Burla *et al.*, 1992). Possible bioconversion processes which can be applied to lignocellulosic materials are presented in Table 1.1.

It is practically and economically feasible to apply bioconversion processes to waste lignocellulosic materials, especially in developing countries (Chang and Steinkraus, 1982; Martinez-Carrera *et al.*, 1991). In Southern Africa vast quantities of unexploited lignocellulosic wastes are generated annually. Maranti sawdust (*Shorea spp.*) is produced in large amounts as a by-product of the construction industry. Furthermore, a large lignocellulose reservoir is derived from the clearing of certain commercially insignificant invasive species. A number of Australian *Acacia* species, namely Black Wattle (*Acacia mearnsii*) and Port Jackson willow (*Acacia cyclops* and *Acacia longifolia*), are economically important invasive species which are widely distributed in, and rapidly spreading through, the Western and Eastern Cape (Nesser, 1993). These weeds are causing the encroachment of valuable farm and conservation land such as the fynbos biome, which is an ecological biome of significant importance owing to its endangered, indigenous nature and to its attraction of ecotourism (Nesser, 1993). Furthermore, the invasion of water catchment areas by these and other such species has serious implications for water restricted areas (Nesser, 1993). **The** control of these weeds is difficult and costly as a result of dense growth patterns and resistance to removal, necessitating the use of both chemical and physical eradication procedures (Nesser, 1993).

Table 1.1 Existing and potential applications for the bioconversion of lignocellulose (Wood, 1985).

Products	Substrates	Technology		Constraints
		Available	Profitable	
Mushrooms	Composted: Straw Sawdust Whole wood	Yes	Yes	Consumer resistance to greater consumption
Feeds or food, SCPa	Solid substrate: Straw Wood	No	No	Product cost. Uniformity of scale up. Rate of treatment
Feeds or foods, SCP	Hydrolysed: Straw Sawdust Wood	Yes	No	Price of pre-treatment. Costs of product.
N <sup>2</sup> -fixation Soil conditioner Biological control	Straw	No		Scale up to farm level. Treatment costs.
Pulp	Wood Straw	Yes		See feeds. Product quality. Product cost.
Bulk chemicals: ethanol Glucose Butanediol	Wood Straw Food by-products	Yes	No	Other feedstock more competitive Nature of feedstock (see feeds)
Speciality chemicals: polymers phenolics	Wood Straw	Yes		Product price (see feeds)
Waste treatment	Pulping by-products	Yes	No	Process cost
Enzymes: cellulases hemicellulases	Wood Straw Sawdust	Yes	No	Product cost compared to liquid fermentation sources

<sup>a</sup> SCP = single cell protein.

Both Maranti sawdust and the invading *Acacia* species have potential application as substrates for bioconversion processes. The only bioconversion process which efficiently utilises the entire lignocellulosic complex is the cultivation of edible mushrooms, resulting in it being one of the most economically viable processes for the exploitation of lignocellulosic wastes (Wood, 1985; Bisaria and Madan, 1983; Buswell *et al.*, 1993). The utilization of the above-

mentioned lignocellulosic materials as substrates for mushroom cultivation could result in the transformation of economic liabilities into important components of a commercially highly lucrative industry.

Mushroom cultivation is dependent upon substrate- and fungal-associated factors (Buswell *et al.*, 1993). Substrate-associated factors are the chemical and structural properties of the lignocellulosic substrate. Fungal determinants are essentially the nutritional and environmental requirements of the cultivated strains, which are predominantly genetically determined, and the ability to produce the degradative enzymes necessary for lignocellulose bioconversion. It is important to have an understanding of these factors for the development of economically feasible cultivation processes.

## 1.2 THE STRUCTURE AND FORM OF LIGNOCELLULOSE

The lignocellulose of woody materials can be divided into macromolecular and minor micromolecular cell wall components (Fengel and Wegener, 1989). The quantity and-type of these components is species specific (Fengel and Wegener, 1989).

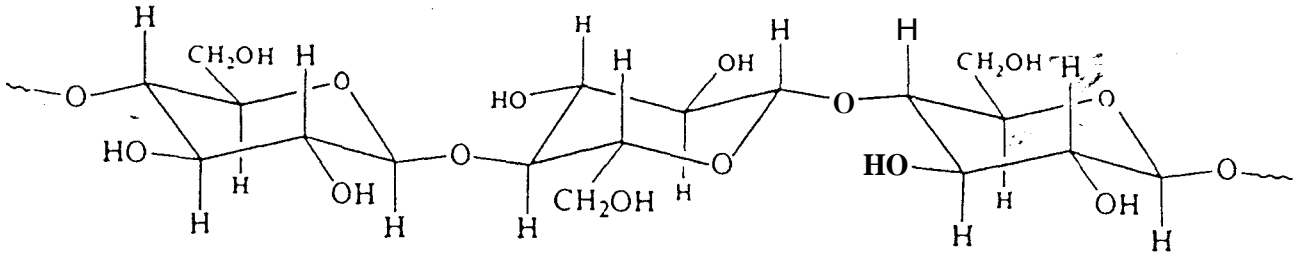
### 1.2.1 Macromolecular components

The macromolecular portion consists of cellulose, hemicellulose and lignin/s in the approximate ratio 4:3:3 (Wood, 1985; Fengel and Wegener, 1989; Cullen and Kersten, 1992). Trees can be divided into hardwood and softwood species depending on their proportional amounts of lignin and hemicellulose. The ratio of cellulose remains constant in both hardwoods and softwoods (Fengel and Wegener, 1989) where, it is believed, it is embedded in a matrix of lignin and hemicellulose (Wood, 1985; Fengel and Wegener, 1989).

#### 1.2.1.1 Cellulose

Cellulose is the most important component of wood (Fengel and Wegener, 1989) and the most abundant renewable resource in nature (Bisaria and Madan, 1983; Wood, 1985; Rajarathnam and Bano, 1989; Fengel and Wegener, 1989; Cullen and Kersten, 1992). Cellulose is a long chain linear homopolymer of linked 1,4 glycosidic anhydroglucose units, which occur in hydrogen bonded parallel strands forming fibrils (Fan *et al.*, 1980; Rajarathnam and Bano,

1989; Fengel and Wegener, 1989; Cullen and Kersten, 1992). The conformational formula of the cellulose chain molecule is shown in Fig. 1.1. -



**Fig. 1.1** Conformational formula of cellulose (Fan *et al.*, 1980).

#### 1.2.1.2 Hemicellulose

Hemicelluloses represent a distinct group of polysaccharides, classified according to their chemically diverse compositions (Aspinale, 1970 cited in Dekker, 1985). This lignocellulosic component is constituted of branched polymers of anhydrosugars linked by various intermonomer bonds (Wood, 1985; Fengel and Wegener, 1989; Raj arathnam and B ano, 1989). The main components of hemicelluloses are therefore either pentoses (xylose), hexoses (glucose, mannose or galactose) hexuronic acids (glucuronic acid) or deoxyhexoses (rhamnose) (Wood, 1985; Fengel and Wegener, 1989; Cullen and Kersten, 1992). Fig. 1.2. indicates the formulae of these sugar components. The main chain of the hemicellulose can either be a homopolymer or heteropolymer, depending on the number of sugar residue types making up its backbone (Fengel and Wegener, 1989).

Hardwood and softwood species vary in their hemicellulose composition in that the former has a higher concentration of this polysaccharide (Fengel and Wegener, 1989; Cullen and Kersten, 1992). In addition, differences in the concentrations and compositions of the individual hemicelluloses are evident between hard- and softwood species (Fengel and Wegener, 1989).

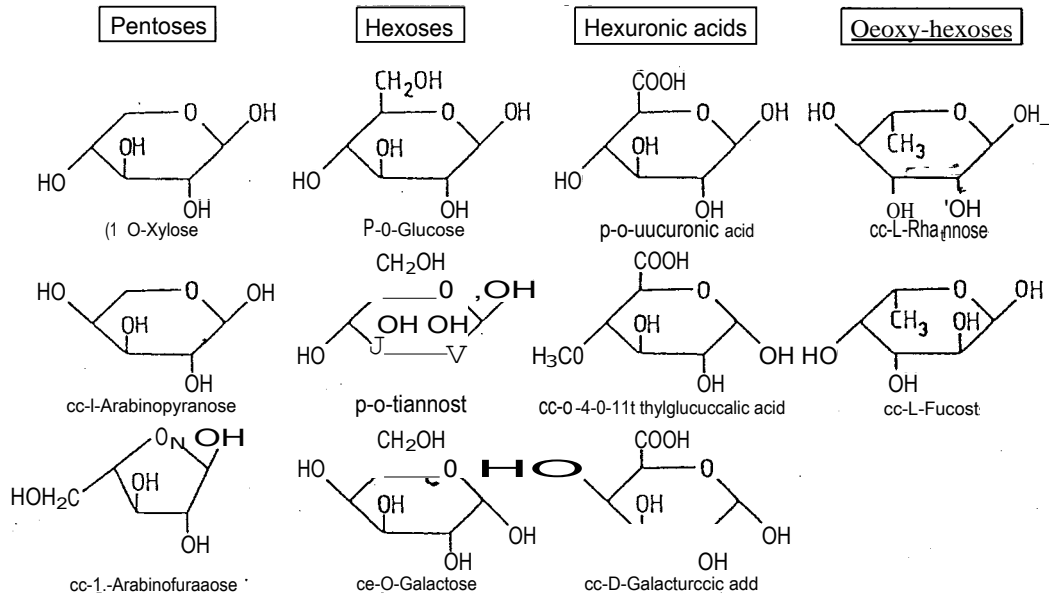
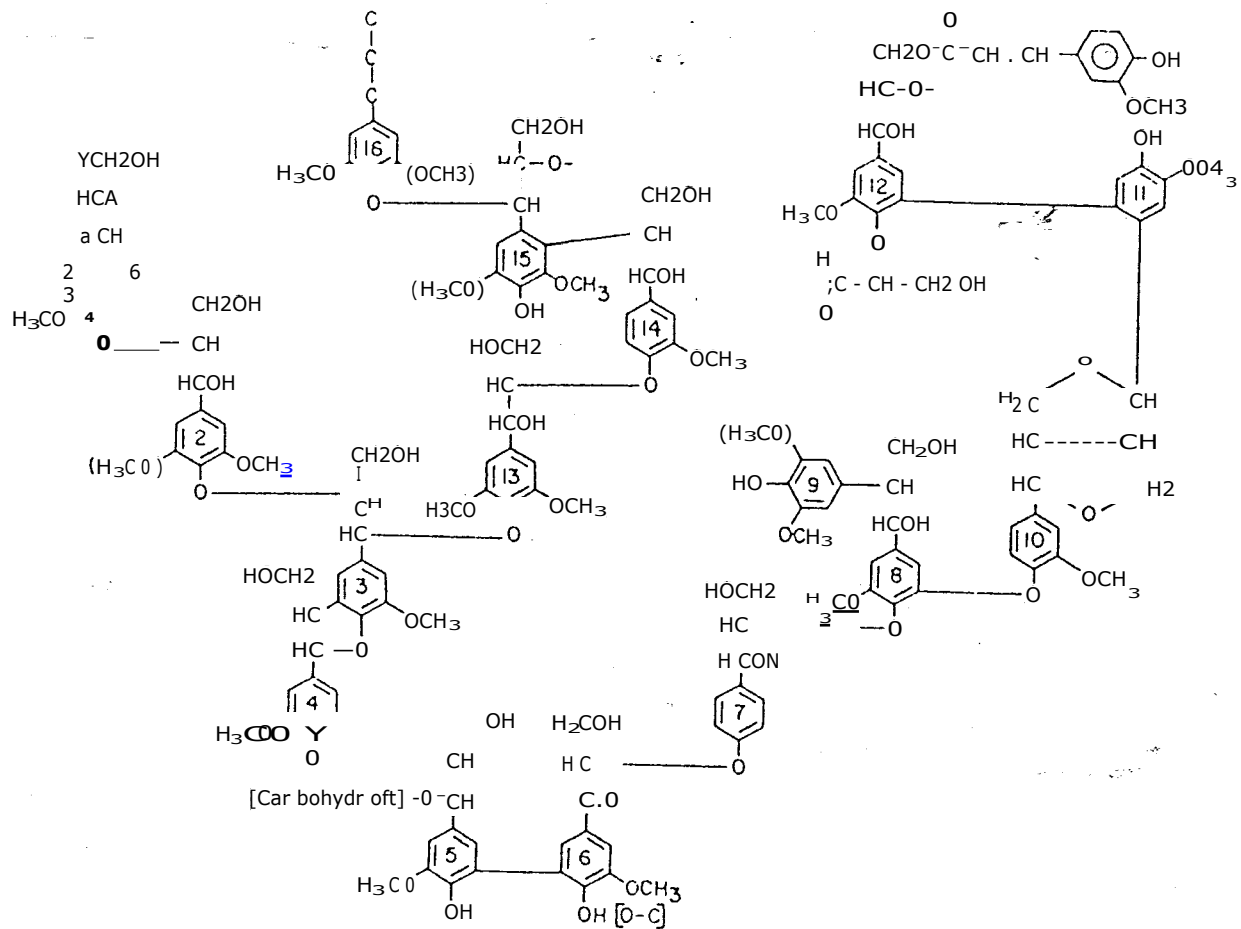


Fig. 1.2 Formulae of the hemicellulose sugar components (Fengel and Wegener, 1989).

### 1.2.1.3 Lignin

The biosynthesis and biodegradation of lignin has been widely reviewed (Janshekar - and Fiechter, 1983; Chen and Chang, 1985; Kirk and Shimada, 1985; Fengel and Wegener, 1989). Lignin is a highly branched, aromatic, three-dimensional polymer of indefinite molecular weight which consists of linked phenylpropane units (Janshekar and Fiechter, 1983; Bourbonnais and Paice, 1988; Rajarathnam and Bano, 1989; Kang *et al.*, 1993). The structure of lignin is illustrated in Fig. 1.3. Several types of intermonomer bonds occur during lignin biosynthesis resulting in the formation of different lignin types (Wood, 1985). Lignin is therefore not a uniform compound but a group of substances with similar chemical properties and differing molecular weights (Janshekar and Fiechter, 1983; Rajarathnam and Bano, 1989). Intra- and interspecific differences in the chemical structure of lignin is common, and is compounded by ecological factors (Janshekar and Fiechter, 1983; Wood, 1985; Rajarathnam and Bano, 1989; Fengel and Wegener, 1989).



**Fig. 1.3.** Structure of lignin (Kirk and Farrell, 1987 cited in Cullen and Kersten, 1992).

### 1.2.2 Micromolecular compounds

Although these compounds only account for a small portion of the total wood composition, they may have a significant effect on the properties and bioprocessing of wood (Fengel and Wegener, 1989). Micromolecular compounds commonly occurring in tree species include organic and minor polymeric substances, as well as minerals. The organic component includes the phenols, terpenoids, alkaloids, aliphatic acids and alcohols (Wood, 1985; Fengel and Wegener, 1989). Minor polymeric substances such as starch, pectin and proteins are also found in small quantities (Fengel and Wegener, 1989; Przybylowicz and Donoghue, 1990). Minerals commonly occurring in wood are potassium, calcium, magnesium and silicon (Fengel and Wegener, 1989).

There is therefore substantial variability between tree species with respect to lignocellulose composition, since the different macro- and micromolecular compounds occur in differing ratios and are of various types. Evidently certain lignocelluloses will be more appropriate for the cultivation of specific mushroom species than others.

### 1.3 MICROBIAL DEGRADATION OF LIGNOCELLULOSIC MATERIALS

The microbial degradation of lignocellulose is influenced by a number of biological restraints imposed by the lignocellulosic and fungal components. Lignocellulosic degradation is dependent on microbial ability to utilize the macromolecular components of this material, an activity which is influenced by a number of factors: 1) the differing degradative potentials of the various fractions; 2) the crystallinity of part of the cellulose fraction; 3) the encrustation by lignin of the cellulose and hemicellulose components; 4) the surface area available for enzymatic activity; 5) the degree of polymerization of the macromolecular components; 6) the moisture content of the wood and 7) the molecular and cellular morphological diversity of wood (Kirk and Connors, 1977; Chang and Steinkraus, 1982; Bisaria and Madan, 1983; Wood, 1985; Rajarathnam and Bano, 1989; Savoie *et al.*, 1994; Moyson and Verachtett, 1991; Buswell *et al.*, 1993). Fungal determinants affecting decomposition rate are the nutrient content of the wood, the temperature, the pH, the oxygen availability, the concentration of toxic or inhibitory compounds, the physiological condition of the fungus and the ability to produce the hydrolytic and oxidative enzymes necessary to degrade wood components into low molecular weight compounds which can be assimilated (Moore-Landecker, 1982; Przybylowicz and Donoghue, 1990; Buswell *et al.*, 1993).

The majority of wood-degrading organisms are either soft-rot, brown-rot or white-rot fungi (Janshekar and Fiechter, 1983; Fengel and Wegener, 1989). Soft- and brown-rot fungal species preferentially degrade wood polysaccharides. They are, however, also capable of degrading lignin (Kirk and Shimada, 1985; Fengel and Wegener, 1989; Przybylowicz and Donoghue, 1990). White-rot fungi, to which lignocellulolytic mushroom species belong, degrade both the lignin and polysaccharide components of wood to varying degrees (Kirk and Moore, 1972; Fengel and Wegener, 1989; Przybylowicz and Donoghue, 1990; Ericksson and Wood, 1985). Although other fungal species, e.g. mould fungi, are also able to grow on

wood, they affect the wood structure negligibly, predominantly by having an inhibitory or stimulatory effect on the propagation of wood-destroying fungi (Fengel and Wegener, 1989).

Although a range of microorganisms degrade hemicellulose and cellulose, it is only those which degrade the recalcitrant lignin component that are able to breakdown intact wood (Kirk and Shimada, 1985). Only white-rot fungi are able to do this efficiently (Hedger, 1982a; Moore-Landecker, 1982; Kirk and Shimada, 1985).

#### 1.4 MUSHROOM BIOLOGY AND CULTIVATION

Of the more than one hundred thousand fungal species known, two thousand are considered edible (Bisaria and Madan, 1983; Przybylowicz and Donoghue, 1990; Rajarathnam *et al.*, 1992) and approximately ten are commercially cultivated on lignocellulosic materials (Food from Wastes, 1981; Bisaria and Madan, 1983; Chu-Chou, 1984).

##### 1.4.1 Mushroom Biology

Mushroom species belong to the group Basidiomycotina (Przybylowicz and Donoghue, 1990). The typical life cycle of Basidiomycetes can be divided into the vegetative and reproductive phases. Haploid, uninucleate basidiospores germinate into the homokaryon or primary mycelium. The sexual phase is characterised by the dikaryon (secondary mycelia), which is formed by hyphal fusion (plasmogamy) of two compatible homokaryons. Mushrooms are produced by the dikaryon as a stress response to suboptimal environmental conditions. Basidiospores are produced, released and the life cycle re-initiated (Tokimoto and Komatsu, 1978; Przybylowicz and Donoghue, 1990; Rajarathnam *et al.*, 1992).

##### 1.4.2 Mushroom Cultivation

Mushroom cultivation is a four-stage process involving: the provision or enhancement of an appropriate substrate; the inoculation of the substrate; the provision and maintenance of environmental conditions to promote mycelial colonization of the substrate; and the encouragement of sexual reproduction resulting in mushroom production (San Antonio, 1981; Wood, 1989; Przybylowicz and Donoghue, 1990). The efficiency and length of the cultivation process, as well as the yield, morphology, size and sensory attributes of the mushrooms produced (Royse and Bahler, 1986; Pettipher, 1988; Przybylowicz and

Donoghue, 1990; Raaska, 1992) are influenced by a number of cultural and environmental factors which are discussed below.

#### **1.4.2.1 Substrate Preparation**

##### **1.4.2.1.1 Lignocellulosic substrate**

The cultivation of edible mushrooms on lignocellulosic materials can be conducted either on whole logs or sawdust derived from these logs; the latter currently being the most commonly used substrate base (Bisaria and Madan, 1983; Wood and Smith, 1988). The physical properties, nutritional composition, moisture content and particle size of the lignocellulosic material will determine its suitability as a substrate for mushroom cultivation, since these factors affect vegetative and reproductive growth (Wood, 1985; Diehle and Royse, 1986; Przybylowicz and Donoghue, 1990; Raaska, 1992). A number of these properties demonstrate inter- and intra-strain specific variation, and are dependent on the age and growing environment of the lignocellulosic material (Przybylowicz and Donoghue, 1990).

The majority of white-rot fungi preferentially degrade hardwood species due to their increased effectiveness in degrading hardwood rather than softwood lignins. Furthermore, softwood species usually contain compounds which are inhibitory to fungal growth, e.g. resins and phenolic compounds (Janshekar and Fiechter, 1983; Fengel and Wegener, 1989; Przybylowicz and Donoghue, 1990).

Domestic production of lignocellulolytic mushrooms requires the testing of locally available, inexpensive substrates in order to obtain the optimal one for cultivation. The choice is dependent upon suitability, cost and availability (San Antonio, 1981; Leatham and Griffin, 1984). Lignocellulolytic fungi vary in their growth responses to different materials. Certain strains, e.g. *Lentinula edodes*, are only successfully cultivated on woody materials, whereas other strains, e.g. *Pleurotus spp.*, are able to grow efficiently on a wide variety of agricultural wastes with differing polysaccharide:lignin ratios (Buswell *et al.*, 1993).

##### **1.4.2.1.2 Substrate formulation**

The nutritional value of the substrate is an indispensable factor when determining the rate of substrate degradation and thus fungal growth, the competitive advantage of the desired

organism, the production time, the yield and its quality (Mee, 1978; Royse, 1985; Rajarathnam *et al.*, 1986; Rajarathnam and Bano, 1989; Przybylowicz and Donoghue, 1990). In sawdust cultivation, the lignocellulosic material is commonly supplemented with a number of additives. High supplementation of media, however, may cause corresponding increases in contamination (Miller and Jong, 1987; Przybylowicz and Donoghue, 1990). A substrate formulation which is frequently used is 80% hardwood sawdust and 20% supplements (dry weight basis). Adaptations of this formulation occur with respect to geographical area and available materials (Mee, 1978; Przybylowicz and Donoghue, 1990).

The efficient growth of fungi requires a number of essential macro- and microelements. Essential macroelements include carbon, nitrogen, sulphur, phosphorus, potassium and magnesium (Moore-Landecker, 1982). Carbon is required in larger amounts than any other element. Fungal species differ in their abilities to utilize certain carbon sources, therefore if more than one carbon source is supplied, preferential utilization occurs (Moore-Landecker, 1982). Nitrogen is required for protein synthesis and is consequently necessary for protoplasm and cell wall construction, and for the production of enzymes (Przybylowicz and Donoghue, 1990). Furthermore, insufficient supply of nitrogen negatively affects the ability of fungi to utilize carbohydrates (Moore-Landecker, 1982). Fungi differ in their abilities to utilize certain nitrogen sources, e.g. many basidiomycetes are unable to utilize nitrates but most are able to utilize organic nitrogen (Moore-Landecker, 1982). High levels of phosphorus increase carbohydrate utilization, and magnesium is involved in the activation of enzyme systems (Moore-Landecker, 1982). A number of microelements are required in trace amounts for fungal growth: iron, zinc, copper, manganese, molybdenum and either calcium or strontium (Moore-Landecker, 1982). Microelements are predominantly associated with enzyme activity, however, they are also structural components of vitamins and other fungal metabolites (Moore-Landecker, 1982).

Nutritional additives commonly incorporated in sawdust cultivation substrates include easily available carbohydrates and nitrogen (Mee, 1978; Przybylowicz and Donoghue, 1990). During vegetative growth a high C/N ratio is optimal, whereas a low ratio is necessary during fructification (Rajarathnam *et al.*, 1986). Potato starch and wheat can be used as carbohydrate sources and usually comprise 7 to 15% of the substrate dry weight (Mee, 1978). In addition,

a sugar source (sucrose or molasses) is often added to a final concentration of 1% to 8% (Mee, 1978). Protein sources optimally constitute 1. to 10% of the dry substrate weight (Mee, 1978). Fats can also be incorporated since they provide a concentrated source of energy, which is degraded slowly and is less available to competing organisms (Przybylowicz and Donoghue, 1990). Furthermore, lipids play a role in the initiation of fructification (Wardle and Schisler, 1969 cited in Rajarathnam *et al.*, 1986).

The incorporation of certain supplements provides the substrate with multiple nutritional factors, e.g. wheat bran, which is carbohydrate based, is a source of nitrogen, vitamins and minerals (Miller and Jong, 1987; Royse *et al.*, 1990). Grains are commonly used as additives in the sawdust cultivation of mushrooms since they contain proteins, carbohydrates and fats (Przybylowicz and Donoghue, 1990). It has further been proposed that some might contain substances which stimulate fruiting (Royse *et al.*, 1990) and which therefore cause significantly increased yields (Rajarathnam and Bano, 1987).

Supplements are also used which have an effect upon the friable and aeratable nature of the substrate e.g. calcium sulphate (Mee, 1978). Finally, the incorporation of calcium carbonate in mushroom medium maintains it at a pH value above 4, which prevents large decreases in pH associated with organic acid production during lignocellulose degradation, which might affect fungal growth negatively (Przybylowicz and Donoghue, 1990).

#### 1.4.2.1.3 Cultivation vessel

The size and shape of the cultivation vessel, the medium from which it is composed and its potential for gaseous exchange are important considerations in obtaining the optimal container for mushroom cultivation (Przybylowicz and Donoghue, 1990). The container must also prevent contamination, the loss of excessive amounts of moisture (Przybylowicz and Donoghue, 1990) and must be resistant to fungal decomposition (Fuzisawa *et al.*, 1978a). Factors which determine container size and shape are: surface area to volume ratio, the time needed for heat penetration, its aeration potential and handling characteristics (Przybylowicz and Donoghue, 1990). Aeration is influenced by the thickness (Fuzisawa *et al.*, 1978a) and chemical composition of the cultivation vessel (Przybylowicz and Donoghue, 1990).

Although the most commonly used containers for mushroom cultivation are polyethylene or polypropylene bags, other containers have been used with varying degrees of success, e.g. glass, vats (Fuzisawa *et al.*, 1978a) and polyvinyl chloride bags (Chang *et al.*, 1981). Polypropylene bags are widely used because they are able to withstand high temperatures (135°C) and are consequently autoclaveable. Furthermore, due to the pliable nature of polypropylene and polyethylene, the container is easily adapted to the cultivation system being used and does not lose excessive amounts of moisture. The gas permeability of polypropylene bags is, however, limited and provision needs to be made for it (Przybylowicz and Donoghue, 1990).

#### **1.4.2.1.4 Substrate sterilization**

Substrate sterilization is used essentially for the prevention and elimination of competing organisms (Lelley and Niehrenheim, 1991; Lanzi, 1991). This is especially important when using particulate lignocellulosic materials since the availability of nutrients for contaminating organisms is greater (Przybylowicz and Donoghue, 1990). Steam sterilization, pasteurization, hot-water treatment and fermentation have been used as pre-treatment techniques (Rajarithnam and Bano, 1987).

Steam sterilization is widely used since it destroys all substrate microflora (Mee, 1978; Fuzisawa *et al.*, 1978a, 1978b; Patrick *et al.*, 1983; Miller and Jong, 1987), and breaks down wood components with a resultant release of easily available soluble matter (Zadrazil, 1978; Zadrazil and Peerally, 1986; Przybylowicz and Donoghue, 1990). It is, however, not economically viable on a commercial scale (Rajarithnam and Bano, 1987). Pasteurization either selectively kills the competing pathogenic microorganisms and allows the survival of symbiotic microflora, or attempts to completely eliminate the substrate microflora (Rajarithnam and Bano, 1987; Lanzi, 1991). The yield of certain wood-degrading fungi on pasteurised substrates has been reported to be lower than that on steam sterilized substrates (Royse *et al.*, 1985). Hot-water treatment destroys certain microbes, increases substrate accessibility to enzymatic degradation by aiding in cellulolytic bond cleavage, and releases phenolic compounds (Rajarithnam and Bano, 1987).

Substrate sterilization can also involve the use of pesticides, e.g. Benlate, Benomyl, Fermente Forte and Sportak Alpha (Houdeau *et al.*, 1991;- Lelley and Niehrenheim, 1991). The inclusion of these fungicides in cultivation media (< 250 ppm) neither influences mycelial growth nor yield (Lelley and Niehrenheim, 1991).

#### 1.4.2.2 Substrate inoculation

##### 1.4./2.1 Mushroom species and strains

White-rot fungal species vary inter- and intra-specifically in their nutritional and environmental requirements (see Table 1.2). The development of an economically feasible cultivation process therefore requires the selection of strains adapted nutritionally and environmentally to a certain locale (Food from Wastes, 1981; Raaska, 1992).

Mushroom strains also differ in their wood-degrading efficiency; in mushroom morphology, size, colour, flavour, quality and yield; in disease resistance; in crop production time and in growth and fructification temperatures (Tokimoto and Komatsu, 1978; Chu-Chou, 1984; Diehle and Royse, 1986; Royse and Bahler, 1986; Przybylowicz and Donoghue, 1990; Khan *et al.*, 1991b; Rinker, 1991; Raaska, 1992; Sermanni *et al.*, 1994).

**Table 1.2** Conditions required for the cultivation of certain lignocellulolytic mushrooms (Food from Wastes, 1981).

Species	Temperature (°C)		Level of environ control required'	Waste substrate
	Spawn run	Fructification		
<i>F.velutipes</i>	18-25	3-8	+++	sawdust-rice bran
<i>L.edodes</i>	20-30	12-20	++	logs/sawdust-rice bran
<i>P.ostreatus</i>	20-27	10-20		straw, paper, sawdust-straw

++++ greatest; + least

#### **1.4.2.2.1.1 Genetics of mushroom strains**

The variability in substrate degradation using different or identical fungal species on different substrates can be accounted for by the interaction between genetic and environmental factors (Rajaratnam and Bano, 1989). The main reason why genetic characterisation and improvement is therefore required is to determine the genetic factors affecting the efficiency of mushroom cultivation.

Large genetic diversity has been found in fungal populations (Clutterbuck, 1994). Basidiomycetes, which belong to the Agaricales group, are highly variable and therefore there is much taxonomic confusion in the classification of this group (Kuo and Wu, 1972). This confusion is primarily associated with their mode of reproduction and natural habitats (Moore-Landecker, 1982; Przybylowicz and Donoghue, 1990; Clutterbuck, 1994).

Mushrooms species are traditionally classified according to three criteria: macroscopic features, e.g fruit body morphology; microscopic characteristics, e.g. spore traits; and intercompatibility studies (Buchanan, 1993). Cultural and physiological characteristics have also been used in classification (Royse *et al.*, 1983; Chiu *et al.*, 1993). The problem associated with all of these means of classification is the possibility of environment/genotype interaction, especially in macroscopic analysis, which limits the reliability of these methods (Royse *et al.*, 1983). Although intercompatibility tests have been successfully used if "the differentiation of fungal strains, often a combination of methods will provide more meaningful results. Recent years have seen the advent of biochemical and genetic markers in the characterisation of mushroom strains.

Biochemical markers which have been widely used have been reviewed by Chiu *et al.*, 1993 and Clutterbuck, 1994. These include protein profiles, enzyme activities, serological properties and most commonly isozyme analysis (Lawson *et al.*, 1975; Iracabal *et al.*, 1991; Ohmasa *et al.*, 1991; Chiu, 1993; reviewed by Royse and May, 1993). These techniques are, however, influenced by physiological and genetic factors and therefore are prone to the same misinterpretations as are prevalent in traditional classification systems (Chiu *et al.*, 1993).

DNA analysis overcomes these problems since the genetic diversity of the species is being directly considered (Chiu *et al.*, 1993). Genetic techniques which have been used for this purpose have been reviewed by Royse *et al.* (1993) and Clutterbuck (1994), and include DNA-DNA hybridization, nucleic acid sequencing, electrophoretic karyotyping (Challen *et al.*, 1991; Chiu, 1993); DNA GC content determination (Kuo and Wu, 1972; Raeder and Broda, 1988) and restriction fragment length polymorphisms (RFLPs) (Raeder and Broda, 1988; Kulkarni, 1991; Molina *et al.*, 1992; Iracabal and Labarere, 1994).

Two methods which have not been used to their full potential in fungal strain differentiation is the polymerase chain reaction (PCR) and the related arbitrarily primed or randomly amplified polymorphic DNA PCR (AP-PCR and RAPDs, respectively). The latter method has been proposed to have great application in mushroom classification and breeding studies (Chiu, 1993). Furthermore, it can be used to identify species specific polymorphisms which are genetically linked to the determinants of important characteristics (Anderson, 1993).

#### 1.4.2.2.2 Spawn Preparation

Spawn is the term given to the inoculum used in mushroom cultivation. The spawn type used is determined by cost, availability and inoculation method (Przybylowicz and Donoghue, 1990). Usually grain or sawdust based spawn is utilised; however, occasionally liquid spawn is used (Leatham and Griffin, 1984; Song *et al.*, 1987; Rajarathnam and Bano, 1987; Przybylowicz and Donoghue, 1990).

Sawdust spawn is composed of sawdust and bran. It is often difficult to separate due to mycelial clumping of the particles; a high inoculation rate is therefore often necessary (Przybylowicz and Donoghue, 1990). The mycelial colonization of this type of spawn takes longer than grain spawn, however, it has a longer storage life (Rajarathnam and Bano, 1987).

Grain spawn can be derived from numerous sources e.g. wheat, millet or rye (Przybylowicz and Donoghue, 1990). It is friable and therefore evenly distributed in the substrate. Rapid colonization at a low inoculum concentration is consequently possible (Przybylowicz and Donoghue, 1990).

Liquid spawn has a high concentration of inoculum particles, is easy to inoculate aseptically and can be uniformly dispersed within the substrate (Song *et al.*, 1987; Przybylowicz and Donoghue, 1990). Furthermore, growth rates in liquid culture are often higher and easily controlled (Leatham and Griffin, 1984; Song *et al.*; 1987). Liquid spawn is not widely used in the mushroom industry primarily because of its cost and inability to adapt efficiently to the solid substrates used (Leatham and Griffin, 1984; Rajarathnam and Bano, 1987). Acclimatization of liquid spawn to sawdust can be achieved by adapting it to wood extract during incubation (Leatham and Griffin, 1984).

#### **1.4.2.2.3 Spawning**

Spawning is the terminology used to describe the inoculation of substrates with spawn. Spawn type and concentration, as well as the method of spawning employed, influences vegetative growth, mushroom size and yield (Raj arathnam and Bano, 1987; Song *et al.*, 1987; Royse, 1989). A positive correlation exists between inoculum size and substrate colonization rate, and between the former and mushroom yield (Kostidinov *et al.*, 1972; Royse, 1989; Przybylowicz and Donoghue, 1990). Mushroom substrates are usually inoculated with a spawn concentration of between 1 and 10% (Mee, 1978; Przybylowicz and Donoghue, 1990).

A number of spawning methods have been successfully used in the cultivation of mushrooms (Rajarathnam and Bano, 1987; Royse, 1989; Fuzisawa *et al.*, 1978b). Shake-up spawning, where the inoculum is thoroughly mixed into the substrate, promotes rapid vegetative growth and high productivities (Royse, 1989).

#### **1.4.2.3 Spawn Run**

Spawn run refers to the vegetative growth of the fungus (Gujral *et al.*, 1987). The spawn run phase of mushroom cultivation is only complete when the substrate nutrients have been accumulated, together with water, as reserves for fruiting (Moore-Landecker, 1982; Przybylowicz and Donoghue, 1990). The amount of time before spawn running is complete can vary depending on the substrate, spawn and environmental conditions implemented during this phase (Ito, 1978). Consequently there are conflicting reports on the effect which spawn run time has on mushroom size, yield and production rate (Zadrazil and Brunnert, 1981; Royse, 1985; Royse and Bahler, 1986; Rinker, 1991; Delpech and Olivier, 1991).

#### **1.4.2.4 Fructification**

Although high nutrient levels have an inhibitory effect upon reproduction, the nitrogen, vitamin and mineral requirements for this phase are higher than those for vegetative growth (Moore-Landecker, 1982). The environmental conditions implemented during fructification are species-specific, but are generally characterised by lower temperatures, higher humidities and greater ventilation than the spawn run phase (Przybylowicz and Donoghue, 1990). The fructification phase can be divided into four stages: induction, pinning, fruiting and resting (Przybylowicz and Donoghue, 1990).

##### **1.4.2.4.1 Induction**

The induction phase indicates the transition from vegetative to reproductive growth, as a consequence of environmental stresses which signal that conditions are no longer favourable for the former (Przybylowicz and Donoghue, 1990). Substrate soaking is a technique commonly used to induce fruiting; however, species-specific responses often occur (Przybylowicz and Donoghue, 1990; Rinker, 1991). Soaking replaces water lost during vegetative growth (Rinker, 1991) and consequently provides a water reservoir for mushroom production (Przybylowicz and Donoghue, 1990). The soaking of substrates further serves to displace carbon dioxide from both inter- and intra-cellular air spaces, thereby reducing the carbon dioxide content of the substrate blocks (Royse, 1989). The soaking time varies according to water temperature, substrate temperature, substrate moisture content, degree of decay, lignocellulosic material and the substrate volume (Przybylowicz and Donoghue, 1990).

##### **1.4.2.4.2 Pinning**

Primordia or pins are "knots" formed by mycelial aggregation, which differentiate into mushrooms. During the pinning phase, it is important to maintain the substrate moisture content and temperature within the optimal range. This growth phase is very sensitive to environmental conditions and high levels of carbon dioxide (Przybylowicz and Donoghue, 1990).

##### **1.4.2.4.3 Fruiting**

The differentiation of primordia into fruiting bodies is dependent upon the supply of water and nutrients by substrate components and undifferentiated primordia (Tokimoto and

Komatsu, 1978). The environmental control required during fruiting is not as stringent as that in the pinning period, and optimal conditions for the two phases are not necessarily similar (Przybylowicz and Donoghue, 1990). Fruiting is affected by temperature, humidity, light and oxygen presence or absence (Przybylowicz and Donoghue, 1990).

