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Genetic Studies on the Edible Mushrooms *Lentinula edodes* and *Pleurotus* Species

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ABSTRACT

Classical and molecular genetic techniques were investigated as approaches to strain improvement in the edible Basidiomycete species *Lentinula* (syn. *Lentinus*) *edodes* (Berk.) Pegler (the Shiitake mushroom) and *Pleurotus* spp. (oyster mushrooms). Central to the theme of breeding these species was the application of protoplasts. Attempts were made to develop a high efficiency isolation and regeneration system for both *L. edodes* and *P. sajor-caju*. Relatively low yields of protoplasts from *L. edodes* and their poor regenerative ability limited their use. However, *Pleurotus* spp. were found to be quite amenable to protoplast isolation, yielding constantly high yields of viable protoplasts.

Extensive mating-type analysis of monokaryotic isolates of *L. edodes* was carried out. Characterization of 17 commercial strains of *L. edodes* were found to have 9 different *A* factors and 10 different *B* factors, whilst four wild-type isolates from China were shown to have 8 different *A* factors and 8 different *B* factors. Analysis of several *L. edodes* strains revealed anomalous ratios of mating-type factors in basidiospore progeny which deviated from the expected 1:1:1:1. Four strains having this imbalance had several *A* and *B* factors in common. Protoplasts were used to obtain neohaplonts from dikaryotic mycelia, however, the ratio of the two nuclear types often deviated from the 1:1 ratio of the dikayon.

Transformation of *P. sajor-caju* was attempted by using several vectors containing bacterial dominant selectable genes under the control of fungal promotor and terminator sequences. Also, 'shot-gun' cloning of promotor sequences from *P. sajor-caju* was attempted by cloning random DNA fragments into a promotorless expression vector. No transformants were obtained by use of any of these vectors.

Molecular karyotyping by clamped homogeneous electric field electrophoresis of several *Pleurotus* spp. revealed differences between species, and strains of *P. sajor-caju*, in terms of chromosome size and number.

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DEDICATION

For my mother, Diana, and grandmother, Martie.

ABBREVIATIONS

a.i.= active ingredient
aq.= aqueous
5AC= 5-azacytidine
ATA= aurintricarboxylic acid
ATP= adenosine triphosphate
bp= base pairs
BSA= bovine serum albumin
CHEF= clamped homogeneous electric field
CTAB= cetyltrimethylammonium bromide
CIP= calf intestinal phosphatase
CLPS= chromosome length polymorphisms
CM= complete medium
CMC= carboxymethyl cellulose
cont.= continued
DMSO= dimethyl sulphoxide
DNA= deoxyribonucleic acid
DTT= dithiothreitol
EDTA= diaminoethantetra-acetic acid
EGTA= ethyleneglycol-bis-(ß-aminoethyl ether) N, N, N', N'tetraacetic acid
Expt.= experiment
5-FI= 5-fluoroindole
Fig.= figure
g= gravity
h= hour
l= litre
Kb= kilobase
LMM= *Lentinula* minimal medium
LSA= lignin sulphonic acid
Mb= megabase
MIC= minimum inhibitory concentration
min= minutes
MM= minimum medium
MYG= malt yeast extract glucose medium
PEG= polyethylene glycol
PFG= pulsed-field gel electrophoresis
pfu= plaque forming units
PMA= potato malt extract agar
RFLP= restriction fragment length polymorphism
RNA= ribonucleic acid
rpm= revolutions per minute
RMYG= regeneration MYG medium
sec= second
SDS= sodium dodecylsulphate
SDW= sterile distilled water
spp.= species
TAE= tris-acetate buffer
TBE= tris-borate buffer
TE= tris-Na_2EDTA buffer
V= volts
VLP= Virus Like Particles
YACs= yeast artificial chromosomes

1. General Introduction

1.1 Cultivation of Edible Fungi

The popularity of the common mushroom *Agaricus bisporus* (Lange) Imbach in Europe and North America, and commercial rewards that have been gained from it over the past century have aroused interest in the cultivation of other forms of edible fungi in these parts of the world. Mushrooms have been seen to be important micro-organisms in the re-cycling of agricultural and industrial wastes (Kirk & Chang, 1981; Bisara & Madan, 1983; Nair, 1983; Schmidt, 1986). Medicinal values of mushrooms is also reported (Cochran, 1978; Claydon, 1985) and the increase in demand for the Shiitake mushroom, particularly in China and Japan, is due in part to the publicity given to the apparent health giving benefits of eating this fungus. What was once considered a delicacy has now become a common foodstuff, and this has led to greater demand and higher production.

Of the edible species of fungi, the vast majority belong to the taxonomic group of the Basidiomycetes (true mushrooms) such as the previously mentioned *A. bisporus* (=*A. brunescens*), *Lentinula edodes* (Berk.) Pegler (=*Lentinus edodes* (Berk.) Sing.) or "Shiitake", and species of *Volvariella* (the paddy straw mushroom) and *Pleurotus* (the oyster mushroom). Additional species such as *Flammulina velutipes* (=*Collybia velutipes*), *Pholita nameko*, *Auricularia* spp., (Jew's ear), *Coprinus comatus* (lawyers wig), *Hypholoma capnoides*, *Stropharia rugoso-annulata*, *Morchella* spp. (morel) and *Kuehneromyces mutabilis* are also cultivated to a lesser extent with the need to promote their individual characteristics (Chang & Miles, 1984). The popular wood blewit (*Lepista nudum*) and truffle (*Tuber* spp., belonging to the Ascomycetes) are yet to be domesticated.

In Europe *A. bisporus* and *P. ostreatus* are the two most important cultivated mushrooms. On a world wide production scale *L. edodes* comes second only to *A. bisporus*, the former being primarily cultivated in countries of South-east Asia (Royse *et al.*, 1985; Campbell & Slee, 1987; Mori, 1987). In 1983 it was estimated that nearly 83% of the total world production of Shiitake was in Japan (the farm value for the previous year reaching $538 million),

followed by China, Taiwan, South Korea and Thailand. Production in the U.S.A. and Canada combined accounted for less than 0.8% (Royse *et al.*, 1985).

Efforts to develop a more efficient, rapid and reliable system for production of the Shiitake mushroom have focussed on the use of enriched sawdust media for its cultivation. This has proved to be a more controlled method attracting much attention in Western countries, and involves growing the mycelium on a sawdust medium contained in polypropylene bags (Przybylowicz & Donoghue, 1988). Traditionally, Shiitake is grown on intact logs of *Quercus serrata* (oak) which requires several years for completion of the fruiting cycle (Harris, 1986). Synthetic substrates have proved to be very useful for cultivation although yield is very much dependent on the strain (Han *et al.*, 1981; Diehle & Royse, 1986; Royse & Bahler, 1986; Campbell & Slee, 1987; Miller & Jong, 1987). Many strains used for the outdoor log method have not proved to be successful under artificial cultivation. *Pleurotus* spp. have been found to have a much wider range of growth substrates which it can utilize. These include agricultural and industrial waste. although straw has proved to be the simplest to use (Zadrazil, 1978). Blocks of substrate are formed and inoculated with a suitable strain, and the type of substrate block used and its dimensions affect the yield and morphology of the fruit bodies obtained.

Efficiencies can be increased by breeding, adding to the genetic diversity of commercial cultures and improving conditions within the environment of the growth rooms (Miller & Jong, 1987). Limitations imposed by use of near monocultures such as with the common white mushroom, *A. bisporus*, have provided an example to growers of Shiitake who will hopefully avoid repeating this mistake with *L. edodes*.

1.2 Strain Characteristics

Requirements for superior mushroom strains could involve selection for any one of numerous traits which are influenced, in part, by responses to environmental stimuli and their underlying genetic controls. Examples include growth rate which is important for

resistance to competing micro-organisms, temperature requirements, the ability to produce extracellular enzymes for the degradation of the growth substrate, and fungicide sensitivity.

The quality of mushrooms which are developed relies on mushroom size and shape, cap and stipe thickness, and texture. Morphological features such as fruit body colour are known to vary with isolates of *A. arvensis* (Fritsche, 1978), *A. bisporus*, *L. edodes* (Liao, 1984), and *F. velutipes*, as are the number of abnormal fruit bodies which develop (Diehle & Royse, 1986). Li & Chang (1978) found differences in radial growth rates of fertile and non-fertile monosporus isolates from *V. volvacea*. However, growth rate in general is not linked to productivity nor to the time taken to fruit (Kinugawa & Hattori, 1974; Li & Chang, 1978; Liao, 1984; Przybylowicz & Donoghue, 1988).

Strain dependent temperature effects can influence vegetative growth (Mori *et al.*, 1974), primordium formation (Liao, 1984), and fruitbody induction in a number of species (Imbernon, 1983; Fultz, 1988; Han *et al.*, 1981). Some strains require cold shock treatment for fruiting to occur whilst others are not affected (Miller & Jong, 1987; Przybylowicz & Donoghue, 1988).

Shiitake cultivation on supplemented sawdust is known to show considerable variation within replicates of a trial and between repeated experiments (Royse *et al.*, 1985). The strain itself is thought to contribute to this effect and has been suggested as being a character which should be selected against. Thus screening during a breeding program could actually use statistical information i.e. "coefficients of variance" from replicates to eliminate any stocks that exhibit a high degree of variation (Royse & Bahler, 1986).

Selection for a high degree of wood decay by *L. edodes* when grown on beech wood was used by Tokimoto *et al.* (1987) to identify commercially viable strains. Strain response to substrate supplements was found to an important character with *Pleurotus* spp. (Royse & Schisler, 1987) as was the amount of "waste" biomass produced during crop harvesting (Imbernon & Labarere, 1989). In addition, the production of sporeless varieties is of great value to the producer

as spores from *Pleurotus* spp. are known to cause human allergies and is also practical in terms of strain protection (Eger, 1978) and mushroom production (Imbernon & Labarere, 1989).

General characteristics such as pinning ability, disease resistance, and production of good sized white fruitbodies were used by Stubnya (1978) as criteria for strain selection with *A. bisporus*. Viral resistance is an important character in the cultivation of *Agaricus* spp., however, resistant species such as *A. bitorquis* have the disadvantage of requiring higher temperatures for fruiting (Elliott & Langton, 1981). Sensitivity of *A. bisporus* to several fungicides has been found to vary with strains (Gandy, 1981) and thus the potential for obtaining resistance in this species could help in the control of fungal pathogens of the crop (Challen & Elliott, 1985).

1.3 Strain Breeding

As mentioned previously, synthetic substrates have proved to be very useful for the cultivation of Shiitake, however, the strain of *L. edodes* used is the major limiting factor affecting production. As with most mushroom species, little is known about how genotypes interact with the substrate regarding yield and quality. Exploitation of genetic variability between isolates is the key to developing desirable traits, and the following will outline the various means by which strain improvement can be carried out.

Classical Breeding Methods

Cultivation of edible mushrooms originally involved isolating cultures from the wild which were found suited to production methods and adopted accordingly (Sinden, 1981) or relied on natural colonization of the growth substrate (Ito, 1978). From here new strains were developed with the aim of improving on the original stocks to suit particular conditions or requirements of the grower, and demands by the consumer.

The first step to greater proficiency involves screening several lines for natural variation. Wild isolates of *Agaricus arvensis* (Fritsche, 1978) and *Lepista nuda* (Fritsche & van Loon, 1978) were found to show great differences when grown on compost under

commercial growth conditions. Han *et al.* (1981) found that only certain strains of *L. edodes* adapted well to a sawdust medium. *In vitro* studies of *Agrocybe aegerita* have also revealed variation in time taken to fruit and yield (Marmeisse, 1989). The best strains demonstrating cultivation requirements could thus be further exploited using a variety of methods.

Natural variation has proved to be an important technique for improvement of the secondary homothallic species, *A. bisporus*. This species has adopted a breeding mechanism that virtually eliminates the homokaryotic phase which is exploited when breeding other basidiomycetes. The white variety of the common mushroom originated from a spontaneous mutation which occurred earlier this century on the mushroom beds of a farmer in the U.S.A. (Sinden, 1981), which is an example of the fortuitous nature linked to breeding.

Fritsche (1972) was able to select for natural variation of single spores of *A. bisporus*, and to obtain several isolates which gave statistically higher yields to the parent. A similar approach was taken by Kneebone *et al.* (1974) to the breeding of a brown variety of *A. bisporus*, and by Li & Chang (1978) to the screening of isolates from *Volvariella volvacea*. Mixed spore suspensions of *A. bisporus* have also been used as a random means of obtaining strains with higher yields (Stubnya, 1978; Sinden, 1981).

It is interesting to note that early this century it was common cultivation practice with Shiitake to inoculate logs with a spore suspension so that colonization could proceed more reliably (Ito, 1978). Thus, farmers may have unknowingly carried out a certain degree of strain selection involving an intensive inbreeding method.

Probably the most widely adopted method of generating new strains involves the isolation of single spore progeny from the species of interest and carrying out crosses between homokaryons of the same strain (i.e. intrastrain) or different strains (i.e. interstrain) to give fertile heterokaryons. Such an approach may vary with species and the strains used, as work with Shiitake has proven. Miller & Jong (1987) found intrastrain hybrids to be "unstable" and greatly

inferior to the original parents. Inbreeding by repeated backcrossing to parents resulted in decreased ability to fruit with *S. commune* (Raper, 1985). Intensive intrastrain crossing does not maintain a level of heterozygosity which is necessary for particular "fruiting genes" to be active (Raper, 1985).

A more detailed study was presented by Tokimoto *et al.* (1987) which involved screening over 2000 intrastrain hybrids for their ability to degrade intact logs of oak and beech. Variation between different strains was encountered which led to the selection of nearly 370 of the best hybrids for further study. Dikaryons produced by outbreeding of *P. ostreatus* were found to yield fruitbodies of higher fresh and dry weight than inbreed strains (Eugenio & Anderson, 1968; Prillinger & Molitoris, 1979).

Strains of *L. nuda* have been selected for growth characteristics on compost by single spore selection and interstrain hybridization (Fritsche & van Loon, 1989). Differences in terms of colony growth rate and fruit body yield have been documented with intrastrain dikaryons of *F. velutipes* (Kinugawa & Hattori, 1974). In this study, variation was found to be attributed to the cytoplasmic background of the dikaryons which is one further variable to be considered when monokaryons are used as the starting material.

Thermotolerant strains of *Pleurotus ostreatus* have been selected using random matings, by mixing germinating spores from fruitbodies of different origins (Imbernon *et al.*, 1983). Controlled interstrain crosses have been applied to develop high temperature tolerant strains of *F. velutipes* with limited success (Fultz, 1988).

All of the cases cited above relied on crosses between random spore isolates , however, Pelham (1967) has emphasized the importance of isolating spores from basidia by micromanipulation. If germination of these progeny was 100% then products of meiotic events could be studied for segregation or linkage of agronomic characters.

An alternative to using two compatible spore isolates to obtain a hybrid involves what is known as "the Buller phenomenon".

Dikaryotization of a monokaryon occurs if challenged with a dikaryon that has component nuclei which are compatible to the monokaryon. This method is known as "di-mon mating" and takes advantage of the novel combination of nuclear components in the cytoplasmic background of the monokaryon. Thus, dikaryons with established characteristics can be further studied without the need to undergo a meiotic event involving recombination and possible spontaneous mutations. This approach has been used to develop hybrids of *L. edodes* for cultivation in areas with higher temperatures (Liao, 1984) and to obtain strains with novel morphological and physiological characteristics (Mori *et al.*, 1984). Modification of the di-mon method to allow more control over the process of heterokaryon formation may prove to be useful and will be discussed below.

Other Breeding Techniques

As mentioned above, the generation of homokaryons from basidiospore germlings results in a varied population. The potential use of homokaryons, however, lies with the ability to obtain homokaryons which is not always feasible for several species and non-sporulating strains. An alternative method of disassociating the component nuclei in a heterokaryotic cell is the formation of "neohaplonts". This technique has been employed for numerous species and involves the use of chemicals (eg. glycine, peptone, sodium taurocholate), microsurgery or protoplast isolation and regeneration (see Table 1.1).

A novel selection system for obtaining heterokaryons of *Aspergillus nidulans* involves introducing both auxotrophic and drug resistance mutations into one strain (Bradshaw & Peberdy, 1984). Forced heterokaryosis can then be followed by selection for prototrophic resistant colonies. These methods utilize the generally dominant, or semidominant, nature of drug resistance mutations but depend on their induction through mutagenesis. Fungicide resistance has been successfully obtained by mutagenesis of hyphal fragments from *A. bisporus* (Challen & Elliott, 1987) and *A. agereita* (Labarere *et al.*, 1989). Resistance to antimetabolites has been suggested to be

TABLE 1.1 Methods of Reported De-dikaryotization of Several Basidiomycete Species

SPECIES	METHOD(S) OF SEGREGATION*	REFERENCE
Agaricus bisporus	1	Dickhardt (1985)
	2, 3	Anderson *et al.* (1984)
	2	Sonnenberg *et al.* (1988)
Agaricus bitorquis	1	Dickhardt (1985)
Collybia velutipes	4	Kinugawa (1978)
	1	Takemaru (1964)
	1	Miles & Raper (1956)
Coprinus macrorhizus	1	Takemaru (1964)
Coprinus radiatus	3	Prud'homme (1965)
Flammulina velutipes	1	Leal-Lara & Eger-Hummel (1982)
Lentinula edodes	1	Takemaru (1964)
	7	Tokimoto *et al.* (1978)
	1	Nishibori & Kinguawa (1978)
Lenzites trabea	1, 4	Kerruish & DaCosta (1963)
Kuehneromyces mutabilis	1	Leal-Lara & Eger-Hummel (1982)
Laccaria bicolor	2	Kropp & Fortin (1985)
Phanaerochaete chrysosporium	2	Gold *et al.* (1983)
Pholiota nameko	1	Takemaru (1964)
	3	Arita (1978)
Pleurotus cornucopiae	1	Leal-Lara & Eger-Hummel (1982)
Pleurotus eryngii	1	Leal-Lara & Eger-Hummel (1982)
Pleurotus ostreatus	1	Leal-Lara & Eger-Hummel (1982)
	1	Miles & Raper (1956)
Polyporus abietinus	3	Fries & Aschan (1952)
Sistotrema brinkmannii	2	Anderson & Cenedese (1984)
Schizophyllum commune	1	Kerruish & DaCosta (1963)
	2	Wessels *et al.* (1976)
	1	Leonard *et al.* (1978)
	2	Raper (1978, 1983)

*Key to methods: 1= Chemical Means; 2= Protoplasts; 3= Mechanically; hyphal tips or fragmentation; 4= Oidia.

important to the identification of novel heterokaryons of *A. bisporus* and for effective disease control during cultivation (Miles & Chang, 1980; Elliott & Langton, 1981).

Mutagenesis has been used to induce non-sporulating mutations into *P. florida* (Chang *et al.*, 1985), *P. ostreatus* and *P. pulmonarius* (Imbernon & Labarere, 1989). In this later example, repeated outcrossing to homokaryons of different origins was carried out by di-mon matings to eliminated unwanted morphological characteristics.

Applications of Fungal Protoplasts to Strain Breeding

Somaclonal Variation

Natural variation found within a population of protoplast regenerants has potential in terms of strain improvement. This phenomenon is known as "somaclonal variation" and is a common feature of plant tissue culture and protoplast/regeneration systems, but little is known about this in fungi. In an extensive review of this subject, Larkin & Scowcroft (1981) consider that plant cell tissue culture induces genetic variability. Chromosomal and gene re-arrangements, transposable elements, and cryptic virus elimination are all considered to be mechanisms whereby plant somaclonal variation originates.

As mentioned above, there are few reports of such variation amongst fungal protoplast regenerants. In a study by Keller (1983) ergot peptide synthesis was not found to vary between the protoplast regenerants of *Claviceps purpurea* although protoclones with altered morphology were common. Protoplast regenerants of antibiotic producing strains of various *Streptomyces* spp. were found to vary in terms of antibiotic production, with up to three-fold increases in antibiotic production observed although some instability was observed in the second generation (Ikeda *et al.*, 1982). Similar instability was observed for secretion of several enzymes by regenerants of *Robillarda* sp. (Kuwabara *et al.*, 1989).

Resistance to streptomycin in sensitive strains of *Streptomyces griseus* was found to occur at a much higher frequency amongst protoplast regenerants than spores (Sugiyama *et al.*, 1983). Cytoplasmic variation was considered to cause the spontaneous loss of

a cytoplasmic incompatibility factor in *Podospora anserina* (Belcour, 1976). Spontaneous auxotrophic mutants have been isolated by de-dikaryotization methods, including protoplasts, from *P. salmoneo-stramineus* (Yoo *et al.*, 1987) and by mechanical methods with *Polyporus abietinus* (Fries & Aschan, 1952). Raper & Oettinger (1963) have suggested that mutations can occur in dikaryotic strains during long periods of continuous sub-culture, and somatic recombination is known to occur in several basidiomycetes (Ellingboe, 1965; Prud'homme, 1965; Shalev *et al.*, 1972), thus it is feasible that somaclonal variation can occur.

Fruiting trials using random dikaryotic protoplast regenerants from *P. ostreatus* were found to yield from 5.7-8.1% more in terms of fruit-body weight than the parent strain in five of the six regenerants tested. No variation was found in terms of stipe length and diameter, and pileus diameter (Magae *et al.*, 1985). This simple approach to strain improvement has much potential for breeding of edible mushrooms, although it has not yet been described for other mushroom species. Kawasumi *et al.* (1987) found a single clamp bearing isolate from protoplasts of a monokaryon of *L. edodes*. Although the fertility of this isolate was not mentioned, it was considered to be the result of a mutation in the mating-type factors.

In all these cases, deviation from the parental type was assumed to originate from genetic variation from within the population of cells isolated by the protoplast isolation technique. From the breeders point of view, protoplasts can be used to isolate single nuclei and the characters associated with it.

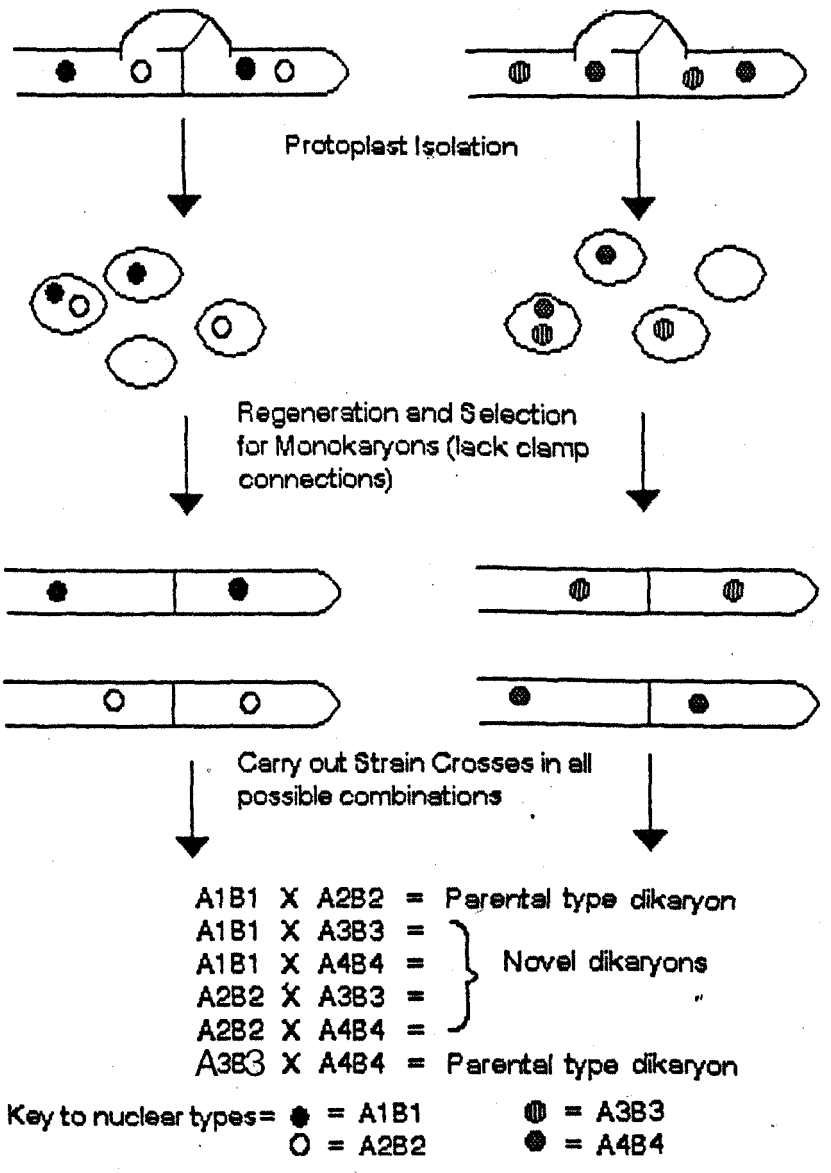
Breeding of the common mushroom *A. bisporus* is hindered by its life cycle (Elliott, 1985). Possibilities in the isolation of homokaryons lies in the release of protoplasts with single nuclei and their regeneration to give homokaryotic mycelia. This strategy has been adopted by Anderson *et al.* (1985) and Sonnenberg *et al.*, (1988) to give homokaryotic lines, and indeed 30-40% of protoplasts from *A. bisporus* have been shown to be uninucleate (N. Curtis pers. comm.).

Isolation of neohaplonts by protoplast regeneration has been applied to only a few species (see Table 1.1) although this application has not been investigated for the development of novel mushroom strains. If one considers an interstrain hybridization programme between two isolates with commercial characteristics, then the number of combinations and potential hybrids is limitless when basidiospore progeny are employed. Protoplasts, however, offer a more directed means of associating characteristics by obtaining monokaryons from the two heterokaryons. Assuming that each combination of neohaplont is compatible with the other then a total of four novel heterokaryons could be screened for required traits and compared to control strains formed by the re-association of the original nuclei (see Fig. 1.1). Success is dependent on full mating-type compatibility between the monokaryons obtained and is limited to a certain degree by the properties required being contained within one of the component nuclei. If the properties of a heterokaryon are dependent on the interaction of the two component nuclei then the products of meiosis should be utilized in a controlled way (Pelham, 1967).

Protoplast Mutagenesis

Treatment of a homogeneous population is an important prerequisite to any mutagenesis programme. Asexual spores are usually treated although hyphal fragments can be used when an organism has no known asexual stage, as is often the case with many Basidiomycetes. Difficulties encountered during induced mutagenesis of certain filamentous fungi can possibly be overcome by protoplast isolation/ reversion. Mutagenesis of *V. volvacea* yielded both auxotrophic and morphological mutants (Mukherjee & Sengupta, 1986). Auxotrophic mutants have also been obtained from *Pleurotus* spp. (Toyomasu *et al.*, 1986; Toyomasu & Mori, 1987a) and the antibiotic producing basidiomycete *Oudemansiella mucida* (Homolka *et al.*, 1988)

Fig. 1.1 Development of Novel Heterokaryons by Crosses Between Neohaplonts



Protoplast Fusion

Protoplast fusion is a powerful technique used to overcome barriers of genetic transfer between isolates of the same species (i.e. intraspecies) or between species (i.e. interspecies). The genetic hybrids produced are of particular value to industry in view to strain improvement of commercially important micro-organism (Ball, 1985). Recently, extensive research has been directed to the potential of protoplast fusion of edible mushrooms as an approach to selective breeding.

Fungal protoplast fusion is now widely induced by treatment of protoplasts with a solution of poly-ethylene glycol (PEG) in the presence of calcium chloride. Electrofusion has been used with the basidiomycete species *S. commune* (Sonnenberg & Wessels, 1986) and *P. ostreatus* (Magae *et al.*, 1986). Chemical fusogens cause random aggregation whereas greater control over the number of fusing protoplasts results with electrofusion. There are, however, difficulties using this procedure and optimum conditions need to be determined for each species.

The PEG-Ca^{2+} system is not fully understood in terms of the mechanism whereby fusion of protoplasts occurs. Calcium ions play a critical role in the fusion of *C. macrorhizus* protoplasts with a 70-fold increase in fusion frequency reported between auxotrophic strains (Kiguchi & Yanagi, 1985). Abe *et al.* (1982) also reported the enhancing effect of calcium ions on fusion of protoplasts from *T. matsutake*. The influence of calcium ions on the perturbation and re-organisation of lipid components has been reported (Cullis & Hope, 1978).

Kiguchi & Yanagi (1985) observed fusion events involving more than two protoplasts of *C. macrorhizus*, with multicellular fusion more common at higher concentrations of PEG. Fusion of *T. matsutake* was observed at a frequency of 1-10% and occurred more commonly between two protoplasts, however, PEG was absent. When treated with PEG these protoplasts were found to be easily damaged (Abe *et al.*, 1982). PEG has been shown to affect viability of protoplasts with only about 10% of the protoplasts surviving treatment (Anné & Peberdy, 1975).

Aggregates of protoplasts remaining after PEG treatment probably vary in size and this clumping effect may influence reversion frequencies.

Protoplast fusion has been used to overcome vegetative incompatibility factors in several species of fungi including *Aspergillus* spp. (Croft, 1985; Ushijima & Nakadai, 1987) and *Mucor* spp. (Ohnuki *et al.*, 1982; van Heeswijck, 1984) and several other filamentous fungi. Protoplast fusion is now considered a more direct method to force heterokaryon formation with a view to strain improvement in a variety of fungi. In basidiomycetes mating-type factors strictly control heterokaryon formation and sexual development, and so protoplast fusion in this group is limited in applications of cross-species hybridization.

Intraspecific protoplast fusion has been applied to several basidiomycete species including *Coprinus macrorhizus* (Kiguchi & Yanagi, 1985), *Phanerochaete chrysosporium* (Gold *et al.*, 1983), *Pleurotus ostreatus* (Ohmasa, 1986) and *P. salmoneo-stramineus* (Toyomasu & Mori, 1987a). In these cases heterokaryons were selected for by complementation of auxotrophic mutations in both of the strains used. Kawasumi *et al.* (1987) selected by dikaryotic intrastrain fusion products of *L. edodes* by the presence of clamp connections.

Intraspecific fusion between strains of *C. macrorhizus* with different morphological features and of compatible mating types produced heterokaryons with characteristics intermediate to the parental types (Kiguchi & Yanagi, 1985). Fruit bodies from hybrid strains were quite uniform in morphology. Fusions carried out between incompatible mating types produced heterokaryons at a rate similar to fusion between compatible strains, however, the products of the incompatible crosses failed altogether to form fruit bodies. Although fusion can overcome physical barriers to hybridization, genetic features controlling compatibility are quite stable (Kawasumi *et al.*, 1987) and cannot be overcome in basidiomycetes which limits beneficial use of these crosses.

Fruitbody formation by a strain of *P. ostreatus* developed by protoplast fusion was found to have characteristics intermediate to both of the parental strains in terms of fruitbody colour, morphology and temperature and light requirement for primordia formation. The strain produced was further characterized by isozyme banding patterns, showing that hybridization between the two strains had occurred (Ohmasa, 1986). Fusion products from crosses between auxotrophic isolates of *P. salmoneo-stramineus* yielded fertile dikaryons, four of seven random isolates had statistically different growth rates (three were faster, and one slower growing). This variation was not present among protoplast regenerants which were treated with, or without, PEG and so was not caused by the protoplast isolation nor the fusion procedure itself and may be due to somaclonal variation within the population (Toyomasu & Mori, 1987a).

The vast majority of reported interspecific crosses by protoplast fusion with basidiomycetes has been carried out using auxotrophic mutants of *Pleurotus* spp. Yoo *et al.* (1984) successfully fused protoplasts from monokaryotic strains of *P. ostreatus* and *P. florida* based on the same principles as with intraspecific fusion. Differences in morphology and growth rate were commonly observed. All of the fusion products possessed clamp connections and yielded fruit bodies ranging in quantity, and were intermediate in colour to the parents. Forty fusion products investigated were found to form no, or only small numbers of, basidiospores (Yoo *et al.*, 1984).

Incompatibility between *P. ostreatus* and *P. salmoneo-stramineus* was overcome by induced protoplast fusion between auxotrophic monokaryons (Toyomasu *et al.*, 1986). Further crosses between *P. ostreatus*, *P. columbinus*, *P. pulmonaris*, and *P. sajor-caju* have been attempted (Toyomasu & Mori, 1987a). Of the six possible combinations which could be induced by protoplast fusion, only four yielded fusion products, the *P. columbinus* x *P. pulmonaris* and the *P. ostreatus* x *P. pulmonaris* crosses failing to yield prototrophs. Hybrids obtained from all the crosses differed in terms of colony morphology and growth rate (Toyomasu & Mori, 1987b). Fruit body formation on a sawdust medium was demonstrated for the *P. ostreatus* x *P. columbinus*

cross, however the other products failed to give fruit bodies (Toyomasu & Mori, 1989).

Interspecific fusion of auxotrophic strains of *Ganoderma lucidum* and *G. applanatum* has been carried out (Park *et al.*, 1988; Um *et al.*, 1988). Although fusants segregated on a complete medium to form mycelia with and without clamp connections, it is possible that the selection pressure for remaining in the heterokaryotic condition is no longer present and so breakdown occurs on the rich medium.

DNA Mediated Transformation

Transformation involves introducing specific DNA sequences into bacterial, fungal, plant or animal cells. In plants and fungi this process is limited by the barrier of the cell wall and necessitates its removal. Protoplasts are ideal for such a procedure, and in fungi this technique is now widely used. Here, addition of plasmid DNA to protoplasts in PEG and calcium chloride mediates transformation (Hynes, 1986).

DNA treated protoplasts are then regenerated on a suitably stabilized medium and transformants selected for by incorporating a phenotypic marker into the plasmid DNA, usually resistance markers or complementation of a nutritional mutation found in the genome of the organism being transformed.

Transformation as a means of increasing gene copy number in basidiomycetes has been shown to occur with a cloned *C. cinereus* isocitrate lyase gene (ISL) (Mellon & Casselton, 1988). Expression of the introduced gene is dependent on the point of insertion in the genome and also copy number. Non-homologous integration of the ISL gene was found to occur in *C. cinereus* (Mellon *et al.*, 1987). Transformation of a leucine auxotroph of *P. florida* has been reported by use of a cloned gene from *F. velutipes*, however, integration of the transforming DNA into the genome was not demonstrated (Byun *et al.*, 1989a & b).

Intergeneric transfer of whole nuclei into protoplasts has been investigated with several edible basidiomycetes, although only

certain crosses were fertile and several fusion products were unstable (Yoo *et al.*, 1987b & 1989a). This approach eliminates the need for introducing nutritional or resistance markers into both strains of a cross.

Thus transformation of protoplasts provides a more targeted and efficient means of genetic manipulation. Cloning and amplification (by increased copy number) of genes involved with processes related to a breeding program can be applied directly. Degradation of the ligno-cellulosic substrate is obviously important in terms of efficiency of breakdown of complex matter and conversion to biomass. Isolation and enumeration of the genes involved in substrate utilization could be amplified by transformation and used to increase nutrient release from specific substrates. In addition, stable resistance, for example to hygromycin B, can be introduced and used as a genetic marker during strain crossing. This would avoid screening a large number of samples for spontaneous resistance to inhibitors, and by-passing the need for mutagenesis. Further discussion on fungal transformation is given in Chapter 5.

1.4 Strain Screening and Identification

Strain Screening Methods

Once initiated, a breeding strategy requires a systematic method for testing isolates for the characteristics required for cultivation. This usually involves small scale fruiting trials (e.g. Kneebone *et al.*, 1974; Tokimoto *et al.*, 1987) which gives information on yield or biological efficiency, or BE (Diehle & Royse, 1986; Royse & Schisler, 1987), or coefficients of variance (Royse & Bahler, 1986). BE is calculated as the percentage conversion of dry substrate to fresh weight mushroom biomass.

A simple method of preliminary screening for suitable strains of *A. aegerita* was applied by Marmeisse (1989). Using dry weight of mushrooms produced per petri dish and the number of days taken to fruit as criteria, reproducible variation was found between strains to warrant interstrain hybridization as a means of further optimizing these characteristics.

An alternative means of utilizing *in vitro* screening methods has been applied to *P. ostreatus* by using primordia formation on artificial medium as a quick and inexpensive means of determining the fruiting ability of an isolate (Eger, 1974). One further extension of this method, which has been used for *L. edodes*, involves screening for resistance to other competing organisms. Great variation was found between isolates in terms of their ability to out grow the invading mycelium of *Hypocrea muroiana* (Kawamura *et al.*, 1980).

Leatham (1990) recently proposed a more ambitious screening strategy for *L. edodes* whereby extensive information is gathered on any one strain concerning enzymatic activities during growth and development, in addition to physiological responses to external stimuli. Degradative enzymes such as ligninases, cellulases, hemicellulases, pectinases and amylases, as well as developmentally regulated enzymes such as lipases and proteases could be quantified for characterizing novel strains. Production of these enzymes is essential for efficient substrate utilization and subsequent conversion to biomass. Increased extracellular laccase production in *P. ostreatus* was selected for by Prillinger & Molitoris (1979) through a gel electrophoretic assay, although no information was given on the utilization of lignocellulsoic substrates.