The number of flushes harvested and the total production time is determined by the strain, substrate formulation and volume, and environmental conditions maintained during cultivation (Przybylowicz and Donoghue, 1990).

#### **1.4.2.4.4 Resting**

The resting stage is the period between flushes or crops of mushrooms. Once a flush of mushrooms has been harvested, the mycelia must once again accumulate nutrients under conditions which promote rapid mycelial growth and prevent contamination (Przybylowicz and Donoghue, 1990). The resting time is determined by mushroom maturity at harvesting (Przybylowicz and Donoghue, 1990).

### **1.4.3 Factors affecting cultivation**

#### **1.4.3.1 Temperature**

Temperature affects vegetative and reproductive growth primarily because enzymes, which are essential for the degradation of wood, have an optimal temperature range within which they are most active. Temperature therefore determines fungal metabolic activity (Przybylowicz and Donoghue, 1990) and the synthesis of various fungal metabolites (Moore-Landecker, 1982). Temperature is also used to direct fungal metabolism from vegetative to reproductive growth and influences mushroom maturation rate, morphology and yield (Tokimoto and Komatsu, 1978; Przybylowicz and Donoghue, 1990).

#### **1.4.3.2 Humidity**

The relative humidity which is maintained during cultivation determines substrate and fruiting body moisture loss and therefore affects mushroom yield, size and quality (Rajarithnam and Bano, 1987; Przybylowicz and Donoghue, 1990).

#### 1.4.3.3 Substrate moisture content

Substrate degradation by various species is significantly affected by the substrate moisture content (Rajarathnam and Bano, 1989). Temperature and humidity conditions must be implemented which maintain the optimal substrate moisture level throughout cultivation. The importance of the substrate moisture content is primarily due to the functional significance of water in the wood decay process (Przybylowicz and Donoghue, 1990). The substrate moisture content also affects the gaseous exchange potential of the substrate since with increased levels there is a resultant decrease in the gaseous phase (Zadrazil and Brunnert, 1981; Raj arathnam and Bano, 1989). Finally, incorrect substrate moisture conditions have a stimulatory effect on the propagation of contaminants (Przybylowicz and Donoghue, 1990).

#### 1.4.3.4 Gas concentration

Gaseous exchange is of significant importance in the degradation of lignocellulosic materials by white-rot fungi (Zadrazil and Brunnert, 1981). Insufficient aeration causes inhibition of growth and limits production (Mee, 1978) since the effective utilization of carbon and nitrogen sources may be affected by oxygen availability (Moore-Landecker, 1982). Carbon dioxide rarely limits growth, provided provision is made for gaseous exchange (Przybylowicz and Donoghue, 1990). The vegetative growth of certain wood-degrading fungi is stimulated by high carbon dioxide levels (Kamra and Zadrazil, 1986; Leatham and Stahmann, 1987; Kinugawa, 1993).

#### 1.4.3.5 Light

Species and sub-species of cultivated mushrooms vary in their light requirements (Moore-Landecker, 1982). In general, light affects fruiting to a greater extent than it does vegetative growth. It may either have an inhibitory or stimulatory effect on the development of reproductive structures and influences mushroom colour and morphology (Moore-Landecker, 1982; Przybylowicz and Donoghue, 1990).

#### 1.4.3.6 pH

The pH of the external environment affects fungal internal pH and, consequently, enzymatic activity (Moore-Landecker, 1982). Enzymes have an optimal pH range within which they are most effective and therefore substrate pH affects fungal growth and productivity

(Przybylowicz and Donoghue, 1990). The permeability of the fungal cell membrane and therefore the absorptive capacity of the fungus is also affected by pH (Moore-Landecker, 1982). Furthermore, the solubility of compounds -and consequently their availability to the fungus is determined by pH (Przybylowicz and Donoghue, 1990).

#### 1.4.3.7 Contamination

Numerous cultivated mushroom species have been defined as weak pathogens of wood (Patrick *et al.*, 1983). Contamination by faster growing organisms is consequently a major problem (Ito, 1978) since it causes decreased yields and production rates (Royse, 1989). Fungal, bacterial and viral contamination is commonly found in mushroom production facilities (Royse, 1989).

Fungal contaminants commonly found during mushroom cultivation can be divided into 3 groups: pathogenic, competitor or weed fungi (Przybylowicz and Donoghue, 1990). The majority of pathogenic fungi are Ascomycetes which produce antifungal compounds or vapours inhibitory to the growth of the cultivated fungus (Fries, 1973; Przybylowicz and Donoghue, 1990). Some of the most common pathogenic fungi belong to the genus *Hypocrea* and their associated asexual phases *Trichoderma* and *Gliocladium*. Competitor fungi are Basidiomycetes which belong to the order Aphyllophorales (commonly known as bracket fungi) (Przybylowicz and Donoghue, 1990). These fungi compete with the cultivated fungus for space and nutrients (Przybylowicz and Donoghue, 1990). Weed fungi can either be Ascomycetes or Basidiomycetes. These fungi are rarely antagonistic and only under conditions of intense contamination do they function as competitors (Przybylowicz and Donoghue, 1990).

Bacterial contamination is common during cultivation; however, these contaminants seldom cause major problems (Royse, 1989; Przybylowicz and Donoghue, 1990). If extensive contamination occurs, the results are similar to those of fungal contaminants and often promotes the presence of these organisms (Royse, 1989). Bacterial contamination during fruiting can either cause morphological abnormalities or spoilage of post-harvest mushrooms (Przybylowicz and Donoghue, 1990; Moore-Landecker and Stotzky, 1973).

The consequence of viral infection on cultivation is largely unknown; however, has been postulated to cause decreased mycelial growth (Yu *et al.*, 1985 cited in Przybylowicz and Donoghue, 1990).

The incidence of contamination can be decreased by the use of sterile cultivation procedures and the maintenance of environmental conditions within optimal ranges, (Ito, 1978; Przybylowicz and Donoghue, 1990; Hordeau *et al.*, 1991). Contamination can also be minimized by using high inocula of suitable varieties which show rapid growth on the selected lignocellulosic substrate (Lanzi, 1991; Delpech and Olivier, 1991).

## 1.5 MUSHROOMS AMENABLE TO CULTIVATION IN SOUTHERN AFRICA

The development of a speciality mushroom production technology for small-scale industries in South Africa, based on locally available lignocellulosic materials, is beneficial in a number of respects. The cultivation of certain broad substrate range wood-degrading fungi, e.g. *L. edodes* (shiitake mushroom), *Pleurotus spp.* (oyster mushroom) and *F. velutipes* (winter mushroom) results in the production of an economically viable agricultural product which has numerous culinary, nutritional and medicinal benefits. Furthermore, the spent substrate has diverse industrial, agricultural and cultural uses (Rajaratnam *et al.*, 1979; Chu-Chou, 1984; Rajaratnam *et al.*, 1987; Rajaratnam and Bano, 1989; Martinez-Carrera *et al.*, 1991; Burla *et al.*, 1992; Moyson and Verachtert, 1993; Okeke *et al.*, 1993; Tharun, 1993; Sermanni *et al.*, 1994).

## 1.6 THE BIOTECHNOLOGY OF EDIBLE MUSHROOM PRODUCTION

Edible mushroom cultivation is a multi-billion dollar industry in many countries (Przybylowicz and Donoghue, 1990; Peberdy *et al.*, 1993). Although mushrooms are produced on a commercial scale in the majority of countries worldwide, the quantities, types and selling prices are geographically variable (Delcaire, 1978; O'Brien, 1989, Wood, 1989).

The common button mushroom (*Agaricus bisporus*) enjoys worldwide production whereas speciality mushrooms, such as the shiitake (*L.edodes*), oyster (*Pleurotus* species) and winter mushrooms (*F.velutipes*), have historically been produced in the Far East. Recent years have,

however, seen production of these mushroom species expanding into America and Europe (O'Brien, 1989, Wood, 1989; Kirchoff and Lelley, 1992). The speciality mushroom market is continually increasing (Table 1.3) due to elevated consumer demand, the beneficial attributes of numerous of these mushrooms and the relatively higher prices received (Patrick *et al.*, 1983; Royse *et al.*, 1990; Royse and Zaki, 1991).

Table 1.3 World production of edible mushrooms (Rajaratnam and Bano, 1987; Peberdy *et al.*, 1993).

Species	Production (metric tonsx1000)a			% Increase 1986-1990
	A	B	C	
<i>Agaricus bisporus</i>	870	1 230	1 420	16.1
<i>Lentinula edodes</i>	170	314	393	25.5
<i>Volvariella volvacea</i>	49	178	207	16.3
<i>Flanuntidina velutipes</i>	60	100	143	43.0
<i>Pleurotus</i> species.	32	169	909	537.9
<i>Auricularia</i> spp.	2	119	400	336.1

<sup>a</sup> A=1979; B=1986 C=1989/1990

The prices at which individual mushroom species are sold vary according to geographical and seasonal factors, market demand and the production costs associated with their cultivation (Delcaire, 1978). Furthermore, the profitability of cultivating these mushroom species vary, e.g. oyster mushroom production shows a greater profitability than shiitake mushroom cultivation (Delcaire, 1978). The economics of mushroom cultivation is primarily dependent on the efficiency of conversion of substrate to harvestable fruiting bodies (Hiromoto, 1991) which is determined by the substrate, strain and environmental conditions employed. It is therefore important to optimize these factors as much as possible in order to make the process as economically viable, and thus commercially attractive, as possible.

## 1.7 RESEARCH OBJECTIVES

Vast quantities of lignocellulosic wastes are generated annually in Southern Africa. Since edible mushroom cultivation is currently the most economically viable process for the utilization of lignocellulosic materials, steps were taken to develop a process for the economic production of speciality mushrooms using locally available wood sources. This would ensure the establishment of a potentially economically viable, low cost, subsidiary industry for many rural communities as well as for a number of major industries.

Research has indicated that both fungal and substrate-associated factors affect the ability of different mushroom species to utilize various substrates. The primary objective of the research was therefore the screening of the most suitable speciality mushroom species for the bioconversion of locally available lignocellulosic wastes. A number of factors which have been identified as important variables affecting mushroom cultivation were also investigated.

The research objectives were therefore as follows:

- 1) The optimization of the *in vitro* production of vegetative cultures for the preparation of spawn.
- 2) The screening of various wood-degrading fungi for their growth and mushroom production potential on locally available lignocellulosic sources. The lignocellulolytic fungi investigated were *L.edodes*, various *Pleurotus* species and *F.velutipes* and these were cultivated on Maranti and Port Jackson based substrates.
- 3) The quantitative and qualitative investigation of enzyme production by *L.edodes* and *Pleurotus* species.
- 4) The use of RAPDs to fingerprint *Pleurotus* at the species and strain level.

## CHAPTER TWO

THE *IN VITRO* GROWTH OF VARIOUS WOOD-DEGRADING FUNGI.

## 2.1 INTRODUCTION

The development of an optimal biotechnological process for the cultivation of edible mushrooms requires optimisation of each phase of the procedure. The initial phase in the cultivation process is the production of a good spawn which primarily requires the *in vitro* growth of the desired fungal strain on synthetic media, under optimal environmental and nutritional conditions.

The environmental requirements for the *in vitro* growth of *Lentinus* and *Pleurotus* strains on synthetic media are well documented (Tokimoto and Komatsu, 1978; Zadrazil, 1978; Song *et al.*, 1987; Khan *et al.*, 1991a; Delpech and Olivier, 1991; Blaich and Esser, 1975). Fungal strains vary in their nutritional requirements; however, *Lentinus* and *Pleurotus* species are generally grown on the following synthetic media, irrespective of the strain: Potato Dextrose Agar (PDA) (Kaneshiro, 1977; Burla *et al.*, 1992; Tan and Moore, 1992; Palmieri *et al.*, 1993; Fasidi and Olorunmaiye, 1994; Sermanni *et al.*, 1994; Di Lena *et al.*, 1994); Malt-Yeast Extract (M3) (Chahal and Khan, 1991; Raaska, 1992); Potato Dextrose Yeast Extract (PDY) (Royse, 1985); Malt Extract (MEA) (Kulkarni, 1990; Kirchhoff and Lelley, 1991; Khan *et al.*, 1991a; Panichajakul *et al.*, 1991; Guillen *et al.*, 1992; Bano *et al.*, 1993; Cedano *et al.*, 1993); Sucrose-Malt-Yeast-Peptone Media (SMPY) (Kulkarni, 1990) and Malt Agar (Zadrazil and Brunnert, 1981; Madan and Bisaria, 1983; Kamra and Zadrazil, 1986; Bourbonnais and Paice, 1988).

Liquid or solid cultures can be used in the determination of fungal vegetative growth on synthetic media. The method of choice for assessing fungal growth in liquid media is dry weight determination which necessitates destructive sampling for an indication of time-related growth (Moore-Landecker, 1982). This method requires a large number of samples making it time-consuming and tedious. However, the measurement of radial growth on agar media allows non-destructive sampling, providing an estimation of fungal vegetative growth over a period of time (Moore-Landecker, 1982). This makes it a preferable method for the preliminary assessment of fungal growth on synthetic substrates.

## 2.2 RESEARCH OBJECTIVES

The rate and density of radial growth on synthetic media has been used as a basis for screening large numbers of strains (Tan and Moofe; 1992). These criteria were used to screen twenty-three species and sub-species of wood-degrading fungi so as to eliminate slow colonisers.

Since fungal growth rates are significantly affected by nutritional factors, it is necessary to determine which synthetic media afford the most favourable growth of the desired strain. In using such media, the initial phase of the cultivation process, i.e. spawn production, is shortened, thus promoting the economic feasibility of the process.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Strain Selection

A preliminary selection was made from twenty-three commercial, dikaryotic mushroom strains (Table 2.3.1) on the basis of mycelial density and radial growth on malt agar plates. Cultures were maintained on agar slants and in sterile water at 4°C. At two month intervals, these were used to start fresh working cultures.

### 2.3.2 Medium Selection

Three *L. edodes* and six *Pleurotus* strains were selected and screened for their vegetative growth on a number of different agar media, using colony radial growth as an indicator. The agar media used in the study were: M3, MA, YG, SMPY, MEPA, B-MEA and GMPY (Appendix A). The pH of all media was adjusted to  $5.6 \pm 0.1$  prior to autoclaving.

For standardized and reproducible testing, agar plugs of 1 cm<sup>2</sup> were cut from the circumference of 2 week-old cultures (Meyrath and Suchanek, 1971; Tokimoto and Komatsu, 1978; Moore-Landecker, 1982), and inoculated onto the relevant media. The plates were incubated at 25°C for eight days and measurements were made every alternate day. The experiment was performed in triplicate.

### 2.3.3 Statistical analysis

One-way Analysis of Variance (ANOVA) was performed on the test results to determine whether significant differences existed between treatment means. The Tukey-Kramer Multiple Comparisons Test was performed to deduce which of the differences amongst the means were significant.

**Table 2.3.1** Strains, origins and recommended growth media

Strains	Source	Recommended media
<i>F. velutipes</i>	DSM 1684	
<i>L. edodes</i>	DSM 1899	MEPA
<i>L. edodes</i>	ATCC 48860	M3
<i>L. edodes</i>	ATCC 58762	M3
<i>P. calyptratus</i>	CCRC 36211	
<i>P. citrinopileatus</i>	CCRC 36239	
<i>P. columbinus</i>	CCRC 36215	
<i>P. cornucopiae</i>	CCRC 36216	
<i>P. cystidiosus</i>	CCRC 36046	
<i>P. cystidiosus</i>	CCRC 36131	
<i>P. cystidiosus</i>	CCRC 36168	
<i>P. cystidiosus</i>	CCRC 36170	
<i>P. cystidiosus</i>	CCRC 36172	
<i>P. cystidiosus</i>	CCRC 36223	
<i>P. cystidiosus</i>	CCRC 36253	
<i>P. eryngii</i>	CCRC 36037	
<i>Plitiscus</i> var. <i>ferulae</i>	CCRC 36214	
<i>P. incarnatus</i>	CCRC 36228	
<i>P. ostreatus</i>	DSM 3344	MEPA
<i>P. ostreatus</i>	DSM 1833	MEPA
<i>P. ostreatus</i>	DSM 1020	MEPA
<i>P. pulmonarius</i>	CCRC 36095	
<i>P. sajor-caju</i>	CCRC 36040	

## 2.4 RESULTS

Species and sub-species varied in radial growth and mycelial density on malt agar plates (results not shown). *P.cornucopiae* 36216 showed a low radial growth presumably owing to the high density cultures produced. All *P.cystididosus* strains showed a slow rate of growth and low mycelial density on the synthetic media tested when compared to the other cultures. These strains were therefore eliminated from further *in vitro* growth studies.

All three *L.edodes* strains; two *P.ostreatus* sub-species (1833 and 3344); *P.calyptratus* 36211; *P. citrinopileatus* 36239; *P.columbinus* 36215 and *Pleurotus spp.* 36031 were selected, and further screened for vegetative growth on various synthetic media. The *L.edodes* and *P.ostreatus* sub-species were selected so as to determine whether intra-specific differences exist in radial growth on various synthetic media, and thus whether sub-species vary significantly in their nutritional requirements.

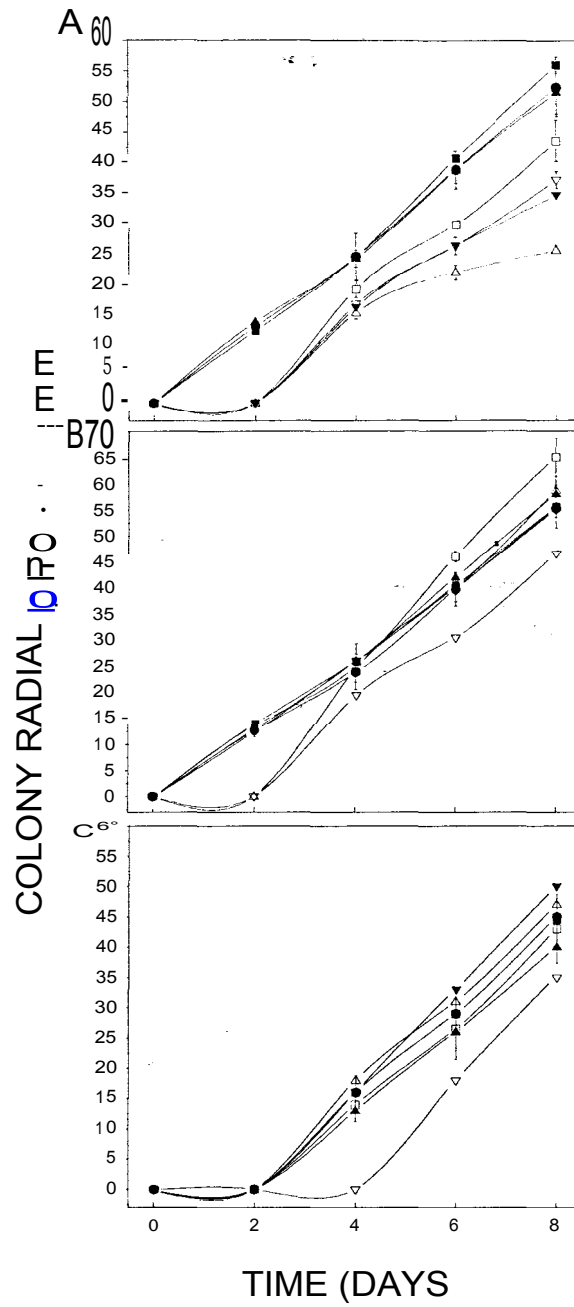
### 2.4.1 *L.edodes* strains

The radial growth of *L.edodes* sub-species on the same media varied, however often not significantly (Table 2.4.1 and Fig. 2.4.1).

Table 2.4.1 Radial growth" of *L.edodes* strains on various agar media formulations

<i>Media</i>	<i>L.edodes</i> 1899	<i>L.edodes</i> 48860	<i>L.edodes</i> 58762
MA	52.3+4.7 [ABCD]	55.3+3.8 [AC]	45.0+0.0 [GHJ]
YG	37.0+1.4 [EF]	46.5+0.7 [BDGH]	35.0+0.0 [F]
SMPY	34.5+0.7 [E]	55.0+1.4 [AC]	50.0+0.0 [ABDJ]
MEPA	43.5+3.5 [FG]	65.0+3.6 [I]	43.0+5.7 [FG]
B-MEA	56.0+1.4 [AC]	55.5+0.7 [AC]	44.5+3.5 [GJ]
GMPY	25.3+0.6 [K]	58.7+3.5 [CI]	47.0+0.0 [DG]
M3	51.5+3.5 [ABDH]	58.0+1.7 [AC]	40.0+0.0 [EFG]

Radial growth is the diameter of colonies (measured in mm) after 8 days incubation at 25°C on various agar media. Entries show the mean and standard deviation of three replicate cultures; means followed by the same letter, in the square brackets, are not considered significantly different at P 0.05. References to the different media are presented in the text.



**Fig. 2.4.1** The growth curves of *L.edodes* strains 1899 (a), 48860 (b) and 58762 (c) grown on various synthetic media. MA (●), YG (v), SMPY (v), MEPA (p, B-MEA (NI), GMPY (A), M3 (A).

The four media which caused a two day lag phase in the mycelial growth of *L.edodes* 1899 (MEPA, YG, SMPY and GMPY) (Fig. 2.4.1a) afforded a significantly lower radial growth of this strain after eight days than the other media Which supported linear growth throughout the experimental period (Table 2.4.1 and Fig. 2.4.1a). After eight days, GMPY supported significantly lower growth of this strain than any of the other synthetic media tested (Table 2.4.1). The radial growth of *L.edodes* 1899 was highest, and did not vary significantly, on the media containing the highest concentration of malt extract (B-MEA, MA and M3) (Table 2.4.1).

The radial growth of *L.edodes* 48860 on all media promoting linear growth (MA, SMPY, B-MEA, M3) did not vary significantly after eight days (Table 2.4.1 and Fig. 2.4.1b). A two day lag phase in growth occurred on YG and GMPY media (Fig. 2.4.1b). MEPA and YG media, respectively, supported the highest and lowest radial growth of this strain.

A two day acclimatization period (lag phase) was required by *L.edodes* 58762 to adapt to synthetic media, thereafter linear growth occurred (Fig. 2.4.1c). On YG medium a four day delay in growth was observed, and this medium also supported significantly less growth after eight days than any of the other media tested (Table 2.4.1 and Fig. 2.4.1c).

#### 2.4.2 *Pleurotus* strains

There were significant differences in the radial growth of *P.ostreatus* strains 3344 and 1833 on all media tested after eight days, with strain 3344 having a consistently higher growth potential (Table 2.4.2). The radial growth of *P.ostreatus* strains on all synthetic media tested did not show a lag phase and was essentially linear (Fig. 2.4.2). The inoculation of these strains on the following media resulted in high radial growth after eight days: MA, SMPY, MEPA, GMPY and M3. The growth of *P.ostreatus* strains on YG and B-MEA media was not significantly different; however, it was significantly lower than on any of the other media tested (Table and Fig. 2.4.2).

Table 2.4.2 Radial growth' of various *P.ostreatus* strains on different agar media.

Media	<i>P.ostreatus</i> 3344	<i>P.ostreatus</i> 1833
MA	76.0±3.6 [A]	54.0±4.2 [C]
YG	65.0±0.0 [B]	35.0±0.0 [D]
SMPY	84.0±2.8 [A]	59.0±0.0 [BC]
MEPA	76.7±1.5 [A]	66.7±7.4 [B]
B-MEA	64.0±3.6 [B]	40.3±3.2 [D]
GMPY	79.3±1.5 [A]	52.0±0.0 [C]
M3	84.0±1.4 [A]	54.5±5.0 [C]

As previously described (Table 2.4.1).

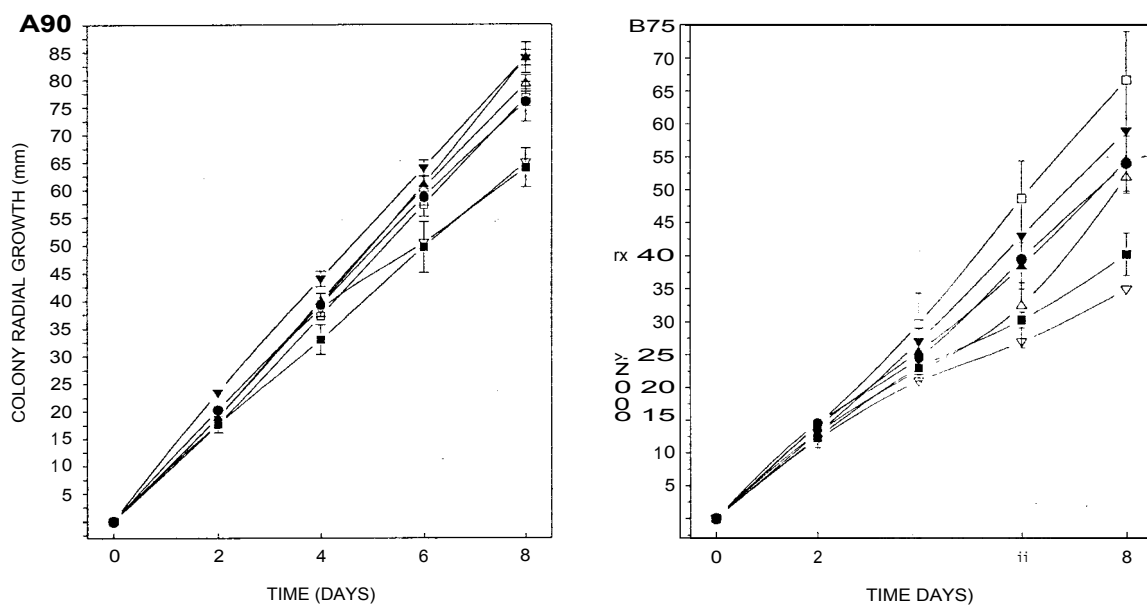


Fig. 2.4.2 The radial growth of *P.ostreatus* strains 3344 (a) and 1833 (b) on synthetic media. MA (lb), YG (v), SMPY (v), MEPA (D), B-MEA (I), GMPY (A), M3 (A).

*P.calypttratus* 36211 showed the lowest radial growth of all *Pleurotus* strains tested after eight days (Table 2.4.2 and 2.4.3). Furthermore, this strain exhibited sigmoidal growth on all media tested (Fig. 2.4.3a). The radial growth of this strain on all media supporting efficient growth (MA, M3, SMPY, GMPY) did not vary significantly after eight days (Table 2.4.3). Measurable growth on YG medium occurred up to day 2, thereafter only a negligible amount of growth occurred (Fig. 2.4.3a). YG is consequently not a suitable substrate for the *in vitro* propagation of *P.calypttratus* 36211 mycelia.

Table 2.4.3 Radial growth" of different *Pleurotus* species on various agar media.

Media	<i>P.calypttratus</i> 36211	<i>P.citrinopileatus</i> 36239	<i>P.columbinus</i> 36215	<i>Pleurotus spp.</i> 36031
MA	54.0±2.8 [ABCD]	54.7±1.5 [ABCDJKL]	69.0±4.6 [GHI]	51.0±3.0 [ABD]
YG	16.0±0.0 [N]	38.0±0.0 [EM]	64.0±0.0 [FGHIJ]	65.0±1.4 [FGHI]
SMPY	50.0±0.0 [ABD]	64.0±0.0 [FGHIJ]	63.0±2.8 [DFGHIJ]	68.5±2.1 [FGHI]
MEPA	N.D <sup>h</sup>	57.7±1.5 [ACDFHJL]	70.7±7.2 [GI]	49.7±3.2 [ABL]
B-MEA	35.5±0.7 [E]	45.7±2.1 [BKLM]	62.3±1.5 [DFGHIJ]	60.0±2.0 [DHIJ]
GMPY	48.0±0.0 [AB]	64.0±5.7 [FGHI]	71.0±4.0 [GI]	69.0±4.4 [I]
M3	52.0±9.9 [ABD]	65.5±2.1 [FGHIJ]	67.7±5.5 [GHI]	N.D

<sup>a</sup> As previously described (Table 2.4.1)  
N.D = not determined.

The growth of *P.citrinopileatus* 36239 on all media was essentially linear up to the sixth day of incubation (Fig. 2.4.3b). The radial growth of this strain on the media promoting the highest (M3, GMPY, SMPY and MEPA) and lowest (B-MEA and YG) growth after eight days did not vary significantly (Table 2.4.3).

The radial growth of *P.columbinus* 36215 on all of the synthetic media tested was linear (Fig. 2.4.3c) and did not vary significantly after an eight day incubation period (Table 2.4.3). Linear growth of *Pleurotus spp.* 36031 occurred up to day 6, thereafter the growth rate,

indicated by the slope of the curve, decreased (Fig. 2.4.3d). The most efficient growth of this strain was sustained on GMPY, SMPY, YG and B-MEA media (Table 2.4.3). These are the only media which contained a carbohydrate source.

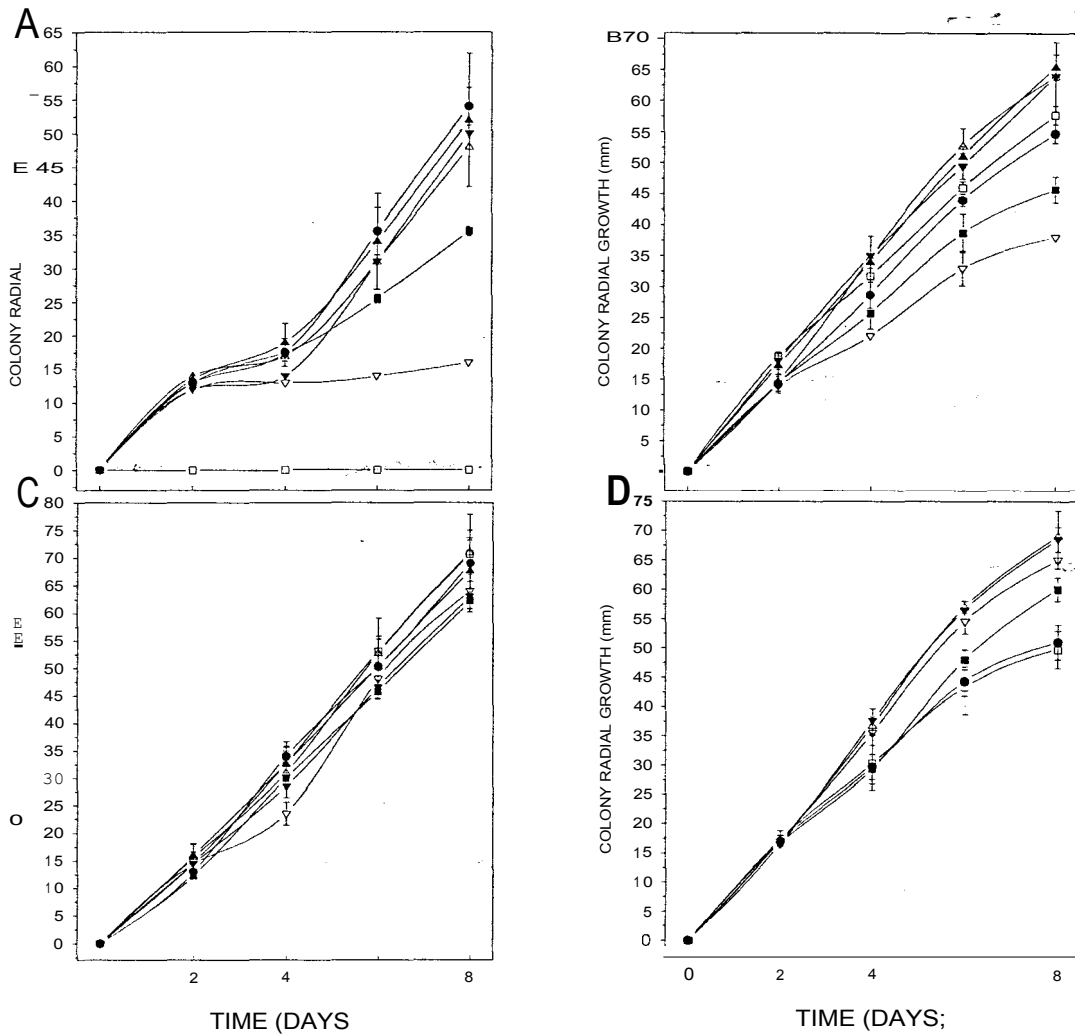


Fig.2.4.3 The growth of *Pleurotus* species 36211 (a), 36239 (b), 36215 (c) and 36031 (d) on various synthetic media. MA (●), YG (v), SMPY (v), MEPA (ET), B-MEA (M), GMPY (A), M3 (A).

### 2.4.3 Optimal synthetic media for the *in vitro* growth of *L.edodes* strains and *Pleurotus* species.

A summary of the optimal media for the *in vitro* growth of the various species examined is shown in Table 2.4.4. *P.ostreatus* 3344 has a significantly higher radial growth after eight days than any of the other species considered (Table 2.4.4). *L.edodes* 1899 did not vary significantly in its radial growth on optimal synthetic media when compared to either *L.edodes* 58762 or *L.edodes* 48860, neither were four of the six *Pleurotus* species investigated significantly different to each other (Table 2.4.4).

Based on these results, no single synthetic medium can be recommended for the general *in vitro* growth of either *L.edodes* strains or *Pleurotus* species since it is evident that strains vary in their nutritional requirements.

Table 2.4.4 Optimal agar media for the *in vitro* cultivation of various *L.edodes* strains and *Pleurotus* species.

Species	Agar media	Radial growth (mm) <sup>a</sup>
<i>L.edodes</i> 1899	B-MEA	56.0±1.4 [AB]
<i>L.edodes</i> 48860	MEPA	65.0±3.6 [AD]
<i>L.edodes</i> 58762	SMPY	50.0±0.0 [B]
<i>P.ostreatus</i> 3344	SMPY	84.0±2.8 [E]
<i>P.ostreatus</i> 1833	MEPA	66.7±7.4 [D]
<i>P.calypttratus</i> 36211	MA	54.0±2.8 [B]
<i>P.citrinopileatus</i> 36239	M3	65.5±2.1 [AD]
<i>P.columbinus</i> 36215	GMPY	71.0±4.0 [D]
<i>Pleurotus</i> spp. 36031	GMPY	69.0±4.4 [D]

<sup>a</sup> As described for Table 2.4.1.

## 2.5 DISCUSSION

Studies have shown that strains vary in their radial growth on different synthetic media (Song *et al.*, 1987; Khan *et al.*, 1991b) and that different strains of the same species have variable growth rates on identical media (Tan and Moore, 1992). The findings of this study support these observations.

The rate and density of mycelial growth on agar media varied according to the medium on which the strain was inoculated, which is in accordance with the literature (Booth, 1971). It was further found that cultures showing a high density mycelial growth, correspondingly showed low radial growth characteristics. Variations occurred in the radial growth of sub-species of *L. edodes* and *P. ostreatus* on the same substrate formulation, however, these differences were not always significant (Table 2.4.1 and 2.4.2). Furthermore, these sub-species varied in the synthetic media which supported their optimal growth (Table 2.4.4) which were not necessarily those which had been recommended for their propagation (Table 2.3.1).

Linear growth of all strains of *L. edodes* tested was observed on MA, B-MEA and M3 (Fig. 2.4.1), which were the media containing the highest concentration of malt extract. Linear growth of strains on media is beneficial where cultivation is considered, as time is an important factor affecting the economics of the process. The lag phase in the growth of certain species on specific synthetic media is probably due to a component of the medium which is inhibitory to growth but which is eventually overcome by biochemical mechanisms. Further studies would, however, need to be conducted in order to test this assumption.

*L. edodes* 48860 showed the greatest radial growth of the *L. edodes* strains tested (Table 2.4.1). Based on the difference in growth observed between the media supporting the highest and lowest growth of these strains, *L. edodes* 48860 and 58762 seem to be less fastidious in their nutritional requirements than 1899. The difference in strains 58672 and 48860 was 15 and 18.5 units respectively, whereas that of 1899 was 30.7 units (Table 2.4.1).

The optimal media for the *in vitro* growth of the three sub-species of *L. edodes* (Table 2.4.4) contained two sources of nitrogen: malt extract and peptone. It would therefore appear that nitrogen is an important factor affecting the vegetative growth of *L. edodes*. The incorporation of peptone in media has been found to stimulate the mycelial growth of certain wood-degrading fungi since it contains various amino acids, low molecular weight polypeptides and trace amounts of minerals and vitamins (Kinugawa, 1993).

*P.columbinus* 36215 appears to be the least fastidious of the *Pleurotus* species tested, whereas *P.calypttratus* 36211 appears to be the most particular in its nutritional requirements on synthetic media (based-on the radial growth range of the synthetic media investigated) (Tables 2.4.2 and 2.4.3). *P.ostreatus* 3344 seems to be less fastidious than strain 1833 (Table 2.4.2). Of the synthetic media used in this investigation; GMPY, SMPY and M3 appear to consistently support efficient radial growth of *Pleurotus* species (Table 2.4.2 and 2.4.3), even though Rajarathnam and Bano (1987) recommended MA medium for the *in vitro* growth of *Pleurotus* species. Based on the results presented in Table 2.4.2 it would appear that SMPY supports the optimal *in vitro* growth of *P.ostreatus* strains.

The study presented here has indicated that the incorporation of glucose or sucrose in synthetic media has a beneficial effect on the *in vitro* growth of the majority of strains investigated. Previous studies have, however, shown that repeated sub-culturing on PDA medium results in a decrease in the *in vivo* colonizing efficiency of *Pleurotus* species, which is possibly due to the poisoning of glucose in the medium (Dade, 1969 cited in Rajarathnam and Bano, 1987). The contradictory nature of these findings can be overcome by storage of cultures on MA or M3 media (both of which do not contain glucose), and the *in vitro* growth of cultures on the above optimal media prior to their inoculation onto spawn substrates, so as to decrease the total cultivation time.