Strain Identification and Registration

Cultivars of Shiitake are registered in Japan to protect the unauthorized use of strains by producers (Anonymous, 1980). Lines are characterized by genetic properties such as the formation of an inhibition zone in culture (which presumably relies on mating compatibility) physiological and cultivation properties, and morphological characteristics of the sporophore (Mori, 1963; Aoshima, 1980; Royse *et al.*, 1985).

Potential use of other markers, such as isoenzyme profiles or RFLP mapping, offer more reliable criteria for cell line authentication. DNA fingerprinting has been used to discriminate isolates of *A. bisporus* and *A. bitorquis*, although no differences were found for several commercially used strains (Castle *et al.*, 1987). This has been suggested as a means of patenting specific cultivars (Horgen &

Anderson, 1987). RFLP techniques have been used with *Coprinus cinereus* to discriminate between strains with different origins, where banding patterns represent the losses or gains of restriction sites present in the genetic material of different isolates (Wu *et al.*, 1983). RFLP's have also been used to distinguish between species, strains, and homokaryons of *A. bisporus* and *A. bitorquis* (Castle *et al.*, 1988 a & b; Loftus *et al.*, 1988; Hintz *et al.*, 1989).

Identification of appropriate genotypes by isozyme analysis has proved to be useful in the identification and strain classification of several basidiomycetes (Royse & May, 1982a & b; Royse *et al.*, 1983a & b; Royse *et al.*, 1987) and between species of *Pleurotus* (May *et al.*, 1988; May & Royse, 1988; Magae *et al.*, 1990). Differences between isolates is based on the distribution pattern of various forms of isoenzymes appearing as bands on a gel. Such a technique has proved to be useful in discriminating stocks of *L. edodes* (Toyomasu & Zennyozi, 1981; Ohmasa & Furukawa, 1986; Royse & May, 1987). However, high resolution and stability of the patterns observed is also dependent on culture conditions and careful control of electrophoresis conditions and thus subject to variation.

1.5 Aims of This Study

Recent interest in the cultivation of the Shiitake and oyster mushrooms in Britain has prompted the strain development programme at Nottingham University. Protoplasts are suggested as being integral to the breeding of new varieties of *L. edodes* and *Pleurotus spp.* Studies on basidiomycete protoplast isolation, reversion, fusion and transformation have recently been seen as a rapidly expanding area of research. There are, however, several barriers to the applications of protoplasts to many species including poor yields with relatively low frequencies of reversion and compared to other well studied organisms.

The original study was to include the isolation of drug resistant strains of *L. edodes* and the development of an efficient protoplast isolation and regeneration procedure. Protoplast fusion and subsequent isolation of drug resistant fusion products would be used as a means of hybridization. Strains characterized in terms of mating

type factors would be selectively crossed to produce novel dikaryons using information from their predicted mating behaviour. Appropriate fruiting trials could thus be carried out as a means of identifying superior cultivars.

Further applications of fungal protoplasts were to be applied to *Pleurotus* spp. to include the development of an efficient DNA mediated transformation system. In addition, karyological studies using pulsed-field gel electrophoresis were to be used for the taxonomic study of *Pleurotus* species identification.

2. Protoplast Isolation from *Lentinula edodes* and *Pleurotus* spp.

2.1 Introduction

Protoplast liberation from microbial and plant cells by digestion of the cell wall has been the focus of much attention over the past 30 years. A protoplast may be described as a naked cell without the support and protection of the cell wall. They are metabolically active, organised units of the cells from which they were derived. To remain intact, protoplasts must be maintained in a suitable osmoticum. Reversion to the normal cellular state, by formation of a cell wall, is brought about by their culture on a suitable osmotically stabilized medium.

Optimum conditions for protoplast release have been described for several commercially important basidiomycete species such as *Agaricus bisporus, Pleurotus* spp., *Volvariella volvacea, Flammulina velutipes* and *Lentinula edodes* (Anderson *et al.*, 1984; Chang *et al.*, 1985a; Ohmasa *et al.*, 1987). A summary of the literature on protoplast isolation from these and other basidiomycete species is given in Table 2.1.

Protoplasts and their potential use in a strain improvement programme of *L. edodes* and *Pleurotus* species has been discussed previously. Attention will now focus on the practical methodology of protoplast isolation and reversion in fungi, in particular basidiomycetes.

Mycolytic Enzymes

A wide range of enzymes have been used for protoplast isolation from edible species (Table 2.1). The combination of different types and the proportions of each has a marked affect on digestion of the mycelium (e.g. Yanagi & Takebe, 1984; Ohmasa *et al.*, 1987). The efficiency of the lytic system employed is likely to be affected by the cell wall chemistry of the species being studied. The ratio and arrangement of cell wall components is generally linked to the taxonomic group to which a species belongs (Bartnicki-Garcia, 1968). In Basidiomycetes the cell wall is composed mainly of chitin and α and ß glucans with a smaller proportion of mannans, lipid and protein

TABLE 2.1 (cont.)

ORGANISM	STABLILIZER/ pH	LYTIC ENZYMES USED*	REFERENCE
G. lucidum	0.5M $MgSO_4$/ 5.8	11	De Vries & Wessels (1973a)
	0.5M Mannitol/ 5.5	6,8,18	Yanagi *et al.* (1985)
	0.6M Sucrose/ 5.8	2,15	Shin *et al.* (1986)
	0.6M Sucrose/ 5.8	2,15	Choi *et al.* (1988)
	0.6M Sucrose/ Not Given	2,15	Um *et al.* (1988)
Grifola frondosa	0.5M Mannitol/ 5.5	6,8,18	Yanagi *et al.* (1985)
	0.5M Mannitol/ 5.5	5,9,14,17	Ohmasa *et al.* (1987)
Hebeloma circinans	0.5M Mannitol/ 5.6	15	Barrett *et al.* (1989)
H. cylindrosporum	0.5M Mannitol/ 5.6	15	Barrett *et al.* (1989)
Hypsizygus marmoreus	0.5M Mannitol/ 5.5	5,9,14,17	Ohmasa *et al.* (1987)
Laccaria bicolor	0.5M Mannitol/ Not Given	15	Kropp & Fortin (1985)
	0.5M Mannitol/ 5.6	15	Barrett *et al.* (1989)
L. laccata	0.5M Mannitol/ 5.6	15	Barrett *et al.* (1989)
Lentinula edodes (=*Lentinus edodes*)	0.5M $MgSO_4$/ 5.8	11	De Vries & Wessels (1973a)
	0.5M $MgSO_4$/ 5.8	4,10,12	Ushiyama & Nakai (1977)
	1.2M $MgSO_4$/ 5.5	11	Kitamoto *et al.* (1984)
	0.5M $MgSO_4$/ 5.8	6,10,17	Toyoda *et al.* (1984)
	0.6M $MgSO_4$/ 5.0	2,5	Woo & Yoon (1985)
	0.5M Mannitol/ 5.5	6,8,18	Yanagi *et al.* (1985)
	0.6M Mannitol/ 4.6	5,8	Kawasumi *et al.* (1987)
	0.5M Mannitol/ 5.5	2,5,9	Ohmasa *et al.* (1987)
	1.2M $MgSO_4$/ 5.5	11	Kitamoto *et al.* (1988)
	0.6M $MgSO_4$/ 5.6	2,6,8,20	Koga *et al.* (1988)
Lentinus tigrinus	0.5M $MgSO_4$/ 5.8	11	De Vries & Wessels (1973a)
Lyophyllum shimeji	0.6M $MgSO_4$/ 5.6	2,6,17	Abe *et al.* (1984)
	0.6M $MgSO_4$/ 5.6	2,6,8,20	Koga *et al.* (1988)
L. ulmarium	0.6M Mannitol/ 5.5	6,8,18	Yanagi *et al.* (1984)
Oudemansiella mucida	0.7M Glucose/ Not Given	16	Holmoka *et al* (1988)
Phanerochaete chrysosporium	0.6M $MgSO_4$/ 5.5	3,15	Gold *et al.* (1983)
	0.7M $MgSO_4$/ 5.9	15	Gaskell *et al.* (1991)
Pholiota nameko	0.5M Mannitol/ 5.5	6,8,18	Yanagi *et al.* (1984)
	0.5M Mannitol/ 5.5	2,5	Ohmasa *et al.* (1987)
	1.2M $MgSO_4$/ 5.5	11	Kitamoto *et al.* (1988)
Pisolithus tinctorius	0.5M Mannitol/ 5.6	15	Barret *et al.* (1989)

TABLE 2.1 (cont.)

ORGANISM	STABLILIZER/ pH	LYTIC ENZYMES USED*	REFERENCE
Pleurotus columbinus	0.7M Mannitol/ Not Given	2,5,9	Toyomasu & Mori (1987a)
P. cornucopiae	0.6M Mannitol/ 5.6	2,5,8,17	Nakabayashi *et al.* (1985)
	0.5M Mannitol/ 5.5	5,9,14,17	Ohmasa *et al.* (1987)
P. corticatus	0.5M $MgSO_4$/ 5.8	11	De Vries & Wessels (1973a)
P. cystidiusus	0.5M Mannitol/ 5.5	5,9,15,17	Ohmasa *et al.* (1987)
P. florida	0.6M $MgSO_4$/ 5.8	1,15,16	Yoo *et al.* (1984)
P. ostreatus	0.6M Mannitol/ Not Given	2,5,8,17	Yamada *et al.* (1983)
	0.4M $MgSO_4$/ 5.5	13	Chang *et al.* (1985)
	Not Given	15	Go *et al.* (1985)
	0.5M Mannitol/ Not Given	2,5,8,19	Yanagi *et al.* (1985)
	0.7M Mannitol/ Not Given	2,5,9	Toyomasu *et al.* (1986)
	0.5M Mannitol/ 5.5	2,5,8,19	Iijima & Yanagi (1986)
	1.2M $MgSO_4$/ 5.5	11	Kitamoto *et al.* (1988)
	0.5M Mannitol/ 5.5	8,15	Ohmasa *et al.* (1987)
	Not Given	1,2,15	Yoo *et al.* (1989b)
P. pulmonaris	0.7M Mannitol/ Not Given	2,5,9	Toyomasu & Mori (1987a)
P. sajor-caju	0.4M $MgSO_4$/ 5.5	13	Chang *et al.* (1985)
	Not Given	15	Go *et al.* (1985)
	0.6M $MgSO_4$/ 5.0	3,15	Lau *et al.* (1985)
	0.7M Mannitol/ Not Given	2,5,9	Toyomasu & Mori (1987a)
P. salmoneo-straineus	0.7M Mannitol/ Not Given	2,5,9	Toyomasu (1986)
	0.5M Mannitol/ 5.5	5,9,14,17	Ohmasa *et al.* (1987)
	0.6M Sucrose/ Not Given	1,2,15	Yoo *et al.* (1989)
P. sapidus	0.6M Sucrose/ 6.0	1,2,15	You *et al.* (1988)
Polystictus versicolor	12.5% Sucrose/ 6.5	16	Strunk (1965)
Rhizoctonia solani	0.6M Mannitol/ 5.2	2,6,14	Hashiba & Yamada (1982)
Robillarda sp.	0.6M Mannitol/ 6.0	2,5,8,20	Kuwabara *et al.* (1989)
Schizophyllus commune	0.6M $MgSO_4$/ 5.8	11	de Vries & Wessels (1972)
	0.5M $MgSO_4$/ 6.75	7,15	Munoz-Rivas *et al.* (1986)
	1.2M $MgSO_4$/ 5.5	11	Kitamoto *et al.* (1988)
Sistotrema brinkmannii			Anderson & Cendese (1984)
Termitomyces clypeatus	0.5M KCl/ 6.0	3,8,15	Mukherjee & Sengupta (1988)

TABLE 2.1 (cont.)

ORGANISM	STABLILIZER/ pH	LYTIC ENZYMES USED*	REFERENCE
Trametes sanguinea	1.2M $MgSO_4$/ 5.5	11	Kitamoto *et al.* (1988)
Tremella fuciformis	0.5M Mannitol/ 5.5	6,8,18	Yanagi *et al.* (1985)
Tricholoma matsutake	0.6M $MgSO_4$/ 5.6	2,6,17	Abe *et al.* (1982)
	0.5M Mannitol/ 5.5	5,9,14,17	Ohmasa *et al.* (1987)
	0.6M $MgSO_4$/ 5.6	6,2,8,20	Koga *et al.* (1988)
Volvariella bombycina	Not Given	2	Stille (1984)
	Several	15	Chang *et al.* (1985)
Volvariella volvacea	0.5M $MgSO_4$/ 5.8	11	De Vries & Wessels (1973)
	0.6M $MgSO_4$/ 5.8	15	Hamlyn *et al.* (1981)
	1.2M $MgSO_4$/ 5.8	11,15	Santiago (1982a)
	1.2M KCl/ 5.8	11,15	Santiago (1982b)
	Not Given	2,15,16	Yoo *et al.* (1985)
	0.6M NaCl/ 6.0	15	Mukherjee & Sengupta (1986)

*KEY TO ENZYME CODES: 1= β-D-Glucanase; 2= β-Glucuronidase; 3= Cellulase CP; 4= Cellulase CP-1500; 5= Cellulase "Onazuka" RS; 6= Cellulase "Onazuka" R10; 7= Cellulase t.v.; 8= Chitinase; 9= Driselase; 10= Helicase; 11=Induced Lytic enzyme; 12= Lytic Enzyme 3; 13= Lywallzyme; 14= Macerozyme R10; 15= Novozym 234; 16= Snail Enzyme; 17= Zymolase 5,000; 18= Zymolase 60,000; 19= Zymolase 100,00; 20= Zymolase 20T.

(O'Brien & Ralph, 1966; Bartnicki-Garcia, 1968). Quantitative analysis of cell walls from *A. bisporus* (Mendoza *et al.*, 1987), *S. commune* (Wessels & Sietsma, 1979) and *Armillaria mellea* (Hernández *et al.*, 1990) indicates a similarity in the organization of the hyphal wall in this group. An inner layer composed of chitin microfibrils covalently linked to ß-glucan, and protein is covered by a layer of alkali soluble α-glucans, with an additional mucilage layer of ß glucans covering the outer surface.

Several companies now produce enzymes, with mycolytic activity, on a commercial scale. Although cellulase activity is often the main component in many of them, they also have side activities such as chitinase, α- and ß-glucanases and protease which are useful for protoplast isolation from fungi. A list of mycolytic enzymes used for cell wall digestion of basidiomycetes is given in Table 2.1, and it is evident that in the majority protoplasts are isolated using more than one product. The observation that product mixtures can enhance or reduce yields, highlights the synergistic effects of various combinations (Hashiba & Yamada, 1982; Hocart *et al*, 1987; Kropp &

Fortin, 1985). Comparison of several commercially available enzymes by Hamlyn *et al* (1981), Hocart *et al* (1987) and Yu & Chang (1987) reveals the striking difference found among different preparations and indeed between batches of the same product.

Osmotic Stabilizers

Support for the naked plasma membrane and its contents after removal of the cell wall is provided by a range of inorganic salts, sugars and sugar alcohols (Davis, 1985). In general, inorganic salts are more suitable for many filamentous fungi (Peberdy, 1979; Davis, 1985), and amongst the basidiomycete species surveyed (Table 2.1) mannitol and magnesium sulphate are most commonly used.

Stabilizer effects on the action of mycolytic enzymes have been investigated by Yu & Chang (1987) with some interesting results. Chitinase activity was greatly affected by the compounds tested, with the actions of α- and β-(1,3) glucanases limited to a lesser extent.

Physiological stabilizers are commonly employed along with an osmotic stabilizer, but at much lower concentrations, usually in the range of 1-100mM. Compounds such as $CaCl_2$ and $MgSO_4$ have been used (e.g. Thomas & Davis, 1980; Skatrud *et al.*, 1986; Homolka *et al.*, 1988) and are believed to aid the stability of the protoplast membrane.

The Organism and Culture Conditions

During the growth of mycelium, both hyphal wall structure and intracellular architecture have been shown to differ with species and with the age of the mycelium (Grove & Bracker, 1970; Trinci & Collinge, 1975; Farkas, 1979). The starting material used for protoplast isolation varies widely amongst the fungi. Asexual spores are a uniform material which would obviously minimise heterogerfity, however, their thick and in many cases, highly melanised wall (Mendoza *et al.*, 1979) is difficult to digest and their use has been limited. Variables encountered when digesting hyphae also exist for protoplast release from asexual spores (Bos, 1985). Pre-germination of fungal spores, producing hyphae in exponential phase of growth, before digestion is the most widespread method for producing material

from species such as *Aspergillus*, *Trichoderma*, *Penicillium* (Hamlyn *et al.*, 1981) and *S. commune* (Ullrich *et al.*, 1985).

Use of homogenized mycelia regrown in liquid culture is used for many basidiomycete species (e.g. Ohmasa *et al.*, 1987) and is also useful for other non-sporulating fungi (Amaral *et al.*, 1983). This method is thought to increase the number of young mycelia which develop by branching from the homogenized fragments (Morinaga *et al.*, 1985), and the age of the regrown culture has been found to affect protoplast yields (e.g. Lau *et al.*, 1985; Kawasumi *et al.*, 1987; Yoo *et al.*, 1989b).

Several factors which influence the physiological status of the material in question include the nutrient content and pH of the medium used, incubation temperature, and the age and source of material to be digested.

Treatment of the cells prior to digestion with lytic enzymes has been found to enhance yields of protoplasts from several species of fungi (Davis, 1985), although there are only a few reports of this being effective with basidiomycete fungi (e.g. Choi *et al.*, 1987). Compounds used vary greatly with respect to their effect on different species, and involves incubating the organism with the appropriate compound(s) for a set period of time (Sietsma & de Boer, 1973; Davis, 1985).

Protoplasts and Their Release

Generally, the first protoplasts released originate from hyphal tips and these are normally non-vacuolated and thus smaller than others released from distal regions of the hypha (Gibson & Peberdy, 1972). In *P. versicolor* protoplasts of partial and whole cells have been observed to be released (Strunk, 1969). Localized digestion of *Fusarium culmorum* results in protoplast extrusion leaving empty cells surrounded by walls which are assumed to be resistant to enzymatic digestion (Rodrigues-Aguirre *et al.*, 1964).

With filamentous fungi more than one protoplast can originate through the digested pore. Santiago (1982) observed repeated constriction of

mycelial cytoplasm of *V. volvacea* to give two or more protoplasts from one cell. Plant cells have been shown by Cocking & Gregory (1963) to produce preformed organised units known as "subprotoplasts" which are released and remain stable in a suitable osmoticum after lytic action. No such compartmentalization has been observed in fungal cells before protoplast release, although electron micrographs of *A. nidulans* reveal small 'outpushings' of the plasmalemma which could be seen to disassociate from the main protoplast (Gibson & Peberdy, 1972).

The nuclear content of each protoplast will obviously vary and so use of nuclear stains in determining the status of a proportion of protoplasts is useful. In *Gliocladium* species the proportion of nucleate to anucleate cells varied with the species, with up to 40% of the protoplasts isolated found to lack a nucleus (Seh & Kenerley, 1988). Heterogeneity in terms of organelle content found in a portion of the protoplasts originates from the variation present with the cells being digested. Nuclear content and the presence of other organelles will vary between protoplasts, however, electron micrographs of *A. nidulans* protoplasts have shown most to contain mitochondria (Gibson & Peberdy, 1972).

Buffer, Temperature and pH Effects

The buffer system used should have certain properties: it should not be an inhibitor of the organism involved and should resist enzymatic degradation. In addition, the range of pH in which it is effective must be considered. Phosphate, maleate and succinate buffers are most commonly used with fungal protoplasts.

Compatibility between buffer and stabilizer is also a prerequisite as shown by de Vries and Wessels (1972) who found that a combination of magnesium sulphate and Sorensen buffer was ineffective for protoplast release from *S. commune*. Phosphate buffers only optimise protoplast release when used at a low concentrations (Davis, 1985).

Buffers are normally of a pH in the range 4.0 to 8.0, with 5.5 being optimum. Although work with *L. edodes* found a pH of 5.8 to be sufficient for protoplast release (Nakai & Ushiyama, 1977; Toyoda *et*

al., 1984), others have found a pH of 4.6 to be best (Kawasumi *et al.*, 1987). *L. edodes* was reported in the later case to be highly sensitive to the external pH, showing a much narrower pH range than other basidiomycetes, although this discrepancy could be due to the differences in strains used.

Cell Wall Formation and Protoplast Regeneration

Isolated protoplasts can be cultured on a suitable medium and growth reverted to the filamentous form. Any protoplast containing a nucleus is capable of reversion, although it is not common for all to do so (Necas & Svoboda, 1985). Reversion is accomplished by repair of the cell wall and continuation of the cell cycle.

Morphological patterns of reversion vary within a population of protoplasts, and between species being dependent on several additional factors. One form of reversion, involves the development of a new cell wall around the protoplast and from this the growth of one or more hyphae (Abe *et al.*, 1982; de Vries & Wessels, 1975; Strunk, 1970). In several fungi a budding chain forms from the original protoplast and from this a string of aberrant hyphae, and eventually one or more normal hyphae develop.

Formation of the various cell wall components in *S. commune* has been extensively investigated (de Vries & Wessels, 1975; van der Valk & Wessels, 1976). Both carbon and nitrogen sources are required for regeneration of *S. commune* protoplasts.

Levels of chitin synthase are influenced by the nutrient content of the regeneration medium of *Aspergillus flavus*. A large increase in chitin synthase activity can de detected if both glucose and *N*-acetyl-glucosamine are added (Moore & Peberdy, 1976). These nutrients would be available immediately as energy sources and structural components thus allowing regeneration to progress rapidly and efficiently.

From the start of regeneration of *S. commune* protoplasts α-(1,3) glucan (or S-glucan) and chitin were found to increase linearly, with ß-(1,3) glucan (or R-glucan) accumulating after 3 hours of

incubation. Abnormal budding structures were less commonly observed in *S. commune*, and up to 50% of the isolated protoplasts were found to revert (de Vries & Wessels, 1975). ß-glucan synthesis is thought to occur in the cell wall or at the outer surface of the plasmalemma (Farkas, 1979). The removal of the cell wall during protoplast isolation, and thus one of the sites of ß-glucan synthesis, could explain why a lag in R-glucan formation is observed.

As protoplasts begin to revert to hyphal growth, changes in the cell wall continue, and thus the deposition of new material is finely balanced with lytic processes involved with hyphal extension. Regeneration conditions are of great importance if isolated protoplasts are to be used further. Thus regeneration frequencies must be maximized, and this seems to be one of the main set-backs of many basidiomycete systems.

Inhibitory effects of the lytic enzymes (Yanagi *et al.*, 1985; Sonnenberg *et al.*, 1988), osmotic stabilizers (Mukerjee & Sengupta, 1986) and toxic effects of polyethylene glycol, commonly used to induce protoplast fusion (Anné & Peberdy, 1975) and DNA mediated transformation, can all reduce viability. Proteolytic and lipolytic activities of digestive preparations have been shown to affect protoplasts of *Fusarium* (Villanueva, 1966). Novozym 234, an enzyme now commonly used to prepare protoplasts from a wide variety of species has been reported to have detrimental effects on regeneration of *P. ostreatus* (Yanagi *et al.*, 1985), *A. bisporus* and *A. bitorquis* (Sonnenberg *et al.*, 1988), *C. bitorquis* (Burrows *et al.*, 1990) and *Neurospora crassa* (Quigley *et al.*, 1987). This enzyme has been found to have very high protease activity (Hamlyn *et al.*, 1981) which, it has been suggested, might interfere with the membrane components at the protoplast surface. The inhibitory effect of Novozym 234 as described is not universal. Skatrud *et al.* (1986) obtained much higher reversion frequency of *Acremonium chrysogenum* protoplasts when Novozym 234 was used in contrast to ß-glucuronidase.

It is obvious from this review that protoplast isolation can be very complex with the interaction of several variables. It was the aim of this study to develop an efficient protoplast system for *Lentinula*

edodes from existing reports and expand on protoplast regeneration from various species of *Pleurotus* with the application to fungal transformation.

2.2 Methods and Materials

2.2.1 Media

Strains of *L. edodes* in general use were maintained at 25°C on PMA containing, gl^{-1}: malt extract (Boots), 8g; sucrose (FSA), 8g; potato flour (Difco), 2g; agar (Sigma) 20g; pH adjusted with HCl to 4.6. Liquid complete medium was prepared as follows, per litre: potato extract, 500ml; malt extract (Boots), 8g; sucrose, 8g; pH to 4.6 with HCl. Potato extract was prepared by boiling 453g of diced potatoes in 500ml demineralized water for 30 min, filtered through nylon mesh and volume to 1l with demineralized water. Both media were sterilized by autoclaving at 121°C for 30 min.

Strains of *Pleurotus* spp. were maintained on malt-yeast-glucose (MYG) at 25°C. MYG consisted of, gl^{-1}: malt extract (Boots), 8g; yeast extract (Oxoid), 4g; glucose (FSA), 20g; agar (Sigma), 20g; pH adjusted to 6.0 with HCl. Glucose was autoclaved separately and the medium sterilized by autoclaving at 121°C for 30 min.

Osmotically stabilized MYG (RMYG) was used routinely for both *L. edodes* and *Pleurotus* spp. RMYG contained, per litre: sucrose, 205.4g; malt extract (Boots), 10g; yeast extract (Oxoid) 4g; glucose, 4g; agar (Sigma) 12g. Both glucose and the osmotic stabilizer were autoclaved separately and added to the medium before use.

Media additives when used, included, ferulic acid (Aldrich Chemical Co.) dissolved in ethanol to give a final concentration in RMYG of $50\mu g ml^{-1}$; Leatham's mineral solution, $10ml l^{-1}$ (Leatham, 1983); Leatham's trace element solution (Leatham, 1983), $1.0ml l^{-1}$; vitamin solution $1.0ml l^{-1}$ (Leatham, 1983); sawdust extract (Leatham & Griffin, 1984); lignin sulphonic acid (LSA); *Pleurotus* spp. amino acid solution included 0.01% of each of the following- aspartic acid, glutamic acid, methionine, leucine, threonine, valine and 0.005% of asparagine and phenylalanine; and peptone (Oxoid).

Kuster's medium was used to maintain Streptomyces cultures, this contained (per 1): glycerol, 10g; KNO_3, 2.0g; NaCl, 2.0g; soluble casein (BDH), 0.3g; KH_2PO_4, 0.2g; $MgSO_4.7H_2O$, 0.05g; $CaCO_3$, 0.02g; $FeSO_4.7H_2O$, 0.01g; agar (Sigma), 12g). In addition, cell wall agar was used to screen for lytic activity. This medium consisted of a 10ml bottom layer containing 1.0gl^{-1} K_2HPO_4, 0.5gl^{-1} $MgSO_4.7H_2O$, 15gl^{-1} Difco agar, and a 10ml top layer of 20gl^{-1} Difco agar and 7gl^{-1} cell walls. All media were sterilized by autoclaving, cell walls were prepared separately as a suspension and added after sterilization.

2.2.2 Strains

Strains of *Lentinula edodes* used in this study included 30-1, 30-2, 30-3, 30-4 and 30-5. For additional information on these strains see appendix A. Species (and strains) of *Pleurotus* used included: 7-4; 31-1; 32-1; 32-2; and 33-1. A complete strain list with full details of strain origin can be found in appendix C.

2.2.3 Lytic Enzymes

Commercial Lytic Enzymes

A complete list of the various commercially obtained lytic enzymes used in this study is presented in Table 2.2. Several of the enzymes used were generously provided by Novo Nordisk Bioindustries Ltd. Novozym 234 batch PPM1961 and Cellulase CP batch 2697/5 were used throughout except where indicated. Lywallzyme L1 was the kind gift of Professor S.T. Chang. In addition to those listed Cellulase (batch 8908/3-6), Glucanex, Lytic Enzyme II, Novozym GX and snail enzyme were tested, but no information regarding enzymatic activities could be obtained.

Enzyme solutions were routinely prepared fresh on the day of use, and centrifuged at 30,000x *g* at 4°C for 30 min. to remove insoluble material and contaminating bacteria. Small enzyme samples were prepared in microfuge tubes and spun at 13,000x *g* at 4°C for 30 min.

TABLE 2.2 Commercially Available Lytic Enzymes Used in This Study

ENZYME	ORIGIN	ACTIVITIES[1,2]	SUPPLIER/ MANUFACTURER
CELLULASE	Not Known	Not Known	Novo Nordisk Bioindustries Ltd.
CELLUCLAST	Not Known	Not Known	Novo Nordisk Bioindustries Ltd.
CELLULASE CP	*Penicillium funiculosum*	1,2,3,4	J. Sturge & Sons, Selby, England.
CELLULASE R10	*Trichoderma viride*	Not Known	Yakult Pharmaceuticals
CEREFLO	*Bacillus subtilis*	2,3	Novo Nordisk Bioindustries Ltd.
CHITINASE	*Trichoderma* sp.	1	Sigma Chemical Co. Ltd
FINIZYME	*Aspergillus niger*	3	Novo Nordisk Bioindustries Ltd.
GLUCANEX	*T. harzianum*	Not Known	Novo Nordisk Bioindustries Ltd.
β-GLUCURONIDASE	*Helix pomatia*	1,2,3,4	Sigma Chemical Co. Ltd.
HELICASE	*Helix pomatia*	1,2,3	L'Industrie Biologique, Francaise, Clinchy, France
LYTIC ENZYME II	Not Known	Not Known	Kyowa Hakko Kogyo Co. Ltd., Japan
LYTIC ENZYME L1	*Cytophaga* sp.	1,2,3,6	BDH, Poole, England
LYWALLZYME L1	*T. longibrachicitum*	1,2,3	Guangdon Inst. Microbiology (China)
MACEROZYM R10	*Rhizopus* sp.	Not Known	Yakult Pharmaceuticals
MEICELASE P	*Trichoderma* sp.	Not Known	Meiji Seika Kasha Ltd., Tokyo, Japan.
NOVOZYM GX	*T. harzianum*	Not Known	Novo Nordisk Bioindustries Ltd.
NOVOZYM 234	*Trichoderma* sp.	1,2,3,6	Novo Nordisk Bioindustries Ltd.
RHOZYME HP150	Not Known	5	Rhom & Haas, Philadelphia, U.S.A.
SNAIL ENZYME	*Helix pomatia*	Not Known	Dept. of Microbiology, University of Szeged, Szeged, Hungary.
ZYMOLASE	*Arthrobacter leuteus*	1,6	Kirin Breweries Co. Ltd., Taskaski, Japan.

1. Information from: Hamlyn *et al.* (1981); Yu & Chang (1987); manufacturer's information. 2. Codes to enzyme activities attributed to cell wall digestion: 1= Chitinase; 2= α-Glucanases; 3= β-Glucanases; 4= Mannanase; 5= Polysaccharidases; 6= Protease.

Induction of Lytic Enzymes in *Streptomyces* spp.

Some 36 soil isolates of *Streptomyces* spp. currently in the Nottingham culture collection were first screened for their growth and lysis on an opaque medium containing *Aspergillus* sp. cell walls. Differential growth rates and the presence of lysis zones around the isolates when grown as a streak on *Aspergillus* cell walls and later on cell wall agar containing *L. edodes* cell walls or ball milled chitin (Sigma Chemical Co.).

Cultures K1, K4 and K29 were found to give good lysis on solid medium and were used in further experiments in shake flasks according to the method of Peberdy & Gibson (1971). The three strains were grown on different media containing chitin, wheat bran or *L. edodes* cell walls as the sole carbon source. After one week the culture filtrates were harvested, dialysed and frozen at -20°C until needed.

The various lytic solutions produced were screened for their ability to digest 4 day old cellophane colonies of *L. edodes* strain 30-1 and yield protoplasts by mixing equal volumes of the sample and 1.2M $MgSO_4$ in 100mM sodium succinate, pH 5.8

4.2.4 Preparation of Fungal Inoculum

Preparation of Cellophane Cultures of *Lentinula edodes*

Plugs of mycelium, 4mm diameter, were taken from the leading edge of an actively growing colony and incubated on 7cm diameter cellophane (Phillip Harris, Ltd.) on PMA, 4 plugs per plate, at 25°C for 4 days. Mycelial growth was removed on a small disk of cellophane carefully sliced from the plate.

Preparation of Mycelial Homogenate of *Lentinula edodes*

Glass Bead Method

12 x 4mm plugs of mycelia were inoculated onto 7cm diameter cellophane disks on PMA and incubated 4 days at 25°C. The growth from 5 plates was harvested by scraping the surface of the cellophane, and placed in 20ml of LCM in a 1oz glass Universal quarter-filled with glass beads (5mm diameter). A macerate was produced by vigorous shaking, and 1.5ml of the mycelial suspension allowed to re-grow in 15ml LCM contained in 9cm petri dishes. Cultures were incubated statically at 25°C.

Silverson Homogenizer

Mycelia produced as described above was homogenized in a Silverson homogenizer (Silverson Machines Ltd.) or a Waring blender. Viability of the hyphal suspension was determined by removing 100µl aliquots at 5 second intervals and serially diluting prior to plating onto PMA. From this experiment 15 secs homogenization time was found to be optimum. With both machines maceration was carried out at the lowest speed possible for 15 secs. The macerate was re-grown as above.

Preparation of Mycelial Homogenate of *Pleurotus* spp.

Protoplast isolation of *P. sajor-caju* was modified from the method used for *L. edodes* and that of Lau *et al.* (1985) 4mm plugs of mycelia were incubated 18-20 hours in 5ml LCM in 9cm petri dishes, 12 plugs were inoculated per plate and the growth from 5 plates harvested.

Mycelium was homogenized for 15 secs using the Silverson and re-grown for 48 h as described previously.

2.2.5 Nuclear Staining of Protoplasts

Protoplasts were fixed in three stages by adding 50µl of 5% glutaraldehyde to 950µl of a protoplast suspension. This was mixed and incubated for 20 min at room temperature. A further addition of 50µl fixative was made followed by repeated incubation. This last step was repeated once. Removal of the glutaraldehyde was brought about by a pulse spin at 6,500x *g* in a MSE microfuge. The pellet was resuspended in water and an equal volume of chromomycin A_3 (Sigma Chemical Co.) added, and stored at 4°C overnight. Chromomycin A_3 was dissolved at a concentration of 0.4mg.ml^{-1} in 30mM $MgCl_2$ and 50% aq. ethanol and stored at -20°C (Slater, 1976).

2.2.6 Protease Assay And Heat Treatment of Novozym 234

Protease activity was determined by a modified method of Hocart (1987). Enzyme activity was assayed by incubating 1ml lytic enzyme solution with 0.5ml 50mM Na succinate, pH 5.8 and 0.5ml 1% denatured Hammarstein casein (heated in a boiling water bath for 15 min). Incubation was carried out at 28°C for 60 min with shaking, and the reaction terminated by adding 2ml ice cold 10% perchloric acid to precipitate the undigested protein. Prior to reading OD_{280} the samples were allowed to stand at room temperature for 15 min, and finally spun at 2,000rpm for 10 min in a MSE bench centrifuge. Trypsin solutions were prepared (1.2-625µgml^{-1}) and used as standard with boiled enzyme solution and buffer only as blanks.

Novozym 234 batch PPM1961 was dissolved in 50mM Na succinate, pH 5.8 at a concentration of 2mg.ml^{-1} and heat treated at 50°C on a shaking platform for 5-25 min. Protease activity was determined as above, and units protease activity expressed as µg trypsin mg^{-1} enzyme.

2.2.7 Total Protein Determination

Protein content was determined using Biorad reagent based on the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Total protein was assayed according to the manufacturer's instructions.

2.2.8 Harvesting and Regeneration of Protoplasts

In the initial experiments on protoplast release 0.6M KCl/ 0.2M phosphate buffer, pH 5.8 was used. However, in subsequent studies involving regeneration this was replaced with 0.6M $MgSO_4$/ 50mM sodium succinate buffer, pH 5.8.

When 0.6M $MgSO_4$ was employed, protoplasts released after 3 to 4 h digestion were harvested in two stages. Initial centrifugation at 1,500 rpm for 10 min had the effect of selectively sedimenting undigested mycelia, lysis debris and contaminating agar. Protoplasts floating in the supernatant were removed and mixed with an equal volume of 0.6M mannitol or 0.6M sucrose and incubated on ice for 10 to 20 min prior to centrifugation at 2,000-3,000rpm for 15 min. The pellet of protoplasts was resuspended and washed once more in 0.6M mannitol or sucrose prior to dilution in osmotic stabilizer and plating.

100µl of each diluted protoplast suspension was spread on each of three plates. Regeneration was routinely carried out on RMYG containing 0.6M sucrose at 25°C, and determined after 14 days for *Pleurotus* spp. and 21 days for *L. edodes*. Frequency of regeneration was defined as the percentage of colonies regenerated from stabilizer solution minus the number of colonies formed from dilutions in water divided by the total microscopic counts.

2.3 Results

2.3.1 Lytic Enzymes on Protoplast Yields

Effectiveness of Commercial Mycolytic Enzymes on Protoplast Release from *Lentinula edodes* 30-1 and *Pleurotus sajor-caju* 32-2

Initial experiments with *L. edodes* were focussed on optimizing the mycolytic enzymes used for cell wall digestion. Eighteen commercial products were screened for ability to release protoplasts from cellophane grown colonies. Of these, eight were further tested in combination with Novozym 234, which was singly the most efficient (Tables 2.3 & 2.4). Higher yields of protoplasts were obtained from a hyphal macerate regrown for 4 days digested with Novozym 234. When used in combination with other enzymes no increase in yield was found despite intensive tests.