The results presented indicate that YG medium is not a good synthetic substrate for the *in vitro* growth of either *Ledodes* or *Pleurotus* strains. Yeast extract (which contains proteins, vitamins and all the essential microelements required for fungal growth (Moore-Landecker, 1982; Fasidi and Olorunmaiye, 1994)) and glucose have been found to be the most stimulatory nitrogen and carbohydrate sources, respectively, for *Pleurotus* growth (Fasidi and Olorunmaiye, 1994). YG medium, which contained only these two compounds, however, did not sustain very good growth of this genus. This could be because it was the only medium of those tested which did not contain malt extract. This emphasizes the beneficial effect of this component in the *in vitro* growth of these wood-degrading fungi.

## 2.6 CONCLUSION

In conclusion, species and sub-species vary in their nutritional requirements, consequently affecting their *in vitro* growth on different synthetic media. In obtaining the optimal *in vitro* combination of strain and synthetic media, "the length of this initial phase of the cultivation process is decreased. Furthermore, a highly positive correlation has been found between mycelial growth on agar media and on sawdust-based media (Bak *et al.*, 1994). The findings of this research can therefore ensure a shorter initial *in vitro* growth period and *in vivo* spawn run time, thereby making the process as a whole more economically feasible.

## CHAPTER THREE

### PRELIMINARY STUDY ON THE CULTIVATION OF THE SHIITAKE MUSHROOM ON LOCALLY AVAILABLE WOOD SOURCES.

#### 3.1 INTRODUCTION

Shiitake, black forest or Chinese mushroom are the common names given to the fruiting body of the non-pathogenic, saprophytic basidiomycete *Lentinula edodes* (Pettipher, 1985), which is widely distributed in the East (Przybylowicz and Donoghue, 1990). *L.edodes* naturally produces mushrooms on dead hardwood logs, especially species of the Fagaceae family (Natalaya and Pataragetvit, 1981; Leatham and Griffin, 1984; Wood and Smith, 1988; Przybylowicz and Donoghue, 1990).

Traditionally, shiitake is cultivated on tree logs ("natural log cultivation"); however, in recent years cultivation on sawdust or agricultural residues ("artificial log cultivation") has increased due to a shortage of hardwood logs, the unpredictability of climatic conditions and the long production time required (Leatham and Griffin, 1984; Ito, 1978; Chu-Chou, 1984; Tan and Moore, 1992). The methodology currently used for artificial log cultivation, which will be referred to in this text as sawdust cultivation, was first mentioned in the late seventies (Fuzisawa *et al.*, 1978a,b; Mee, 1978). Both methods of cultivation have, however, advantages and disadvantages.

##### 3.1.1 Log Cultivation

Log cultivation requires low capital investment, is labour intensive and has minimum requirements for space and equipment (Royse, 1985; Worrall and Yang, 1992; Ferchak and Croucher, 1993). Furthermore, the mushrooms produced are of a superior quality to sawdust cultivated mushrooms: they are often denser, have a stronger flavour and a longer storage life (Przybylowicz and Donoghue, 1990; Raaska, 1992). No pesticides are used, and since there is an increasing market for organic products, there could be a future demand for such mushrooms (Przybylowicz and Donoghue, 1990).

Log cultivation is, nevertheless, characterised by low productivity and a prolonged production time due to seasonal and erratic yields (Fuzisawa *et al.*, 1978a; San Antonio, 1981; Leatham, 1982; Royse *et al.*, 1985; Chu-Chou, 1984; Hiromoto, 1991). Furthermore, the cost of wood has increased due to a shortage thereof and consequently log cultivation is becoming increasingly expensive (Fuzisawa *et al.*, 1978a). Finally, log cultivation can be considered wasteful since an

environmentally renewable resource is employed when alternative raw materials can be used with greater economic returns.

### 3.1.2 Sawdust cultivation

Sawdust cultivation typically has a high production rate since a short growth cycle results in high productivity (Royse, 1985; Royse *et al.*, 1985; Royse and Bahler, 1986; Royse, 1989; Hiramoto, 1991). Sawdust cultivation is not seasonal and consequently continuous production is possible (Royse *et al.*, 1990). This mode of cultivation, therefore, ensures a constant cash flow and is able to adapt to changing market trends (Przybylowicz and Donoghue, 1990).

The major disadvantage of sawdust cultivation is the relatively high initial investment costs associated with setting up such an operation since special facilities are required for cultivation, sterilization, air filtration and environmental control (Royse and Bahler, 1986; Przybylowicz and Donoghue, 1990).

### 3.1.3 Important considerations in shiitake cultivation

#### 3.1.3.1 Substrate

##### 3.1.3.1.1 Log cultivation

Shiitake is usually commercially cultivated on logs of *Quercus* (Oak) species (Ito, 1978; Wood and Smith, 1988). A number of growers have, however, been able to cultivate it successfully on a variety of hardwood species (San Antonio, 1981; Chu-Chou, 1984; Leatham and Griffin, 1984; Raaska, 1992).

The season during which trees are felled for shiitake production is important, since this influences the nutritional and physical properties of the wood. Trees cut at the beginning of spring support optimal growth because of their high concentration of easily available carbohydrates (Ito, 1978; Przybylowicz and Donoghue, 1990).

The presence of bark and its thickness is of great significance during log cultivation of shiitake mushrooms. The bark prevents water loss, physical damage and contamination of the log substrate (Przybylowicz and Donoghue, 1990). Furthermore, it has been proposed by Fuzisawa *et al.* (1978a) that hyphal differentiation into fruiting bodies occurs at the bark.

The substrate diameter used in shiitake log cultivation significantly affects production rate and fruiting body size (Royse, 1989; San Antonio, 1981). Logs ranging in diameter from 5 to 20 cm, and in length from 0.9 to 1.2 m, are usually used (Wood and Smith, 1988; Przybylowicz and Donoghue, 1990).

#### 3.1.3.1.2 Sawdust cultivation

A wide range of sawdusts have been used with varying degrees of success in the sawdust cultivation of shiitake (Chu-Chou, 1984; Royse and Bahler, 1986; Dare *et al.*, 1988; Diehle and Royse, 1991; Khan *et al.*, 1991a; Kirchhoff and Lelley, 1991; Rinker, 1991). These sawdusts are usually derived from hardwood species; if softwood sawdust is used it usually comprises less than 40% of the total sawdust component (Mee, 1978). Particles of between 1 to 5 mm are the most rapidly degraded by *L.edodes* (Mee, 1978; Przybylowicz and Donoghue, 1990).

The pre-treatment method supporting the optimal growth and productivity of *L.edodes* is strain dependent. Commonly used methods are pasteurization and steam sterilization (Royse *et al.*, 1985; Rinker, 1991).

#### 3.1.3.2 Strain

The single most important factor affecting shiitake mushroom production is the strain which is used (Miller and Jong, 1987). Significant genetic differences have been reported in *L.edodes* strains (Diehle and Royse, 1986; Przybylowicz and Donoghue, 1990). There is currently, however, a limited amount of knowledge on the growth, productivity and quality of these different strains (Diehle and Royse, 1986). *L.edodes* strains are divided into three groups; warm-weather, wide-range and cold weather strains (Table 3.1.1) on the basis of their fructification temperature range (Tokimoto and Komatsu, 1978).

#### 3.1.3.3 Spawn run conditions

The optimal temperature for the vegetative growth of *L.edodes* is 25°C; however, growth will occur between 4°C and 35°C (Tokimoto and Komatsu, 1978; Royse, 1989; Przybylowicz and Donoghue, 1990; Raaska, 1992). The relative humidity should be maintained between 50% and 70% (Przybylowicz and Donoghue, 1990) to sustain the substrate moisture content within the optimal range: 55% to 70% for sawdust cultivation (Miller and Jong, 1986; Royse, 1989; Przybylowicz and Donoghue, 1990) and between 35% and 75% for log cultivation (Przybylowicz and Donoghue, 1990; Raaska, 1992).

Table 3.1.1 Characteristics and fruiting temperature requirements of *L.edodes* strains (Przybylowicz and Donoghue, 1990).

Production phase	Warm-weather strains	Wide-range strains	Cold-weather strains
Spawn run time <sup>a</sup>	8-12 months	6-12 months	16-20 months
Induction: Soak water temp.	10-16°C	12-20°C	4-12°C
Pinning	16-20°C	12-25°C	4-16°C
Fruiting	10-22°C	10-18°C	7-18°C

<sup>a</sup> These are the spawn run time requirements during log cultivation.

There is much contradiction in the literature concerning the illumination requirements of *L.edodes*. Light has been found to be inhibitory to the vegetative growth of *L.edodes* (Tokimoto and Komatsu, 1978); however, exposure to light during this phase is essential for the fruiting of shiitake (Miller and Jong, 1987; Royse, 1989; Przybylowicz and Donoghue, 1990). Various light intensities have been proposed as being optimal for shiitake cultivation (Tokimoto and Komatsu, 1978; Royse, 1989; Miller and Jong, 1987; Przybylowicz and Donoghue, 1990). Light wavelengths between 370 nm and 420 nm (fluorescent) are reported to be appropriate for shiitake mushroom cultivation (Tokimoto and Komatsu, 1978; Przybylowicz and Donoghue, 1990).

Spawn run time is 1 to 2 years for log cultivation (Chu-Chou, 1984), and 30 to 180 days for sawdust cultivation (Przybylowicz and Donoghue, 1990). Long spawn run times are required during log cultivation since the cellular organization of wood retards the vegetative growth of *L.edodes* (Royse, 1989). Sawdust medium has a high nutrient content and is particulate, which implies that it has a large surface area for enzymatic degradative action. Consequently shorter spawn run times are sufficient to ensure complete colonization of the substrate (Przybylowicz and Donoghue, 1990).

#### 3.1.3.4 Fruiting conditions

The end of the vegetative phase in sawdust cultivation is indicated by the presence of a clear or brownish fluid, and the browning of certain substrate areas (Przybylowicz and Donoghue, 1990). Evidence of the complete colonization of logs is the appearance of white mycelial growth on the log ends, and a softening of the wood (Przybylowicz and Donoghue, 1990).

Optimal temperatures for the induction of fruiting are strain dependent (Przybylowicz and Donoghue, 1990). In sawdust cultivation, induction is initiated by either altering the temperature, soaking the substrate blocks at the optimal induction temperature for 12 to 24 hours, or cold shocking the blocks by soaking them at 5°C to 8°C for 5 to 12 days (Przybylowicz and Donoghue, 1990).

Pinning temperatures are strain dependent. Most *L.edodes* strains produce primordia at temperatures ranging from 10°C to 20°C in three to ten days (Przybylowicz and Donoghue, 1990). The relative humidity should be maintained at 85% to 95%, and the substrate moisture content between 55% and 65% (Przybylowicz and Donoghue, 1990). Fructification requires the maintenance of a relative humidity between 75% and 95%, and a temperature relevant to the strain under cultivation (Przybylowicz and Donoghue, 1990).

The recovery period requires the provision of warm conditions (15°C to 25°C) for 4 to 10 days, and a substrate moisture content of between 30% and 40% (Przybylowicz and Donoghue, 1990). Once mycelial recovery is complete, the fruiting process is once again initiated (Przybylowicz and Donoghue, 1990).

Flushes are generally harvested every two to three weeks for a period of three to six months during the sawdust cultivation of *L.edodes* (Przybylowicz and Donoghue, 1990). Under natural environmental conditions logs only produce two crops of shiitake per year, in spring and autumn, for three to seven years (Chu-Chou, 1984). The manipulation of environmental conditions during log cultivation (termed forced fruiting) results in higher yields with shorter fruiting cycles (Przybylowicz and Donoghue, 1990). Three to four crops can be harvested per year by means of forced fruiting; however, if logs are induced to fruit too often, lower yields are produced due to insufficient accumulation and storage of nutrients (Przybylowicz and Donoghue, 1990).

#### 3.1.4 Nutritional and health benefits of the shiitake mushroom

The shiitake mushroom is one of the most important commercially produced mushrooms in the world (San Antonio, 1981) due to its culinary, nutritional and medicinal attributes. Shiitake contains lenthionine and guanosine 5'-monophosphate, unique aroma and flavour enhancing compounds, respectively (Royse and Bahler, 1986; Tokimoto and Komatsu, 1978). Nutritionally shiitake is a good source of amino acids, proteins, lipids, carbohydrates, minerals and B, D and E vitamins (Bano *et al.*, 1963; Leatham, 1982; Przybylowicz and Donoghue, 1990; Flynn, 1991).

Compounds have been isolated from shiitake which reduce blood cholesterol levels (Tokita *et al.*, 1972), stimulate the immune system activity (Flynn, 1991), act as antiviral or antitumour agents (Leatham, 1982; Ito, 1-978; Flynn, 1991) and prevent platelet aggregation (Flynn, 1991). The chemotherapeutic agents which have been isolated from shiitake, unlike others, are non-toxic at high concentrations (Przybylowicz and Donoghue, 1990). The biologically active compounds produced by *L.edodes* are presented in Table 3.1.2.

Table 3.1.2 Biologically active compounds isolated from shiitake mushrooms (Przybylowicz and Donoghue, 1990).

<u>Compound</u>	<u>Effects</u>	<u>Type of compound</u>	<u>Activity</u>
Eritadenine	Lowers blood cholesterol levels; antiviral	adenine derivative	accelerates cholesterol metabolism and excretion
Ac2P	antiviral	polysaccharide	inhibits viral replication
Virus like particles	antiviral; antitumour	double-stranded RNA	induces interferon production
KS-2	antitumour; antiviral	polysaccharide	induces interferon production
Lentinan	antitumour	polysaccharide	stimulates T-helper cells in immune system
LAP 1	antitumour	polysaccharide	immune system modulator
Polyphenol oxidase	antitumour	protein	unknown
Unknown	reduces blood coagulation	possibly nucleosides or -tides	inhibits platelet aggregation
Cortinellin	antibacterial	unknown	broad spectrum antibiotic
Unknown	antifungal	disulphide	unknown
FBP	antiviral	protein	inhibits viral infection in plant

### 3.2 RESEARCH OBJECTIVES

The primary objective of the study was to ascertain whether locally available lignocellulosic wastes (Maranti and Port Jackson) are suitable for the cultivation of shiitake mushrooms. Oak was used as the control wood source since it is widely used for the commercial cultivation of shiitake. Log cultivation is more suited to the rural communities of Southern Africa, therefore both log and sawdust cultivation were attempted to compare the production efficiencies of the two cultivation systems.

Growth studies were also conducted to determine the influence of strain, spawn and substrate formulation on vegetative propagation.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Log cultivation

##### 3.3.1.1 Preparation of inoculum

Plug and sawdust spawn was prepared according to the method proposed by San Antonio (1981) (Appendix B; 1.1). Once cool, it was inoculated with a two week old culture of *oftedodes* 1899 (prepared as described in Section 2.3.2 on MA medium) and incubated at 25°C for 21 days.

##### 3.3.1.2 Felling of trees and preparation of logs for inoculation

Oak logs (*Quercus spp.*) which had been felled in summer, were supplied by Mukwa Timbers in Stutterheim, Eastern Cape, South Africa. Logs were prepared for inoculation 30 days after felling since the high sugar content present promotes rapid mycelial propagation (Tokimoto and Komatsu, 1978, Wood and Smith, 1988; Royse, 1989). Logs with diameters between 9 and 17.6 cm, lengths ranging from 250 to 570 cm, and a bark thickness of 3 to 7 mm were used as substrates for cultivation. These were soaked at 4°C for 11 days prior to inoculation to increase the log moisture content. Inoculum holes, with a diameter of 12.5 mm and a depth of 1 to 2 cm, were drilled in a diamond pattern corresponding to the method of Leatham (1982).

##### 3.3.1.3 Inoculation

Thirty logs were used in the study, half of which were sterilized (121°C x 180 minutes). The logs were spawned in a sterile chamber with one of the two inocula, so as to provide five replicas of each treatment. Ten control logs were also included in the study.

Once the logs had been inoculated with the appropriate spawn, the inoculation holes were sealed with bark plugs, using hot paraffin wax, which minimized moisture loss and contamination.

##### 3.3.1.4 Spawn run conditions

The lean-to stacked logs (Przybylowicz and Donoghue, 1990) were incubated in a concrete constant environment room under ambient laboratory illumination (7300 lux), and at a temperature of 25°C. The relative humidity was maintained at between 50% and 70%. Logs were soaked every three weeks, at 4°C for 24 hours, to maintain the log moisture content within the optimal range. The logs were treated in the above manner for six months.

### 3.3.2 Sawdust media

#### 3.3.2.1 Strains

Two-week old agar cultures of *L.edodes* strains 1899k, 58762 and 48860 were prepared as previously reported (Section 2.3.2), using synthetic media optimal for their *in vitro* growth (Table 2.4.4). These agar cultures were either used as inoculum for growth study substrates, or in the preparation of liquid and grain-sawdust spawn.

##### 3.3.2.1.1 Liquid spawn

B-MEA broth aliquots of 100 ml (Appendix A; 1.6) were placed in 500 ml Erlenmeyer flasks containing glass beads, and inoculated with agar-derived *L.edodes* mycelia. The inoculated media was incubated in the dark at 25°C and shaken at 150 rpm. After an incubation period of two weeks an equal volume of nutritive media, prepared as previously described by Kostadinov *et al.* (1972) (Appendix B; 1.3), was aseptically added and the cultures further incubated for two days. The resultant liquid spawn was used for the inoculation of growth study and production substrates.

##### 3.3.2.1.2 Grain/sawdust spawn

Grain/sawdust spawn was prepared according to the method adapted from Diehle and Royse (1986) (Appendix B; 1.2). Agar plugs were used to inoculate the cooled spawn substrates, which were then incubated in the dark at 25°C for 14 days. Spawn flasks were shaken regularly to prevent mycelial clumping. The spawn was stored at 4°C until required. Spawn of all three *L.edodes* strains were used for the inoculation of production media.

##### 3.3.2.2 Wood Source

The rate of *L.edodes* infectivity was evaluated on various sawdusts: Oak (*Quercus spp.*), Pine (*Pinus spp.*), Yellowwood (*Podocarpus spp.*), Maranti (*Shorea spp.*) and Port Jackson (*Acacia longifolia*). Oak, Pine and Yellowwood sawdusts were supplied by Mukwa Timbers in Stutterheim, Eastern Cape, South Africa. Maranti was supplied by the East London Joinery and Port Jackson by Professor R. Kirby of Hermitage Farm, Grahamstown. Port Jackson was supplied as logs and chipped by the Parks Department of the Grahamstown Municipality. The sawdusts were soaked for two days prior to incorporation into substrates to increase the moisture content. Based on *L.edodes* rate of infectivity of the different wood sources, only certain were used in the growth and production studies.

### 3.3.2.3 Substrate formulation

A variety of formulations were tested as potential substrates for shiitake mushroom cultivation (see Table 3.3.1). The substrate compositions used for the growth studies were composed of 100 g amounts of sawdust, whereas 400 g and 1000 g amounts were used for production studies. Irrespective of the sawdust content, the substrates were amended with appropriate quantities of organic and inorganic additives. Table 3.3.1 provides greater detail on the composition of these media.

Wood sources were either used separately or in combination, e.g. Port Jackson was either used as the sole wood source in the substrate or in combination with Maranti or Oak. The proportions of the individual sawdust components ranged from 1:9 to 9:1.

All sawdust combinations and substrate formulations (Table 3.3.1) were used in the production studies. In the growth studies only a representative number of these substrate formulations were used to indicate the effect of wood source and substrate formulation on *L.edodes* growth.

### 3.3.2.4 Medium Preparation and Inoculation

The substrate components were thoroughly mixed and placed in autoclavable containers, either nylon polyester (NP) bags (Multifoil, Johannesburg) or polyvinyl chloride (PVC) tubes (N.T.C., Port Elizabeth) sealed with NP film. Both types of containers contained a PVC "neck" stoppered with cotton wool. All substrates to be used in growth studies were bagged in NP bags, whereas both containers were used for productivity analyses. Substrates were sterilized at 121°C for 120 minutes and spawned with a 10% inoculum (wet weight basis).

### 3.3.2.5 Spawn run conditions

All inoculated substrates were incubated in the dark at a temperature of 25°C and a relative humidity of 60% to 80%. The spawn run time was approximately one month for all inoculated substrates. Once colonization was complete, substrate containers were partially removed. Substrates were then induced to fruit by soaking them at 4°C for 48 hours.

**Table 3.3.1** The sawdust substrate compositions investigated for the cultivation of shiitake mushrooms.

<b>Substrate Components</b>	<b>Mee (1978)<sup>a</sup></b>	<b>Patrick <i>et al.</i> (1983)</b>	<b>Rinker (1991)</b>	<b>Kirby (Pers. Comm.)</b>
Wood source	85%	80.0%	77.0%	77.0%
Potato starch	8.5%	-	-	-
Yeast extract	2.1 %	1.5%		
CaSO <sub>4</sub> .2H <sub>2</sub> O	4.2%			
Sucrose	1.2%		1.2%	1.0%
Molasses	-	1.0%	-	
Wheat bran		17.0%	10.9%	20.0%
White millet		-	10.9%	
CaCO <sub>3</sub>		0.2%	-	1.5%
Fertilizer <sup>c</sup>			-	0.5%
dH <sub>2</sub> O <sup>e</sup>	600 ml	65.0%	1:1.2	1:1.2

<sup>a</sup> The substrate formulation proposed by Mee, 1978 was altered by the incorporation of 1.2% sucrose.

<sup>b</sup> The fertilizer used contained a 3:2:1 ratio of nitrogen, phosphate and potassium.

<sup>c</sup> Water was added, in the above ratios, according to the substrate dry weight.

### 3.3.2.6 Fructification conditions

A number of different temperature and lighting conditions were tested. Substrates were incubated at 4°C, 10°C, 15°C, 17°C or 20°C either in complete darkness or with a photoperiod of 12 hours. An illumination intensity of 35 lux (measured by a Lutron LX-101 Digital Lux Meter) was supplied by automatically controlled overhead fluorescent lights. The relative humidity in the production room was maintained between 60% and 100%. The water balance in the substrate blocks was maintained by daily irrigation and bi-weekly soaking at 4°C for 48 hours.

### 3.3.3 Analytical methods

#### 3.3.3.1 Moisture Determination

A pre-weighed quantity of substrate was dried at 80°C for 24 hours. The values obtained were used to determine the substrate moisture content, which was expressed as a percentage, according to the following formula:

$$X = a/b \times 100 \quad (3.1)$$

X= wood moisture content (%)  
 difference between wet and dry substrate weight (g)  
 b= substrate wet weight (g)

### 3.3.3.2 Visual inspection

Segments (5 cm) were cut from logs at monthly intervals to ascertain the level or extent of *L.edodes* infectivity.

### 3.3.3.3 Growth determinations

The separation of fungal mycelia from solid substrates is difficult, necessitating the use of indirect methods of growth assessment which either measure fungal cell components or factors involved in fungal metabolism (Boyle and Kropp, 1992; Di Lena *et al.*, 1994). One of the simplest means of estimating fungal degradation of substrates is the determination of the loss in substrate organic matter content (Rajaratnam and Bano, 1989). This decrease in organic matter, measured as a decrease in substrate dry weight, is owing to the production of carbon dioxide and water by the fungus as it degrades carbohydrates (Rajaratnam and Bano, 1989; Boyle and Kropp, 1992).

Destructive sampling was employed in ascertaining fungal growth over time. Substrate dry matter contents were determined by drying at 100°C for 48 hours. The percentage decrease in substrate dry weight was then determined:

$$Y = M_c - M_s / M_c \times 100 \quad (3.2)$$

Y = percentage decrease in substrate dry weight  
 $M_c$  = mass of the uninoculated control sample  
 $M_s$  = mass of the sample

## 3.4 RESULTS

### 3.4.1 Log cultivation

The logs were soaked prior to inoculation since the moisture content was not within the optimal range, possibly as a consequence of log incubation at 25°C for 30 days. The inoculated substrate moisture content varied from 25.5% to 41.7%, depending on the bark and log diameter.

Visual inspection of the inoculated logs indicated that contamination became established soon after incubation. Unsterilized logs were generally contaminated earlier than sterilized logs. After an incubation period of 6 months, however, 80% of t4 logs were visibly contaminated. The most prolific contaminant was a green mould, identified as *Trichoderma spp.* Contamination was essentially external and restricted to the bark and exposed areas. Contamination was also evident in the inoculation holes; however, no *L.edodes* mycelial growth was observed. It therefore seemed unlikely that mushroom production would occur and the study was discontinued.

### 3.4.2 Sawdust cultivation

#### 3.4.2.1 Growth studies

In all growth studies agar inoculum and medium prepared according to Mee *et al.* (1978) was used, unless otherwise reported. The effect of strain, wood source, substrate formulation and inoculum type on substrate utilization were investigated.

##### 3.4.2.1.1 Strain

Oak substrates inoculated with *L.edodes* 48860 showed a noticeably lower percentage decrease in substrate dry matter content than those colonized with *L.edodes* 1899 and 58762 (Fig. 3.4.1). Strains 1899 and 58762 were equally well adapted to Oak substrate cultivation, and had decreased the dry matter content to a comparable level after a 23 day growth period (Fig. 3.4.1).

*L.edodes* 58762 showed a considerably higher initial growth rate than strain 1899. This -strain decreased the substrate dry weight by 4.1% by day 7, whereas 1899 only showed a 0.4% decrease. The growth of strain 1899 from day 7 to 23, however, caused a 7.22% decrease in substrate dry weight as compared to 3.8% with strain 58762.

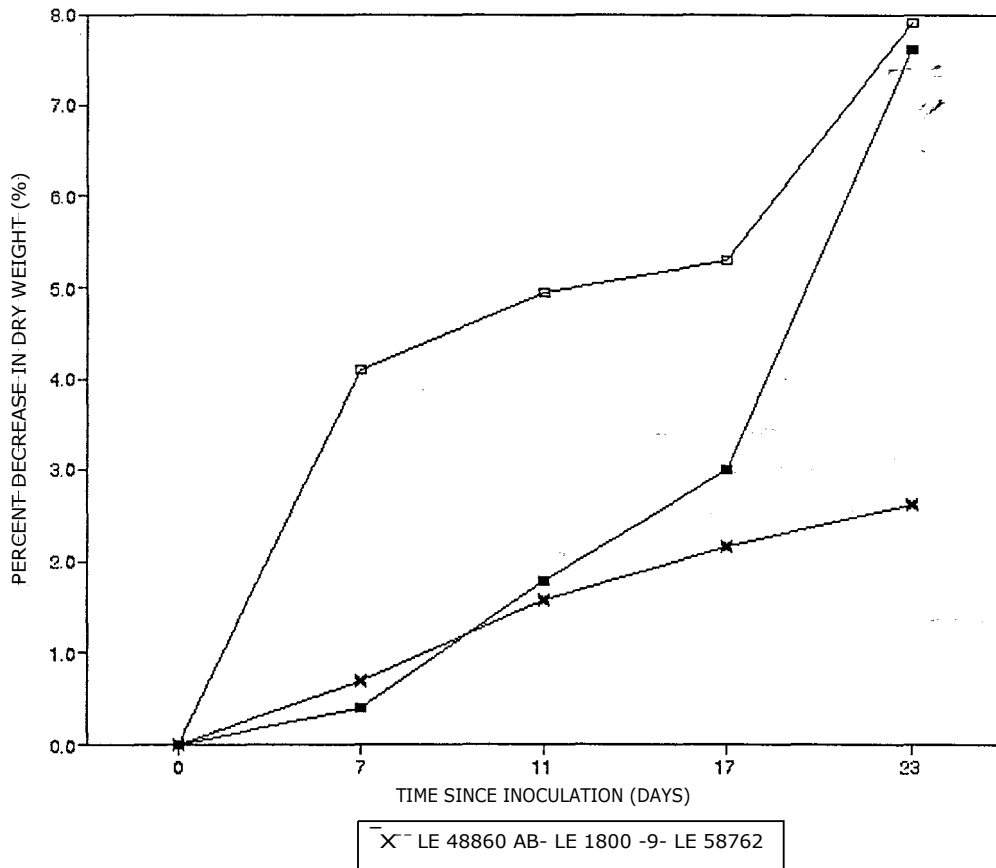


Fig. 3.4.1

The percentage decrease in Oak substrate dry weight after incubation with *L.edodes* strains 1899, 48860 and 58762.

#### 3.4.2.1.2 Wood source

The infectivity rate of *L.edodes* 1899 was used as a preliminary means of screening wood sources as suitable substrates for shiitake cultivation. The amount of time before growth became established was ascertained by visual inspection (Table 3.4.1)

The rate of infectivity of *L.edodes* 1899 on Pine and Yellowwood was slow when compared to that on Maranti and Oak. Maranti, Oak and Port Jackson were used in subsequent *L.edodes* growth and production studies. Maranti was used since it is easily available and supported the greatest infectivity rate of *L.edodes*. Port Jackson is abundant in the Eastern Cape. Oak is commercially used for the production of shiitake mushrooms and had a comparable infectivity rate to Maranti.

Table 3.4.1 Rate of infectivity of *L.edodes* strain 1899 on various sawdust media.

Sawdust Source	Time (Days) <sup>o</sup>
Oak	5
Pine	10
Yellow Wood	8
Maranti	3

<sup>a</sup>

Amount of time before growth became visually perceivable.

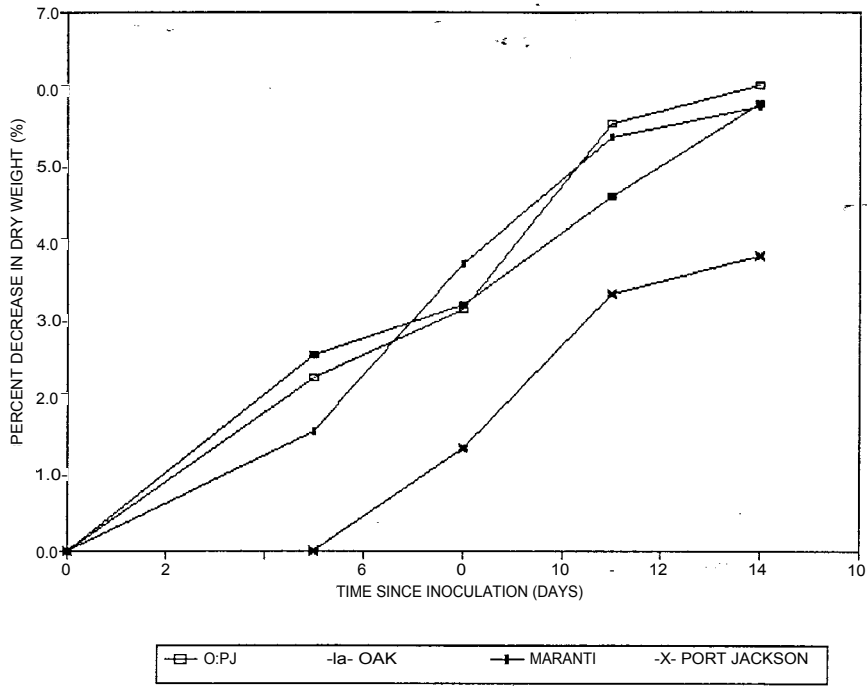
The inoculation of *L.edodes* 1899 onto various substrates showed preferential utilization of a 1:1 ratio of Port Jackson:Oak sawdust medium (Fig. 3.4.2a). This sawdust mixture was utilized with greater efficiency than media containing the individual sawdust components, as indicated by the greater percentage decrease in substrate dry weight. The percentage utilization of Oak, Maranti and Port Jackson:Oak media does not vary greatly after 14 days, however, all were considerably higher than that of the Port Jackson medium.

*L.edodes* 58762 utilized Oak sawdust more efficiently than a 1:1 Port Jackson:Oak sawdust combination (Fig. 3.4.2b). The difference was, however, not considerable (12.7% and 11.1%, respectively, after 17 days).

#### 3.4.2.1.3 Substrate formulation

The addition of sucrose to substrate formulations has been found to have a beneficial effect on the growth of *L.edodes* 1899 on Port Jackson medium (Fig. 3.4.3a). This effect was most evident in the initial growth phase, where medium containing sucrose was decreased by 1.33% whereas that without sucrose showed no decrease in dry matter content.

A



B

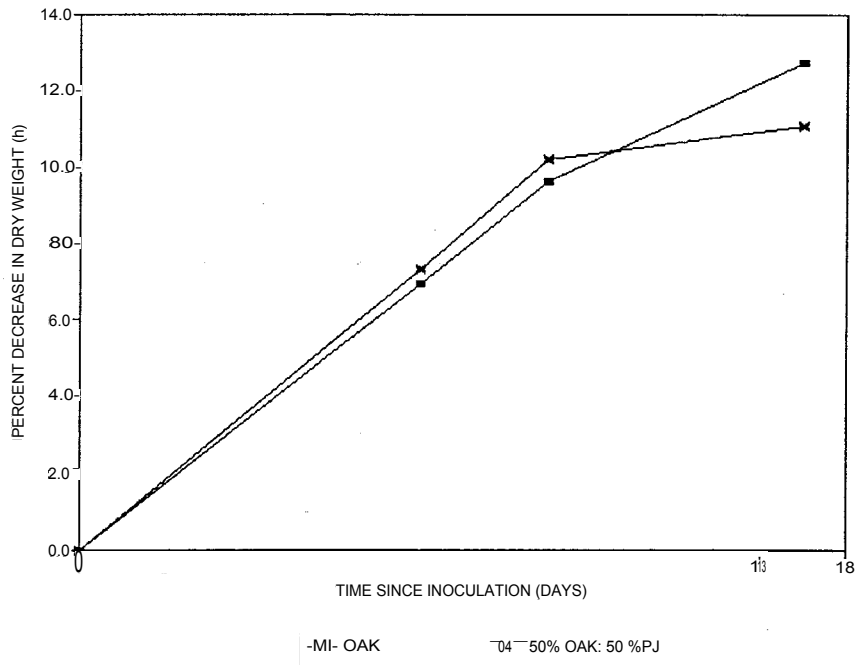


Fig. 3.4.2

The utilization of various wood sources by *L. edodes* strains 1899 (a) and 58762(b).

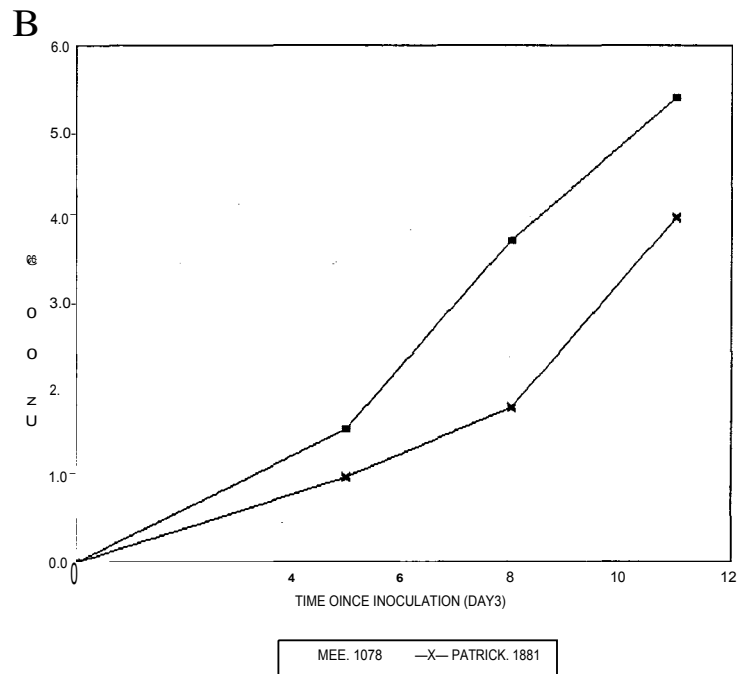
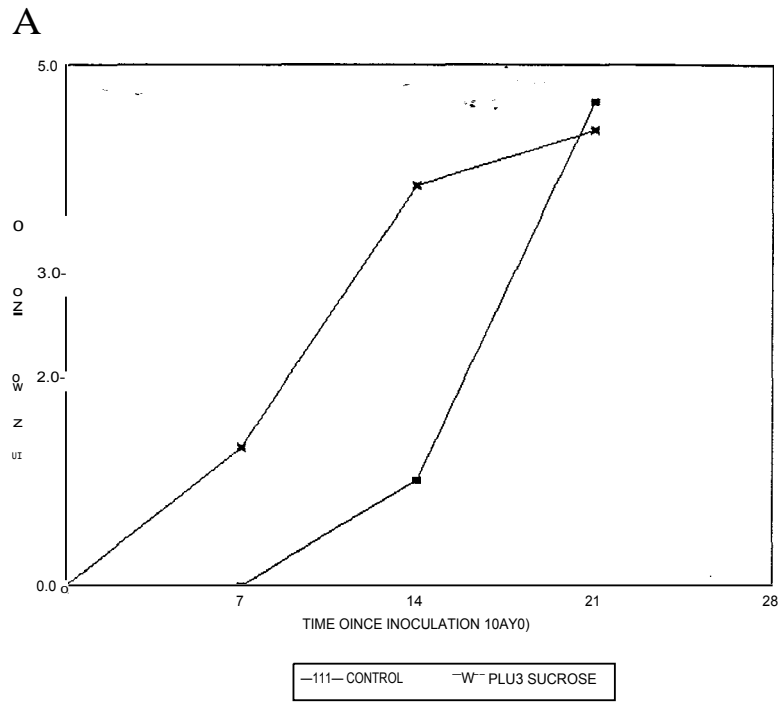


Fig. 3.4.3

Substrate formulation and its effect on *L.edodes* 1899 growth on Port Jackson medium (Mee, 1978) prepared with and without the addition of sucrose (A) and Maranti medium prepared according to Mee (1978) and Patrick *et al.* (1983) (B).

The utilization of Maranti substrate prepared according to the formulations of Mee (1978) (with 1.2% sucrose) and Patrick *et al.* (1983) varied, however not noticeably, when inoculated with *L.edodes* 1899 (Fig. 3.4.3b).

#### 3.4.2.1.4 Inoculum

Inoculum type has been found to have a considerable effect upon the growth of *L.edodes* strain 1899 (Fig. 3.4.4). Oak substrates inoculated with liquid spawn showed a decrease of 7.2% in their dry matter contents after 17 days, whereas those spawned with agar inoculum showed a decrease of only 3.0%.