TABLE 2.3. Effect of Various Mycolytic Enzymes on Protoplast Yield from *Lentinula edodes*

ENZYME	ENZYME CONCENTRATION	PROTOPLAST YIELD* 2 hr	3hr	4hr
Celluclast	0.5%v/v	0	0	0
Cellulase CP	10mg.ml^{-1}	0	+	+
Cellulase R10	5mg.ml^{-1}	+	+	+
Cereflo	0.5%v/v	0	0	0
Finizyme	0.5%v/v	0	0	0
β-Glucuronidase	5%v/v	0	+	+
Helicase	10mg.ml^{-1}	+	+	+
Lytic Enzyme L1	5mg.ml^{-1}	+	+	+
Lytic Enzyme II	5mg.ml^{-1}	+	0	0
Lywallzyme L1	5mg.ml^{-1}	+	++	++
Macerozyme R10	5mg.ml^{-1}	+	+	+
Miecelase	10mg.ml^{-1}	+	+	+
Miecelase P	10mg.ml^{-1}	0	0	+
Novozym	0.5%v/v	+	++	+
Novozym 234	2.5mg.ml^{-1}	+	+	+
Novozym 234	5mg.ml^{-1}	+++	++++	++++
Novozym 234	7.5mg.ml^{-1}	++++	++++	++++
Novozym 234	10mg.ml^{-1}	++++	++++	++++
Novozym 234	15mg.ml^{-1}	++++	++++	++++
Rhozyme	0.5%v/v	0	0	0
Snail Enzyme	5mg.ml^{-1}	+	+	+
Zymolase 5,000	5mg.ml^{-1}	+	+	+

*Protoplast yield as protoplasts per ml lytic solution. One 4 day old cellophane grown colony of strain 30-1 was digested in 1ml 0.6M KCl, 0.2M phosphate bufer, pH 5.8. Key: +=<10^5 protoplsts ml^{-1}; ++= 1.0-4.9 x 10^5 protoplsts ml^{-1}; +++= 5.0-9.9 x 10^5 protoplasts ml^{-1}; ++++= > 1.0 x 10^6 protoplasts ml^{-1}.

Very efficient lysis of *P. sajor-caju* was obtained using Novozym 234 alone and in combination with Cellulase CP with 0.6M $MgSO_4$, 50mM $CaCl_2$/ 50mM sodium succinate buffer, pH 5.8. In addition, highest yields were obtained by digesting 1g mycelium in 10ml enzyme solution (equivalent to 50mg of each Novozym 234 and Cellulase CP), see Figs. 2.1 and 2.2.

Effects of Induced Strepzymes on Protoplast Release

No lytic activity was detected from any of the isolates grown on any of the substrates tested. No protoplasts were formed and no digestion of the mycelium was detected. Further concentration of the samples by freeze drying also failed to lyse *L. edodes* mycelia.

TABLE 2.4. Effect of Selected Lytic Enzymes used in Combination with Novozym 234 on Protoplast Release from *Lentinula edodes*

ENZYMES USED*	CONCENTRATION	PROTOPLAST YIELD** METHOD A	METHOD B
Novozym 234	10mg.ml^{-1}	+	++++++
Cellulase CP	10mg.ml^{-1}	+++	NA
β-Glucuronidase	5%v/v	+++	NA
Helicase	10mg.ml^{-1}	+	NA
Lysallzym L1	5mg.ml^{-1}	+	NA
Macerozym R10	5mg.ml^{-1}	+	NA
Snail Enzyme	5mg.ml^{-1}	+++	NA
Zymolase 5,000	5mg.ml^{-1}	+++	NA
Cellulase CP	10mg.ml^{-1}		
& β-Glucuronidase	5mg.ml^{-1}	+++	++++++
Cellulase CP	10mg.ml^{-1}		
& Helicase	10mg.ml^{-1}	NA	+++++
Cellulase CP	10mg.ml^{-1}		
& Snail Enzyme	5mg.ml^{-1}	+++	+++++
Cellulase CP	10mg.ml^{-1}		
& Zymolase 5,000	5mg.ml^{-1}	+++	+++++
Helicase	10mg.ml^{-1}		
& β-Glucuronidase	5%v/v	NA	+++++
Helicase	10mg.ml^{-1}		
& Zymolase 5,000	5mg.ml^{-1}	NA	++++++
Helicase	10mg.ml^{-1}		
& Snail Enzyme	5mg.ml^{-1}	NA	++++++
Snail Enzyme	5mg.ml^{-1}		
& β-Glucuronidase	5%v/v	+++	+++++
Zymolase 5,000	5mg.ml^{-1}		
& β-Glucuronidase	5%v/v	+++	++++++
Zymolase 5,000	5mg.ml^{-1}		
& Snail Enzyme	5%v/v	+++	++++++

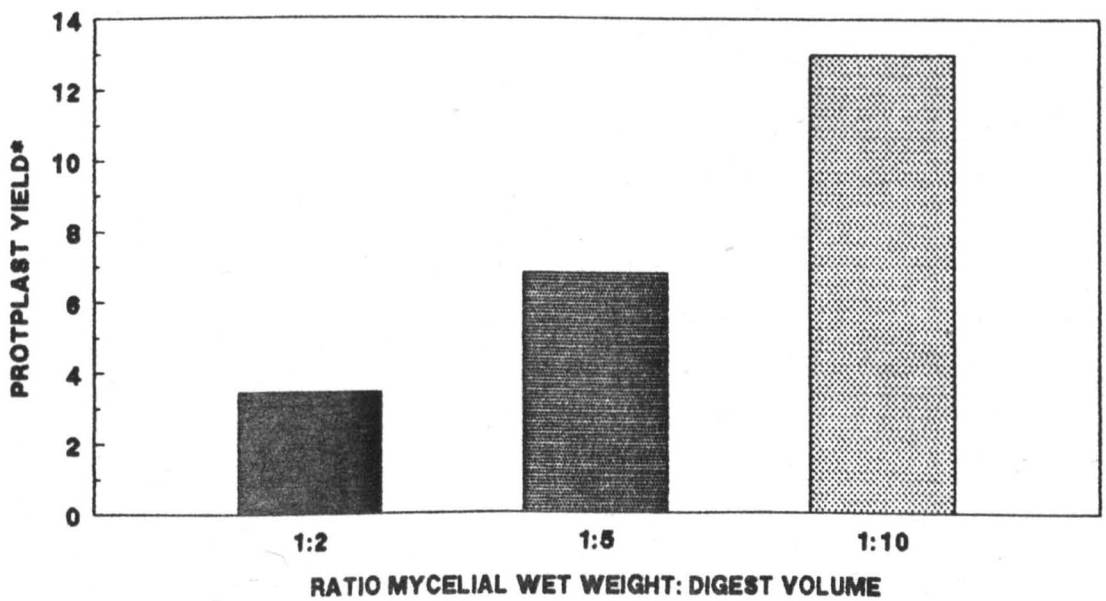
*Novozym used in combination with other enzymes at a concentration of 10mg.ml^{-1}.**Method A: one 4 day old cellophane grown colony of strain 30-1. Key: += 1-4.9 x 10^5 protoplsts ml^{-1}; ++= 5.0-9.9 x 10^5 protoplasts ml^{-1}; +++= 1.0=4.9 x 10^6 protoplasts ml^{-1}. Method B: 4 day re-grown hyphal macerate. Key: +++++= 1-4.9 x 10^7 protoplasts/ gram wet weight mycelia; ++++++= 5-9.9 x 10^7 protoplasts/ gram wet weight mycelium. NA= Not attempted. Digestion was carried out in 1ml 0.6M KCl, 0.2M phosphate buffer, pH 5.8, and yield determined after 3 hours.

TABLE 2.5. Effect of Lytic Enzymes on Protoplast Release from *Pleurotus sajor-caju* 32-2

LYTIC ENZYMES USED	CONCENTRATION	PROTOPLAST YIELD*
Novozym 234 [PPM1961]	$10mg.ml^{-1}$	8.22×10^8
Cellulase CP [2697/5]	$10mg.ml^{-1}$	1.1×10^8
Cellulase CP & Novozym 234	$5mg.ml^{-1}$ $5mg.ml^{-1}$	6.58×10^8
Cellulase CP & β-Glucanase	$5mg.ml^{-1}$ $5mg.ml^{-1}$	9.9×10^7
Cellulase CP & β-Glucuronidase	$5mg.ml^{-1}$ 5% v/v	1.9×10^8
Cellulase CP & Helicase	$5mg.ml^{-1}$ $5mg.ml^{-1}$	1.67×10^8

*Protoplasts/ gram mycelium/ 5 hours.

Fig. 2.1 The Effect of Mycelial Fresh Weight and Lytic Volume on Protoplast Release from *Pleurotus sajor-caju* 32-2

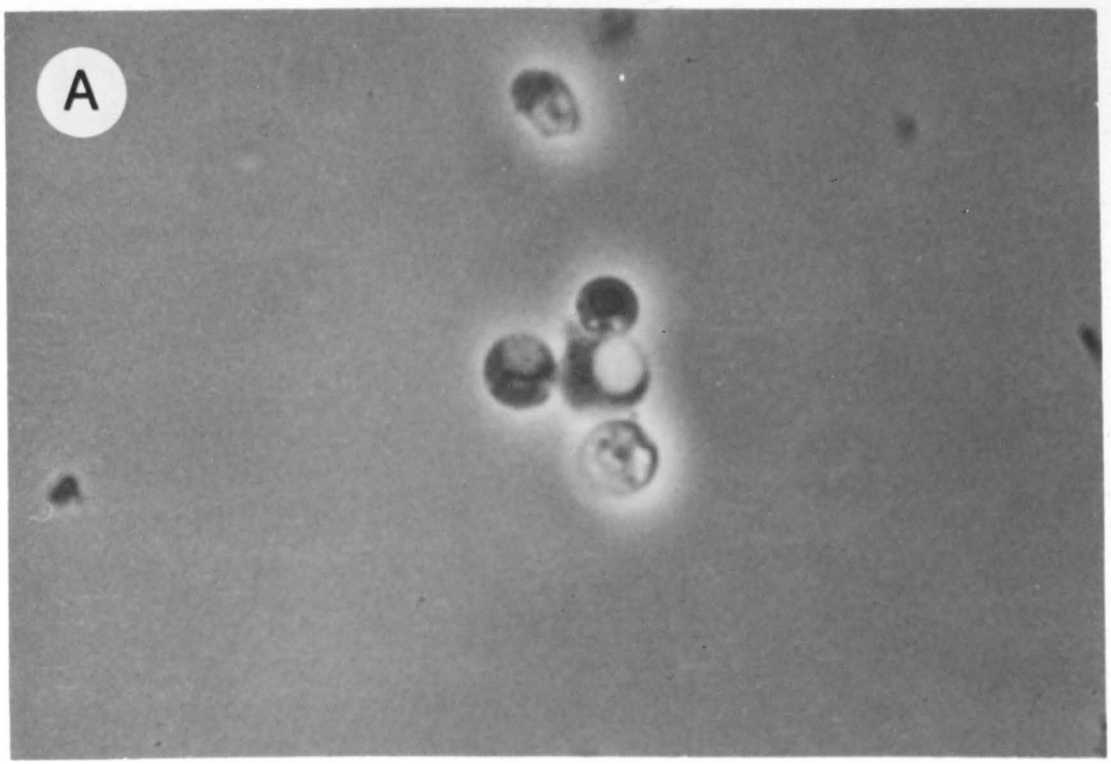


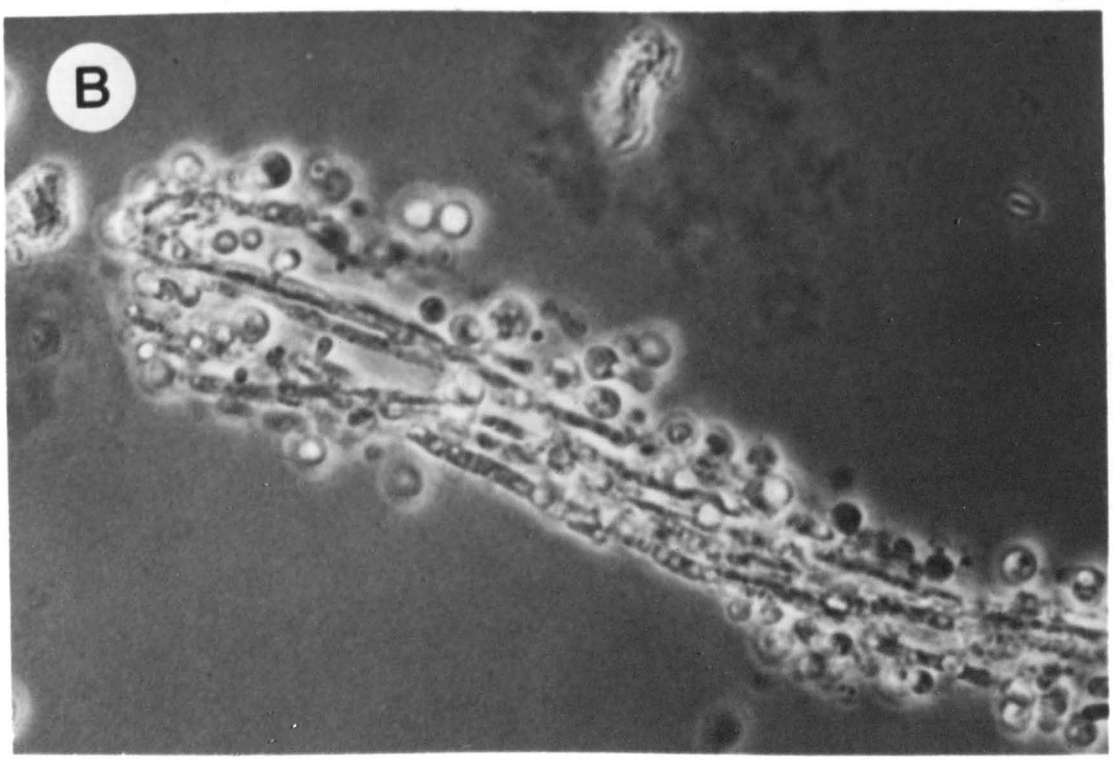
*Protoplasts x 10^8 per gram mycelial fresh weight/ 5 hours.

Figure 2.2 Protoplasts from *Lentinula edodes* and *Pleurotus sajor-caju*

A. Protoplasts of *L. edodes* strain 30-1 suspended in 0.6M sucrose after isolation in 0.6M $MgSO_4$ and purification (x400).

B. Protoplasts released from *P. sajor-caju* strain 32-2 in 0.6M $MgSO_4$/ 50mM $CaCl_2$, after 1 h digestion with Novozym 234 and Cellulase CP (x400).


A


B

2.3.2 Influence of Osmotic Stabilizers on the Timed Release of Protoplasts from *Lentinula edodes* 30-1

Two salts, one sugar and one sugar-alcohol were compared for their ability to stabilize protoplasts from cellophane grown cultures of *L. edodes*. Microscopic counts were made hourly and results are presented in Fig. 2.3. Sucrose and mannitol were found to have an equally good stabilizing effect, and it was noted that at higher concentrations (above 0.6M) protoplasts became highly vacoulated. KCl was found to be relatively unsuitable for *L. edodes*, with 0.6M $MgSO_4$ being the most efficient. Hence, all further experiments with *L. edodes* were carried out using this stabilizer.

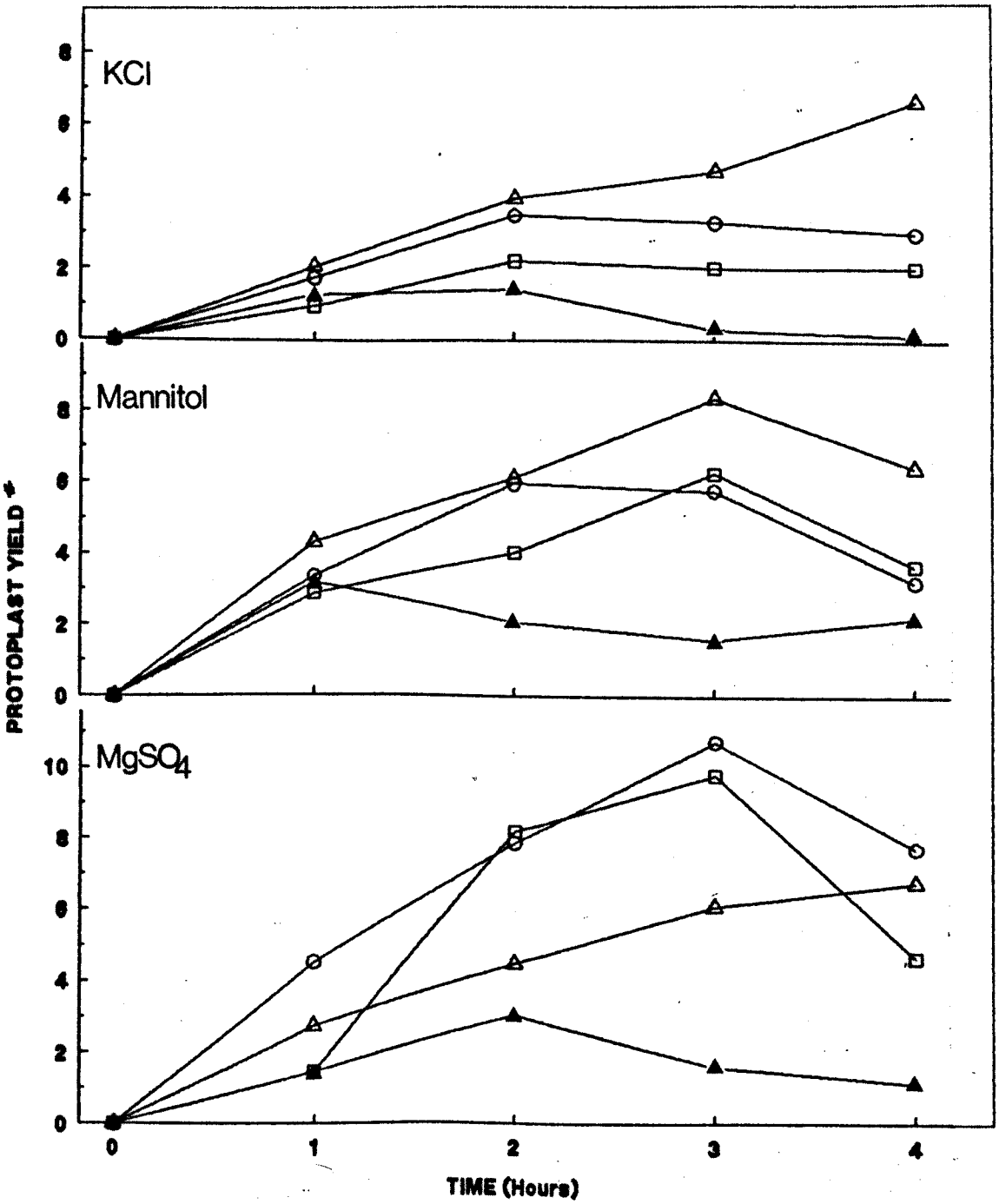
2.3.3 Viability of *Lentinula edodes* Hyphae in Response to Homogenization Time

Results on the effect of maceration time on the number of colony forming units from *L. edodes* are given in Fig. 2.4.

2.3.4 Mycelial Age and its Effect on the Release of Protoplasts from *Lentinula edodes* 30-1

Preliminary results with strain 30-1 had shown that a 4 day regrown hyphal macerate produced using glass beads as being optimum for protoplast release. However, great variation was encountered with this method in terms of mycelial fragmentation which in turn affected digestion. Attempts to standardize the homogenate used as inoculum led to the adoption of the Silverson homogenizer. This had the effect of producing a very homogeneous suspension and was further tested with strain 30-2, 30-3 and 30-4 (see Fig. 2.5). Protoplasts were released using Novozym 234 ($10mg.ml^{-1}$) and stabilized in 0.6M $MgSO_4$ and 50mM sodium succinate, pH 5.8. Once again the optimum age of mycelial regrowth for protoplast liberation was 4 days.

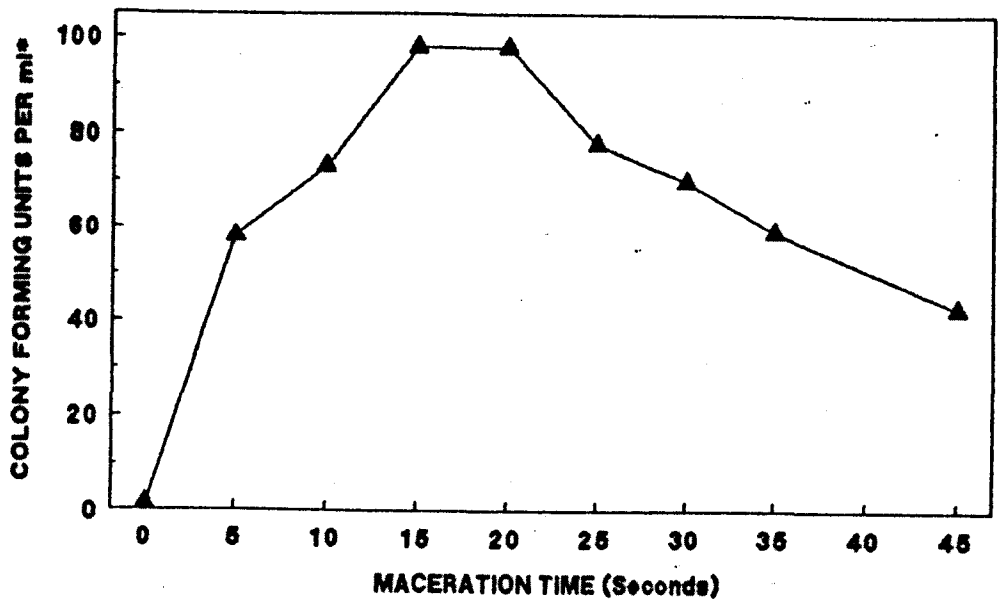
Fig. 2.3 Effect of Osmotic Stabilizers on the Timed Release of Protoplasts from *Lentinula edodes* 30-1



*Protoplasts per ml x 10^{4}.

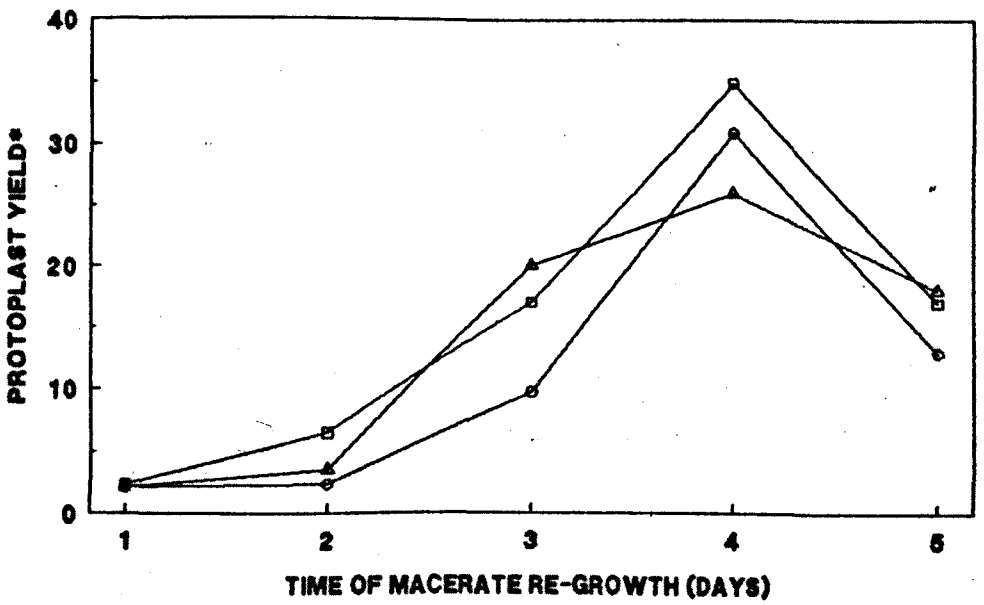
Key to solute concentrations:
—▲— = 0.4M; —○— =0.6M; —□— = 0.8M; —△— = 1.0M.

Fig. 2.4 The Viability of Hyphal Fragments of *Lentinula edodes* Produced over Different Maceration Times



*$_{x10^3}$.

Fig. 2.5 The Effect of Mycelial Age on Protoplast Release from *Lentinula edodes*



Key to strains:
—○— = strain 30-2; —□— = strain 30-3; —△— = strain 30-4.

*Protoplasts x10^6 per gram fresh weight mycelia/ 4 hours.

2.3.5 Optimizing Protoplast Regeneration in *Lentinula edodes* and *Pleurotus* spp.

Regeneration of *L. edodes* protoplasts was initially carried out on osmotically stabilized media by plating protoplasts after centrifugation, as removal of the lytic enzymes was not possible due to the high buoyancy of the protoplasts and their inability to sediment. These initial experiments gave extremely low regeneration frequencies of 0.001-0.09%. Further experiments had shown that embedding the suspension in RMYG containing 0.6% agarose doubled regeneration frequencies. However, attempts to further improve regeneration led to a change in osmotic stabiliser as adopted by Amaral *et al.* (1983). Initial experiments revealed that protoplasts harvested in this manner regenerated at a rate 5 to 10 times greater, thus giving regeneration rates of between 0.5-1%. With *Pleurotus* spp. this method was also used and found most suitable.

Several experiments were carried out on the regeneration of other *L. edodes* strains including 30-2, 30-3, 30-4 and 30-5. Great variation was encountered between repeated experiments for each strain with regeneration ranging from 0.07-0.35%. Regeneration of *L. edodes* protoplasts was found to occur by the formation of an aberrant chain from which a hypha would emerge. Direct growth of a hypha from the protoplast was rarely seen. The various morphologies observed during protoplast regeneration of *L. edodes* 30-1 are shown in Fig. 2.6

Variation of Lytic Enzymes on Protoplast Regeneration of *Lentinula edodes* 30-1

In this series of experiments, various enzyme combinations were tested for their effect on regeneration frequency. Protoplasts were liberated in 0.6M $MgSO_4$ and debris removed by centrifugation. Protoplasts were diluted in 0.6M sucrose and plated directly onto RMYG.

Figure 2.6 Protoplast Regeneration in *Lentinula edodes* Strain 30-1

Morphological variation observed in regenerating protoplasts after 48 h incubation on osmotically stabilized medium (all x400).

A. Hyphal growth direct from a regenerating protoplast.

B. Irregular protoplast regeneration.

C. Abnormal protoplast regeneration.

D. Aberrant chain formation prior to reversion to hyphal growth.

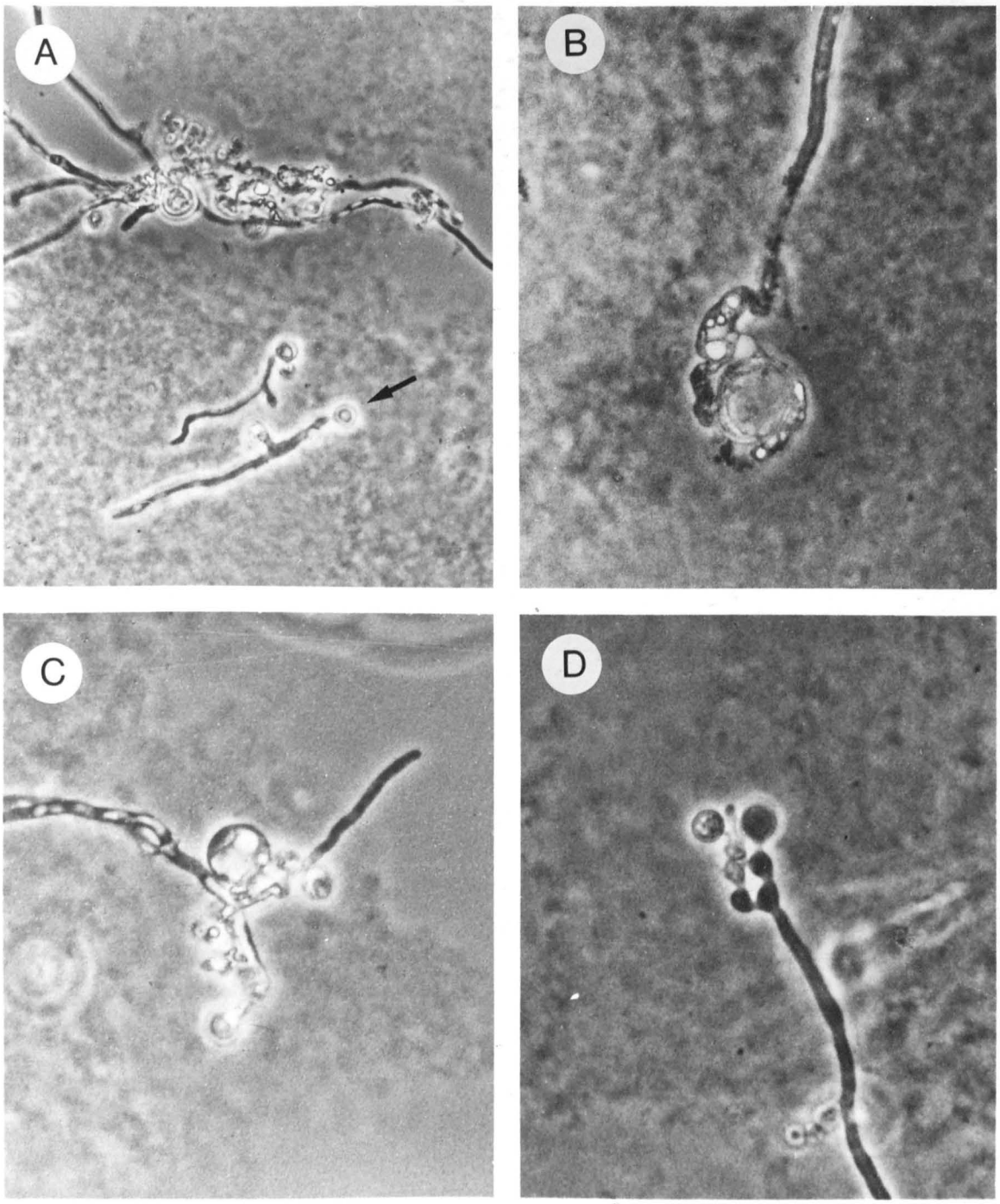

A
B
C
D

TABLE 2.6 The Influence of Mycolytic Enzymes on Protoplast Reversion

EXPERIMENT NUMBER	ENZYMES USED	ENZYME CONCENTRATION	PROTOPLAST YIELD*	REGNERATION FREQUENCY
1	Novozym 234	15mg.ml^{-1}	3.2 x 10^{7}	0.02%
1	Cellulase CP	30mg.ml^{-1}		
	Helicase	10mg.ml^{-1}		
	Chitinase	1mg.ml^{-1}	7.8 x 10^{6}	0.23%
2	Novozym 234	15mg.ml^{-1}	7.4 x 10^{7}	0.03%
2	Cellulase CP	30mg.ml^{-1}		
	β-Glucuronidase	5%v/v	3.0 x 10^{7}	2.64%
3	Novozym 234	10mg.ml^{-1}	1.4 x 10^{8}	0.01%
3	Cellulase CP	20mg.ml^{-1}		
	Helicase	10mg.ml^{-1}		
	Rhozyme	10mg.ml^{-1}	2.0 x 10^{7}	0.77%

*Protoplasts per gram wet weight mycelia/ 4 hours.

Osmotic Stabilizer Effect on the Regeneration of *Lentinula edodes* 30-1 and *Pleurotus florida* 31-1

As a means of further increasing reversion, several osmotic stabilisers at different concentrations were tested. Of those used with *L. edodes* $MgSO_4$, KCl, NaCl and glucose were found totally unsuitable and gave no regeneration at all. Even allowing for the possibility of slower growth, prolonged incubation of up to 6 weeks yielded no regenerants. 0.8M sucrose was found to have a slight improvement on regeneration as compared to 0.6M sucrose. However, with *P. florida*, 0.6M sucrose was found most suitable. As a matter of convenience the lower concentration was used for all further experiments (see Figs. 2.7 and 2.8).

Fig. 2.7 Regeneration Frequencies of *Lentinula edodes* 30-1 on Various Osmotic Stabilizers

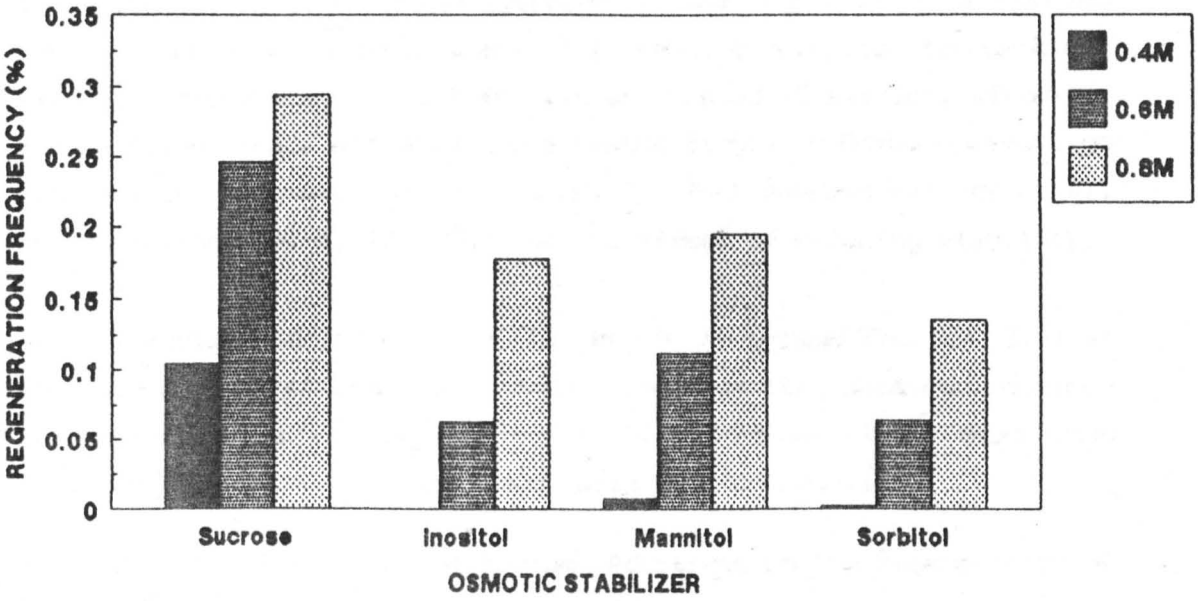
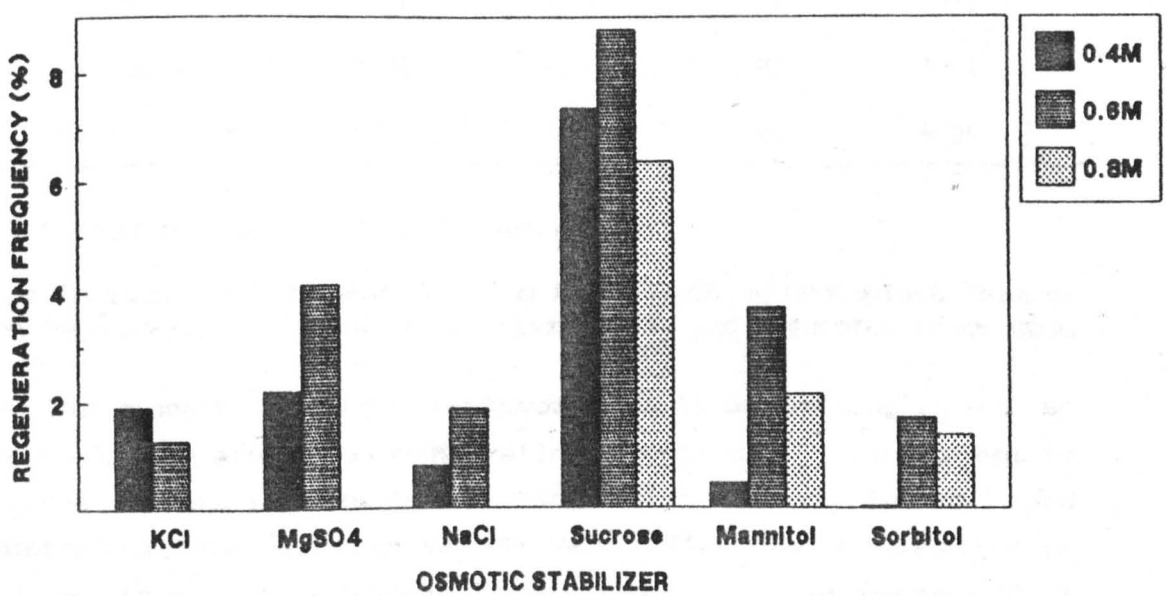


Fig. 2.8 Osmotic Stabilizer Effects on the Regeneration of *Pleurotus florida* 31-1



Influence of Medium Additives on the Regeneration of *Lentinula edodes* and *Pleurotus* spp.