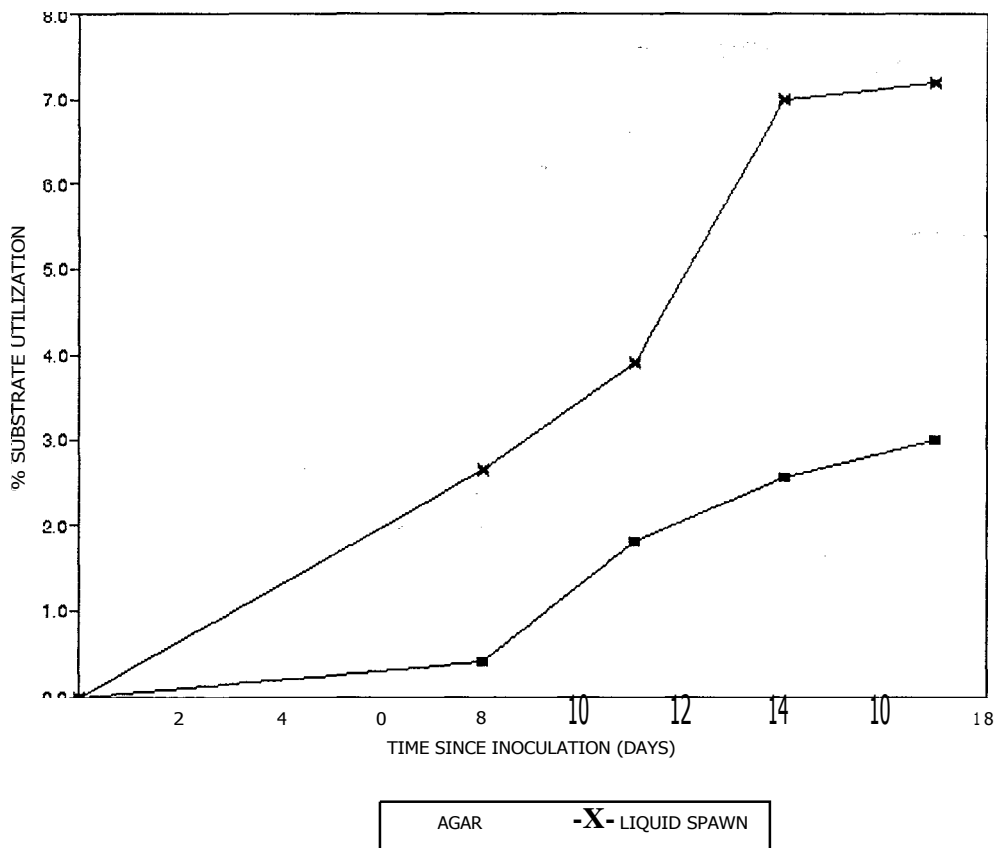


Fig. 3.4.4

Inoculum effect on substrate utilization by *L.edodes* 1899.

#### 3.4.2.2 Production studies

A number of different physical, nutritional and environmental conditions were investigated in attempting to achieve shiitake mushroom production. All were unsuccessful. A range of different sawdust combinations were tested as components of a variety of substrate formulations (Table 3.3.1); different inocula of all *L.edodes* strains were tested and two different containers were investigated.

The environmental conditions during vegetative growth (spawn run) were identical for all substrates inoculated and complete colonization was evident after three to four weeks. Approximately 40% of all substrates, however, were extensively contaminated. Pin formation and browning of substrate surfaces was apparent on only those substrates packaged in PVC tubes (the possible reason for which is provided in Chapter 4). Although a range of temperatures and two different lighting regimes were investigated in the fructification phase; no primordia, and consequently no mushrooms, were produced.

### 3.5 DISCUSSION

The success of mushroom cultivation is determined by the interaction of physiological and environmental parameters. A large number of factors therefore, either independently or in combination, could be responsible for the failure in cultivating shiitake mushrooms. Vegetative growth of the various *L.edodes* strains did, however, occur on sawdust media as evidenced by the white mycelial growth and decreases in substrate dry matter contents.

Temperature and moisture (within and surrounding the substrate) have a major impact upon mushroom cultivation, with the latter having a considerably greater effect than the former (Raaska, 1992). Incorrect moisture conditions during the spawn run period have been proposed as the major reason associated with the failure of shiitake log cultivation (Ito, 1978; Przybylowicz and Donoghue, 1990). Wood is not decayed if its moisture content is below 20% (Moore-Landecker, 1982). Optimal substrate moisture content is therefore essential for the production of shiitake fruitbodies (Mee, 1978; Royse, 1989). The initial moisture content of the inoculated logs in this study was the minimum limit required for the cultivation of shiitake mushrooms; whereas the optimal is from 65% to 70% (Royse, 1989). Since optimal substrate and moisture conditions are required at the commencement of the cultivation process to enable the rapid growth and establishment of the desired fungus; the low initial moisture content could have been a major contributing factor to the failure in producing log cultivated mushrooms in this study.

It has been proposed that light is not a critical factor affecting shiitake mushroom cultivation (San Antonio, 1981). Therefore the sub-optimal lighting conditions used in this study would not, solely, have been responsible for the failure of the cultivation process. San Antonio (1981) successfully produced log cultivated mushrooms in lighting conditions ranging from 500 to 90 000 lux.

The two most important physical factors affecting mushroom production are the substrate and strain used (San Antonio, 1981; Hiromoto, 1991). *L.edodes* 1899 was probably an unsuitable strain to use for log cultivation as a result of its fastidious nature and slow vegetative growth (refer to Chapter2).

Although Oak logs are the preferred species for the cultivation of shiitake mushrooms (Leatham and Griffin, 1984), inter- and intraspecific differences may occur in wood density, moisture and nutrient levels (Przybylowicz and Donoghue, 1990). Furthermore, certain species of Oak contain water-soluble substances which are inhibitory to the growth of *L.edodes* (Leatham and Griffin, 1984). The logs used in this study were felled in late summer and although trees felled in this season do not have the desired sugar content, mushrooms should still be harvested, however at lower yields and higher contaminant levels (Przybylowicz and Donoghue, 1990).

The failure in the log cultivation of shiitake mushrooms was therefore probably caused by a number of factors; e.g. an unsuitable strain/substrate combination together with sub-optimal environmental conditions resulted in the establishment of high levels of contaminants, which further inhibited mycelial growth.

A range of substrates and environmental conditions were investigated, and all were unsuccessful in promoting the sawdust cultivation of shiitake mushrooms. Although it could be argued that this is as a result of the substrates used, it is unlikely that all sawdusts, including Oak, could be inhibitory to shiitake mushroom production, especially when growth occurred so efficiently on them.

Warm-temperature and wide-temperature range strains were used in this study, thus the fruiting temperatures implemented should have been favourable to fruiting. Many of the commercially available strains, however, are suited to log cultivation and are not amenable to sawdust cultivation (Patrick *et al.*, 1983; Miller and Jong, 1987; Przybylowicz and Donoghue, 1990). Furthermore, only a limited number of strains produce economically viable crops (Miller and Jong, 1987).

Although the strains used in this study were not suited to sawdust cultivation, they were able to utilize a range of substrates efficiently, indicating the effect which a number of cultural parameters have on growth. Growth on sawdust media was influenced by the strain, spawn type and substrate formulation used.

Strains varied considerably in their growth on, and consequently consumption of, different substrates. *L.edodes* strains 1899 and 58762 showed greater growth and Oak sulistraie consumption than, 48860 (Fig. 3.4.1). This was in direct contrast to the *in vitro* growth studies previously reported (Chapter 2), where strain 48860 showed considerably greater growth than the other two strains. Growth on synthetic media, therefore, does not always correlate with that on lignocellulose-based substrates. Both studies, however, grouped strains 58762 and 1899 together in terms of their growth potential.

In accordance with the literature (Royse and Bahler, 1986), this study found that growth is significantly influenced by the interaction between strain and substrate formulation. Of the wood sources tested, *L.edodes* 1899 grew best on the Port Jackson:Oak based medium (Fig. 3.4.2b) whereas strain 58762 showed most efficient utilization of the Oak based substrate (Fig. 3.4.20). The growth of *L.edodes* 1899 on different substrate formulations varied; however, this difference was not considerable (Fig. 3.4.3). Although the addition of sucrose did not have a significant effect upon the growth of *L.edodes* 1899 after 21 days, it did increase initial growth substantially (Fig. 3.4.3a). Saccharides are not essential for fungal growth, however, they do provide high initial growth rates since they afford an easily available source of carbohydrate (Mee, 1978). Furthermore, it has been reported that the incorporation of this nutrient source ensures consistent crops which mature synchronously (Royse *et al.*, 1990).

Spawn type has been found to influence the rate of mycelial colonization of substrates (Kostidinov *et al.*, 1972). The findings of this study indicate that substrate colonization occurs more rapidly when using nutritive liquid inoculum than when using agar inoculum (Fig. 3.4.4), which corresponds to previous reports (Kostidinov *et al.*, 1972).

### 3.6 CONCLUSIONS

*L.edodes* has been found to be extremely fastidious in its nutritional and environmental requirements, especially during fructification. Although, a wide array of cultivation conditions were used in this study, none were found to be conducive to shiitake mushroom production.

Vegetative growth of all strains on a number of sawdust media did, however, occur indicating the influence which strain, substrate formulation and spawn type have on growth. Growth occurred on all sawdust media tested, including Port Jackson and Maranti, implying that the use of an appropriate strain under the correct fructification conditions could result in the production of shiitake mushrooms on locally available lignocellulosic wastes.

## CHAPTER FOUR

### OYSTER AND WINTER MUSHROOM GROWTH AND PRODUCTION ON LOCALLY AVAILABLE WOOD SUBSTRATES FROM SOUTH AFRICA.

#### 4.1 INTRODUCTION

##### 4.1.1 Cultivation of *Pleurotus* species

The cultivation of *Pleurotus* species has recently increased due to their ability to produce mushrooms (commonly known as oyster mushrooms) of good organoleptic quality in varying climatic conditions and on a wide range of inexpensive agricultural and industrial byproducts (Bano *et al.*, 1963; Delcaire, 1978; Rajarathnam and Bano, 1989; Buswell *et al.*, 1993; Iracabal *et al.*, 1995). The wide temperature and substrate range as well as short cultivation time required for oyster mushroom cultivation (Bano *et al.*, 1963; Pettipher, 1988), have resulted in this being one of the most extensively cultivated mushrooms worldwide (Pettipher, 1988; Upadhyay and Vijay, 1991).

The growth characteristics, size, colour, morphology and yield of mushrooms produced by *Pleurotus* species are variable depending on the strain and cultural conditions implemented (Rajarathnam and Bano, 1987). Substrate formulation, strain, temperature, light, air composition and cultivation technique have been identified as important factors affecting cultivation (Zadrazil, 1978; Kamra and Zadrazil, 1986).

##### 4.1.1.1 Substrate

*Pleurotus* species are naturally found on a wide range of lignocellulosic materials, which they are able to metabolise, due to their extensive production of a large number of extracellular enzymes (Eger, 1978; Bano and Rajarathnam, 1982; Garcha *et al.*, 1984; Rajarathnam and Bano, 1987). Oyster mushrooms have therefore been cultivated, with varying degrees of success, on a number of lignocellulosic wastes (Bano *et al.*, 1963; Kostidinov, 1972; Kaneshiro, 1977; Chang *et al.*, 1981; Zadrazil and Brunnert, 1981; Bano and Rajarathnam, 1982; Patrick *et al.*, 1983; Rajarathnam *et al.*, 1983; Garcha *et al.*, 1984; Kamra and Zadrazil, 1986; Rajarathnam *et al.*, 1986; Zadrazil and Peerally, 1986; Gujral *et al.*, 1987; Pettipher, 1988; Bononi *et al.*, 1991; Ferchak and Croucher, 1993; Hordeau *et al.*, 1991; Lelley and Niehrenheim, 1991; Worrall and Yang, 1992; Sermanni *et al.*, 1994).

The substrate carbon:nitrogen ratio affects the growth of *Pleurotus* species (Fasidi and Olorunmaiye, 1994). Although substrate degradation rates are not critically influenced by nitrogen concentration (Rajaratnam and Bano, 1989), high substrate nitrogen levels can have a negative effect during cultivation, possibly due to catabolite repression of degradative enzyme biosynthesis (Kaneshiro, 1977). Furthermore, certain minerals and trace elements have been found to be essential for the growth of *Pleurotus*, e.g. calcium, potassium, copper and zinc (Fasidi and Olorunmaiye, 1994).

Optimal growth of *Pleurotus* species is promoted by substrate moisture contents between 65% and 75% (Chang *et al.*, 1981; Rajaratnam and Bano, 1987). Species of this genus are highly saprophytic and are able to grow on unsterilized, sterilized, pasteurized and fermented substrates (Bano *et al.*, 1963; Chang *et al.*, 1981; Stolzer and Grabbe, 1991).

#### **4.1.1.2 Strain**

A range of different *Pleurotus* species are cultivated worldwide (Delcaire, 1978; Eger, 1978). Geographical factors determine the strains which are cultivated in a certain locale (Delcaire, 1978). Optimal fructification temperatures are intra- and interspecies specific, as are macro- and microscopic characteristics (refer to Table 4.1.1). *Pleurotus* species can be divided into three groups on the basis of their fructification temperatures. The cold weather or European strains require fructification temperatures below 15°C (Eger, 1978; Hauser *Pleurotus* Documentation 6.84, Pers. comm.); warm weather strains produce mushrooms at temperatures above 15°C (Hauser *Pleurotus* Documentation 6.84, Pers. comm.) and the so called "American" strains have a wide fruiting temperature range (from 4°C to 27°C) (Eger, 1978).

*P.ostreatus* is one of the most widely cultivated oyster mushroom species due to its high quality yield (Hauser *Pleurotus* Documentation 6.84, Pers. comm.) and high saprophytic colonization ability (Zadrazil, 1978; Kamra and Zadrazil, 1986). Sub-species are either cold (European or winter group) or warm (Florida or summer group) weather strains requiring fructification temperatures ranging from 10°C to 15°C or 18°C to 23°C, respectively (Patrick *et al.*, 1983).

**Table 4.1.1** Physical characteristics of certain *Pleurotus* species (Peng *et al.*, 1990)a.

Species	Cap, flesh and taste	Growth Habit	Fruiting Temperature
<i>P. calypratus</i> 36211	5.5-12 cm Cap-white Flesh-white. Taste pleasant.	Gregarious	20 - 25°C
<i>P. citrinopileatus</i> 36239	1.5-7.0 cm Cap-pale yellow Flesh-pale yellow,thin. Taste pleasant	Caespitose	16 - 20°C
<i>P. columbinus</i> 36215	1.5-8.5 cm Cap-light glaucous Flesh-white,thin Taste pleasant	Caespitose	16 - 18°C
<i>P. cystidiosus</i> <sup>b</sup> 36046 36131 36168 36170 36171 36172 36223 36253	3.0-15.0 cm; Cap-buff,hazel,white or grey Flesh-white Taste pleasant	Gregarious (produces asexual spores)	16 - 32°C
<i>P. cornucopiae</i> 36216	1.5-6.0 cm Cap-cream Flesh-white Taste pleasant	Caespitose	16 - 18°C
<i>P. dryinus</i> 36217	2.2-9.0 cm Cap-white Flesh-white,thick Taste pleasant	Solitary or gregarious	16 - 18°C
<i>P. eryngii</i> 36037	2.0-11.0 cm Cap-buff Flesh-white,thick Excellent edibility (taste of almond)	Caespitose	15 - 18°C
<i>P. flabellatus</i> 36222	1.6-6.5 cm Cap-light flesh Flesh-light flesh,thin Taste pleasant	Caespitose	16 - 20°C
<i>PfUscus</i> var. <i>ferulae</i> Lanzi 36214	6.0-18.0 cm Cap-buff Flesh-white Taste of almond	Caespitose	16 - 18°C
<i>P. incarnatus</i> 36228	3.0-16.0 cm Cap-salmon Flesh-pale flesh Taste pleasant	Gregarious	18 - 22°C
<i>P. pulmonarius</i> 36095	2.0-15.0 cm Cap-whitish/cream Flesh-white,thin Edible (odour of ammonia)	Gregarious	16 - 32°C
<i>P. sajor-caju</i> 36040	4.5-15.0 cm Cap-pale mouse grey Flesh-white,thin Taste pleasant	Gregarious	16 - 22°C

The information supplied is relevant to CCRC cultures, some of which have been mentioned in Table 2.3.1. The information furnished is a generalization of the characteristics of the various sub-species of *P. cystidiosus* mentioned.

*P. eryngii* shows slow vegetative growth and low contaminant resistance which limits its commercial cultivation, even though the yield obtained can be comparable to that of *P. ostreatus* (Zadrazil, 1978). Commercially, this species has the potential of being more popular than other *Pleurotus* species due to its superior consistency, pleasant aroma and culinary qualities (Zadrazil, 1978).

### **Cultivation conditions**

The gaseous requirements of different *Pleurotus* species vary (Kamra and Zadrazil, 1986). Oxygen presence positively influences substrate degradation, as does certain levels of carbon dioxide (up to 20%) (Kamra and Zadrazil, 1986). The oxygen requirements of *Pleurotus* species are lower than those of many other fungi (Rajarathnam and Bano, 1987).

Light has been found to be necessary for the production of good quality fruiting bodies at high yields (Kamra and Zadrazil, 1986). Although it has been postulated that light plays a role in the initiation of primordia development (Zadrazil, 1978), longer exposure to light is necessary during fructification than pinning (Eger, 1978). *Pleurotus* species vary in their light requirements (Eger, 1978). The lighting intensity that has been prescribed for oyster mushroom cultivation varies from below 40 lux (Bisaria and Madan, 1983) to between 500 and 1000 lux (Patrick *et al.*, 1983). Spawn run conditions should be maintained between 25°C and 30°C and at a relative humidity from 75% to 80% (Zadrazil, 1978; Patrick *et al.*, 1983). Spawn run time ranges from 16 to 30 days, depending on the strain being cultivated (Hauser *Pleurotus* Documentation 6.84, Pers. comm.).

There is currently a limited amount of knowledge on the factors affecting the fructification process of *Pleurotus* species (Rajarathnam and Bano, 1987). Strain-specific fructification temperatures are usually used and the relative humidity should be maintained between 90% and 95% (Patrick *et al.*, 1983). The induction mechanism which is most effectively used in the initiation of oyster mushroom production is partial substrate desiccation, followed by heavy watering and illumination (Rajarathnam and Bano, 1987). The effectiveness of the process can be improved in some species by using a cold temperature shock treatment (Zadrazil, 1978).

The harvesting time varies from 6 to 12 weeks, with a variable amount of flushes being cropped throughout that period, depending on the strain (Hauser *Pleurotus* Documentation 6.84, Pers.comm.).

#### **4.1.2 *Flammulina velutipes* cultivation**

The fruitbodies produced by *F.velutipes* have a number of common names: enokitake (Delcaire, 1978; Chu-Chou, 1984); velvet stem (Mee, 1978), winter mushroom (Tonomura, 1978; Wood and Smith, 1988) and golden mushroom (Delcaire, 1978).

##### **4.1.2.1 Substrate**

Sawdust cultivation is the method of choice in the commercial production of winter mushrooms (Tonomura, 1978; Wood and Smith, 1988). The common substrate used is a 4:1 mixture of sawdust and rice bran at a 58% to 60% moisture level (Tonomura, 1978). The sawdust is often softened by storage of 6 to 12 months prior to use (Tonomura, 1978; Food from Wastes, 1981). *F.velutipes* is able to utilize a wide range of carbon sources, however, with variable efficiency (Tonomura, 1978; Kinugawa, 1993). Nitrogen, potassium, phosphorus, magnesium and sulphur are essential for the growth of *F.velutipes* (Kinugawa, 1993). Polypropylene bottles are commonly used as cultivation vessels (Tonomura, 1978; Wood and Smith, 1988), and steam sterilization is the most commonly used pre-treatment method (Tonomura, 1978).

##### **4.1.2.2 Environmental Requirements**

Temperature and humidity conditions are fundamentally important (Tonomura, 1978; Food from Wastes, 1981), and are optimally altered four times during winter mushroom cultivation (Tonomura, 1978; Wood and Smith, 1988). The optimal temperatures for mycelial growth range from 22°C to 25°C (Tonomura, 1978; Wood and Smith, 1978; Kinugawa, 1993), for primordia development from 10°C to 20°C and for fruiting from 10°C to 15°C (Tonomura, 1978, Kinugawa, 1993). Although exposure to light is not essential during cultivation, it is necessary for the maturation of fruiting bodies (Tonomura, 1978; Kinugawa, 1993).

## 4.2 RESEARCH OBJECTIVES

The nutritional and environmental requirements vary-for the vast number of *Pleurotus* species cultivated commercially. A number of *Pleurotus* species, which are amenable to cultivation under South African environmental conditions, were thus evaluated for their substrate utilization and fruitbody production potential on a range of locally available wood sources, using a novel cultivation method. A strain of *F.velutipes* was similarly investigated. Factors affecting mushroom cultivation such as the cultivation vessel, strain, substrate formulation, water source and age of the spawn employed, were also investigated.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Strains

*F.velutipes* and a group of representative *Pleurotus* species were selected from 22 commercial strains. The *Pleurotus* species tested were: *P.calyptratus* (36211); *P.citrinopileatus* (36239); *P.columbinus* (36215); *P.cystidiosus* (36131, 36168, 36170, 36172); *P.eryngii* (36037); *P.fuscus* var. *ferulae* Lanzi (36214); *P.ostreatus* (1020, 1833, 3344); *P.pulmonarius* (36095) and *Pleurotus* spp. (36031). Strains were obtained from the ATCC, DSM and CCRC (refer to Table 2.3.1).

The fungal strains were inoculated onto either MA or agar media promoting their optimal *in vitro* growth (Table 2.4.4) and incubated at 25°C for fourteen days. These two-week old cultures were used in the preparation of grain-sawdust spawn (Section 3.3.2.1.2).

### 4.3.2 Lignocellulosic material

The wood sources used were Maranti (*Shorea* spp.), Port Jackson (*A.longifolia*) and Black Wattle (*A.mearnsii*). Maranti and Port Jackson were obtained from sources previously described (Section 3.3.2.2) and Black Wattle was supplied by Professor R. Kirby of Hermitage Farm, Grahamstown, South Africa. The lignocellulosic component of the substrates was comprised of woodshavings (< 5 mm<sup>2</sup>) which had been soaked for two days.

### 4.3.3 Substrate preparation

Two different substrate formulations were tested with regard to their fruitbody production potential: medium adapted from Rinker, 1991 (Appendix B; 2.4) and that of R.Kirby (pers.

comm.) (Appendix B; 2.5). Unless otherwise specified, the latter substrate formulation was used. The substrate ingredients were combined, thoroughly mixed and dry substrate weights determined by drying at 80°C overnight. Either tap or distilled water was added to ensure a substrate dry weight:water ratio of 1:1.2. The substrates were placed in one of the following autoclaveable containers: wide-mouthed glass jars (Consol), polyvinylchloride tubes (PVC supplied by N.T.C, Port Elizabeth), nylon polyester bags (NP provided by Multifoil, Johannesburg) and polypropylene bags (PP provided by Mr J. Davies of the Ichthyology Department, Rhodes University, Grahamstown, South Africa). The containers used all had a diameter of 7 to 8 cm and a length of either 10 or 15 cm. The containers were sealed to prevent contamination and a PVC neck, stoppered with cotton wool, was used to provide aeration to the growing fungus.

All substrates were sterilized at 121°C for 120 minutes and spawned with a 10% inoculum (wet weight basis). The inoculated substrates were thoroughly mixed and, the bagged substrates, modelled into log forms.

#### **4.3.4 Spawn run conditions**

The inoculated substrates were incubated in the dark at 25°C at a relative humidity of between 60% and 80%. Uninoculated culture vessels were also placed under the above conditions and examined on a daily basis. All experiments were performed in triplicate thus the results shown are the mean values from three parallels.

Once the mycelia had completely colonized the substrates (after approximately 3 weeks), the containers were partially opened and the substrates soaked at 4°C for 48 hours. The substrates were then placed under production conditions.

#### **4.3.5 Fruiting conditions**

The partially exposed substrates were incubated at 17°C and at a relative humidity between 80% and 100%. A photoperiod of 12 hours (35 lux) was provided daily by automatically controlled overhead fluorescent lights.

The water balance was maintained in the substrate blocks by irrigating twice daily. After the harvesting of each flush, or every three weeks, substrate blocks were soaked at 4°C for 48 hours. Cultivation studies were conducted over a 100 or 200 day cropping period.

#### **4.3.6 Analytical methods**

##### **4.3.6.1 Growth assessments**

The ability of the various fungal strains to degrade and thus utilize the different wood sources was ascertained by determining the loss in organic matter of the substrate over a period of time. Dry weight measurements were used for this purpose.

Small amounts of substrate (2.6 g) (Appendix B; 2.5) were placed in McCartney bottles and autoclaved for 30 minutes. The cooled substrates were inoculated and placed under vegetative growth conditions as previously described (Section 4.3.4). Destructive sampling was employed with samples being analyzed every two days for an experimental period of six days. Sample dry weights were determined by drying at 160°C until constant weights were obtained (approximately 10 hours), and substrate utilization was determined as previously described (Section 3.3.3.3).

##### **4.3.6.2 Harvesting, Biological Efficiency and Basidiome size**

Mushrooms were harvested (immediately after the cap had opened and the margin had become flat), counted and weighted. The data obtained was used in the calculation of biological efficiency and basidiome size.

The term biological efficiency is used to describe the conversion efficiency from substrate to mushroom product (Royse and Zaki, 1991), and is determined by the following equation:

$$\%BE = M_{FM} / M_s \times 100 \quad (4.1)$$

%BE = percentage biological efficiency

$M_{FM}$  = fresh weight of mushrooms harvested (g)

$M_s$  = dry substrate weight (g)

Basidiome size defines the average weight of mushrooms harvested (Royse and Zaki, 1991), and is calculated from the following equation:

$$BS=M/N \quad (4.2)$$

BS=Basidiome size (g)

M=Total weight of mushrooms harvested in the cropping period (g)

N=Total number of mushrooms harvested in the cropping period

#### 4.3.6.3 Statistical analysis

Treatment effects were quantified statistically where possible (i.e. when results were obtained from more than one substrate block) using one-way Analysis of Variance (ANOVA) and the Tukey-Kramer Multiple Comparisons Test. The significance of treatment conditions were confirmed at the 5% level of error.

The coefficient of variation (CV) was determined to ascertain the variability within treatment means. This value expresses the sample standard deviation as a percentage of the sample mean.

## 4.4 RESULTS

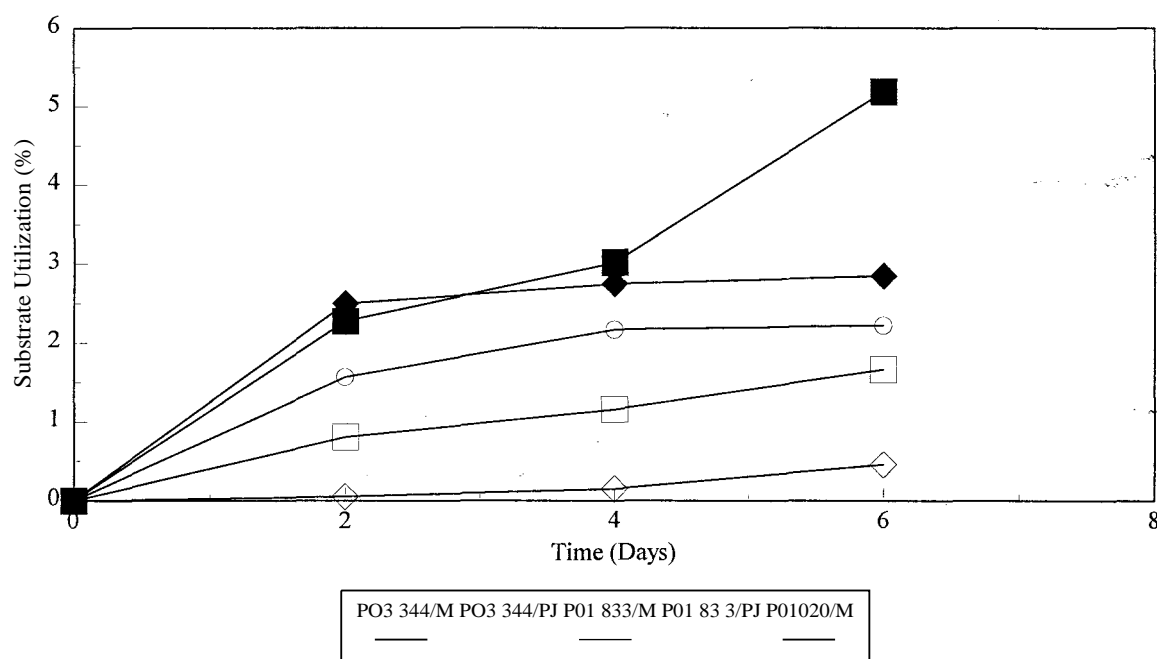
### 4.4.1 Growth studies

The results presented in Fig. 4.4.1 and Table 4.4.1 indicate the differing abilities of *P.ostreatus* strains to utilize two different wood sources. *P.ostreatus* strains do not differ significantly in their abilities to utilize a certain wood source; however, they do vary in their utilization of different wood sources. From this preliminary investigation it was decided to continue the remainder of the growth studies using *P.ostreatus* 3344, since this strain showed the greatest ability to utilize Port Jackson.

**Table 4.4.1** The percentage utilization of different wood sources' by three strains of *P.ostreatus* following a six day spawn run time.

Strain	Port Jackson	Maranti
<i>P.ostreatus</i> 3344	5.28±1.39 [A] <sup>h</sup>	1.74±0.06 [B]
<i>P.ostreatus</i> 1833	2.83±0.94 [AB]	0.46±0.43 [B]
<i>P.ostreatus</i> 1020	N.a	2.19±0.4 [B]

<sup>a</sup>  
<sup>h</sup> The substrate used was sawdust based and contained no additives. Entries show the mean±standard deviation of three replicates. Values, and therefore treatments, followed by the same letter, in the square brackets, are not considered significantly different at P=0.05. N.D = not determined.



**Fig.4.4.1** The utilization of Maranti and Port Jackson by three strains of *P. ostreatus*.

If one compares the growth results for *P.ostreatus* 3344 presented in Tables 4.4.1 and 4.4.2, it is evident that media supplementation significantly affects the ability of this species, and one can assume others, to utilize lignocellulosic materials. The use of supplements increased Port Jackson and Maranti utilization by factors of 2.5 and 10, respectively.

**Table 4.4.2** Percentage substrate utilization by various *Pleurotus* species after a six day spawn run time.

Species	Maranti	Port Jackson	Black Wattle
<i>P.citrinopileatus</i> 36239	20.5716.20 [AB]{a} <sup>h</sup>	9.5511.56 [A]{b}	8.1610.68 [A]{D}
<i>P.columbinus</i> 36215	23.8110.61 [A]{a}	8.1110.87 [A]{b}	0.0010.00 [C]{c}
<i>P.eryngii</i> 36037	14.0814.38 [B]{a}	7.4811.60 [AB]lab}	6.0011.07 [13]{b}
<i>P.fuscus</i> var. <i>ferulae</i> Lanzi 36214	17.7412.43 [AB]{a}	4.0310.72 [BC]{b}	0.0010.00 [C]{c}
<i>P.ostreatus</i> 3344	17.1711.60 [AM]{a}	13.3512.14 [D]{a}	7.7710.87 [AB]{b}
<i>Pleurotus spp.</i> 36031	16.0410.65 [AB]{a}	2.9110.78 IC]{b}	1.8211.04 [C]{b}

<sup>a</sup> The substrate used was prepared as detailed in Appendix B, Section 2.5.

<sup>h</sup> Entries show the mean±standard deviation of three replicas. Treatment means followed by the same letter in the same column (square brackets), and same row (curvy brackets) are not considered significantly different at P=0.05.

Interspecies-specific variability in substrate utilization by *Pleurotus* species was evident (Table 4.4.2 and Fig. 4.4.2). The variability was greater between, than within, wood sources. - All *Pleurotus* species investigated showed an identical trend in substrate utilization: Maranti was utilized more efficiently than Port Jackson which was degraded, as indicated by its decrease in dry matter content, more rapidly than Black Wattle. The utilization of Black Wattle and Port Jackson by individual species was often comparable, which is understandable since they are both *Acacia* species. *P.columbinus* and *P.fuscus* var. *ferulae* Lanzi, however, did not utilize Black Wattle at all (Table 4.4.2) which implies an inability to grow on this wood source. Since *Pleurotus* species utilized Black Wattle less efficiently than the other wood sources investigated, it was not tested as a substrate for mushroom cultivation.

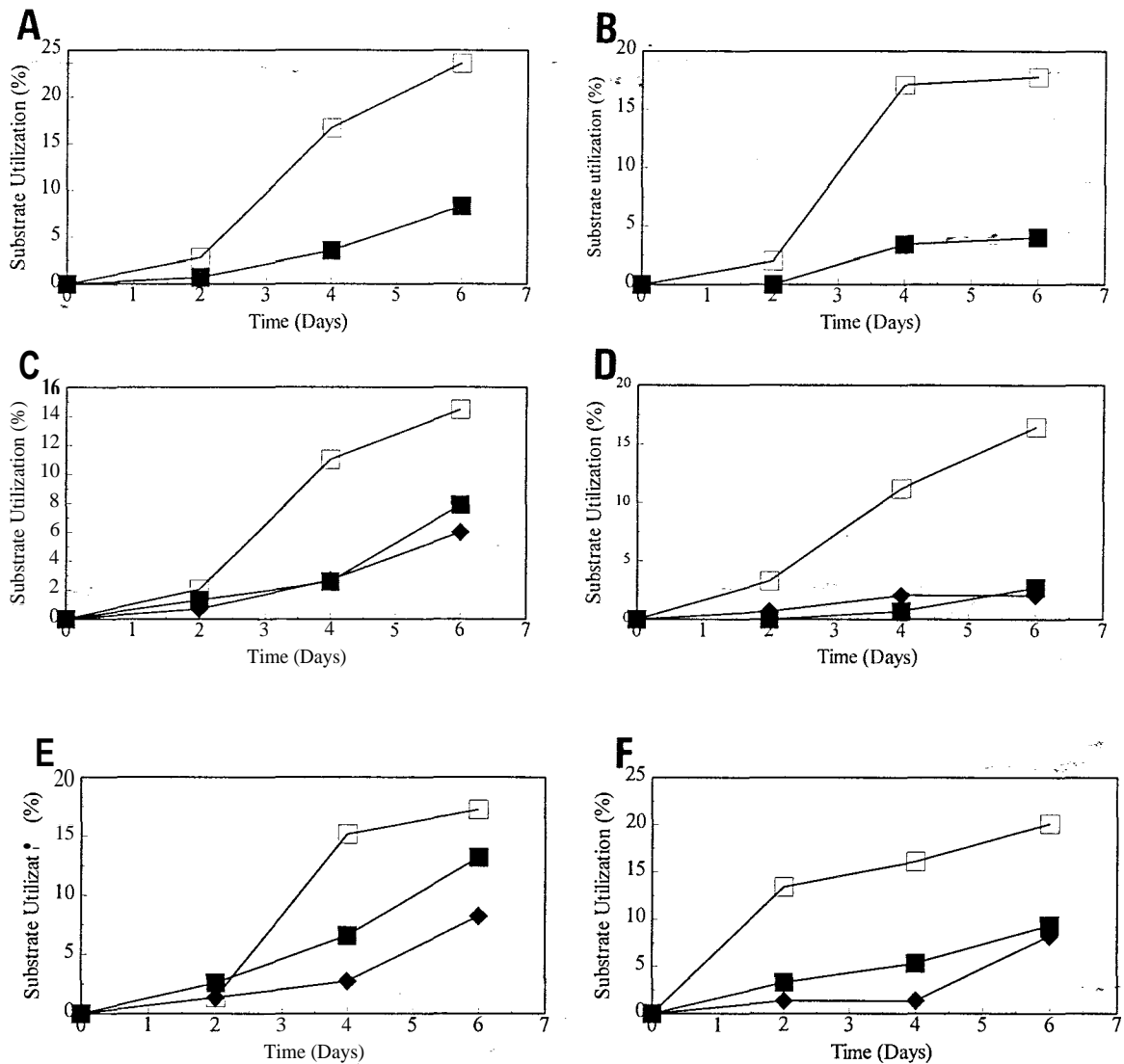


Fig. 4.4.2 The utilization of three sawdust-based substrates (□ Maranti, ■ Port Jackson and • Black Wattle) by *P. columbinus* 36215 (A), *P. fuscus* var. *ferulae* 36214 (B), *P. eryngii* 36037 (C), *Pleurotus* spp. 36031 (D), *P. ostreatus* 3344 (E) and *P. citrinopileatus* (F). 36239.

It is therefore important to consider a number of different species to determine which utilizes the desired wood source most efficiently. Of the species tested *P. columbinus*, *P. ostreatus* and *P. citrinopileatus* showed the most efficient utilization of Maranti, Port Jackson and Black Wattle, respectively. From these preliminary results it appears that *P. ostreatus* 3344 would be the most suitable oyster mushroom species for the utilization of invading *Acacia* species.

#### 4.4.2 Production studies

The different stages involved in the development of fruiting bodies are shown in Fig. 4.4.3. Bag cultivation is used to depict this since substrate visibility is higher.

The production characteristics of *P.ostreatus* 3344 on Port Jackson based media, using different container vessels, is indicated and graphically represented in Table-44.3 and Fig. 4.4.4, respectively. Photographic images of mushroom production using the different cultivation vessels are shown in Fig. 4.4.5.

**Table 4.4.3** A comparison of different container vessels and the effects which these have on biological efficiency, basidiome size, production timea and the amount of substrate moisture" lost during *P.ostreatus* 3344 cultivation.