Inclusion of various medium additives in the regeneration medium was investigated as a possible means of increasing reversion frequencies. With *L. edodes* strain 30-1 ferulic acid, sawdust extract, vitamins, minerals and trace elements were tested singly in RMYG, however, no increase in regeneration was found. In fact sawdust extract at all concentrations (0.1%, 1%, 10%) had the effect of reducing viability.

Various medium additives were tested on *Pleurotus florida* 31-1 at several concentrations and from the results obtained optimum concentrations were then applied to *P. ostreatus* 7-4, *P. sajor-caju* 32-1 and *P. pulmonarius* 33-1, see Table 2.7 for results.

TABLE 2.7 The Influence of Medium Additives on the Regeneration of *Pleurotus* species

SUPPLEMENT USED	SPECIES AND STRAIN/ REGENERATION FREQUENCY 7-4	31-1	32-1	33-1
Control	4.20%	4.30%	1.60%	3.69%
0.2% Peptone	4.00%	4.30%	1.54%	3.35%
0.2% LSA	4.93%	4.51%	1.35%	5.47%
Amino Acids	4.95%	4.70%	1.90%	3.93%
Vitamins	4.93%	5.10%	1.65%	4.25%

2.3.6 Protease Inactivation of Novozym 234

Heat Treatment of Novozym 234 and its Effects on Protoplast Release and Regeneration of *Lentinula edodes* 30-1 and *Pleurotus sajor-caju* 32-2

Previous experiments had shown Novozym 234 to be limiting in its use because of an effect on regenerating protoplasts. In an attempt to improve regeneration, protease inactivation was carried out and adapted from the method of van der Valk (1984). Heat treated Novozym 234 was found to have a variable effect on both yield and protoplast viability (see Tables 2.8 and 2.9). Mycelia digestion was monitored hourly as protoplasts were release by lytic action on *L. edodes* 30-1. Protease activity was limited by the addition of trypsin inhibitor or by 10 min heat treatment at 50°C (see Fig. 2.9).

TABLE 2.8 The Effect of Heat Treated Novozym 234 on the Regeneration of Protoplasts from *Pleurotus sajor-caju* 32-2

HEAT TREATMENT (50°C)	PROTOPLAST YIELD*	REGENERATION FREQUENCY
0 min	1.38 x 10^7	1.96%
5 min	1.48 x 10^7	3.08%
10 min	1.50 x 10^7	2.48%
15 min	1.08 x 10^7	3.00%

*Protoplasts/ gram wet weight mycelium/ 3 hours digestion.

TABLE 2.9 Yield and Regeneration of *Lentinula edodes* 30-1, Protoplasts Using Heat Treated Novozym 234

EXPERIMENT NUMBER	HEAT TREATMENT (50°C)	PROTOPLAST YIELD	REGENERATION FREQUENCY
1	0min	2.08 x 10^{8**}	0.2%
	10min	4.64 x 10^{8**}	0.12%
2	0min	3.36 x 10^{8**}	0.24%
	15min	3.04 x 10^{8**}	0.11%
3	0min	2.6 x 10^{7**}	0.38%
	15min	9.5 x 10^{6**}	0.79%
4	0min	1.1 x 10^{6**}	0.28%
	15min	1.6 x 10^{6**}	0.70%

*Protoplasts/ gram wet weight mycelia/ 3 hours.
**Protoplasts/ gram wet weight mycelia/ 4 hours.

Effect on Protease Activity

Preliminary experiments on protease determination of Novozym 234 showed activity to be far higher than the range of trypsin standards used. It was then found that heat treatment dramatically reduced protease levels to below the range of the standards. The results presented in Fig. 2.10 demonstrate the effectiveness of heat inactivation.

2.3.7 Batch Variation of Novozym 234: Consequences for Protoplast Release and Regeneration

It has been well noted in our laboratory that batch differences occur with the product Novozym 234. A series of experiments revealed a striking difference between five batches tested. In addition, the

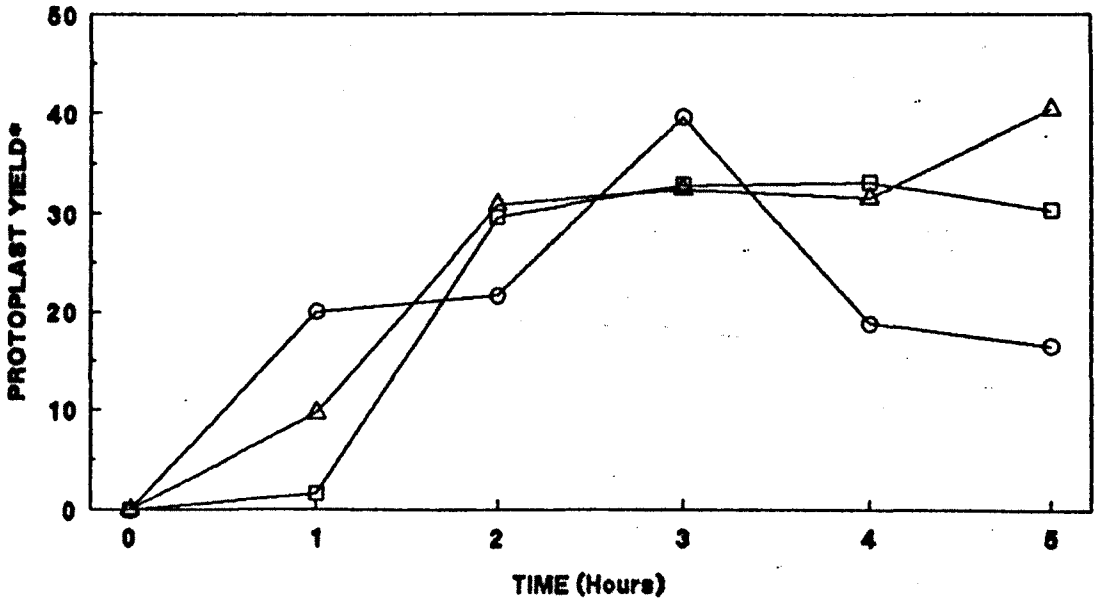
opportunity to test other products manufactured by the same company was given (see Fig. 2.11 and Tables 2.10 and 2.11).

TABLE 2.10 The Influence of Lytic Enzyme and Batch Variation on the Yield and Regeneration of Protoplasts from *Lentinula edodes* Strain 30-1

LYTIC ENZYME USED*	PROTOPLAST YIELD**		PROTOPLAST REGENERATION FREQUENCY (%)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Novozym 234 PPM1906	1.8×10^8	NA	0.16%	NA
Novozym 234 PPM1961	2.08×10^8	3.36×10^8	0.20%	0.24%
Novozym 234 PPM2934	4.28×10^8	2.34×10^8	0.19%	0.29%
Novozym 234 SP-C25-BRT0789	3.28×10^8	3.68×10^8	0.12%	0.58%
Novozym 234 SP-C25-BRT01289	4.4×10^8	1.92×10^8	0.06%	0.11%
Glucanex	NA	4.4×10^7	NA	0.11%

*Used at 10mg.ml^{-1}.
**Protoplasts/ gram wet weight mycelium/ 3 hours digestion.
NA = Not attempted.

Fig. 2.9 The Consequences of Protease Inhibition on the Release and Stability of Protoplasts from *Lentinula edodes* 30-1



Key to treatment:
—○— = Novozym 234; —□— = Novozym 234 + 0.02% trypsin inhibitor;
—△— = Novozym 234 heat treated at 50°C for 15 min.

*Protoplasts per gram fresh weight mycelium x10^7.

Fig. 2.10 Protease Specific Activity of Heat Treated Novozym 234

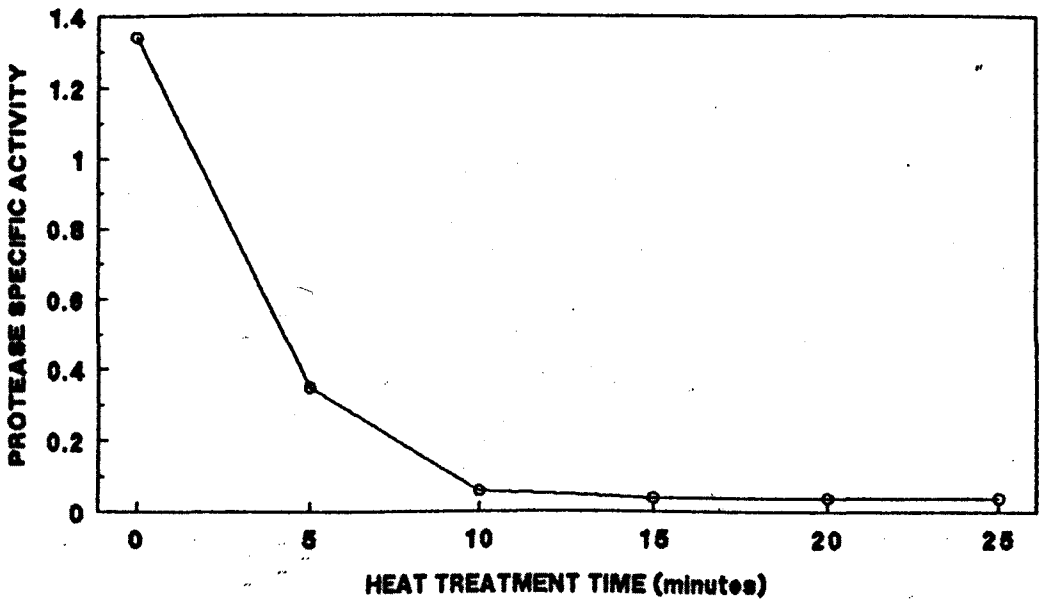
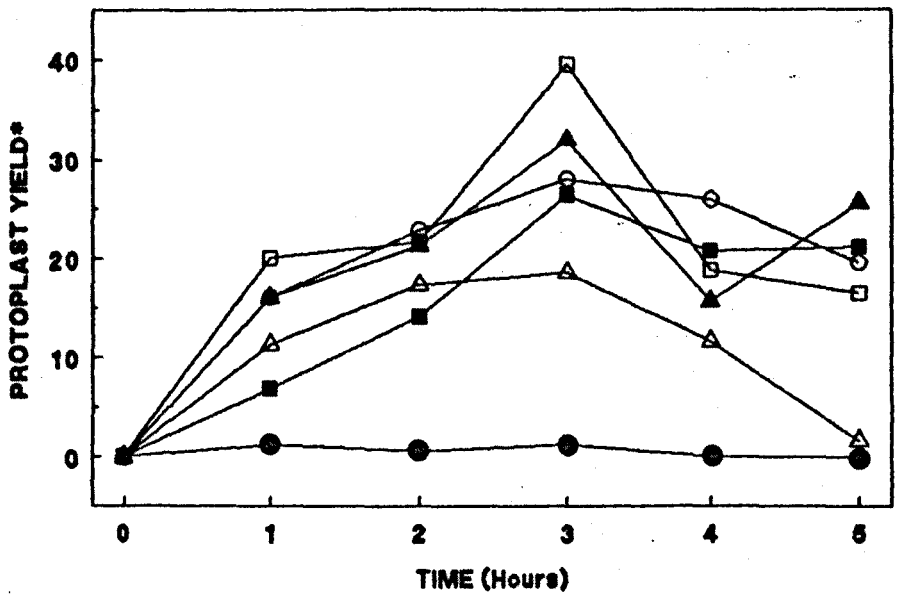


Fig. 2.11 Batch Variation of Novozym 234 on the Timed Release of Protoplasts from *Lentinula edodes* 30-1



Key to enzymes: —●— = Novozym 234 [PPM1523]; —○— = Novozym 234 [PPM1906]; —□— = Novozym 234 [PPM1961]; —△— = Novozym 234 [PPM2934]; —■— = Novozym 234 [SP-C25-BRT0789]; —▲— = Novozym 234 [SP-C25-BTR1289].

*Protoplasts $x10^7$ per gram fresh weight mycelia.

TABLE 2.11 The Effect of Lytic Enzyme and Batch Variation on the Yield and Regeneration of Protoplasts From *Pleurotus sajor-caju* Strain 32-2

LYTIC ENZYMES USED (5mg.ml^{-1} each)	PROTOPLAST YIELD* Expt. 1	Expt. 2	PROTOPLAST REGENERATION FREQUENCY (%) Expt. 1	Expt. 2
Novozym 234 [PPM1523] & Cellulase CP [2697/5]	4.29 x 10^8	4.95 x 10^8	1.46%	1.37%
Novozym 234 [PPM1906] & Cellulase CP [2697/5]	1.24 x 10^9	9.45 x 10^8	3.74%	2.11%
Novozym 234 [PPM1961] & Cellulase CP [2697/5]	1.35 x 10^9	9.92 x 10^8	3.3%	2.63%
Novozym 234 [SP-C25-BRT089] & Cellulase CP [2697/5]	1.15 x 10^8	1.50 x 10^8	1.22%	1.07%
Novozym 234 [PS/NZ234HP] & Cellulase CP [2697/5]	2.65 x 10^8	2.34 x 10^8	6.04%	6.94%
Novozym GX & Cellulase CP [2697/5]	1.29 x 10^8	2.1 x 10^8	2.89%	2.65%
Glucanex & Cellulase CP [2697/5]	NA	9.85 x 10^7	NA	0.45%
Novozym 234 [PPM1961] & Cellulase [8908/3-6]	1.05 x 10^9	1.11 x 10^9	5.57%	5.60%

*Protoplasts/ gram wet weight mycelium/ 3 hours digestion.
NA = Not attempted.

2.3.8 Protoplast Nuclear Frequency

During the course of routine protoplast isolation, samples were fixed and stained to ascertain the proportion of protoplasts containing nuclei. Samples were fixed prior to harvesting to eliminate the possibility of selection for a population of protoplasts. For each experiment, or batch, between 100 and 150 protoplasts were randomly chosen before the nuclear status was determined (see Figs. 2.13 & 2.14 and Table 2.12).

Fig. 2.12 Nuclear Frequency of Protoplasts from *Lentinula edodes* 30-1 Isolated using Different Batches of Novozym 234

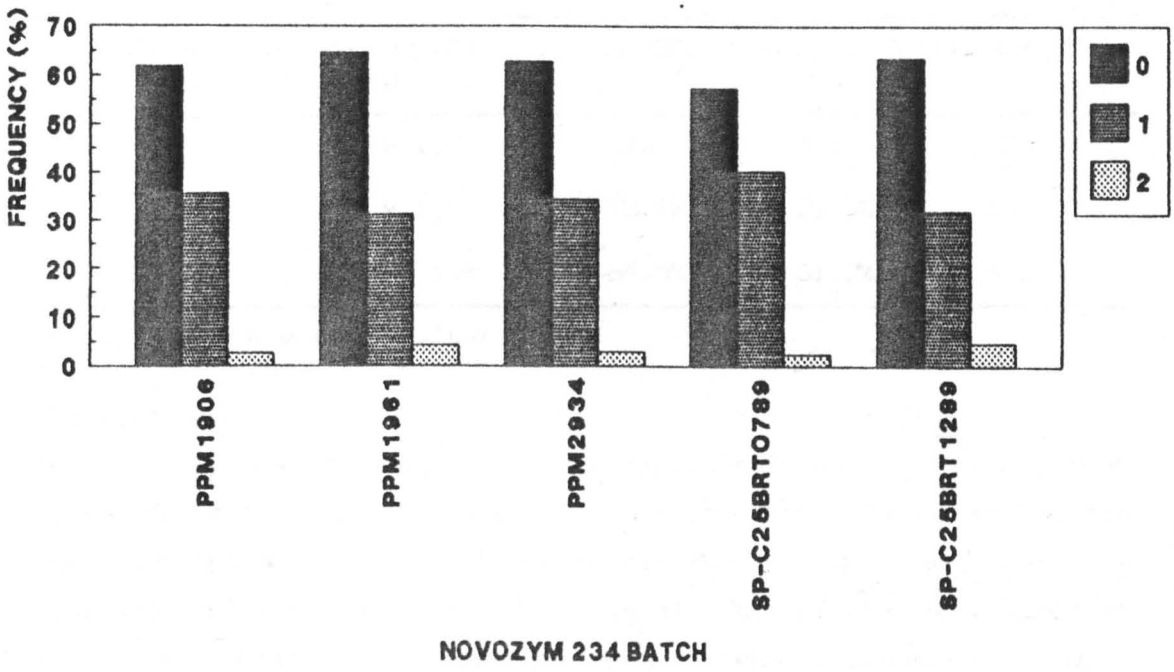


Fig. 2.13 Nuclear Frequency of Protoplasts from *Pleurotus sajor-caju* 32-2 Isolated using Different Batches of Novozym 234

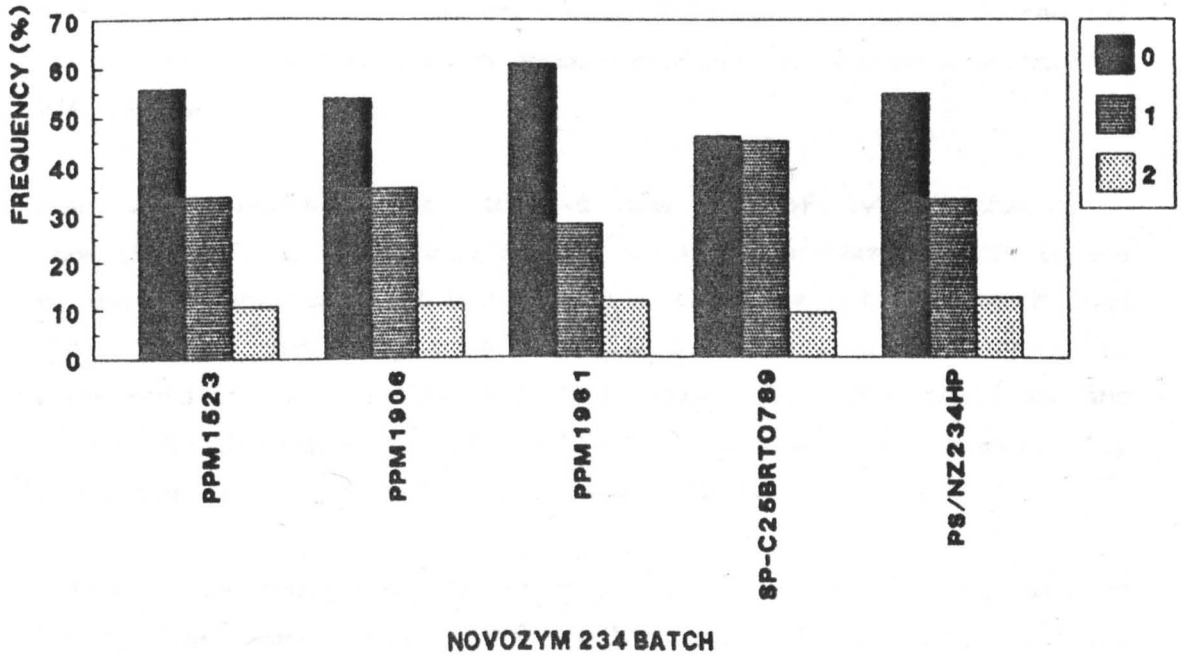


TABLE 2.12 Nuclear Frequency of Protoplasts Isolated from *Lentinula edodes* 30-1

EXPERIMENT NUMBER	PROTOPLAST YIELD*	NUMBER OF NUCLEI/ FREQUENCY 0	1	2
1	4.4×10^7	52%	45%	3%
2	2.2×10^{8}	75.5%	21.5%	3%
3	2.08×10^{8}	64.5%	31.2%	4.3%

*Protoplasts/ gram fresh weight mycelium/ 4 hours.