Container	Production Time (Days)	BE (%)	BS (g)	Moisture Loss (%)
Glass	161±3 [A]	77.0±9.1 [A]	3.26±1.09 [A]	6.89±3.20 [A]
PVC	196±44 [A]	37.7±13.1 [B]	1.39±0.21 [A]	12.61±2.97 [A]
PP	244±35 [A]	37.4±3.2 [B]	1.20±0.42 [A]	8.89±3.55 [A]
NP				70.61±4.50 [B]

<sup>a</sup> The production time is the period from inoculation through to the harvesting of 7 flushes. The moisture lost from uninoculated containers when incubated under spawn run conditions for a period of 28 days. Entries show the mean±standard deviation of three replicates. Treatment means followed by the same letter, in the same column, are not considered statistically different at P=0.05.

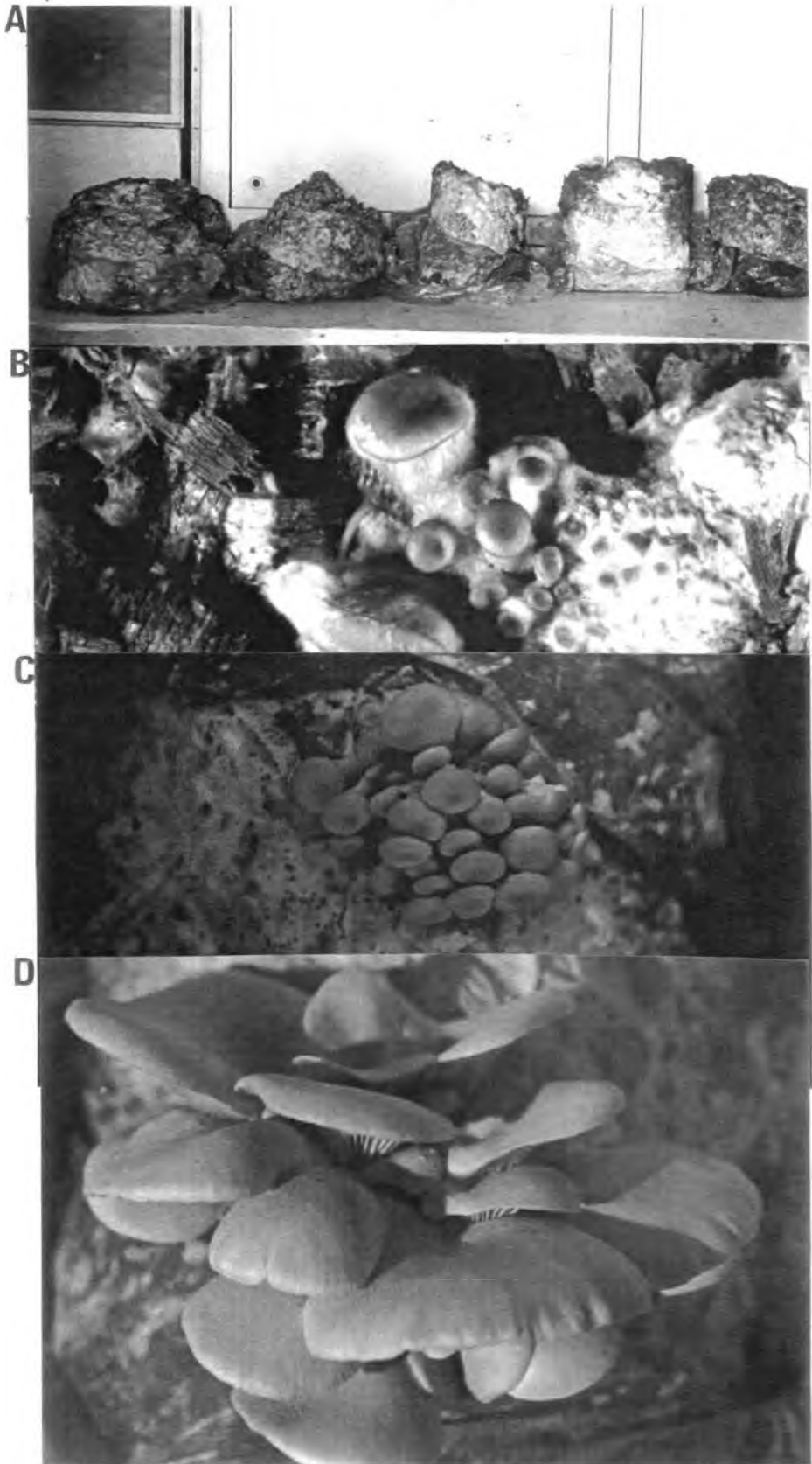


Fig. 4.4.3 Stages in o., stir mushroom cultivation: (A) vegetative growth; (B) primordia development; (C) immature mushrooms; (D) mature mushrooms.

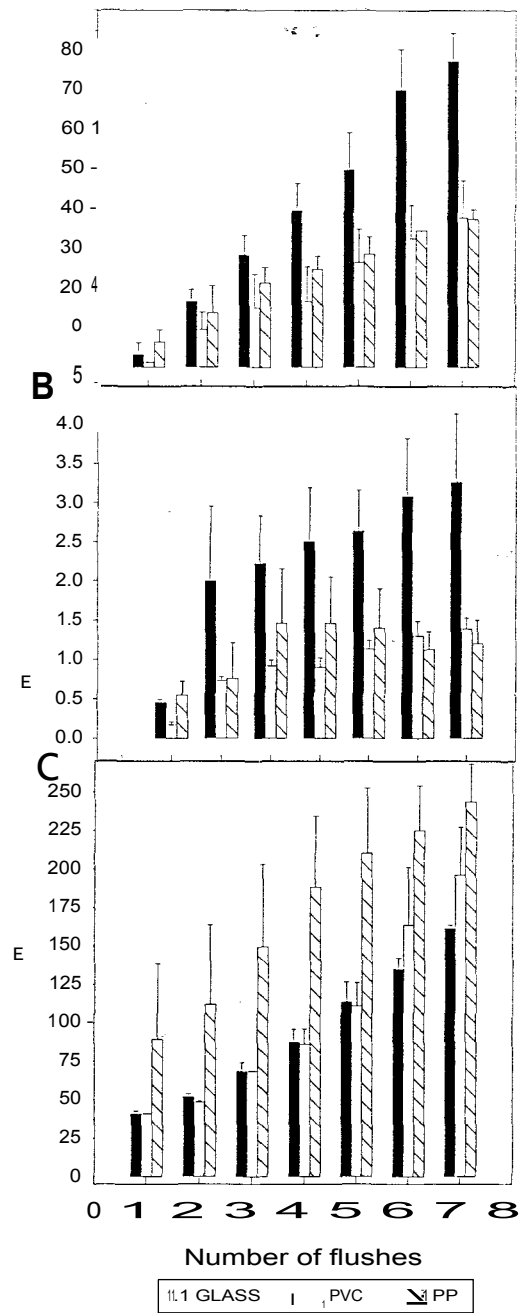


Fig. 4.4.4

The effect which different containers have on the biological efficiency (A), basidiome size (B) and production time (C) of *P.ostreatus* 3344 during cultivation on Port Jackson medium.



**Fig. 4.4.5** *P.ostreatus* primordia and mushroom production on Port Jackson medium using different cultivation vessels: (A) Glass; (B) PVC tubes and (C) PP bags.

From the results presented, it is evident that the type of container used in the cultivation of edible mushrooms affects the cultivation time, yield and quality of mushrooms harvested. A relationship exists between the amount of substrate, moisture lost and the yield and average weight of mushrooms produced. Attempts to cultivate *P.ostreatus* in nylon polyester bags proved to be unsuccessful. It can be postulated that one of the main reasons for this is the fact that this container medium allowed the loss of significantly more moisture from the substrate block than any of the other containers tested (Table 4.4.3).

The results obtained indicate that glass is the most favourable container, of those investigated, for oyster mushroom production. The cultivation of *P.ostreatus* 3344 in glass vessels resulted in higher yields, larger mushrooms and lower production times than when the other container media were used. Glass, however, is more expensive and has reduced versatility compared to the other container media investigated. PP bags are the most commonly used cultivation vessels during edible mushroom cultivation. PVC, however, is widely available and has comparable effects to PP on the production criteria examined. In an attempt to develop an alternative method for lignocellulolytic mushroom cultivation, PVC tubes were used in the remainder of the production experiments.

Both Maranti and Port Jackson based substrates are suitable for the cultivation of numerous *Pleurotus* species. Cultivation of these species on Port Jackson based medium, however, generally resulted in the production of higher yields of mushrooms (Table 4.4.4 and Fig. 4.4.6).

**Table 4.4.4** The number of flushes harvested and cumulative biological efficiency (BE) of various *Pleurotus* strains on Port Jackson and Maranti based medium, using a 100 day cultivation period.

Strain'	Port Jackson		Maranti	
	BE (%) <sup>b</sup>	Flushes	BE (%)	Flushes
<i>P.ostreatus</i> 3344	60.7±8.9 (A) [14.7%]	4	0	
<i>P.ostreatus</i> 1833	51.0±4.4 (ACB) [8.7%]	3	21.9±11.1 (C) [50.6%]	1
<i>P.ostreatus</i> 1020	20.0±5.8 (B) [29.1%]	2	6.6	1
<i>P.columbinus</i> 36215	27.6±12.5 (ACB) [45.2%]	2	30.3	3
<i>P.eryngii</i> 36037	27.6±5.3 (ACB) [19.1%]	3	17.3	3
<i>Pleurotus spp.</i> 36031	25.7	2	26.42	2
<i>P.calypttratus</i> 36211	38.9±5.7 (ACB) [14.69%]	1	0	
<i>P.pulmonarius</i> 36095	54.3±5.1 (AC) [9.5%]	4	26.8±19.7 (C) [73.5%]	1
<i>P.citrinopileatus</i> 36239	20.7±10.1 (B) [48.6%]	1	0	
<i>P.fuscus</i> 36214	0		0	
<i>P.cystididosus</i> 36170	0		0	
<i>P.cystididosus</i> 36131	0		0	
<i>P.cystididosus</i> 36168	0		0	
<i>P.cystididosus</i> 36172	0		0	
<i>F. velutipes</i>	0		0	

<sup>a</sup> The origins of the various strains are depicted in Table 2.3.1.

<sup>h</sup> Entries indicate the mean±standard deviation of replicate experiments where more than one substrate block per treatment was productive. Entries followed by the same letter, in the curved brackets, are not considered significantly different at P=0.05.

The values in the square brackets represent the CVs obtained within treatment means.

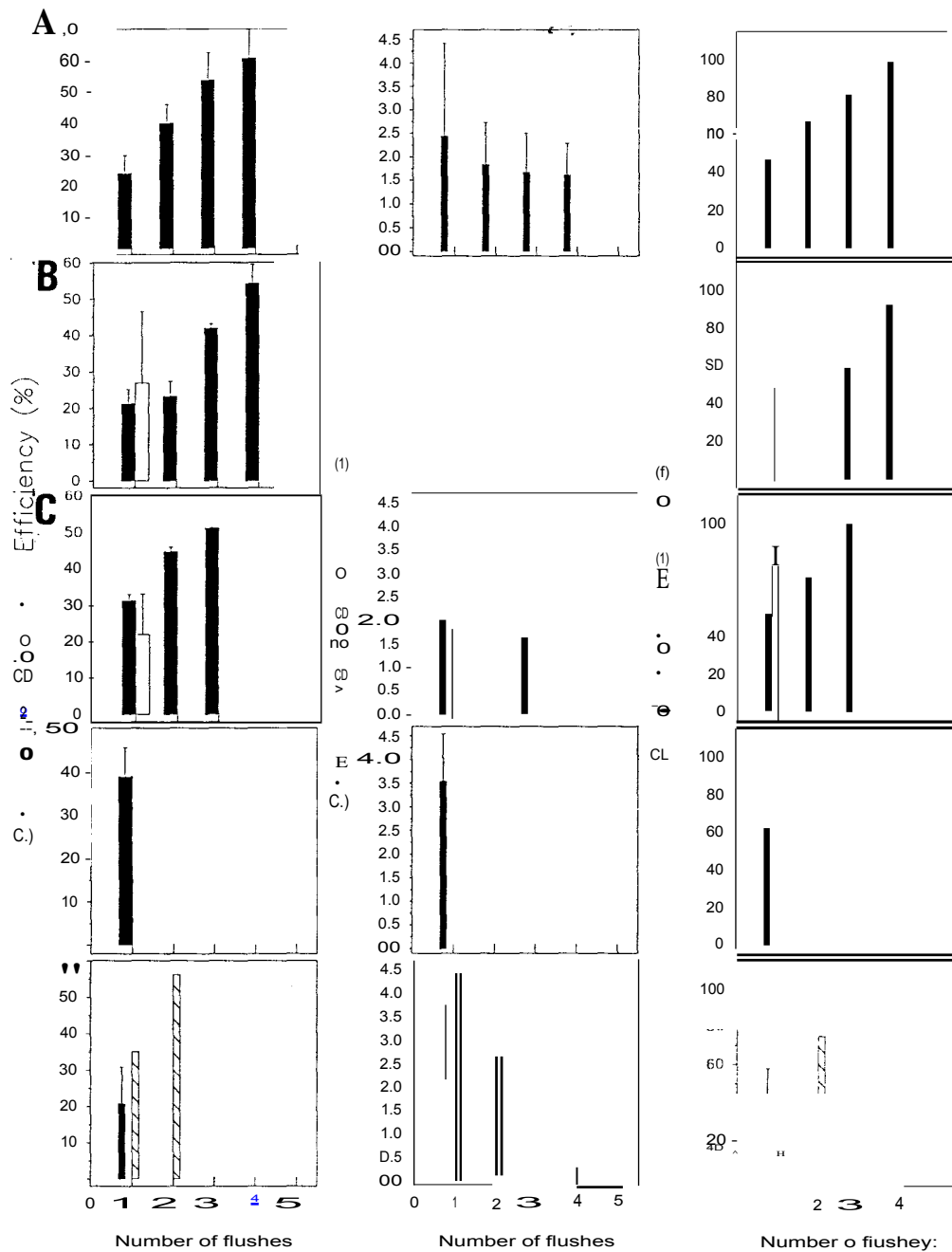


Fig. 4.4.6 Production characteristics of various *Pleurotus* strains on Port Jackson (E) and Maranti (O). Situations where only one substrate block was productive or showed noticeably better production characteristics than other blocks, are indicated by (D) on Port Jackson media and (O) on Maranti substrate (A) *P.ostreatus* 3344; (B) *P.pulmonarius* 36095; (C) *P.ostreatus* 1833; (D) *P.calypratus* 36211; (E) *P.citrinopileatus* 36239.

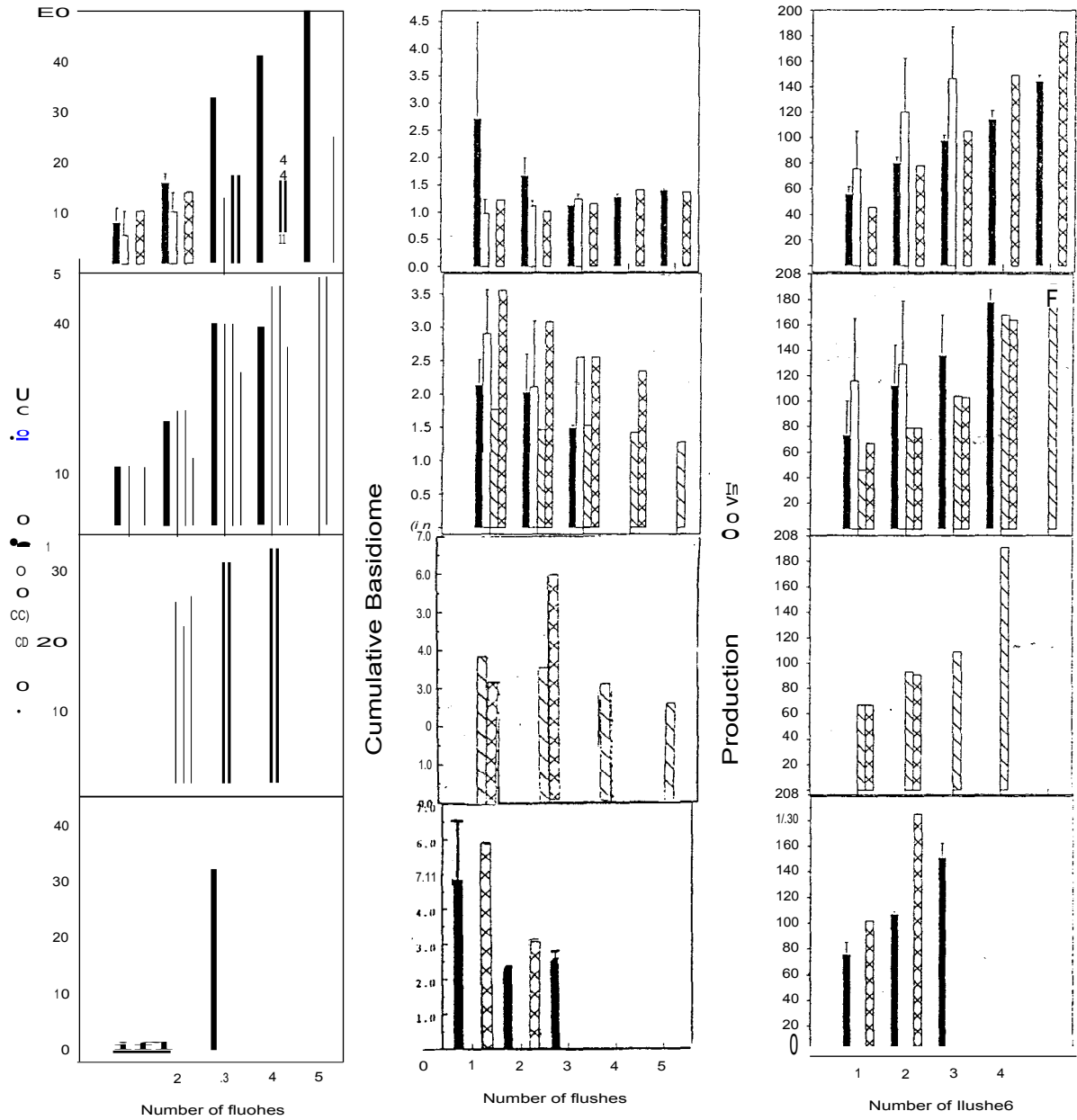


Fig.4.4.6 Cont. (F) *P. eryngii* 36037; (G) *P. columbinus* 36215; (H) *Pleurotus* spp. 36031; (I) *P. ostreatus* 1020.

As is evident from Table 4.4.4 and Fig. 4.4.6, intra- and interspecies specific variation in the production of oyster mushrooms occurred on Maranti and Port Jackson based media, often with treatment means being statistically different.' Only six of the fourteen *Pleurotus* strains used in this study produced mushrooms on Maranti medium, whereas nine efficiently converted Port Jackson substrate with high resultant yields of mushrooms. Three of the species tested, *P.ostreatus* strains 3344 and 1833 and *P.pulmonarius* 3605, showed high conversion efficiencies of Port Jackson substrate, which were often significantly greater than those exhibited by other species on the same substrate. Although the yields obtained on Port Jackson were generally higher than those on Maranti; in certain species the productivities on the two media were comparable (*P.columbinus* 36215 and *Pleurotus spp.* 36031). A number of species which showed high conversion efficiencies of Port Jackson, did not produce mushrooms on Maranti based medium (*P.ostreatus* 3344, *P.calypttratus* 36211, *P. citrinopileatus* 36239). In attempting to determine the effect which prolonged cultivation has on yield, certain low yielding mushroom strains( 1020, 36215, 36037 and 36031) were cultivated for an additional 100 day period. Although the yields were increased substantially, these strains still produced lower yields than the high producing strains previously mentioned.

Growth experiments indicated (Table 4.4.2) higher Maranti than Port Jackson media utilization, which does not correlate with the production studies conducted (Table 4.4.4). Furthermore, although all strains had shown an ability to utilize Maranti based substrates, not all produced mushrooms on this substrate, e.g. *P.ostreatus* 3344, *P.citrinopileatus* 36239 and *P.fuscus* var. *ferulae* 36214. A relationship was, however, found between the strains showing most efficient Port Jackson and Maranti utilization and those producing the highest yields on these substrates (*P.ostreatus* 3344 and *P.columbinus* 36215, respectively). Information obtained from dry weight measurements has therefore got limited application in determining species specific production potential on different substrates.

Certain of the strains employed in this study namely *F. velutipes*, *P.fuscus* var. *ferulae* Lanzi 36214 and all of the *P.cystididosus* strains, were not successfully cultivated on either of the wood sources tested. *P.fuscus* var. *ferulae* Lanzi completely colonized both Port Jackson and Maranti-based substrates after a period of three weeks; however, no mushrooms were produced under fructification conditions. *F. velutipes* and *P.cystididosus* strains were not

aggressive colonizers of either the spawn or production media utilized. Since these observations were consistent between experiments, it can be concluded that these mushroom strains are not suitable for cultivation under the nutritional and environmental conditions investigated.

The spawn run time for all fungal species investigated was approximately 13, three weeks. Variations in the interval between substrate inoculation and the harvesting of the first crop of mushrooms was, however, evident between strains (refer to Fig. 4.4.6). Substrates colonized with *P. pulmonarius* 36095 produced mushrooms within 22 days, whereas other high yielding strains generally produced mushrooms within 40 to 60 days. Certain strains, usually those associated with low yields, only produced fruiting bodies 70 to 80 days after inoculation. The yields produced in the first crop were high and provided an indication of the productivity of the cultivation systems being investigated.

Correlations were evident between fruit body production potential, number of flushes harvested and the variability within treatments (indicated by the CV value). In the development of economically feasible cultivation methodologies, it is desirable to have as low a variability in productivity as possible. This study has shown that high yielding strain/substrate combinations are generally associated with low CVs and a large number of flushes, whereas the converse is true of low yield potential combinations (Table 4.4.4 and Fig. 4.4.6).

A general trend among the majority of low yielding production substrates was unreliability in the number of productive substrate blocks (indicated in Fig. 4.4.6). In certain instances substrate blocks inoculated with identical strains would show highly variable production characteristics: only one substrate block would be productive or produce significantly higher yields or number of flushes than other inoculated substrates.

The growth habits and macroscopic characteristics of strains used in this study are depicted in Fig. 4.4.7 and 4.4.8, respectively.

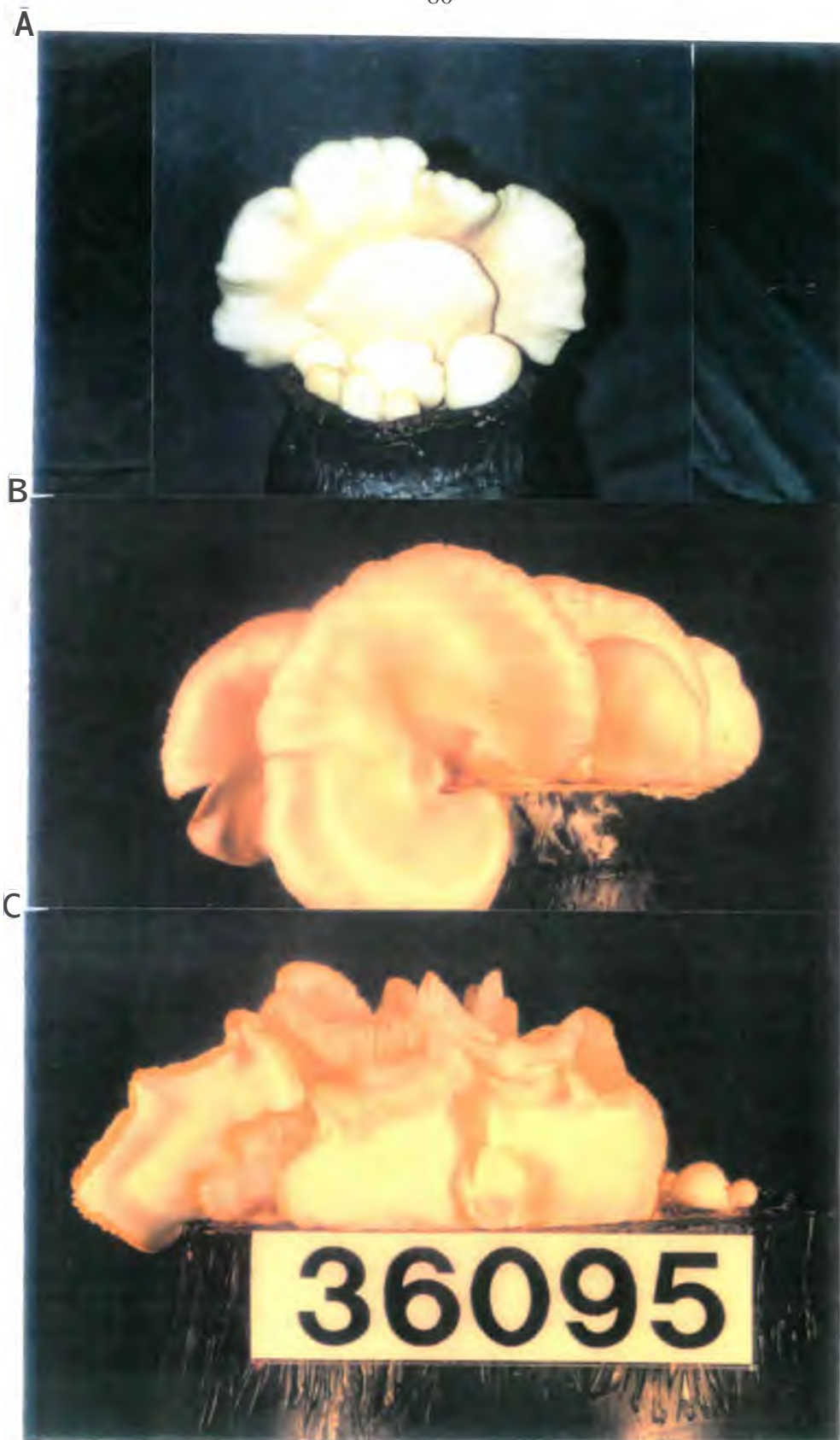
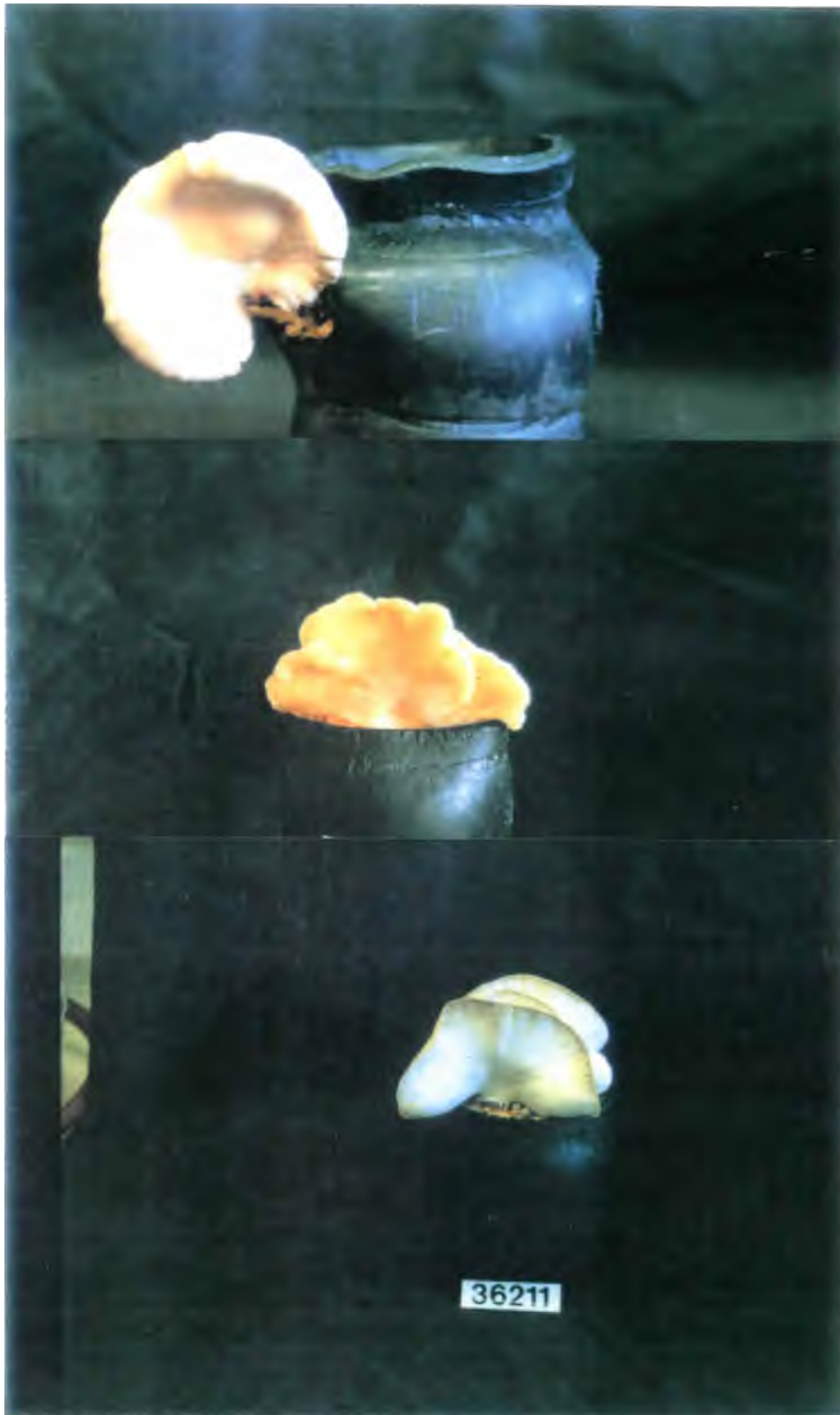


Fig. 4.4.7 Growth habits of various *Pleurotus* species: (A) *P.ostreatus* 3344, (B) *P.ostreatus* 1833; (C) *P.pulmonarius* 36095.



**Fig. 4.4.7** Cent. (D) *P.eryngii* 36037, (E) *P.calyptratus* 36211 and (F) *P.citrinopileatus* 36239.

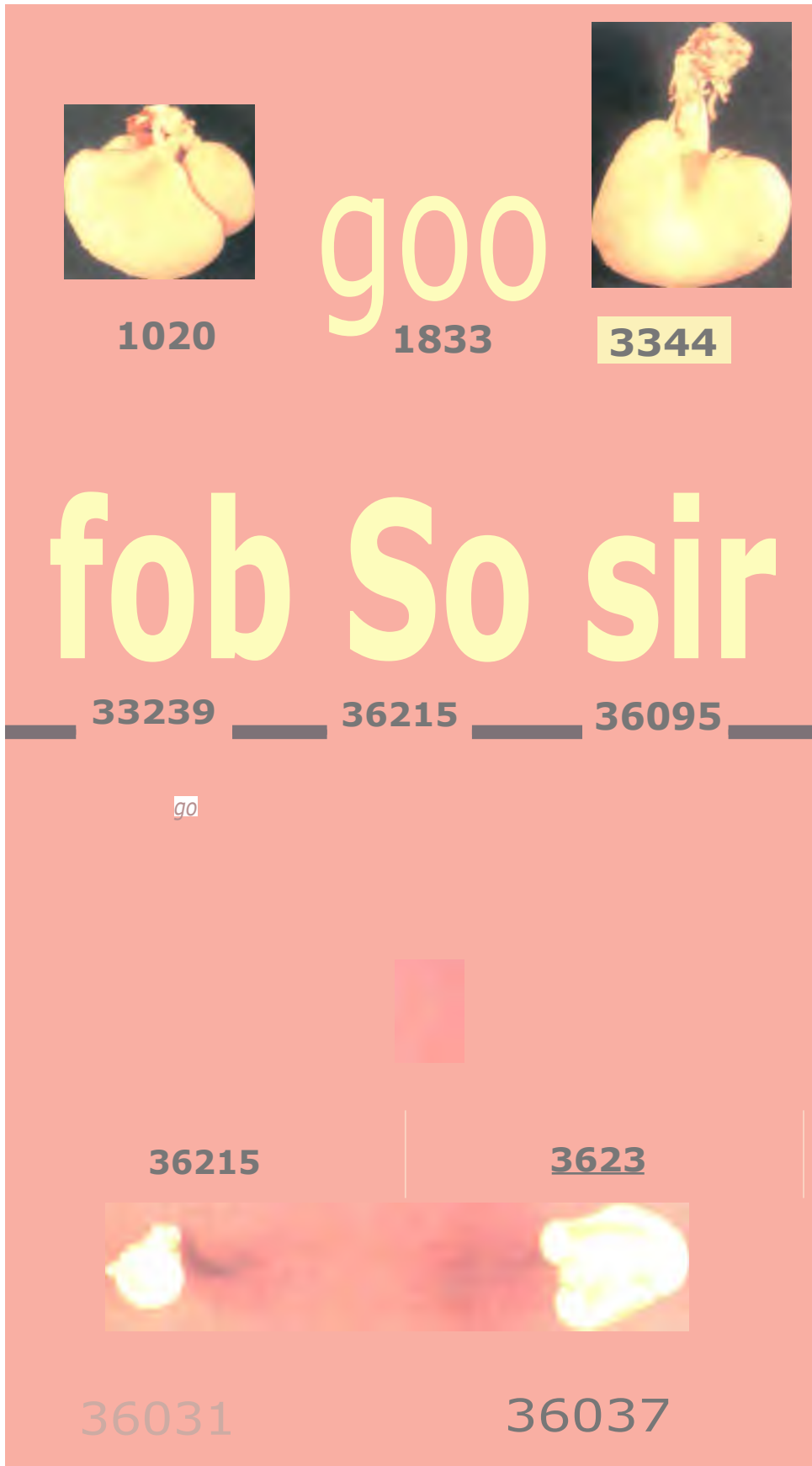


Fig. 4.4.8 Morphological characteristics of *Pleurotus* fruiting bodies.

The majority of strains had caespitose or gregarious modes of growth, with the exception of *Pleurotus spp.* 36031 which grew solitarily and had a significantly longer stem than the other strains. Morphologically all strains were similar and produced white/cream fruiting bodies.

Fungal and substrate-associated factors, i.e. the strain and wood source used for cultivation, affected the size and number of mushrooms produced, evident in the values for basidiome size (BS) presented in Table 4.4.5 and represented in Fig. 4.4.6. The results obtained were, however, not significantly different between treatment means.

Table 4.4.5 The effect of fungal and substrate-associated factors on cumulative basidiome size"

Strain	Port Jackson	Maranti
<i>P.ostreatus</i> 3344	1.61±0.7 [42.2%]	0
<i>P.ostreatus</i> 1833	1.62±0.2 [10.2%]	1.80±0.8 [42.4%]
<i>P.ostreatus</i> 1020	2.38±0.02 [0.6%]	6.07
<i>P. columbinus</i> 36215	2.02±0.5 [24.8%]	2.55
<i>P. eryngii</i> 36037	1.12±0.02% [1.8%]	1.15
<i>Pleurotus spp.</i> 36031	3.57	6.12
<i>P.calyptratus</i> 36211	3.54±1.0 [28.0%]	0
<i>P. citrinopileatus</i> 36239	2.13±1.6 [76.9%]	0

As for Table 4.4.4. *P.pulmonarius* 36095 has a highly gregarious growth habit which makes it difficult to determine its basidiome size.

No significant differences in the yield of oyster mushrooms was evident when *P. ostreatus* 3344 and *P.columbinus* 36215 were cultivated on different Port Jackson based substrate formulations (Table 4.4.6). The cumulative basidiome size of *P.columbinus* was significantly

greater on the medium adapted from Rinker (1991) than on the other medium tested (Table 4.4.6 and Fig. 4.4.9). The number of flushes harvested, however, was lower on the former than on the latter medium investigated.

Table 4.4.6 The effect of substrate formulation on biological efficiency and basidiome size of various *Pleurotus* species.

- Strain	BE (%) <sup>a</sup>	BS (g) <sup>a</sup>	BE (%) <sup>b</sup>	BS (g) <sup>b</sup>
<i>P.ostreatus</i> 3344	60.7±10.9	1.61±0.83	33.4±18.5	0.87±0.37
<i>P.columbinus</i> 36215	27.6±17.6	2.02±0.71	29.9	9.24

Kirby (pers. comm.)

<sup>h</sup>

Rinker (1991).

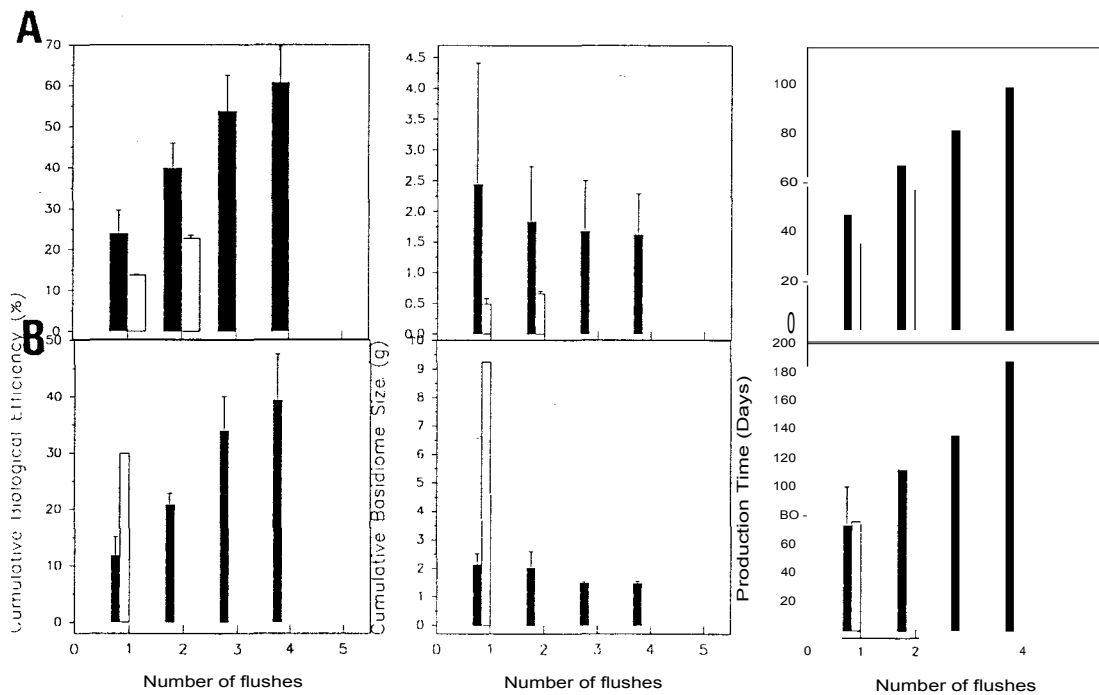


Fig.4.4.9 Different substrate formulations [a (■); b (□)] and their effect upon the production characteristics of *P.ostreatus* 3344 (A) and *P.columbinus* 36215 (B).

Comparable yields, numbers and masses of mushrooms (as indicated by the BE and BS values presented in Table 4.4.7) were obtained when incorporating either tap or distilled water in Port Jackson-based substrates. It can therefore be postulated that locally available water is suitable for the cultivation of oyster mushrooms without prior treatment.

Table 4.4.7 Differences in the cumulative biological efficiency and basidiome size of various *Pleurotus* species with the addition of distilled or tap water to substrate components.

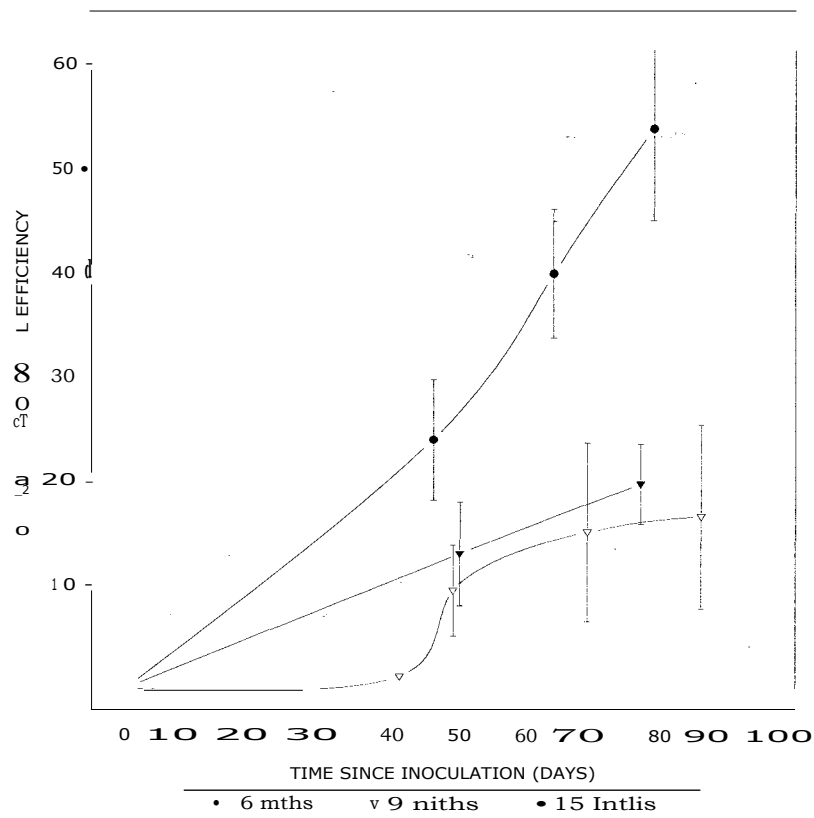
Strain	BE (%) <sup>a</sup>	BS (g) <sup>a</sup>	BE (%) <sup>b</sup>	BS (g) <sup>b</sup>
<i>P.ostreatus</i> 3344	60.7±10.9	1.61±0.83	55.1±21.5	1.36±0.18
<i>P.ostreatus</i> 1833	51.0±5.4	1.62±0.20	44.3±9.3	1.87±0.13
<i>P. calypratus</i> 36211	30.0±16.4	3.54±1.22	24.6±22.6	3.38±2.28
<i>P.pulmonarius</i> 36095	54.3±7.3	2.07±0.58	46.4±11.1	2.59±0.62
<i>P. citrinopileatus</i> 36239	27.8±24.5	1.54±1.00	66.7±2.6	2.41±0.57
<sup>a</sup>	Distilled Water			
<sup>b</sup>	Tap Water			

The age of the spawn which is used to inoculate substrates has been found to have a significant effect upon the yields produced (Table 4.4.8 and Fig. 4.4.10). The use of young spawn (6 months old) results in the production of significantly higher yields than old spawn (9 and 15 months old). The yields produced from substrates inoculated with 9 and 15 month old spawn did not vary significantly. Furthermore, the production trends varied between substrates inoculated with the different aged spawns. Substrates inoculated with the young spawn showed a consistently high accumulation of mushrooms, whereas those inoculated with the older spawns showed a gradual accumulation of mushrooms (Fig. 4.4.10).

**Table 4.4.8** The effect of spawn age on cumulative biological efficiency and basidiome size during *P.ostreatus* 3344 cultivation on Port Jackson medium.

Spawn Age (months)	BE (%) <sup>a</sup>	BS (g)
6	60.7±10.9 [A]	1.61±0.83 [A]
9	16.6±12.5 [B]	0.90±0.17 [A]
15	19.7±4.5 [B]	2.97±1.14 [A]

<sup>a</sup> Entries show the mean±standard deviation of three replicates. Values in columns followed by the same letter, are not considered significantly different at P=0.05.



**Fig. 4.4.10** The effect of spawn age on the conversion efficiency of Port Jackson medium by *P.ostreatus* 3344.

#### 4.5 DISCUSSION

The main objective of the study presented was essentially to cultivate oyster and winter mushrooms on locally available wood sources under environmental conditions which are conducive to high productivity. Although oyster mushrooms were successfully produced, winter mushrooms were not harvested under any of the conditions investigated. Based on the

research presented, it can be postulated that the reason for this was probably the strain or substrate formulation used. Although the strain used produced mushrooms successfully on grain-based substrates (results not shown), no mushrooms were harvested from sawdust-based substrates. This implies the importance of grain as a substrate component in winter mushroom cultivation. Grain based substrates, with the exclusion of sawdust, have been successfully used in the cultivation of certain mushroom species (Hirornoto, 1991). *F.velatipes* could therefore be cultivated in South Africa on grain-based substrates, but not on locally available wood sources.

The cultivation process used for the production of oyster mushrooms affects its efficiency and hence economic viability. This study has focused on the physical parameters affecting production, since the environmental requirements of *Pleurotus* species have been widely documented (Zadrazil, 1978; Patrick *et al.*, 1983; Rajarathnam and Bano, 1987; Kamra and Zadrazil, 1986; Rajarathnam and Bano, 1992; Hauser *Pleurotus* Documentation 6.84, Pers.. comm.). Factors affecting oyster mushroom production include the wood source, substrate formulation, strain, spawn age and container vessel used.

Mycelial growth and productivity studies indicated intra- and interspecies specific differences in the utilization of various substrates, illustrating the dependence of yield, quality and crop production time on the strain and substrate used. This is consistent with previous reports (Chu-Chou, 1984; Diehle and Royse, 1986; Royse and Bahler, 1986; Raj arathnam and Bano, 1989; Przybylowicz and Donoghue, 1990).

Vegetative growth determinations, based on dry weight measurements, can be used to determine the appropriateness of the substrate/strain combination used. They do not, however, illustrate the potential productivity of the components being used. Although Maranti based substrates promoted efficient mycelial growth of various *Pleurotus* species (Table 4.4.2); they did not support high yields of oyster mushrooms (Table 4.4.4). The use of wood which has been used in certain industries, e.g. Maranti, for the cultivation of edible mushrooms usually causes problems. This is because chemicals are often incorporated which prevent the degradation of the wood by fungi (Moore-Landecker, 1982). The efficient utilization of the Maranti based substrate, indicated in Table 4.4.2 and Fig. 4.4.2, is probably due to the

utilization of easily available carbon sources e.g. sucrose, rather than to Maranti sawdust utilization. This view is supported by the fact that growth on Maranti sawdust without the addition of various supplements was low (Table 4.4.1 and Fig. 4.4.1).

This study has indicated that of the locally available wood sources tested, Port Jackson is the most applicable to oyster mushroom production. The different substrate degradative potentials can possibly be caused by the presence of toxic or inhibitory compounds, differences in substrate C:N ratios or dissimilar lignin structures (Rajarithnam and Bano, 1989). Port Jackson supported the production of mushrooms by various *Pleurotus* species. The yields produced were generally higher than when Maranti was used as a substrate component and the cultivation process was less variable, as indicated by the lower CV values obtained (Table 4.4.4).

The use of different Port Jackson based substrate formulations does not affect the biological efficiency of the process significantly when cultivating *P.ostreatus* 3344 and *P.columbinus* 36215 (Table 4.4.6). It does, however, have an effect upon the basidiome size of the mushrooms produced by *P.columbinus*. From these findings it can be postulated that the wood source used is the substrate component affecting cultivation most significantly, which is understandable since it comprises 77% of the total substrate composition and therefore is the major source of nutrition to the growing fungus.

In the development of an economically feasible cultivation process, it is important to choose a strain adapted to a certain locale in terms of available wood sources and environmental conditions (Food from wastes, 1981). Although all strains investigated produced mushrooms on Port Jackson based medium, the efficiency with which this was accomplished varied (Table 4.4.4). Two *P.ostreatus* strains (1833 and 3344) and *P.pulmonarius* 36095 showed the highest productivity on this substrate. *P.ostreatus* 3344 is a 'Florida' strain which implies an ability to fruit at temperatures ranging from 18°C to 23°C (Patrick *et al.*, 1983), whereas *P.pulmonarius* has a wide fruiting temperature range (refer to Table 4.1.1). Both strains are therefore appropriate candidates for cultivation under South African conditions. *P.cystidioides* strains also have a wide fruiting temperature range and therefore would be

suitable for cultivation under local conditions. They were, however, not successfully cultivated under the conditions described.

The age of the spawn used has been found to be an important factor affecting mushroom cultivation and consequently its commercial viability. It was found that the older the spawn, the less productive the process (Table 4.4.7 and Fig. 4.4.10) which corresponds to previous findings (Tonomura, 1978). The reason for this can be ascribed to the following: a negative relationship exists between metabolic activity and the age of the mycelia (Tokimoto and Komatsu, 1978) and, spawn which has been stored for extended periods of time has lower colonization rates due to substrate nutrient depletion (Przybylowicz and Donoghue, 1990). It has been reported that both the spawn storage time and temperature have an effect upon the growth and productivity of *Pflabellatus* (Rajarithnam and Bano, 1987).

The strain and substrate used in the cultivation process not only has an effect upon yield, as indicated by the biological efficiency values, but also on the period of time before the first crop of mushrooms is harvested, the number of flushes harvested, the number and Weight of mushrooms produced (basidiome size) and the variability of the process (CV values) (Table 4.4.4 and Fig. 4.4.6). In general the higher the biological efficiency of the process, the more favourable its effect on these factors will be and therefore the greater its economic feasibility and applicability to small scale industries.

In agricultural communities the water used to increase substrate moisture content would be tap water since it would not be viable to use distilled water. The research conducted initially used distilled water so as to eliminate the possibility of variability associated with changing water conditions and the possible negative effects of chlorine ions. The finding that tap water can be used in the cultivation process with comparable results to those obtained with distilled water (Table 4.4.7), implies that the production process described can be applied to local, small-scale, rural farming enterprises without the pre-treatment of water being necessary. It has previously been observed that chlorinated water supplied by municipal systems generally has a low chlorine concentration which will not hamper the development of fungal mycelia (Przybylowicz and Donoghue, 1990).

Irrespective of the positive interaction between strain and substrate, if the container vessel used is inappropriate, the production of mushrooms will not occur. This study has indicated the importance of the container medium used in the cultivation of oyster mushrooms, which has been ascribed to its water retention capacity based on its evaporative potential. Container media showing high evaporative potentials have a consequent negative effect upon substrate productivity, e.g. nylon polyester bags (Table 4.4.3). Although glass was found to be the most favourable container medium of those investigated, PVC tubes promoted the production of comparable yields to PP bags, which are the most commonly utilized containers. Furthermore, PVC tubes are widely available to the majority of farming communities and the method of cultivation described in this study provides an alternative to conventional mushroom farming processes.

The effect of container medium on production further illustrates the importance of substrate moisture content on growth and productivity. Low substrate moisture contents did not support mushroom production, as observed in substrates incubated in nylon polyester bags. The maintenance of substrate moisture levels within the optimal range therefore necessitate the use of containers having low evaporative potentials and the sustainment of high environmental humidity levels. A short decrease in humidity below 80% had a major effect on the yield of mushrooms harvested, affecting both the primordia and maturation stages of mushroom development.

Finally, a problem experienced with this study as a whole is the high CV values obtained in a number of the experiments. A mushroom cultivation process should aim to have CVs between 5% and 10% to make the process more consistent and predictable (Royse, 1985), which is obviously desirable in its commercial application. If the CV value is too high, it means the results are too variable to indicate differences, significant or otherwise, if they truly exist (Royse and Bahler, 1986).

#### 4.6 CONCLUSION

The efficient cultivation of oyster and winter mushrooms has been found to be dependent on a number of factors, the most important being the strain, substrate and container vessel used. The strains promoting the most efficient production of oyster mushrooms were identified as

being *P.ostreatus* strains 3344 and 1833 and *P.pulmonarius* 36095. Of the waste lignocellulosic materials tested, Port Jackson was found to be the most favourable for oyster mushroom cultivation. This wood source is easily available, "cheap" and promotes the reliable production of fruiting bodies, which ensure its applicability in the cost-effective production of oyster mushrooms (Royse and Zaki, 1991). Furthermore, since Port Jackson is a widely occurring invasive weed, the control of its expansion could be encouraged by publicising its applicability in speciality mushroom production. Oyster mushrooms can be successfully cultivated in either glass bottles, polypropylene bags or polyvinylchloride tubing. Although glass bottles promoted highly productive cultivation systems, polyvinylchloride tubing could be used as an alternative to traditional cultivation vessels.

## CHAPTER FIVE

THE PRODUCTION OF LIGNIN AND CELLULOSE-DEGRADING ENZYMES BY *L.edodes* AND *Pleurotus* SPECIES DURING VEGETATIVE GROWTH ON WOOD.

## 5.1 INTRODUCTION

Lignocellulose degradation involves the breakdown of insoluble compounds into soluble components which can eventually be used during metabolism (Fengel and Wegener, 1989). The microbial degradation of lignocellulose by white-rot fungi is essentially enzymatic, although the mechanisms involved are poorly understood. The growth and fruiting of mushroom strains on woody substrates is therefore dependent upon the ability to produce the enzymes required to utilize the substrate components as nutritional sources (Buswell *et al.*, 1993). It is consequently understandable that the levels of certain enzymes have been correlated with growth processes in basidiomycetes, e.g. mushroom development (Panichajakul *et al.*, 1991).

Inter- and intraspecies specific variability in the enzyme systems of white-rot fungi effect their proportional degradation of the lignocellulosic components of different wood species (Rajaratnam and Bano, 1989; Fengel and Wegener, 1989; Royse, 1985; Sermanni *et al.*, 1994). Efficient enzyme production and activity in these fungi is dependent upon the substrate moisture content, pH, temperature and illumination (Rajaratnam and Bano, 1989; Bano *et al.*, 1993), with different enzymes becoming active at different stages in the cultivation process.

Although *Pleurotus* species have a faster growth rate than *L.edodes* (Moyson and Verachtert, 1991), both fungal types have a high initial growth rate on lignocellulosic materials due to the availability of easily utilizable carbon sources, and low molecular weight compounds released during sterilization (Burla *et al.*, 1992, Moyson and Verachtert, 1991). Once these exploitable nutritional sources have been depleted, there is a resultant decrease in growth rate since it becomes necessary to utilize the complex polymers of the lignocellulosic material (Burla *et al.*, 1992). *L.edodes* decomposes lignin in the initial growth stages with a steady increase in cellulose degradation over time (Babasaki and Ohmasa, 1991). During *L.edodes* cultivation there is a greater consumption of cellulose and hemicellulose than lignin (Moyson

and Verachtert, 1991). *Pleurotus* species preferentially degrade lignin, followed by hemicellulose and cellulose (Rajaratnam and Bano, 1989). Certain species (*P.pulmonarius* and *P.sajor-caju*) have been found to simultaneously degrade lignin and hemicellulose with only a slight decrease in the cellulose fraction (Moyson and Verachtert, 1991); whereas other species (*P.flabellatus*) have shown preferential utilization of cellulose over hemicellulose (Rajaratnam *et al.*, 1979). The vegetative growth of *Pleurotus* is associated with high lignin degradative activity, ensuring the availability of large amounts of cellulose for degradation during fructification (Rajaratnam *et al.*, 1979; Sharma, 1987). An illustration of the enzymes involved in the degradation of lignocellulose by *Pleurotus* is presented in Fig. 5.1.1.

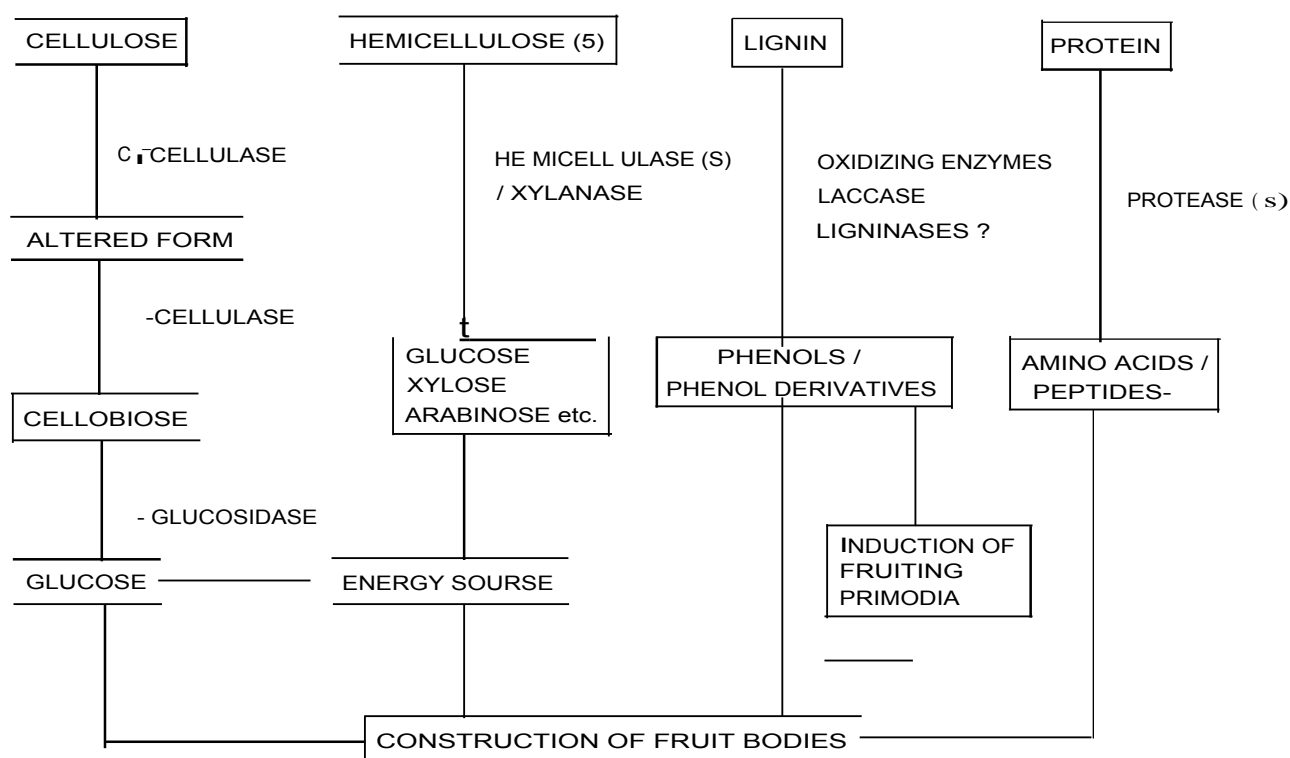


Fig. 5.1.1 The degradatory enzymes involved during *P.ostreatus* utilization of, and fructification on, lignocellulose (Rajaratnam and Bano, 1989: p.65).

### 5.1.1 Lignin Degradation

The chemistry and biochemistry of lignin degradation has been widely reviewed (Janshekar and Fiechter, 1983; Chen and Chang, 1985; Kirk and Shimada, 1985; Fengel and Wegener, 1989). The mechanisms involved in lignin degradation, and the potential to degrade this compound, varies between species due to the variability in the type and activity of degradative enzymes produced (Fengel and Wegener, 1989; Boyle *et al.*, 1992). The lignin degrading activity of white-rot fungi is dependent on the reductive and oxidative intra- and extracellular enzymes produced (Ruel and Barnoud, 1985; Chen and Chang, 1985). The most important enzymes involved in lignin degradation are the extracellular peroxidases (lignin peroxidase [LiP] and manganese peroxidase [MnP]) and phenol oxidases (aryl or veratryl alcohol oxidase [AAO or VAO, respectively] and laccases). Peroxidases and phenol oxidases have similar substrate specificities, however, they vary in their co-substrate requirements (oxygen and hydrogen peroxide, respectively) (Blaich and Esser, 1975). These enzymes catalyse oxidative reactions which are thought to be non-specific and non-stereoselective (Keyser *et al.*, 1978; Janshekar and Fiechter, 1983). Recent research has indicated that lignin degradation requires a combination of either MnP and LiP or MnP and laccase (De Jong *et al.*, 1992). MnP and laccase oxidise phenolic units only, which account for less than 10% to 20% of wood lignin; whereas LiP degrade non-phenolic compounds (Lapierre and Monties, 1989 cited in Guillen *et al.*, 1992; Delgado *et al.*, 1992).

The degradation of lignin is not solely dependent upon enzymatic activity but also, indirectly, on radical mechanisms through the action of activated oxygen species such as hydrogen peroxide, singlet oxygen, a superoxide radical and the hydroxyl radical (Ruel and Barnoud, 1985; Kirk and Shimada, 1985; Bourbonnais and Paice, 1988; Guillen *et al.*, 1994). Only singlet oxygen and the hydroxyl radical are reactive enough to be considered lignin oxidants in the natural situation; hydrogen peroxide and the superoxide radical are biochemical precursors to these reactive species (Kirk and Shimada, 1985). It has been postulated that the activity of enzymes is localized in the mycelial biomass, whereas that of radicals is often at locations removed from its immediate vicinity (Ruel and Barbound, 1985).

Degradation of low molecular weight aliphatic and aromatic products of lignin degradation must occur within the fungal mycelia. There is, however, a limited amount of knowledge on this aspect of lignin degradation (Kirk and Shimada, 1985).

#### 5.1.1.1 Lignin Peroxidases

Lignin peroxidases are extracellular, oxidising peroxidases which are thought to play a central role in the degradation of lignin (Kirk *et al.*, 1990; Cullen and Kersten, 1992). One of the most extensively studied lignin-degrading fungi, *Phanerochaete chrysosporium*, has been found to produce two extracellular peroxidases; ligninase (which is commonly called lignin peroxidase (LiP)) and manganese peroxidase (MnP) (Paszczynski *et al.*, 1988; Forrester *et al.*, 1988; Guillen *et al.*, 1992).

It has been suggested that chelated  $Mn^{3+}$  (a product of manganese peroxidase activity) and LiP function synergistically in lignin degradation (Forrester *et al.*, 1988). Boyle *et al.* (1992) have shown that in *P.chrysosporium* LiP is important in the solubilization of lignin and that MnP is important in the consequent formation of carbon dioxide, indicating that solubilization and mineralization of lignin are two distinct processes which are regulated separately. It is, however, not known which of the enzymes are active during the initial and latter stages of lignin degradation (Boyle *et al.*, 1992).

##### 5.1.1.1.1 Ligninase (LiP)

The ligninase from *P.chrysosporium* has been characterised and has the ability to oxidise a range of structurally diverse aromatic compounds (Kirk *et al.*, 1990). Ligninases depolymerise lignin and degrade lignin model compounds in the presence of hydrogen peroxide and absence of manganese (Paszczynski *et al.*, 1988; Forrester *et al.*, 1988; Cullen and Kersten, 1992).

Veratryl alcohol is an extracellular aromatic compound which is synthesised by *P.chrysosporium* as a secondary metabolite during its lignolytic phase (Gutierrez *et al.*, 1994). This compound induces the production of ligninase which is therefore a product of secondary metabolism (Asther *et al.*, 1988). Ligninases oxidise veratryl alcohol to veratryldehyde, which acts as an intermediate in lignin biodegradation (Gutierrez *et al.*, 1994).

A lignin peroxidase has been purified and characterised in *P.ostreatus* which differs from that of *P.chrysosporium* in molecular mass, carbohydrate content and substrate specificity (Kang *et al.*, 1993). The oxidative nature of this enzyme is also dependent on the presence of hydrogen peroxide and it exhibits activity on a large number of phenolic compounds (Kang *et al.*, 1993). This peroxidase, however, showed no affinity for non-phenolic compounds or veratryl alcohol, which is in direct contrast to the lignin peroxidases of *P.chrysosporium* (Kang *et al.*,1993). From the differences observed between the lignin peroxidases isolated from the two species, it has been postulated that they may play alternative roles in lignin degradation (Kang *et al.*, 1993).

#### 5.1.1.1.2 Manganese Peroxidase (MnP)

MnP is a secondary metabolite which has been isolated and characterised in *P. chrysosporium* (Gold and Glenn, 1988). Mn(II) peroxidase oxidises  $Mn^{2+}$  to  $Mn^{3+}$ , using hydrogen peroxide as a oxidant (Paszczytiski *et al.*, 1988; Forrester *et al.*, 1988; Cullen and Kersten, 1992).  $Mn^{3+}$  is a non-specific oxidant which oxidises a variety of organic compounds (Gold and Glenn, 1988), and has been postulated as playing a role in the oxidation of phenolic and non-phenolic lignin residues (Cullen and Kersten, 1992). MnP is, however, not strong enough an oxidant to oxidise veratryl alcohol or degrade chemical linkages commonly found in lignin (Forrester *et al.*, 1988). It has further been proposed that  $Mn^{3+}$  plays a role as a highly mobile compound capable of extending the lignin degradative system beyond that which is allowed by lignolytic enzymes (Forrester *et al.*, 1988).

#### 5.1.1.2 Phenol Oxidases

Although phenol oxidases have been the most extensively studied class of lignolytic enzymes, their complete role in lignin degradation, albeit important, is surrounded by much controversy (Kirk and Connors, 1977; Janshekar and Fiechter, 1983; Platt *et al.*, 1984).

##### 5.1.1.2.1 Laccases

Laccases commonly occur in wood degrading organisms (Blaich and Esser, 1975; Galli *et al.*, 1991). These enzymes are polyphenol oxidases in which the reduction of oxygen to water is typically accompanied by the oxidation of phenolic substances (Thurston, 1994). Laccases have a non-specific substrate range which varies between different laccase types. The

following have been identified as good substrates: catechol, hydroquinone, guaiacol and 2,6-dimethoxyphenol (Kirk and Kelman, 1965; Thurston, 1994).

The role of laccases in lignin degradation remains largely unclarified, although a number of postulations have been made (Kirk and Connors, 1977; Thurston, 1994). Although laccases are not essential for lignin degradation, as indicated by their absence in one of the strongest lignolytic fungi known (*P.chiysosporium*), research has shown that they play a role in many lignolytic reactions, possibly including Me-catalysed degradation (Thurston, 1994). The oxidation of phenolic compounds has been proposed to occur via the action of multiple laccases (Blaich and Esser, 1975). Laccases have been implicated in both lignin depolymerization and polymerization, since it has been suggested that they polymerise highly reactive, toxic products of degradation prior to entry into the fungal mycelia (Janshekar and Fiechter, 1983; Thurston, 1994).

The findings of numerous reports indicate the production of laccases by *Pleurotus* species (Platt *et al.*, 1984; Matcham *et al.*, 1985; Rajarathnam *et al.*, 1987; Sharma, 1987; Bourbonnais and Paice, 1988; Burla *et al.*, 1992; Delgado *et al.*, 1992; Garzillo *et al.*, 1992; Guillen *et al.*, 1992; Boyle *et al.*, 1992; Buswell *et al.*, 1993; Palmieri *et al.*, 1993; Gutierrez *et al.*, 1994; Sermanni *et al.*, 1994) and by *L.edodes* (Panichajakul *et al.*, 1991; Boyle *et al.*, 1992; Sermanni *et al.*, 1992; Buswell *et al.*, 1993).

#### 5.1.1.2.2 Aryl Alcohol Oxidases (AAO)

AAO degrade a number of lignin-related alcohols (e.g. veratryl alcohol) and aromatic compounds synthesized by the fungus itself (e.g. anisaldehyde) with the resultant production of hydrogen peroxide (Bourbonnais and Paice, 1988; Delgado *et al.*, 1992; Gutierrez *et al.*, 1994; Guillen *et al.*, 1994). AAO has been shown to have a wide substrate range, being able to degrade both polymeric lignin and simple aromatic compounds which are released during lignin degradation (Guillen *et al.*, 1994; Gutierrez *et al.*, 1994). Extracellular hydrogen peroxide acts as a co-substrate for lignin peroxidase activity, therefore the activity of extracellular hydrogen-peroxide producing enzymes, such as AAO, is important (Guillen *et al.*, 1992; Gutierrez *et al.*, 1994).

AAOs have been purified and characterised in a number of *Pleurotus* species, e.g. *P.sajorcaju* (Bourbonnais and Paice, 1988), *P.eiyingii* (Guillen *et al.*, 1992) and *P.ostreatus* (Garzillo *et al.*, 1992). The AAOs of the different *Pleurotus* species vary in the number of isoenzymes produced and have molecular masses ranging from 71 KDa to 81 KDa. Although the significance of the extracellular AAO in *Pleurotus* is not known, it has been postulated that it functions in lignin degradation, together with laccase, in the dehydrogenation of aromatic compounds (Bourbonnais and Paice, 1988). Although produced in primary metabolism (Bourbonnais and Paice, 1988; Asther *et al.*, 1988), AAO activity has been found to be highest during secondary metabolism and is influenced by light; therefore implying that it plays an important role in light-induced fruiting reactions (Delgado *et al.*, 1992).

#### 5.1.1.3 Factors affecting lignin degradation

Lignin degradation can be regulated by a variety of conditions, independent from their effects on fungal growth (Janshekar and Fiechter, 1983; Boyle *et al.*, 1992).

Lignin degradation in numerous white-rot fungi only commences under conditions of nitrogen limitation, e.g. *P.claysosporiunt* (Boyle *et al.*, 1992). This is understandable if one considers the natural, nitrogen-limited environment of wood-degrading organisms (Kirk and Connors, 1977; Keyser *et al.*, 1978). It has been proposed that the sensitivity to nutrient nitrogen is due to its biochemical repression of the enzymes involved in lignin degradation (Janshekar and Fiechter, 1983). The degradation of lignin by certain white-rot fungi, e.g. *L.edodes* and certain *Pleurotus* species, is not influenced by nitrogen content (Kirk and Shimada, 1985; Boyle *et al.*, 1992).

Oxygen is essential for the microbial degradation of lignin, indicating the oxidative nature of lignin degradation (Kirk and Kelman, 1965; Kirk and Connors, 1977; Burla *et al.*, 1992). It has been proposed that oxygen does not necessarily induce lignin degradation, but that it definitely enhances it once it has commenced (Bar-Lev and Kirk, 1981 cited in Janshekar and Fiechter, 1983). An increase in molecular oxygen increases both the titre of the total lignolytic system, and its rate of action (Bar-Lev and Kirk, 1981 cited in Kirk and Shimada, 1985). There is a species specific response to oxygen in lignin degradation: *P.chrysosporium* is influenced to a greater extent than *P.ostreatus* (Janshekar and Fiechter, 1983). This can

be due to the fact that only certain of the enzymes involved in lignin degradation are dependent upon oxygen.

Although the presence of manganese increases MnP activity, it decreases LiP activity and the rate of lignin degradation. This indicates the importance of LiP in lignin degradation (Boyle *et al.*, 1992). Laccase activity in *P.sajor-caju* has been found to be greater in high manganese media (Boyle *et al.*, 1992).

### 5.1.2 Wood polysaccharide degradation

There is a gradual increase in polysaccharidase activity during the vegetative growth of *Pleurotus*, which increases significantly during fructification (Rajarathnam and Bano, 1989). Cellulases are inductive enzymes (Ryn and Mandels, 1980 cited in Dekker, 1985) whereas hemicellulases are both constitutively and inductively produced (Dekker and Richards, 1976 cited in Dekker, 1985).

*L.edodes* and *Pleurotus* species produce the various classes of cellulases essential for the degradation/hydrolysis of cellulose: exo-B-1,4-glucanase, endo-B-1,4-glucanase and B-glucosidase (Rajarathnam and Bano, 1989; Leatham, 1985 and Tokimoto *et al.*, 1987 cited in Shisido, 1992). Exo- and endo-glucanases hydrolyse native cellulose into long, linear anhydroglucose chains and then into disaccharide units (cellobiose) which are hydrolysed by B-glucosidase into glucose (Patterson, 1975 cited in Rajarathnam and Bano, 1989).

Hemicelluloses are hydrolysed into hexoses, pentoses and often uronic acid through synergistic enzyme action (Moore-Landecker, 1982; Cullen and Kersten, 1992). Since hemicellulose is a structurally more complex compound than cellulose, the enzyme systems operative are similarly more complex (Dekker, 1985; Cullen and Kersten, 1992). Hemicellulases act specifically on those anhydrosugars making up the backbone of the hemicellulose polymer (Dekker, 1985), and therefore the enzymes and biochemical processes involved are determined by the hemicellulose being degraded (Moore-Landecker, 1982). Enzymes involved include xylanases, arabinases and mannases (Rajarathnam and Bano, 1989). *L.edodes* and *P.sajor-caju* have been reported to produce high levels of xylanase (Buswell *et al.*, 1993).

## 5.2 RESEARCH OBJECTIVES

The degradation of lignocellulosic material requires the concerted action of a number of enzymes varying in substrate specificities. Lignin agar, with p-anisidine incorporated, was used as an initial indication of the lignin degrading ability of various wood-degrading fungi. The semi-quantitative assessment of the cellulolytic activities of the various species were determined on acid swollen cellulose agar.

Since lignin is one of the most important components of wood affecting degradation and therefore substrate utilization, due to its encrustation of wood polysaccharides, the enzymes involved in its degradation during the growth of *L.edodes* and *Pleurotus* species on Port Jackson based medium were investigated.

## 5.3 MATERIALS AND METHODS

### 5.3.1 *In vitro* enzyme studies

Agar plugs of two-week old cultures of *F.velutipes*, various *L.edodes* strains and a number of *Pleurotus* species were inoculated onto lignin agar (LA) (Appendix A; 2.1) and acid-swollen cellulose agar (ASCA) (Appendix A; 2.2). The LA had 2.5 mM of p-anisidine incorporated (W.Leukes, Pers. comm.). The agar cultures were incubated at 25°C, which is the optimum temperature for the vegetative growth of the various fungal species. Measurements of the precipitation and hydrolysis zones, on LA and ASCA respectively, were conducted at appropriate time intervals.

### 5.3.2 Wood substrate preparation for enzyme quantitation

Port Jackson wood chips (<5 mm) were soaked in distilled water for 48 hours at room temperature and used in the preparation of media (Appendix B; 2.5). The substrate components were thoroughly mixed and 2.6 g amounts (wet weight basis) placed in McCartney bottles. The substrate dry weight was determined by drying to constant weight at 80°C, and distilled water was added to provide a 1:1.2 ratio of substrate:water (dry weight basis).

The medium was sterilized by autoclaving (121°C) for 15 minutes, allowed to cool and inoculated with a 10% spawn inoculum (see Appendix B; 1.2). The following fungal species

were used in the study: *P.ostreatus* 3344, *P.columbinus* 36215, *P.eryngii* 36037 and *L.edodes* 58762.

Samples were incubated in the dark at 25°C under relative humidity conditions of 60% to 70%. Destructive sampling was employed with samples being removed at certain vegetative growth phases: Day 0 (after inoculation), Day 6 (in the middle of vegetative growth) and Day 15 (at the end spawn run). Samples were frozen with liquid nitrogen and placed at -70°C until the completion of the experimental period.

Samples were prepared for protein and enzyme analysis by homogenizing in 40 ml of Tris Buffer (50 mM; pH 7.5) for 1.5 minutes (Unitrax Homogenizer).

### **5.3.3 Protein measurements**

The method used for sample preparation was modified from Boyle and Kropp (1992). Sample aliquots (1.5 ml) of the colonized wood-chip preparations were transferred into 1.5 ml Eppendorf tubes and centrifuged for 15 minutes using a bench top microfuge. 0.8 ml amounts of the supernatant were added to 0.08 ml of a 0.15% aqueous solution of sodium deoxycholate. The samples were incubated at room temperature (20°C), with occasional mixing, for 10 minutes and then cooled on ice. To the cold samples was added 0.16 ml cold 70% trichloroacetic acid (TCA). Samples were further incubated on ice for approximately 10 minutes and thereafter centrifuged for 15 minutes in a microfuge. The pellets were washed in cold 7% TCA and then resuspended in 0.5 ml 0.05 M NaOH by incubating at room temperature for 2 hours. Following incubation, the samples were neutralised with the addition of 0.5 ml 0.05 M HCl.

Protein concentrations were determined according to a method adapted from Lowry *et al.* (1951). Reagents used are described in Appendix C. Aliquots (1 ml) of crude protein were placed in test tubes to which was added 15 ml Reagent A, 0.75 ml Reagent B and 0.75 ml Reagent C. The samples were thoroughly mixed and incubated at room temperature for 15 minutes. Three ml of Reagent E was added to each sample and thoroughly mixed. Samples were incubated for 45 minutes in the dark. The absorbance of each sample at 750 nm ( $A_{750}$ ) was determined as soon as possible after incubation.

A protein standard curve, using a known BSA concentration, was used in the determinations of sample protein concentrations.

### **5.3.4. Enzyme assays**

#### **5.3.4.1 Preparation of enzyme extract**

1.5 ml volumes of the colonized wood-chip slurries were centrifuged for 15 minutes (in a bench top microfuge). The supernatant was used as a crude enzyme extract and was used for subsequent enzyme analyses. Where necessary, the supernatant was stored at -20°C and -70°C to maintain the activity of the enzymes.

#### **5.3.4.2 Phenol oxidases**

Phenol oxidase activity was determined using guaiacol and catechol as substrates. The method in which guaiacol was used was performed according to Rajarathnam *et al.* (1987). A 200  $\mu$ l aliquot of the crude enzyme solution was incubated with an equal volume of guaiacol (40 mM) and 600  $\mu$ l of sodium phosphate buffer (0.1 M, pH 5.6). Readings were taken at 465 nm at time 0 and after 240 minutes ( $E_{465}=12\ 100\ M^{-1}.cm^{-1}$ ) (Guillen *et al.*, 1992). Catechol oxidase activity was determined by the method of Platt *et al.* (1984). Activity was measured by following the change in absorbance at 475 nm of a solution containing 6 mM catechol, 1 mM  $CuSO_4$  and 100  $\mu$ l crude enzyme.

#### **5.3.4.3 Lignin peroxidase**

Lignin peroxidase activities were determined using established methods. Manganese peroxidase activity was determined according to the method of Gold and Glenn (1988). The reaction mixture consisted of 500  $\mu$ l reagent A (sodium lactate buffer, 100 mM, pH 4.5 containing 100  $\mu$ M  $MnSO_4$ ) and 500  $\mu$ l reagent B (a freshly prepared solution of 100  $\mu$ M  $H_2O_2$  in distilled water). The reaction was initiated at room temperature by the addition of a 200  $\mu$ l aliquot of crude enzyme extract. The rate of Mn (III) lactate formation was determined spectrophotometrically by following the initial increase in absorbance at 240 nm for 5 minutes.

The hydrogen peroxide-dependent oxidation of veratryl alcohol to veratryldehyde was measured by the method of Tien and Kirk (1984) which has been described by various

authors (Kirk *et al.*, 1986; Guillen *et al.*, 1992). The alcohol is not absorbed at 310 nm, whereas the aldehyde is (molar extinction coefficient = 9300 M<sup>-1</sup>cm<sup>-1</sup>) (Bourbonnais and Paice, 1988). The absorbance of the reaction mixture was determined at 310 nm for 20 minutes. The reaction mixture was composed of 0.4 ml dH<sub>2</sub>O, 0.3 ml Na-tartrate buffer (0.25 M, pH 2.5), 0.1 ml of a 10 mM stock solution of veratryl alcohol, 0.2 ml crude enzyme extract and 6 μl H<sub>2</sub>O<sub>2</sub>.

All enzyme assays were expressed as AOD.min<sup>-1</sup>.ml<sup>-1</sup> enzyme extract. Where molar extinction coefficients were available, enzyme activity units were determined which is defined as the amount of enzyme which releases 1 μmole of oxidised product per minute per ml.

## 5.4. RESULTS

### 5.4.1 Lignolytic enzyme assays

Wood-degrading basidiomycetes, which produce extracellular phenoloxidas, can be identified on phenol containing media by the formation of coloured oxidation products (Kirk and Kelman, 1965). The production of these enzymes are detected by the formation of dark diffusion zones (Kirk and Kelman, 1965). Enzymes which are capable of producing these coloured products are laccases, catechol oxidases and peroxidases (Kirk and Kelman, 1965). The black precipitation zones produced during wood-degrading fungal colonization of lignin agar with p-anisidine incorporated is indicated in Table 5.4.1 and photographically illustrated in Fig. 5.4.1. The diameter of the zone is taken as an indication of the concentration of enzymes; it increased substantially during the growth phase without, necessarily, being accompanied by mycelial growth.

Table 5.4.1 The *in vitro* lignin degrading ability of various edible basidiomycetesu

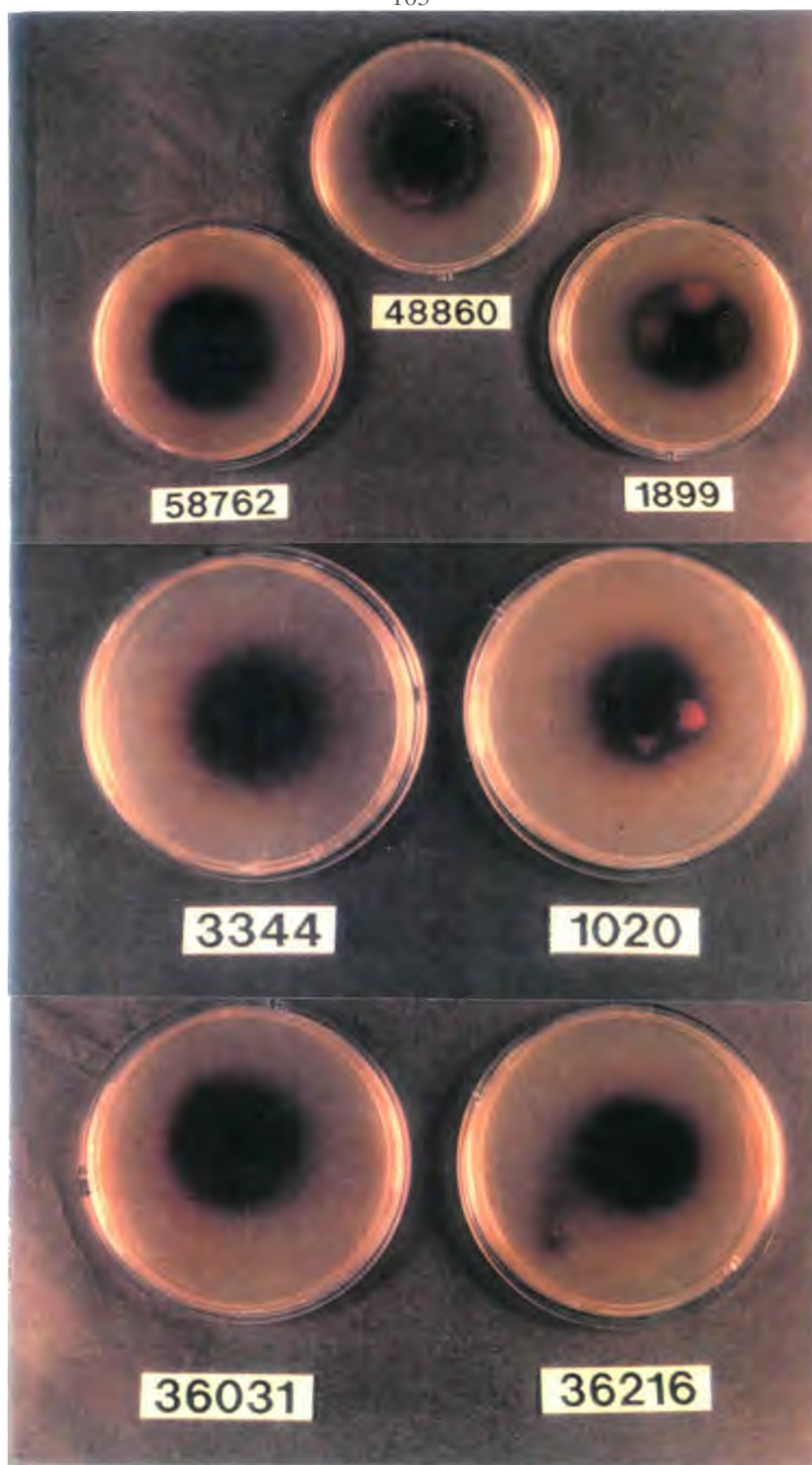
Strain <sup>o</sup>	Zone of precipitation (min)
<i>L.edodes</i> 58762	45.3±0.6 [A]
<i>L.edodes</i> 48860	41.7±1.2 [B]
<i>P.ostreatus</i> 3344	38.7±3.8 [BC]
<i>P.ostreatus</i> 1833	35.5±0.7 [CD]
<i>Pleurotus spp.</i> 36031	34.3±5.5 [D]
<i>P.cornucopiae</i> 36216	32.8±3.2 [DE]
<i>F. velutipes</i>	32.0±0.0 [DE]
<i>P.incarnatus</i> 36228	31.0±0.0 [DEF]
<i>L.edodes</i> 1899	30.7±2.1 [DEF]
<i>P.ostreatus</i> 1020	30.0±1.0 [DEF]
<i>P.eryngii</i> 36037	29.0±0.0 [EFG]
<i>P.cystididosus</i> 36170	26.0±0.0 [FGH]
<i>P.calypratus</i> 36211	24.7±2.5 [GH]
<i>P.cystididosus</i> 36131	24.3±2.1 [GH]
<i>P.columnbinus</i> 36215	22.5±0.7 [H]
<i>P.fuscus</i> var. <i>ferulae</i> Lanzi 36214	15.7±1.2 [I]
<i>P.cystididosus</i> 36223	0.00±0.0 [J]
<i>P.cystididosus</i> 36172	0.00±0.0 [J]

<sup>a</sup> The lignin degrading ability of the various strains was determined by measuring the precipitation zone formed after 8 days incubation on lignin/p-anisidine agar.

<sup>b</sup> Entries indicate the mean ± standard deviation of three replicates; means followed by identical letters are not considered statistically different at  $P=0.05$ .

Inter- and intraspecies specific variation in the zones of black precipitates formed are evident in Table 5.4.1. Certain strains of *P.cystididosus*, namely 36223 and 36172, did not produce black precipitates on the agar medium tested, which would imply an inability to degrade lignin.

*L.edodes* 58762 showed a significantly larger zone of precipitation than any of the other strains tested, implying a higher production of lignolytic enzymes. This was followed by *L.edodes* 48860. Subspecies (e.g. *P.ostreatus* strains 1833 and 3344) showed comparable zones of precipitation and one can therefore conclude that they often produce similar quantities of lignolytic enzymes.



**Fig. 5.4.1** The response of various wood-degrading fungi to incubation on Lignin agar with p-anisidine incorporated.

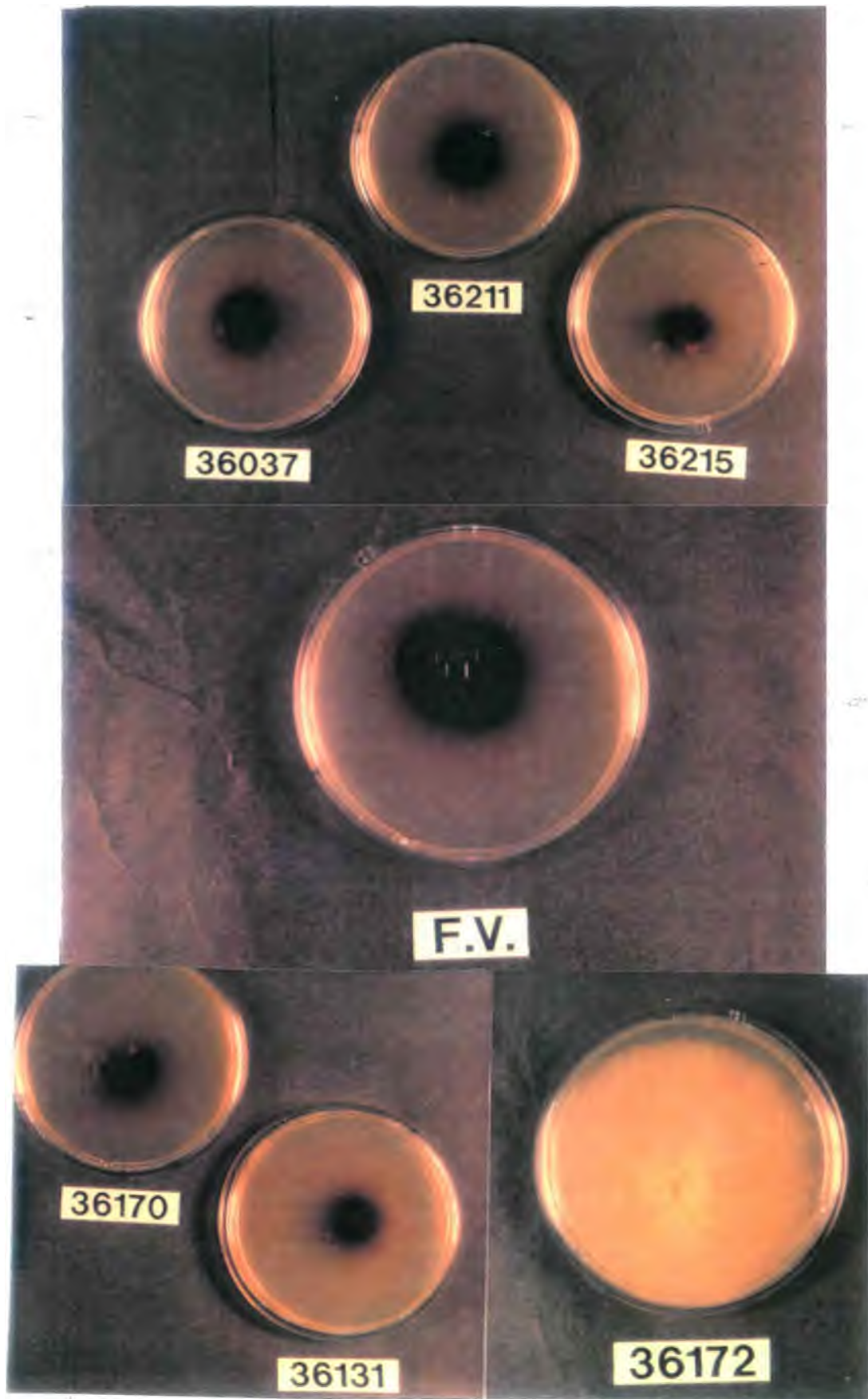


Fig. 5.4.1 Cont.

In the quantitation of certain lignin degrading enzymes produced by four wood-degrading fungi, it was discovered that enzyme activity levels varied between species and during different stages of the cultivation process. The same trend was observed in the protein concentration of the different species during cultivation (Table 5.4.2).

Table 5.4.2 Phenol oxidase and peroxidase activities' of various white-rot fungi during vegetative growth.

Strain	Time (Days)	Total protein (mg/ml)	Phenol oxidases		Lignin peroxidases			
			Guaiacol		Catechol	MnP	Oxidation of veratryl alcohol	
			A	B	A	A	A	B
L.edodes 58762	0	0.70	0	0.36	0.08	0.02	0.03	2.69
	6	2.36	0.01	0.44	0.10	0	0.06	6.18
	15	3.67	0.03	2.68	0.78	0	0	0
P.ostreatus 3344	0	0.81	0.01	0.77	0.04	0	0	0"
	6	3.13	0.03	2.78	0.17	0	0.12	12.90
	15	5.28	0.03	2.73	0.23	0.02	0.21	22.20
P.columbinus 36215	0	1.24	0.03	2.73	0.18	0	0.04	3.76
	6	2.98	0.03	2.77	0.62	0	0.03	2.69
	15	3.81	0.04	2.88	0.47	0	0.10	10.48
P.eryngii 36037	0	1.03	0	0.19	0.04	0	0	0
	6	4.88	0.04	2.90	1.02	0	0.11	11.83
	15	4.99	0.03	2.16	0.82	0	0.18	18.98

"

A= AOD.min-1.ml<sup>-1</sup> enzyme extract.

B= Activity Units (umol.min<sup>-1</sup>.ml<sup>-1</sup>).

From the results presented it appears that catechol is a better substrate for the detection of phenol oxidases than guaiacol, since in this study it has been shown to be more sensitive than the latter. Two trends were evident in catechol oxidation levels: a constant increase in enzyme activity level from inoculation through to the end of the spawn run period (strains 58762 and 3344); or an increase during vegetative growth followed by a decrease prior to fruiting (strains 36215 and 36037). These different trends show varying degradative activities of these four mushroom strains, based on the cultivation phase at which the measurement was conducted.

No MnP, or negligible levels, were detected in all fungi investigated. The formation of veratryldehyde from veratryl alcohol oxidation increased throughout the cultivation period in all *Pleurotus* species tested; during *L.edodes* cultivation, however, the level decreased at the end of vegetative growth.

#### 5.4.2 The screening of cellulolytic enzyme production

*F.velutipes* showed significantly greater cellulolytic activity, as measured by its zone of hydrolysis on ASCA, than any of the other species investigated with the exception of *L.edodes* 1899. The three sub-species of *L.edodes* did not vary greatly in their cellulose degrading activity. It is interesting to note that none of the *Pleurotus* species investigated showed cellulose hydrolysing activity after a months incubation period (Table 5.4.3).

Table 5.4.3 Detection of the cellulolytic activity of various fungi using acid-swollen cellulose agar.

Strain	Zone of hydrolysis onmY
<i>F.velutipes</i>	19.5±2.5 [A]
<i>L.edodes</i> 1899	16.0±0.0 [AB]
<i>L.edodes</i> 48860	11.0±0.0 [C]
<i>L.edodes</i> 58762	13.0±3.0 [BC]
<i>P.citrinopileatus</i> 36239	0
<i>P.columbinus</i> 36215	0
<i>P.cornucopiae</i> 36216	0
<i>P.cystidiosus</i> 36170	0
<i>P.cystidiosus</i> 36172	0
<i>P.eryngii</i> 36037	0
<i>P.fuscus</i> var. <i>ferulae</i> 36214	0
<i>P.ostreatus</i> 1020	0
<i>P.ostreatus</i> 3344	0
<i>P.pulmonarius</i> 36095	0
<i>Pleurotus</i> spp. 36031	0

Entries, which are the means±standard deviation of three replicates, indicate the diameter of the zone of hydrolysis (mm) produced after incubation at 25°C for one month. Values followed by the same letter are not significantly different at P=0.05.

## 5.5 DISCUSSION

Wood-degrading fungi have been found to vary, often significantly, in their enzyme production and activity. This can initially be determined using *in vitro* assays. These assays indicate whether the enzyme is produced in detectable amounts and what the expected titre is, e.g. lignin agar with p-anisidine incorporated and ASCA allowed a detection of the lignocellulolytic activity of various fungi. Although these *in vitro* studies are not accurate and do not differentiate between the different classes of enzymes produced, they indicate the general degradative potential of the fungi under investigation. Enzyme quantitation studies enable the determination of specific enzyme activities during *in vivo* growth.

The *in vitro* lignocellulolytic activity of various species showed great variability, as indicated in Tables 5.4.1 and 5.4.3. *L.edodes* strains and *F.velutipes* showed detectable enzyme activity levels on both ASCA and lignin agar with p-anisidine. All *Pleurotus* strains showed high lignin degradative activity but an inability to *in vitro* hydrolyse cellulose, which is understandable since cellulolytic activity in this group is primarily associated with reproductive growth (Rajaratnam and Bano, 1989).

*P.ostreatus* strains 3344 and 1833 produced the largest precipitation zones of all *Pleurotus* strains investigated, on lignin agar with p-anisidine. This correlates with the high yield potentials of these strains when cultivated on Port Jackson based substrates (Chapter 4), which indicates the potential application of *in vitro* plate assays for the selection of high yielding strains.

In certain instances, a correlation was observed between *in vitro* and *in vivo* enzyme assays. *L.edodes* 58762 produced the largest precipitation zone on agar medium and showed the highest detectable catechol oxidase activity. *P.ostreatus* 3344 also showed high precipitate formation on lignin agar and the most efficient oxidation of veratryl alcohol; however, it produced low levels of catechol oxidases (Tables 5.4.1 and 5.4.2). *P.eryngii* 36037 showed a different trend in that it produced a small diameter precipitate on lignin agar; nevertheless, it was shown to oxidise catechol and veratryl alcohol with greater efficiency than most of the other strains investigated.

This study has indicated that the different species investigated vary in their enzyme activity levels, which is consistent with previous reports (Platt *et al.*, 1984), and the stage at which maximal activity occurs (Table 5.4.2). Furthermore, the sensitivity of the enzyme assay method on the substrate used has also been illustrated. The detection of phenol oxidase activity in this investigation was higher on catechol than guaiacol substrates, and the trends in enzyme activity levels of certain strains varied depending on the substrate used. The phenol oxidase levels of *L.edodes*, using both guaiacol and catechol as substrates, increased substantially towards the end of spawn run time. *P.columbinus* 36215 showed a gradual increase in guaiacol oxidation throughout the cultivation period, whereas catechol oxidation decreased with the onset of fructification (day 15). In *P. ostreatus* an opposite trend to *P.columbinus* was found with reference to substrate oxidation. *P.eiyingii* showed high initial guaiacol and catechol oxidation during spawn run which then decreased at the onset of fructification. This decrease in phenol oxidase activity at the end of vegetative growth corresponds to previous findings cited in the literature (Platt *et al.*, 1984; Galli *et al.*, 1991; Delgado *et al.*, 1992) and could possibly be due to end product inhibition. Garzillo *et al.* (1992) found that nutritional changes occurring during substrate colonization by *P.ostreatus* result in the production of different phenol oxidases with varying substrate specificities which evidently function in the efficient utilization of available nutrients. It has been postulated by Garzillo *et al.* (1992), that in *P.ostreatus* phenol oxidases play an essential, indirect function in the initial phases of lignin depolymerization which is then continued by other enzymes, such as AAO.

All of the fungi investigated in this study have been found to have higher phenol oxidase than peroxidase activity (Table 5.4.2). This has been reported for *Pleurotus* species (Platt *et al.*, 1984).

MnP activity was only detected in low amounts in *L.edodes* 58762 and *P.ostreatus* 3344 on days 0 and 15, respectively. The lignin degrading ability of *L.edodes*, however, has been found to be dependent upon the presence of manganese, and therefore on a Mn-dependent peroxidase (Forrester *et al.*, 1988). Although LiP is produced by *L.edodes* (Forrester *et al.*, 1990 cited in Archer and Wood, 1994), neither LiP nor MnP have been detected in *Pleurotus* using common assay methods (Bourbonnais and Paice, 1988; Guillen *et al.*, 1992). The

nature of the enzyme which was detected in the oxidation of veratryl alcohol is therefore questionable. It has been proposed that in *Pleurotus* species the oxidation of veratryl alcohol is catalysed by AAO (Bourbonnais and Paice, 1988). Both AAO and LiP catalyse the oxidation of veratryl alcohol; however, whereas LiP requires hydrogen peroxide for this reaction AAO does not (Guillen *et al.*, 1992). It has been proposed that AAO can often be mistaken for lignin peroxidase in *Pleurotus* species since AAO efficiently oxidises veratryl alcohol at pH 3 in the presence of hydrogen peroxide (Bourbonnais and Paice, 1988), which are the assay conditions used in this study.

Since LiP has been detected in *L.edodes* and not in *Pleurotus* species, it is possible that the assay used in this study was detecting two types of enzyme: LiP and AAO, respectively. Furthermore, previous reports on the production of AAO in *P.ostreatus* (Garzillo *et al.*, 1992) and *P.eryngii* (Guillen *et al.*, 1992) and the lack of LiP detection using the veratryl alcohol assay method lend support to this hypothesis. Alternatively the enzyme assay employed could be simultaneously detecting a combined LiP/AAO activity. Further research is required to determine whether the proposed enzyme systems are indeed present in the strains studied, e.g. the oxidation of veratryl alcohol in the absence of hydrogen peroxide would support the presence of VAO in the *Pleurotus* species investigated. Irrespective of the enzyme being studied, it appears as though *L.edodes* 58762 produces low amounts of phenol peroxidase, whereas *P.ostreatus* 3344 and *P.eryngii* 36037 produce significant amounts of this enzyme group (Table 5.4.2). In contrast to the phenol oxidases, lignin peroxidase levels increased throughout the cultivation period in all species investigated and reached maximal activity levels at the onset of fructification (Table 5.4.2).

No mushrooms were produced by *L.edodes* 58762 (refer to Chapter 3) which had high phenol oxidase levels, but no detectable peroxidase activity, at the onset of fructification (day 15). In contrast *P.ostreatus* 3344, which had the highest yield potential of all the strains investigated, had relatively low phenol oxidase levels but high peroxidase activity at the end of vegetative growth. These observations suggest that the peroxidase group of enzymes plays an important role in fruiting body formation and may be a limiting factor in the number of mushrooms produced by a given strain.

Protein determinations provide an indication of nitrogen-based metabolism and the rapidity and extent of protein synthesis and accumulation during the colonization of wood (Boyle and Kropp, 1992). Since fungal cells contain more protein than does wood, one would expect the concentration of protein to increase as the colonization process continues (Boyle and Kropp, 1992). Total protein levels increased throughout the incubation time, and hence with the accumulation of fungal biomass (Table 5.4.2).

## 5.6 CONCLUSION

The production of lignocellulolytic enzymes varies between species and according to the growth phase being investigated. Phenol oxidases and peroxidases are produced by both *L.edodes* and *P.ostreatus*, however in varying activity levels. Although high quantities of lignin degrading enzymes have been detected in *Pleurotus* species, using both *in vitro* and *in vivo* studies, no vegetative production of cellulolytic enzymes was identified. *L. edodes*, however, produced both lignolytic and cellulolytic enzymes during vegetative growth.

The lignin agar and enzyme assays provide a clearly useful approach to screening both wild-type and mutated fungal isolates, e.g. *Pleurotus* species, for superior utilization of substrates and improved mushroom production, as supported by the correlation between certain enzymes and productivity on Port Jackson based substrates.

## CHAPTER SIX

THE USE OF RAPDS TO FINGERPRINT *Pleurotus* AT THE SPECIES AND STRAIN LEVEL.

## 6.1 INTRODUCTION

The rapid development of cultivation systems for numerous *Pleurotus* species, often resulting in the misinterpretation and misapplication of species names (Buchanan, 1993), has resulted in much taxonomic confusion in this genus (Eger, 1978; Rajarathnam and Bano, 1987; Royse and May, 1993). The complexity of *Pleurotus* classification is further compounded by its cosmopolitan nature and variability in macro- and microscopic features (Eger, 1978; Buchanan, 1993). The differentiation of *Pleurotus* species based on structural characteristics (macro- and microscopic features) is therefore often not feasible (Rajarathnam and Bano, 1987). The recent classification of *Pleurotus* has consequently been based on intercompatibility studies. Compatible strains are considered to constitute a species; morphological and colour variations are of secondary importance in these studies and are related to cultural variations (Rajarathnam and Bano, 1987). Although intercompatibility studies have been successfully applied in the classification of *Pleurotus* species, the application of additional tools might provide more reliable and useful information about the genus as a whole.

The wide applicability of the polymerase chain reaction (PCR), indicated by the numerous papers published in this field, implies a possible use for it in the resolution of the taxonomic confusion associated with *Pleurotus*. The PCR is a molecular technique involving the *in vitro* enzymatic amplification of specific DNA sequences, using a pair of highly specific oligonucleotide primers which flank the target DNA by hybridising to opposite strands (Mullis and Faloona, 1987; Caetano-Anolles *et al.*, 1991). The methods, principles and applications of this technique have been widely reviewed (Erlich, 1989; Innis *et al.*, 1990; Anderson, 1993).

Numerous speciation studies of wood-degrading fungi have utilized the PCR. RFLP analysis of specific PCR products has been used in the differentiation of *Lentinus*, *Neolentinus*, *Pleurotus* and *Lentinula edodes* (Molina *et al.*, 1992); and in the systematic investigation of

*Pleurotus* species (Iracabal *et al.*, 1995). Furthermore, PCR amplification of two regions of the nuclear rDNA locus has been used in the phylogenetic analysis of *Pleurotus* species (Vilgalys and Sun, 1994). This study differentiated between two phylogenetic groups: an ancient group which is dispersed worldwide and a more recently evolved group within the Northern hemisphere.

A related technique, named the arbitrarily primed polymerase chain reaction (AP-PCR), has potential application in classification, microbial screening, genetic mapping, population genetics and breeding studies (Welsh and McClelland, 1990; Williams *et al.*, 1990; Fujimori and Okuda, 1994). AP-PCR has also been named RAPD fingerprinting (Random Amplification Polymorphic DNA) since it uses primers of arbitrary nucleotide sequence to detect polymorphisms in random genomic DNA segments (Williams *et al.*, 1990). RAPD fingerprinting has been extensively applied to species classification (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anolles *et al.*, 1991; Khush *et al.*, 1992; Tham *et al.*, 1994; Huang *et al.*, 1994; Kaukas *et al.*, 1994); including that of *L.edodes* strains (Chiu *et al.*, 1993).

The advantages associated with using RAPD fingerprinting for taxonomic studies are: it provides species specific fingerprints, enabling the differentiation of even closely related individuals; no prior knowledge of the DNA sequence is required; DNA cloning and hybridization is not required, as with RFLP analysis; a large number of loci are targeted comparable to isozyme analysis; and, since primers of arbitrary sequence are used, coding and non-coding regions of the genome are sampled (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anolles *et al.*, 1991; Khush *et al.*, 1992; Clark and Lanigan, 1993). Consequently, RAPD fingerprinting is more rapid and simple, as well as less expensive, than other DNA differentiation techniques (Clark and Lanigan, 1993).

A number of disadvantages are inherent in RAPD fingerprinting. An awareness of these factors, and their impact on RAPD fingerprinting, is important in understanding the implications of results obtained in speciation studies (Clark and Lanigan, 1993). RAPDs are significantly influenced by experimental parameters and therefore standardization of all variables is of importance in obtaining reproducible results (Chiu *et al.*, 1993; Tham *et al.*,

1994). RAPD fingerprints are dominant and therefore do not differentiate between heterozygous and homozygous individuals (Royse *et al.*, 1993); RAPDs do, however, identify monokaryon-dikaryon relationships (Chiu *et al.*, 1993). Since RAPD markers are dominant, gene frequency estimates for such loci are less accurate than those obtained with codominant markers such as isozyme analysis and RFLPs. RAPD fingerprints do not necessarily indicate independent loci or allelic forms since the products of different loci might have similar molecular weights and therefore might comigrate on gels (Chiu *et al.*, 1993; Royse *et al.*, 1993; Clark and Lanigan, 1993). Furthermore, it has not been determined whether RAPD bands which are common between species, and which can therefore be used as genetic markers, have identical genetic sequences (Chiu *et al.*, 1993). Another consideration when analysing RAPD data is that polymorphisms between species is considered to occur from the amplification of single nucleotide substitutions in the genomic region being amplified (Clark and Lanigan, 1993); however, mispriming could be the cause of the observed polymorphisms.

## 6.2 RESEARCH OBJECTIVES

The extensive application of RAPDs in the differentiation of species implies its potential use in overcoming the problems currently experienced in *Pleurotus* taxonomy. This study therefore attempted to ascertain the feasibility of using such a method for clarifying the taxonomic relationships between *Pleurotus* species and strains, and consequently the intra- and interspecies specific variation inherent in *Pleurotus*.

## 6.3 MATERIALS AND METHODS

### 6.3.1 RAPD Fingerprinting

#### 6.3.1.1 Fungal cultures

The *Pleurotus* strains which are cited in Table 2.3.1 were used in this investigation. Strains were initially grown for two weeks on GMPY agar (Appendix A; 1.7) and then transferred into 20 ml volumes of GMPY broth medium. Cultures were incubated at 25°C with agitation (200 rpm) for 14 days. Prior to DNA extraction, the purity of cultures was ascertained by visual inspection on agar medium.

### 6.3.1.2 DNA Extraction

Standard buffers used in the extraction protocol, which was adapted from Khush *et al.* (1992), are cited in Appendix D; 1. Aliquots of the fungal cultures (1.5 ml) were pelleted and the cells washed twice in 500 µl TE buffer. The pellets were resuspended in 500 µl cold extraction buffer, 67 µl 10% SDS, 2.5 µl Proteinase K (Boehringer Mannheim from a stock of 20 mg/ml) and 1 µl RNase A (from a 10 mg/ml stock solution); and incubated at 65°C for 60 minutes. Samples were mixed with 170 µl 5 M potassium acetate and incubated on ice for 20 minutes. Samples were then extracted with a phenol:chloroform:isoamyl alcohol solution (25:24:1) followed by chloroform:isoamyl alcohol (24:1). The DNA was ethanol precipitated and dissolved in TE buffer. The final DNA concentration was estimated spectrophotometrically (Shimadzu UV-160A UV visible recording spectrophotometer) at 260 nm according to the method of Sambrook *et al.* (1989).

### 6.3.1.3 RAPD amplification

A number of oligonucleotide decamers (synthesised by the Department of Biochemistry, University of Cape Town, South Africa), from the primer collection of the Department of Biochemistry and Microbiology, Rhodes University, were used in this study:

Primer 2: 3'-5' TCACATGGCA	5'-3' ACGGTACACT
Primer 6: 3'-5' AACCGATGCT	5'-3' TCGTAGCCAA
Primer 7: 3'-5' ACGTAGCACT	5'-3' TCACGATGCA
Primer 11: 3'-5' GTGATGAAGG	5'-3' GGAAGTAGTG
Primer 12: 3'-5' ACCTGCGTTA	5'-3' ATTGCGTCCA

The above primers all had a GC content of 50% which is similar to the GC ratio of *Pleurotus ostreatus* (Kuo and Wu, 1972).

Amplification reactions consisted of: 5 µl 10 x Promega PCR reaction buffer (50mM Tris-HCl, pH 8.3 and 15 mM MgCl<sub>2</sub>); 1.5 µM MgCl<sub>2</sub>; 100 µM of each dNTP; 0.1 µM primer; 100 ng genomic DNA and 1 unit *Taq* DNA polymerase (Biolab). The total reaction volume used was 50 µl which was made up using double distilled water. The reaction mixture was overlaid with sterile oil to prevent evaporation. Amplifications were conducted in a JDI Model 8012 High Performance Temperature Profiler programmed for 40 cycles of 30 seconds

at 94°C; 30 seconds at 36°C and 60 seconds at 72°C. Initial denaturation was conducted at 94°C for 270 seconds, and final extension at 72°C. for 240 seconds.

All PCR reactions were conducted using standard preparation and experimental procedures. All equipment used in DNA extraction and amplification reactions was washed with 1 N HCl and sterilized at 121°C prior to use. Only double distilled water was incorporated into reaction mixtures. Negative controls were amplified with the samples to ensure the absence of reagent contamination or primer artifacts and the attainment of strain-specific fingerprints.

Amplification products were size-fractionated by electrophoresis on 10% polyacrylamide slab gels (Appendix D; 2) and polymorphisms detected by silver staining (Appendix D; 3). In all gels, a molecular mass marker was included as a reference (Appendix D; 2.1.6).

Photographic data capture of banding patterns was used for cluster construction by UPGMA analysis (unweighted pair-group method with arithmetic average), using the GelManager\* Biosystematical Programme (Jackman 1991/1992). UPGMA clustering forms groups by successively pairing similar molecular phenotypes according to the extent of their observed distances (Sneath and Sokal, 1973 cited in Iracabal *et al.*, 1995). The dendograms obtained were used to infer relatedness between different strains.

#### 6.4 RESULTS

The optimization and standardization of the PCR reaction is important in the fingerprinting of species for classification purposes since it ensures accurate, reproducible results. The optimized procedure described provided the most suitable fingerprint patterns of *Pleurotus* species. A large number of bands, over a wide size range, were obtained using the proposed amplification and staining method, indicating the high degree of sensitivity of both systems. It was observed that extractions containing brown pigment were not amplified under the above conditions, e.g. DNA from *L.edodes* 1899. Furthermore, amplification did not occur when using PCR reaction buffer free of MgCl<sub>2</sub>. The MgCl<sub>2</sub> concentration in the PCR buffer, however, was not sufficient to ensure efficient strain amplification and therefore additional MgCl<sub>2</sub> incorporation was necessary. Although lower concentrations of DNA were amplified, 100 ng amounts resulted in successful amplification throughout the species range. A total of

5 primers were screened in this study, however, only primers 2, 6 and 11 were used for subsequent analysis since they provided the greatest reproducibility and consistency in fingerprint patterns.

RAPD fingerprinting of *Pleurotus* species was found to be variable between PCR reactions and when using samples from different extractions. As is evident in Fig. 6.4.1, although the fingerprints of *P.cystidiosus* 36253 and *P.ostreatus* 1020 between PCR reactions are very similar (lanes 3,4 and 7,8, respectively), the absence of certain bands and differences in band intensity can be observed. Simultaneous extractions of duplicate cultures using identical extraction buffers, resulted in variable DNA fingerprints of *P.cystidiosus* 36253 (Fig. 6.4.1 - lanes 2 and 3).

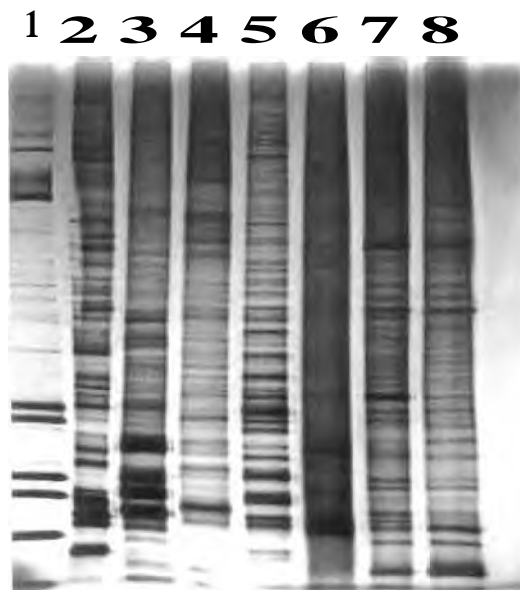


Fig. 6.4.1 RAPD fingerprinting patterns of various species of *Pleurotus* using primer 2. illustrating variability associated with different extractions and amplification reactions. pBR 3221Hinf I marker (Lane 1); different extractions of *P.cystidiosus* 36253 (lanes 2 and 3); different amplifications of identical extracts of *P.cystidiosus* 36253 (Lanes 3 and 4), *P.cystidiosus* 36168 (Lanes 5 and 6) and *P.ostreatus* 1020 (Lanes 7 and 8).

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<sup>1</sup>Please Note: Marker (Lane 1) contaminated by overspill from Lane 2 during the loading of the gel.

A representative number of fingerprint patterns obtained using the three arbitrary primers previously described (Section 6.3.1.3) are shown in Fig. 6.4.2 (Primer 2), 6.4.3 (Primer 11) and 6.4.4 (Primer 6). Primer-specific fingerprints were obtained when using the different primers. With a particular primer, dramatically different fingerprints were obtained throughout the species range, indicating a high degree of polymorphism, and therefore a large sequence diversity, in *Pleurotus*. The fingerprints obtained were strain specific and were not conserved, even amongst sub-species.

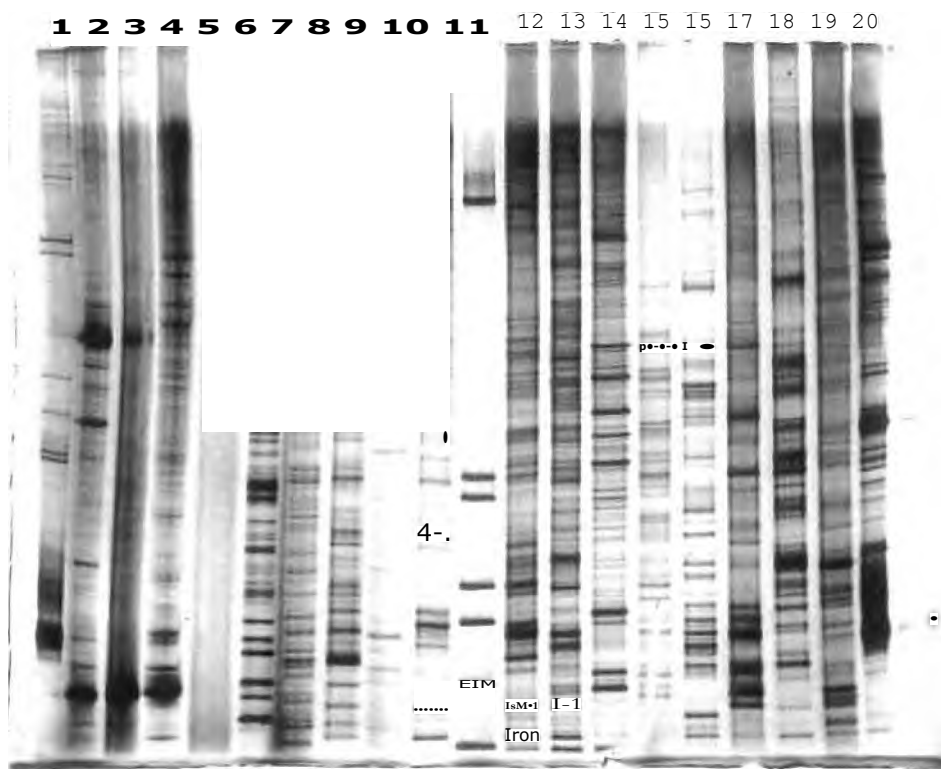
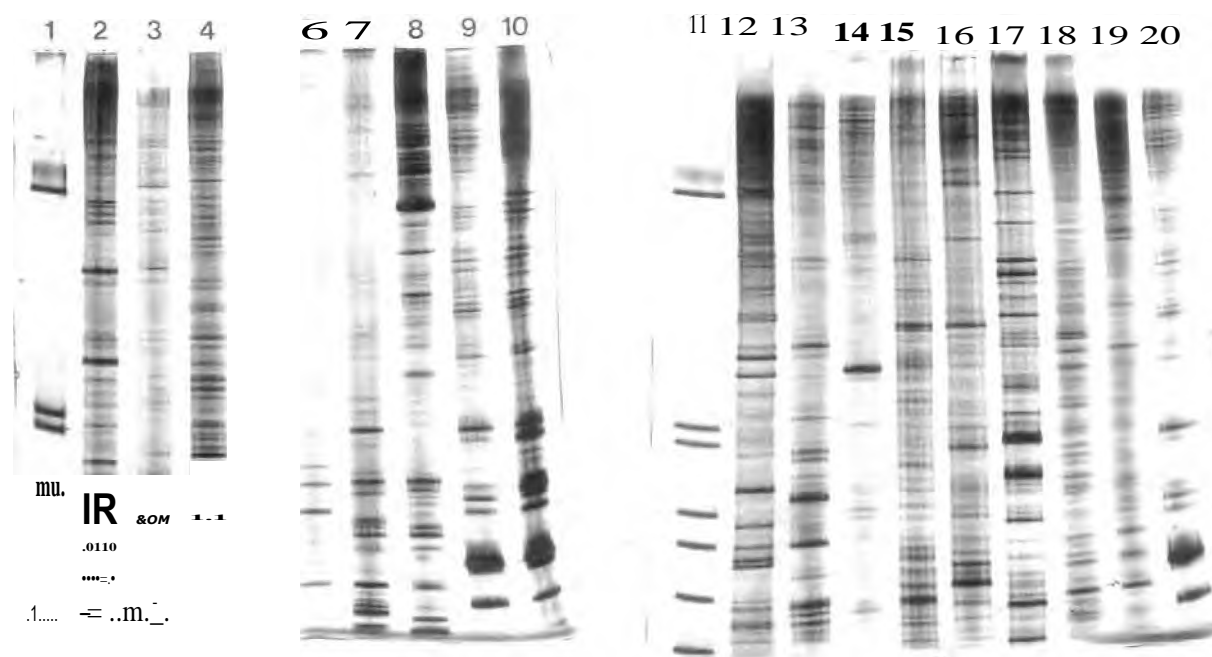


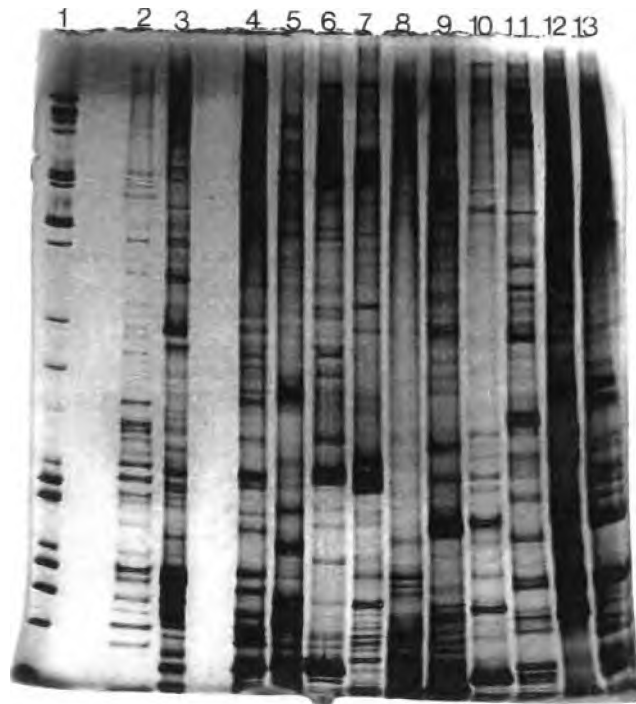
Fig. 6.4.2

The use of Primer 2 in the generation of RAPD fingerprints of numerous *Pleurotus* species and *L edodes* strains. From the left: 1-*Ledodes* 48860; 2-*P.ostreatus* 3344; 3-*P.ostreatus* 1833; 4-*P.ostreatus* 1020; 5-negative control; 6-*P.cystidiosus* 36223; 7-*P.cystidiclosus* 36131; 8-*P.cystidiosus* 36170; 9-*P.cystidiosus* 36172; 10-*P.cystidiosus* 36253; 11-Molecular mass marker (pBR 322/Hhlf 1); 12-*P.flahellatus* 36222; 13-*P.pulmonarius* 36095; 14-*P.calypo-crtus* 3621 I; 15-*P.citrinopileatus* 36239; 16-*P.incarnatus* 36228; 17-*Pluscus* var. *ferulae* 36214; 18-*P.cornucopiae* 36216; 19-*P.columbinus* 36215; 20-*L edodes* 58762.



**Fig. 6.4.3** Amplification of DNA from certain *Pleurotus* and *Ledodes* strains using Primer 11. 1-Molecular mass marker (pBR 322/ *Hinf* 1); 2-*P.ostreatus* 3344; 3-*P.ostreatus* 1020; 4-*P.cystidiosus* 36253; 5-*Pcystidiosus* 36131; 6-*P.cystidiosus* 36170; 7-*Pcystidiosus* 36372; 8-*P.flabellatus* 36222; 9-*P.pulmonarius* 36095; 10-*Ledodes* 58762; 11-Molecular mass marker (pBR 322/*Hinf* 1); 12-*P.calyptratus* 36211; 13-*P.citrinopileants* 36239; 14-*P. incarnatus* 36228; 15-*P.fuscus* var. *ferulae* 36214; 16-*P.cornucopiae* 36216; 17-*Peolumbinus* 36215; 18-*P.eryngii* 36037; 19-*L.edodes* 58762; 20-*L-edodes* 48860.

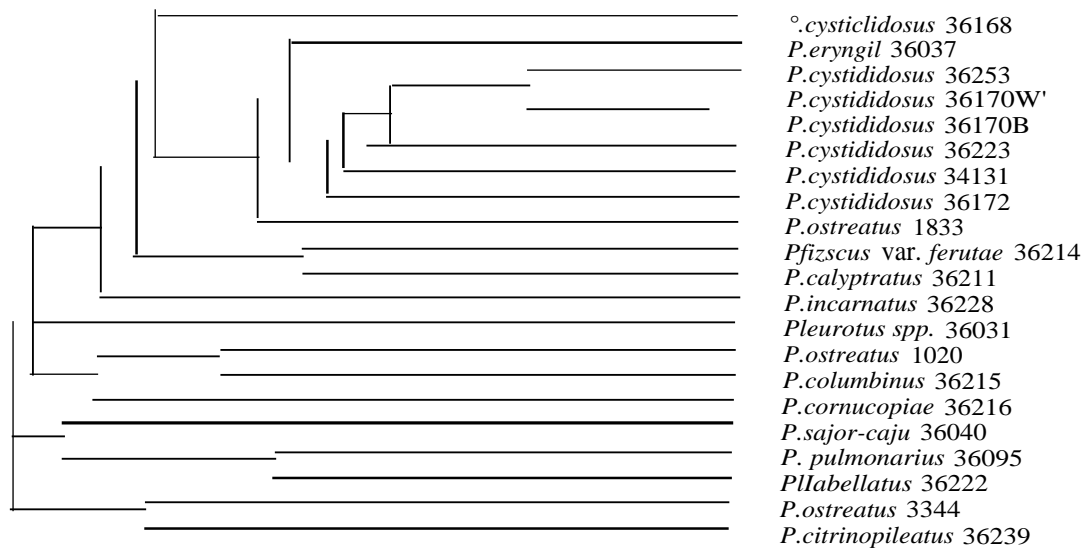
A number of other fungal species (*Ledodes* and *P.notatum*) were included in the study to determine whether the *Pleurotus* group forms a large cluster separate from these 'unrelated' species. As can be seen from Fig. 6.4.2 to 6.4.4, the DNA profiles of these unrelated species did not vary noticeably from those of the *Pleurotus* group. Furthermore, UPGMA analysis did not separate the *Pleurotus* group from these species (results not shown).



**Fig. 6.4.4** DNA amplification of numerous fungal species using Primer 6. From the left: 1- Molecular mass marker (pBR 322I<sub>Hinf</sub> 1); 2-*P.ostreatus* 3344; 3-*P.ostreatus* 1833; 4-*P.cystidiosus* 36170B; 5-*P.eystidiosus* 36170W; 6-*P.cystidiosus* 36253W; 7-*P.cystidiosus* 36172; 8-*P.puirnonarius* 36095; 9-*P.citrinopileatus* 36239; 10-*P.cornucopiae* 36216; 11-*P.columbinus* 36215; 12-*P.kurotus* spp. 36031; 13-*P.notatum*.

Since RAPD analysis indicates differing banding patterns for the various *Pleurotus* species investigated, it can be used successfully for the differentiation of members of this genus. DNA-profiles for each of the species were analyzed by UPGMA and the dendograms obtained for primers 2 and 11 are shown in Fig. 6.4.5. The dendogram obtained from primer 6 did not indicate consistent groupings with primers 2 and 11 or with the literature and therefore is not presented. Although the dendograms indicate species relatedness, they further signify the high genetic variability between members of this genus.

The dendograms obtained from these two primers vary greatly, with primer 2 indicating relationships which are more consistent with the current taxonomic status of the species. Certain strains group together consistently between primers, e.g. *P.ostreatus* 1020 and *P.columbinus* 36215; *P.pulmonarius* 36095 and *P.flabellatus* 36222 (Fig. 6.4.5). Primer 2 analysis indicates a separate *P.cystidiosus* cluster (with the exception of strain 36168 which is separated from the cluster by *P.eryngii* 36037), whereas in the alternative dendogram this is not evident. Isolates of *P.cystidiosus* 36253 and 36170 which vary in cultural characteristics (asexual conidia production) group very closely together. *P.ostreatus* sub-species do not group together, except for strains 1833 and 3344 in primer 11; however, the degree of similarity is low.



13

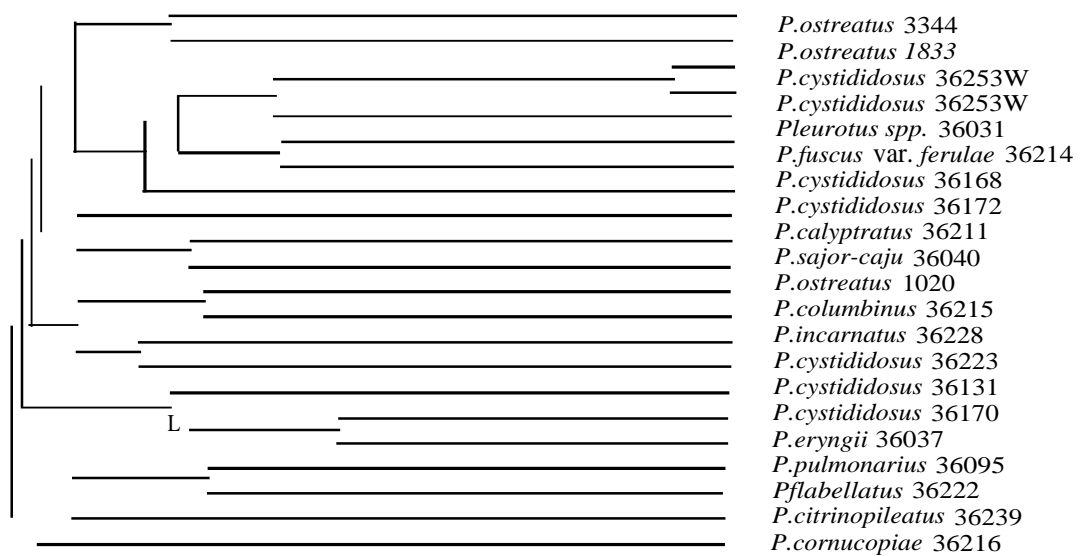


Fig. 6.4.5 Relationships among *Pleurotus* species as visualized by UPGMA clustering:(A) Primer 2; (B) Primer 11. W indicates cultures lacking in conidia production and B, cultures in which conidia are produced.

## 6.5 DISCUSSION

RAPD analysis of numerous *Pleurotus* strains has indicated a high level of variability, which is consistent with previous reports (Royse and May, 1993; Vilgalys and Sun, 1994; Iracabal *et al.*, 1995). This high variability suggests an ancient evolutionary divergence across this group (Vilgalys and Sun, 1994).

Primer specific fingerprints were obtained (Fig. 6.4.2, 6.4.3 and 6.4.3), which is in agreement with the literature (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anolles *et al.*, 1991; Khush *et al.*, 1992; Chiu *et al.*, 1993; Kaukas *et al.*, 1994). This is to be expected when considering the variability in template/primer interactions, depending on the primer sequence. Furthermore, one would expect variable clustering since the degree of polymorphisms present will vary with the sequence being targeted. Clustering with greater reliability could be achieved by the pooling of all DNA profiles obtained from the different primers, since a wider DNA range would be considered. Pooling of primer data---was, however, not possible with the analytical system used in this study.

The taxonomic status of *Pleurotus* has recently been reviewed (Rajaratnam and Bano (1987) and Buchanan (1993)) and has been determined by the collective use of numerous techniques, including macroscopic observations and intercompatibility studies. Some of the data obtained in this study is consistent with these reviews.

A "group" consisting of *P.ostreatus*, *P.pulmonarius*, *P.sapidus*, *P.florida* and *P.columbinus* is at the centre of much controversy based on whether to differentiate them as separate species, or to group them together as *P.ostreatus* with the current species being varieties thereof (Rajaratnam and Bano, 1987; Buchanan, 1993; Peng *et al.*, 1990; Peberdy *et al.*, 1993). The confusion is based on morphological similarities or dissimilarities and compatible or incompatible reactions between isolates of the individual species. In this study *P.ostreatus* 1020 and *P.columbinus* 36215 consistently grouped together; however *P.ostreatus* 3344, which is a 'Florida' (DSM Catalogue) strain, did not form part of this group (Fig. 6.4.5). Furthermore, *P.pulmonarius* 36095 did not group with any of the *P.ostreatus* strains; however it consistently clustered with *P.flabellatus* 36222 which does not correlate with previous

reports (Iracabal *et al.*, 1995). In the taxonomic analysis of various *Pleurotus* species, it has been found that *P.pulmonarius* and *P.sajor-caju* form a separate, related group (Iracabal *et al.*, 1995). Although the same relationship is evident in dendrogram 6.4.5(A), it is of a very low genetic similarity.

*P.citrinopileatus* and *P.cornucopiae* are intercompatible (Buchanan, 1993) and consequently the former has been named *P.cornucopiae* var. *citrinopileatus* (Peng *et al.*, 1990). However, in this investigation, as in other similar studies (Vilgalys and Sun, 1994), these two strains did not group together (Fig. 6.4.5).

The morphological characteristics of the fruiting bodies produced by *Plitscus* var. *ferulae* Lanzi (36214) are very similar to those produced by *P.eryngii* (36037) (Peng *et al.*, 1990). Consequently *P.fuscus* var. *ferulae* Lanzi should actually be named *P.eryngii* (DC:Fr) Quel.var.eryngii (Peng *et al.*, 1990). The dendograms obtained from the research conducted (Fig. 6.4.5), however, do not group these two strains together indicating the limitation of macroscopic characterization of *Pleurotus* strains.

Although a separate *P.cystidiosus* group is formed when amplification with primer 2 is conducted, this is not the case with the use of primer 11; however, certain strains still group together (Fig. 6.4.5). *P.cystidiosus* produce conidia on agar and sawdust media (Peng *et al.*, 1990). However, it has been discovered in this study that repeated sub-culturing results in the loss of this characteristic. The amplification of both 'variants' of *P.cystidiosus* 36253 and 36170 results in very close groupings (between 90 and 95%) (Fig. 6.4.5) which illustrates the internal consistency of the experimental methodology used. The absence of asexual spore production, therefore, does not appear to be caused by large scale genetic rearrangements, such as deletion, but rather by a single or few mutations or in response to environmental factors.

In primer 11 analysis *P.ostreatus* strains 1833 and 3344 group together, as do *P.ostreatus* 1020 and *P.columbinus* 36215 (Fig. 6.4.5). This is consistent with their comparable productivities on sawdust media (see Chapter 4). This implies that the efficiency of the cultivation process is greatly dependent on the genetic characteristics of the strains under

investigation, which is in agreement with the literature on other edible mushrooms (Diehle and Royse, 1986; Royse and Bahler, 1986; Rinker, -1991).

A number of reasons, apart from the inherent variability between *Pleurotus* species, could be responsible for the results obtained; namely the analytical method employed and the origins of the strains used. RAPD analysis is greatly dependent upon the data capturing equipment used if this is inadequate, then unreliable results are obtained. The reliability of RAPD results are also highly dependent upon band inclusion during analysis (Clark and Lanigan, 1993; Tham *et al.*, 1994). In the analysis performed by GelManager, relationships between species were inferred from both shared absences and presences; however, the absence of a band does not necessarily imply similarity. It has been discovered that species specific groupings occur only when strains from identical locations are used (Vilgalys and Sun, 1994). This could explain the inconsistent grouping in this study, and in general the taxonomic disarray of *Pleurotus* species.

## 6.6 CONCLUSION

RAPD analysis does not provide an overall taxonomic structure for the genus *Pleurotus* since the differences between species is too great. AP-PCR, therefore, does not have potential for higher taxonomic studies of *Pleurotus*; mitochondrial or nuclear sequencing, as well as RFLP analysis, should be appraised for this purpose.

RAPDs can, however, be used for strain identification, the elimination of duplicate strains and the assignment of *Pleurotus* to crude sub-species levels, if appropriate reference strains are available. Furthermore, RAPDs could possibly be used in breeding studies since a relationship has been found between genetic relatedness and comparable productivity on sawdust media. In traditional breeding programs it can be used in preventing crosses between two strains which are genetically identical but which have been inappropriately named, and in indicating strains which are most genetically dissimilar and therefore which are most likely to vary at loci which control quantitative traits such as production characteristics.

## CHAPTER SEVEN

## FINAL DISCUSSION AND CONCLUSION, AND FUTURE RECOMMENDATIONS

## 7.1 GENERAL DISCUSSION AND CONCLUSION

South Africa is a source of vast quantities of lignocellulosic wastes which currently have no economic use. A number of these materials were considered as substrates for the cultivation of edible mushrooms, since this technology represents one of the most commercially feasible processes for the utilization of waste lignocellulose. Edible mushroom cultivation is a particularly attractive option in developing countries such as South Africa, which have high unemployment rates and large rural communities. The application of this technology, which is essentially rurally based and highly labour intensive, would facilitate in overcoming the movement towards urbanization and would promote the generation of wealth in those rural communities where unemployment and poverty is a major problem. The application of mushroom cultivation in such communities, however, requires extensive training programmes to enable the development of small scale industries ensuring the efficient use and management of local resources.

This study has indicated that speciality mushroom cultivation is a potential subsidiary industry for South Africa. A consideration of the cost and profitability of the process is, however, necessary in determining its feasibility and therefore applicability. The establishment of a mushroom cultivation facility requires high initial investment costs. Areas in which sufficient ventilation occurs and in which temperature, humidity and lighting conditions can be controlled, are required. Furthermore, equipment which can be used for the microbial control of the substrate prior to inoculation is necessary, e.g. a sterilizing unit. Once the production facility has been established, the factor having the most profound effect upon the profitability of the process is the yield produced. This is predominantly determined by the interaction between the substrate/strain combination used and the environmental conditions implemented.

This investigation has indicated the importance of optimizing the substrate/strain combination used, from the initial *in vitro* propagation of the strain to the final inoculation onto the production substrate. Intra- and interspecies specific variability in growth on different artificial media, and growth and productivity on various lignocellulosic substrates was found

in this study. The selection of a production substrate based on negative or low value, locally available lignocellulosic materials is beneficial. This study has predominantly concentrated on wood sources widely available in the Eastern and Western Cape, namely Maranti (*Shorea spp.*) and Port Jackson willow (e.g. *A.longifolia*). Both wood sources have advantages and disadvantages associated with their use as substrates in mushroom cultivation. Maranti sawdust and woodshavings, a waste product of the timber industry, can be easily applied to edible mushroom cultivation. Its use is, however, subject to availability and the establishment of a reliable supplier. Transportation and storage costs are associated with the cultivation of mushrooms on this substrate. Furthermore, this study has indicated that the number of fungal species capable of producing yields on this substrate was limited (9 out of 18) and of those that were productive, erratic and low yields were produced. The yield potential and therefore utility of this substrate in mushroom cultivation could be increased by the identification of strains tolerant to the chemicals incorporated into it to ensure its functionality in the timber industry. Port Jackson willow is an exotic invasive weed which is widely dispersed in the Western and Eastern Cape; therefore availability in the rural areas would not be a problem, hence eliminating the need for transportation and storage. This plant species, which is difficult and costly to eliminate, causes the encroachment of valuable agricultural land and therefore its utilization in edible mushroom cultivation would convert a liability into an important component of a potentially profitable industry. Port Jackson willow proved to be a better substrate than Maranti in speciality mushroom production (9 of the 18 species studied produced mushrooms). There is, however, an important factor limiting its use: the cost of felling the trees and chipping them into sizes which are optimal for use in edible mushroom cultivation. This added cost would affect the profitability of the process since it would increase the initial investment cost of setting up a mushroom production facility.

Neither *L.edodes* nor *F.velutipes* produced fruiting bodies when cultivated on Port Jackson and Maranti based media. A number of the fourteen *Pleurotus* species investigated were successfully cultivated, however, with variable efficiency depending on the strain/substrate combination used. Yields on Port Jackson were generally higher and less erratic than on Maranti. From this study the strains most suitable for cultivation on Port Jackson are *P.ostreatus* strains 3344 and 1833 and *P.pulmonarius* 36095. These strains could therefore be used in the cultivation of oyster mushroom under South African conditions.

Biochemical and genetical studies were performed in an attempt to understand the variability in growth and yield production of various strains. Biochemical studies of *Pleurotus spp.* and *L.edodes* have indicated that intra- and interspecies specific variability in quantitative and qualitative enzyme production occurs. This can be loosely correlated with variability in the yield and quality of mushrooms produced by the different *Pleurotus* strains. Furthermore, genetical studies using RAPD fingerprinting have identified the genus *Pleurotus* as being highly variable, which explains the great variability in fruitbody production potential under the different substrate conditions investigated. A relationship has been observed between groupings produced from cluster analysis of the different RAPD profiles, and strains which showed similar productivities on the different lignocellulosic substrates considered. This investigation has therefore indicated the potential value of using biochemical and genetical studies as a means of acquiring a better understanding of the biology of mushroom cultivation and an indication of the productive potentials of certain strains, if appropriate reference strains are employed.

In conclusion, edible mushroom cultivation on locally available lignocellulosic materials could potentially become established as a highly lucrative industry in Southern Africa, if an appropriately productive substrate/strain combination is used and if cultivation is conducted under optimal environmental conditions.

## 7.2 FUTURE RECOMMENDATIONS

The increased economic viability of the cultivation process described in this study requires a more comprehensive understanding of the biology of edible mushroom cultivation. This necessitates extensive research on enzymatic reactions occurring, and the importance of these, in lignocellulosic degradation. Furthermore, a better understanding of the impact of wood chemistry in fungal degradative potential, and therefore on fungal growth and yield production, is required. A comprehension of the influence of various environmental factors, e.g. light, on the different stages of edible mushroom cultivation would assist in the optimization of the process. Lastly, genetical studies are powerful vehicles for improving the production potential of strains. Studies aimed at identifying the genes coding for the various enzymes involved in lignocellulose degradation and their expression, would facilitate in mutational studies aimed at increasing their activity levels.

**APPENDIX A**  
**SYNTHETIC MEDIA**

*1. IN VITRO GROWTH MEDIA*

The formulations presented below were obtained from the ATCC and DSM Strain Catalogues, except for that of GMPY medium. The broth media were prepared as described below, however, agar was excluded from the formulations. All media was made up to volume using distilled water.

**1.1 M3 media (g/l)**

Malt Extract	24.0
Yeast Extract	5.0
Commercial Agar	15.0

**1.2 Malt agar (MA) (g/l)**

Malt Extract	30.0
Bacto-agar	15.0

**1.3 Yeast-glucose media (YG) (g/l)**

Yeast Extract	5.0
Glucose	20.0
Commercial Agar	15.0

**1.4 SMPY media (g/l) (Kulkarni, 1990)**

Sucrose	10.0
Malt Extract	10.0
Peptone	5.0
Yeast Extract	5.0
Commercial Agar	15.0

**1.5 MEPA media (g/l)**

Malt Extract	10.0
Soya Peptone	3.0
Commercial Agar	15.0

**1.6 Malt extract agar (Blakeslee's formula) (B-MEA) (g/l)**

Malt Extract	20.0
Glucose	20.0
Peptone	1.0
Commercial Agar	20.0

**1.7 GMPY media (g/l)**

Malt Extract	11.3
Yeast Extract	3.3
Soya Peptone	1.0
Glucose	6.7
Commercial Agar	15.0

**2. ENZYME SENSITIVE MEDIA****2.1 Lignin Agar (LA) (g/l) (Hedger, 1982)**

Sucrose	30.0
NaNO <sub>3</sub>	2.0
KCl	0.5
KH <sub>2</sub> PO <sub>4</sub>	1.0
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5
FeSO <sub>4</sub>	0.1
Yeast Extract	0.5
Lignin*	0.5
Commercial Agar	15.0

The lignin which was used was Indulin AT (Westvaco Polychemicals).

2.2 Acid-swollen cellulose agar (ASCA) (g/l) (Tansey, 1971 cited in Hedger, 1982).

2.2.1 Acid-swollen cellulose

The entire preparation procedure was conducted at 4°C. A 30 g amount of air-dried Whatman cellulose powder was added to 400 ml of 88% orthophosphoric acid and the mixture stirred for 2 hours. 2 l of distilled water was then added to the mixture. The suspension was filtered through five layers of cheesecloth and thereafter two layers of Whatman Number 1 filter paper. The cellulose residue was resuspended in 2 l of distilled water and filtered as previously described. The resultant residue was then resuspended in 1 l of 2% Na<sub>2</sub>CO<sub>3</sub> and homogenized for 5 minutes in a Waring Blender. The homogenate was stored at 4°C for 12 hours, washed with 5 l distilled water and filtered once again. The residue was further clarified by centrifugation (10 000 rpm x 5 minutes) and the pellet homogenized for 5 minutes (pH value of 6.5±0.1). The wet acid-swollen cellulose was dried to constant weight at 80°C and used in the preparation of ASCA.

2.2.2 Preparation of acid-swollen cellulose agar.

NH <sub>4</sub> H <sub>2</sub> P O <sub>4</sub>	2.0
KH <sub>2</sub> P O <sub>4</sub>	0.6
K <sub>2</sub> HP O <sub>4</sub>	0.4
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.8
Thiamine	0.0001
Yeast Extract (Biolab)	0.5
Adenine	0.004
Adenosine	0.0008
Acid-swollen cellulose	5.0
Commercial Agar	17.0

The above components were mixed and 10 ml volumes dispensed into test tubes prior to sterilizing at 121°C for 15 minutes. The settling out of cellulose particles was prevented by rolling the tubes as the agar cooled.

**APPENDIX B****SPAWN AND SUBSTRATE PREPARATION****1. Spawn preparation****1.1 Sawdust/plug spawn** (San Antonio, 1981)

Wheat bran	30.0 g
Oak sawdust	120.0 g
Oak wood chips	30 (1x2x2 cm, 1.5 cm thick)

The components were placed in 1 l home-canning glass Consol jars (7.5 cm diameter x 15 cm length). The spawn substrate was then sterilized at 100°C for 90 minutes followed by autoclaving at 121°C for 90 minutes.

**1.2 Grain/sawdust spawn** (adapted from Diehle and Royse, 1986)

Sawdust	10.4 g
White millet	200.0 g
Wheat bran	2.6 g
dH <sub>2</sub> O	87.0 ml

The spawn substrate components were placed in 500 ml Erhlenmeyer flasks and autoclaved for 45 minutes.

**1.3 Nutritive media** (g/l) (Kostadinov *et al.*, 1972).

Cane molasses	50.0
Ammonium nitrate	3.0
Ammonium sulphate	1.0
Potassium phosphate	1.0

The ingredients were placed in Erhlenmeyer flasks and sterilized at 121°C for 45 minutes.

## 2. Sawdust media

### 2.1 Mee, 1978 (*L.edodes*)

Wood source	85.0%
Potato starch	8.5%
Yeast powder	2.1%
CaSO <sub>4</sub> .2H <sub>2</sub> O	4.2%
Water	600.0 ml

### 2.2 Mee, 1978 (other mushroom species)

Wood source	80.0%
Potato starch	9.0%
Yeast extract	9.0%
Sucrose	2.0%
Water	600.0 ml

### 2.3 Patrick *et al.*, 1983

Wood source	80.0%
Wheat bran	17.0%
Sugar cane molasses	1.0%
Yeast extract	1.5%
Calcium carbonate	0.2%

Water was added to provide a total substrate moisture content of between 60% and 65%

### 2.4 Cultivation medium adapted from Rinker, 1991

Wood source	77.0%
Sucrose	1.2%
Wheat bran	10.9%
White millet	10.9%

Water was added to provide a 1:1.2 ratio of substrate to water (dry weight basis).

**2.5 Cultivation formulation obtained from R.Kirby (Pers. comm.)**

Wood source	77.0%
Sucrose	1.0%
Wheat bran	20.0%
CaCO <sub>3</sub>	1.5%
Fertilizer [3:2:1 (28): N:P:K]	25.0%

Water was added to provide a 1:1.2 ratio of substrate (dry weight basis) to water.

**APPENDIX C****REAGENTS USED FOR PROTEIN DETERMINATIONS****1 Reagent A**

10% (w/v)  $\text{Na}_2\text{CO}_3$  in 0.5 N NaOH

**2 Reagent B**

1% (w/v)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in distilled water

**3 Reagent C**

2% (w/v) potassium tartrate in distilled water

**4 Reagent D**

0.03% BSA in distilled water

**5 Reagent E**

5 ml of 2 N Folin Ciocalteu reagent in 50 ml distilled water (freshly prepared).

**APPENDIX D****STANDARD METHODS, REAGENTS AND BUFFERS FOR DNA STUDIES****1. DNA Extraction Buffers****1.1 TE Buffer** (Khush *et al.*, 1992)

Tris-HCl (pH 8.0)	50 mM
EDTA	10 mM

**1.2 Extraction Buffer** (Khush *et al.*, 1992)

Tris-HCl (pH 8.0)	100 mM
EDTA	50 mM
NaCl	500 mM
Sodium bisulfate	20 mM

**1.3 RNase Preparation** (Sambrook *et al.*, 1989)

Pancreatic RNase A was dissolved, at a concentration of 10 mg/ml, in a solution containing 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. The solution was heated to 100°C for 15 minutes and slowly cooled to room temperature. The RNase was stored at -20°C until required.

**1.4 Phenol preparation** (Sambrook *et al.*, 1989)

Phenol was melted at 68°C, and hydroxyquinoline was added to a final concentration of 0.1%. An equal volume of 1 M Tris-HCl (pH 8.0) was added and the solution mixed for 15 minutes. The aqueous layer was removed, once phase separation had occurred, and the process repeated with 0.1 M Tris-HCl (pH 8.0) until the pH of the phenol was below 7.8. The phenol was stored under 0.1 M Tris-HCl at 4°C until required. The storage time was limited to one month.

## 2 Polyacrylamide Electrophoresis (PAGE)

The method used was similar to the Laemmli system with the exception that TBE was used as the running buffer (adapted by Mr C. Bezuidenhout; Pers. comm.). Basic procedures for the preparation of solutions and buffers used in PAGE are described in Sambrook *et al.*, 1989. All chemicals were supplied by Sigma, unless otherwise specified.

Tall<sup>™</sup> Mighty Smalls from Hoefer Scientific Instruments were used to perform slab polyacrylamide gel electrophoresis of the RAPD products.

### 2.1 Solutions and Buffets

#### 2.1.1 Acrylamide Stock Solution

Acrylamide	30.0%
NN-methylene-bis-acrylamide	0.5%

The above chemicals were dissolved in distilled water and stored at 4°C until required.

#### 2.1.2 Resolving Gel (10%)

1.5 M Tris-HCl (pH 8.8)	9.0 ml
Acrylamide stock	12.0 ml
TEMED (Merck)	20.0 $\mu$ l
10% Ammonium persulphate	300.0
10% SDS	<b>1.4 ml</b>
dH <sub>2</sub> O	13.6 ml

#### 2.1.3 Stacking Gel (6%)

0.5 M Tris-HCl (pH 6.8)	<b>9.0 ml</b>
Acrylamide stock	<b>6.0 ml</b>
TEMED (Merck)	20.0 $\mu$ l
10% Ammonium persulphate	300.0 $\mu$ l
10% SDS	<b>1.4 ml</b>
dH <sub>2</sub> O	<b>19.6 ml</b>

#### 2.1.4 **Sample Buffer**

Bromophenol Blue	0.25%
Sucrose	- 40.0%
EDTA	0.1 M

#### 2.1.5 **TBE Buffer (5x solution)**

Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20.0 ml

#### 2.1.6 **Marker preparation (Sambrook *et al.*, 1989)**

pBR 322	5.0 µl
<i>Hinf</i> 1	10.0 ul
Buffer B (10x)	10.0 ul
dH <sub>2</sub> O	75.0 ml

The mixture was incubated at 37°C for 2 to 5 hours and the reaction stopped with 25 µl sample buffer.

## 2.2 **PAGE procedure**

The gel apparatus was assembled according to the manufacturers' instructions and the resolving and stacking gels allowed to polymerise separately. RAPD products were diluted in sample buffer, loaded and electrophorised in TBE buffer at 150 V for 2 hours (or until the dye front reached the gel end). All gels had a marker (pBR 322 digested with *Hinf* 1) incorporated which acted as a reference strain.

## 3 **Silver Staining (Quigen protocol)**

### 3.1 **Buffers**

#### 3.1.1 **Buffer A**

Ethanol	10.0%
Acetic acid	0.5%

**3.1.2 Buffer B**

AgNO <sub>3</sub>	0.1%
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This buffer is reusable.

**3.1.3 Buffer C**

NaOH	1.5%
NaBH <sub>4</sub>	0.01%
Formaldehyde	0.15%

This buffer was prepared immediately before use.

**3.2 Silver staining protocol** (adapted from the Quigen protocol for TGGE)

The gel was removed from the gel mould and submerged in Buffer A for 6 minutes. The solution was discarded and the gel covered with Buffer B for 10 minutes. The gel was then washed twice with double distilled water and developed in Buffer C for 20 to 30 minutes. Incubation with all buffers were conducted under linear agitation conditions.

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