2.4 Discussion

Numerous applications of fungal protoplasts require a consistent system for efficient release of viable protoplasts. Thus a method for obtaining optimum yields of protoplasts from *L. edodes* and *Pleurotus* spp. was established. Initially several lytic enzymes were screened for their ability to release protoplasts from mycelium, and Novozym 234 was found to be the most suited to both *L. edodes* and *P. sajor-caju*. Although Novozym 234 was found to be more efficient in terms of protoplast yield, a mixture of Novozym 234 and Cellulase CP was adopted for use with *Pleurotus* spp. This was to avoid potential regeneration problems as was encountered with *L. edodes* when Novozym 234 was used.

Most basidiomycete systems involve the use of lytic "cocktails" consisting of up to four different products, and Novozym 234 is one of the few enzymes that has been used alone for optimum yields (see Table 2.1). ß-Glucanase and chitinase are the key mycolytic components of Novozym 234, and this product has higher chitinase and α- and ß- glucanase specific activities compared to commercially available purified enzymes (Yu & Chang, 1987).

Following the results of Hamlyn *et al.* (1981) batch variation between Novozym 234 samples was verified. In addition, new highly purified experimental batches were tested and found to have variable effects with both *L. edodes* and *P. sajor-caju*. Information obtained from work in our laboratory on Novozym 234 has shown that batch numbers PPM1523, SP-C25BRT0789 and SP-C25BRT1289 have about half the chitinase activities of batches PPM1906, PPM1961 and PPM2934 (C. Ullhoa, pers. comm). In addition, limited information from the

manufacturer's of Novozym 234 shows batch PPM1523 as having lower ß-glucanase activity than PPM1906 and PPM1961. From this information and the results gathered in this study it would seem that protoplast yield from *P. sajor-caju* 32-2 is related to chitinase and ß-glucanase activities present. This is in contrast to *L. edodes* which gave comparable yields with batches PPM1961, PPM2934, SP-C25-BRT0789 and SP-C25BRT01289. Low protease batches (SP-C25BRT0789 and SP-C25BRT01289) gave extremely good yields with *L. edodes* and it is possible that protoplasts from this species are more sensitive to external protease activity which may affect stability. This was further verified by monitoring protoplast numbers during digestion with and without reduced protease levels in the lytic solution.

It is a common observation that actively growing areas of cell walls are the first to be degraded by lytic action. Protoplasts from *Polystictus versicolor* were seen to be released from both the tips of growing hyphae and also clamp connections, i.e. where the cell wall is thinnest (Strunk, 1965). Protoplasts from *Pleurotus* spp. were more commonly found to be released from all parts of the cell, and it was not unknown for complete dissolution of the hyphal wall to occur giving rise to chains of protoplasts. Release from *L. edodes* was mainly confined to hyphal tips. Repeated constriction of the cytoplasm may account for the low proportion of nucleated protoplasts in both *L. edodes* and *P. sajor-caju*.

L. edodes was found to give relatively low yields of protoplasts despite optimization of several variables. Mycelium was observed to be poorly digested with relatively large hyphal fragments present in the lysis mixture. Mycelia of *L. edodes* was always found to be highly pigmented in comparison to many of the *Pleurotus* species. Thus it is possible that the active components of these lytic mixtures are inhibited to a certain extent by the nature of the cell wall. Bull (1970b) showed that melanin present in cell walls of *Aspergillus nidulans* inhibited both chitinase and ß-glucanase activity, although no study has been made on this relationship with protoplast release. Combined action of chitinase and ß-glucanase for cell wall lysis was also demonstrated by Bull (1970b) in *A. nidulans* where a ß-glucan was found to protect chitin, and a certain proportion of chitin present

was resistant to hydrolysis. Association of chitin and ß(1,3) glucan in cell walls of *S. commune* has been demonstrated by Siesma & Wessels (1979) and in *Armillaria mella* (Hernandez *et al.*, 1990)

Attempts to reduce melanin deposition in *L. edodes* by Na_2EDTA following the method of Chet & Henis (1969) were limited as the mycelium failed to grow when 1mM or 10mM Na_2EDTA was incorporated into the medium. Inhibitors of melanin biosynthesis such as tricyclazole have been found to be highly effective amongst species of Ascomycetes and some Fungi Imperfecti, although ineffective against Basidiomycetes (Wheeler, 1983). It is yet to be investigated whether high protoplast yields can be obtained by inhibiting melanin biosynthesis or its deposition in fungal cell walls

Using Novozym 234 to then test the variable effects of osmotic stabilizer and concentration on protoplast stability and yield, found 0.6M $MgSO_4$ as being a suitable osmoticum. Magnesium sulphate or mannitol have each been used previously for protoplast release from *L. edodes* and numerous *Pleurotus* spp. (see Table 2.1). Selection of the optimum osmoticum and concentration for protoplast stability is a key variable affecting protoplast yield from many fungal species (e.g. Amaral *et al.*, 1983; Lynch *et al.*, 1985; Hocart *et al.*, 1987). However, the influence of the osmoticum is not limited to providing support to the protoplast but has been shown to affect component activities of several enzymes including α- and ß-glucanases (Yu & Chang, 1987), chitinase (Thomas & Davis, 1980; Yu & Chang, 1987), celluases (Muller & Ryscnka, 1987) and snail enzyme (Arnold & Garrison, 1979).

Of the osmotic stabilizers tested on *L. edodes* all but KCl gave rise to highly vacuolated protoplasts. Chang *et al.* (1985) also observed this feature when protoplasts of *L. edodes* were isolated in sucrose. This property was found very useful for harvesting protoplasts from digests as they remain in suspension after centrifugation. In addition, this avoided filtration through sintered glass, which greatly reduced the number of protoplasts harvested. This effect is not universal as Sonnenberg *et al.* (1988) were unable to float protoplasts of *A. bisporus* and *A. bitorquis* when magnesium sulphate

was used and this stabilizer was shown to inhibit protoplast regeneration of *V. volvacea* when used during isolation (Mukherjee & Sengupta, 1986)

The buffer solution used during lysis was not investigated for variation, and so it is possible that further optimization would require screening other buffers and a range of pH's for greatest yield. You *et al.* (1988) found higher protoplast yields were obtained from *P. sapidus* without the use of a buffer and several commonly used buffers such as phosphate, McIlvaine and sodium maleate were found to dramatically reduce protoplast yields. Although early workers with *L. edodes* found a pH of 5.8 to be sufficient for protoplast release (Nakai & Ushiyama, 1977; Toyoda *et al.*, 1984), others have found a pH of 4.6 to be optimum (Kawasumi *et al.*, 1987).

From this point considerable attention was given to the preparation of the fungal inoculum. As yields from cultures grown on cellophane were found to be limiting, work concentrated on protoplast isolation from macerated mycelia growth in static liquid culture. This method is thought to increase the number of young hyphae which develop by branching from the homogenized fragments (Morinaga *et al.*, 1985), and presumably increases the number of cells undergoing extension and thus more prone to lytic action. The age at which the mycelium is digested has a profound effect on yields with an optimum culture age giving peak yields. Before or after this time, reduced protoplast numbers are found (e.g. Yamada *et al.*, 1983; Ohmasa *et al.*, 1987).

Numerous methods were employed to find the most suitable homogenization method, time and regrowth period. Lack of attention to detail for the preparation of mycelial homogenates is common amongst literature for basidiomycete protoplast isolation, e.g. Santiago, 1982; Toyomasu *et al.*, 1986; Kawazumi *et al.*, 1987. The method developed although standardized as far as possible did lead to considerable variation in the final yields and regenerative ability of protoplasts obtained. The cause of the variation experienced is still not clear.

Several homogenization methods were tested by Kawasumi *et al.* (1987) with *L. edodes* and the best involved fragmentation by use of a razor. When this was attempted in our laboratory it was found to be prone to contamination, yielded very little fresh weight mycelium for the effort, and was very difficult to standardize. However, protoplasts obtained from mycelia are not necessarily optimum for these studies. Protoplasts from basidiospores of *L. edodes* were found to have a ten-fold regenerative ability over protoplasts with hyphal origin (Woo & Yoon, 1985). Pre-germinated basidiospores of *S. commune* have been used successfully and found to require a short digestion period, with each spore seen to liberate a single protoplast (Ullrich *et al.*, 1985). Alhough the spores are the result of a meiotic division, heterogenity in terms of the number of nuclei per protoplast was found to be minimized and higher regneration frequencies obtained. Attempts to obtain fruit bodies of *L. edodes* for basidiospore isolation were limited in success and highly prone to contamination (see Chapter 3) which restricted alternative inocula for these studies. Akamatsu *et al.* (1983) were successful in converting 90% of oidia into protoplasts from *C. cinereus*, however, *L. edodes* and the *Pleurotus* spp. studied here do not form oidia and so this further confined experiments to use with hyphal macerates.

Regrowth of the hyphal macerate of *L. edodes* was required to obtain high yields of protoplasts. The medium used was not investigated for variable effect on protoplast yields , although the use of rich liquid media is most commonly used for the species listed in Table 2.1. Yamada *et al.* (1983) found protoplast release from *P. ostreatus* to vary greatly with the nutrient content of the medium used. A complete medium supplemented with sulphite pulp waste compounds has been used to produce material for optimum protoplast isolation from *P. ostreatus* (Iijimia & Yanagi, 1986) and *L. edodes* (Kawasumi *et al.*, 1987). Addition of other compounds such as ferulic acid (Kawamura *et al.*, 1983) which are known to increase the growth rate of *L. edodes* could possibly have the same effect.

Regeneration of protoplasts was shown to be highest when sucrose was used as the osmotic stabilizer during regeneration of both *L. edodes* and *P. florida*. Sucrose is commonly used for this purpose with many

other species, e.g. *C. macrorhizus* (Kiguchi & Yanagi, 1985) and *F. velutipes* (Yea *et al.*, 1988). Other media and additional supplements could have been tested to obtain greater reversion frequencies, which in the case of *L. edodes* were considerably lower than previous reports (Kawasumi *et al.*, 1987).

Regeneration of *L. edodes* protoplasts was observed to follow one of two morphological patterns. The most frequent involved the formation of irregular budding structures which varied in length, with the ultimate appearance of a regular shaped hypha. The direct formation of normal hypha was less commonly seen. In numerous fungi a budding chain forms from the original protoplast and eventually one or more normal hyphae develop. Abe *et al.* (1982) found that this form of reversion was only observed with protoplasts from *Tricholoma matsutake* when incubated on a solid medium. Gibson and Peberdy (1972) have observed a reduction in the length of the abberant hypha in reverting protoplasts of *A. nidulans* if N-acetyl-glucosamine was added to the regeneration medium. These patterns of growth are dependent on the relationship between cell volume and the rate of cell wall repair. Thus a balance in the deposition of wall material and an increase in cytoplasmic volume will allow for rapid reversion. If cell wall repair occurs at a rate slower than the increase in cellular volume, abnormal morphologies result (Necas & Svoboda, 1985).

Protoplast reversion of *L. edodes* was found to be highly sensitive to the lytic enzymes used. Novozym 234 was shown to have adverse effects on regeneration compared to other enzyme combinations. This was initially thought to be due to the high protease activity present in this product (Hamlyn *et al.*, 1981). Attempts were made to reduce protease activity by heat inactivation which was found to have variable effects. Addition of trypsin inhibitor and use of low protease batches of Novozym 234 were also used to test protoplast release and reversion.

Further work could have included optimizing concentrations of trypsin inhibitor or bentonite (Kitamoto *et al.*, 1988) to the lytic solution. An alternative to increasing regeneration after lysis with Novozym

may be the development of optimum conditions for protoplast release by use of other commercial products. Although found not to be as suitable, the combined use of several lytic enzymes and the pre-treatment (e.g. DTT, 2-mercaptoethanol, or Na_2EDTA) of mycelia prior to digestion may overcome the limitations of lower yields. Choi *et al.* (1987) obtained three-fold increases in protoplast yields by pre-treatment of mycelia from *G. lucidum* with Na_2EDTA or 2-mercaptoethanol. Protoplasts from basidiomycetes tend to suffer from low yields and regeneration frequencies and thus it may be that a more complex stabilizer system needs to be employed as have been in use with plant systems (e.g Finch *et al.*, 1990).

Nuclear determination of both *L. edodes* and *P. sajor-caju* protoplasts revealed a large proportion lacked a nucleus which is not unusual (Lynch *et al.*, 1985; Seh & Kennerley, 1988; Barrett *et al.*, 1989). An interesting trend is seen in that the highest yields from *L. edodes* rendered fewer nucleated protoplasts and the reverse was true when lysis was less efficient. This might have consequences for other aspects of this study. Work by Isaac (1987) revealed that the nuclear frequency in protoplasts of *A. nidulans* was influenced by both the digestion period and the osmoticum used. The relatively low proportion of nucleated protoplasts (0-27%) from several mycorrhizal fungi was considered to be a reflection of their sparse distribution pattern in growing hyphae (Barrett *et al.*, 1989).

3. A Breeding Strategy for *Lentinula edodes*

3.1 Introduction

Classical techniques of strain breeding involve the combination of specific inheritable traits from pre-existing cultivars. However, hybridization ultimately depends on limitations imposed by incompatibility factors of the mating system. Adoption of a mating system in higher fungi is a means of limiting self-fertilization and the promotion of out-breeding (Mather, 1942). Fertility mechanisms and structures have been extensively studied amongst basidiomycetes and the following is a brief review of the literature relating to mating systems as they apply to natural, and potentially, commercial breeding.

Fungal Incompatibility Systems and Their Genetic Structure

Self-fertility and self-sterility is controlled by a variety of mechanisms in different species including primary homothallism, secondary homothallism, and heterothallism. A summary of these systems is given, with examples, in Table 3.1.

Of the heterothallic and secondary homothallic species, one of three mating systems is employed with different genetic organization and varying consequences for breeding. Unifactorial, or bipolar, systems are controlled by a single factor which is either biallelic (in lower fungi) or multiallelic (in higher fungi). Heterokaryotic mycelia results only if two isolates, carrying different factors, mate. The potential for cross-fertility with siblings in heterothallic species is a maximum 50%. In higher fungi the *A* factor is thought to comprise of a single locus although relatively little is known about its structure.

Bifactorial, or tetrapolar, systems are regulated by two unlinked incompatibility factors and a fertile heterokaryon is formed only if two isolates are heteroallelic for both incompatibility factors. In theory 25% of siblings are compatible in heterothallic species thus promoting out-breeding. A few examples of tetrapolarity amongst edible basidiomycetes include *Agrocybe aegerita* (Meinhardt *et al.*, 1980), *L. edodes* (Nisikado & Yamauti, 1935; Takemaru, 1961),

Pleurotus ostreatus (Eugenio & Anderson, 1968), and *Pleurotus sajor-caju* (Roxon & Jong, 1971).

TABLE 3.1 Genetic Structures of Fungal Mating Systems

FERTILITY MECHANISM	POLARITY	STRUCTURE	EXAMPLE(S)
Primary Homothallism			*Coprinus sterilinus* (Raper, 1966) *Phanerochaete chrysosporium* (Alic *et al.*, 1987)
Secondary Homothallism	Bipolar	1 locus/2 alleles	*Neurospora tetrasperma* (Fincham & Day, 1971) *Coprinus sassii* (Kemp, 1975)
	Bipolar	1 locus/3 alleles	*Coprinus bisporus* (Kemp, 1980) *Podospora anserina* (Bernet, 1965)
	Tetrapolar	2 loci	*Coprinus plagioporus* (Fincham & Day, 1971) *C. subpurpureus* (Fincham & Day, 1971) *Clitocybe litus* (Raper, 1966) *Marasmius limosus* (Raper, 1966) *Mycena tenella* (Raper, 1966)
Heterothallism	Bipolar	1 locus/2 alleles	*Neurospora crassa* (Perkins *et al.*, 1982) *Saccharomyces cerevisiae* (Ahmad, 1953)
	Bipolar	1 locus/ multiple alleles	*Pholiota nameko* (Arita & Takemaru, 1962) *Polyporus palustris* (Flexer, 1969)
	Tetrapolar	2 loci (*A* and *B* each with multiple alleles	*Schizophyllum commune* (Raper & Miles, 1958) *Coprinus cinereus* (Day, 1960) *P. ostreatus* (Eugenio & Anderson, 1968)
	Tetrapolar	2 loci (*a* and *b*) *a* has two alleles *b* had multiple alleles	*Ustilago maydis* (Rowell & DeVay, 1954) *Tremella mesenterica* (Bandoni, 1963)
	Octapolar	3 loci (A, B & C)	*Psathyrella coprobia* (Jurand & Kemp, 1973)

Natural populations of most tetrapolar species have a large number of both *A* and *B* factors (Raper *et al.*, 1958a; Eugenio & Anderson, 1968); Tokimoto *et al.*, 1973; Meinhardt & Leslie, 1982). Miles & Chang (1987) are currently using mating factors of *L. edodes* to determine the percentage germplasm that is present in a strain collection with the aim of conserving the species. Relatively few mating type factors of *L. edodes* in the Japanese wild population have been suggested to be due to "contamination" by cultivated strains (Tokimoto & Komatsu, 1978).

In *Schizophyllum commune* each *A* and *B* factor comprises two linked loci known as Aα and Aß and Bα and Bß respectively. Compatibility for either factor requires allelic difference at α, ß, or both. Analysis of the frequency of these loci in nature has predicted the occurrence of 9 Aα and 32 Aß alleles (i.e. 288 different *A* factors) and 8 Bα and 8 Bß alleles (81 different *B* factors) (Raper *et al.*, 1960; Parag & Koltin, 1971; Stamberg & Koltin, 1973). Little evidence exists for the two locus structure in other tetrapolar species although it has been implied as being characteristic of bifactorial species (Koltin *et al.*, 1972). This assumption is based on the appearance of non-parental factors amongst spore progeny of several species including *Flammulina velutipes*, *L. edodes*, *P. ostreatus*, *P. spondelicus* and *S. commune* (Takemaru, 1961; Eugenio & Anderson, 1968).

Functions of the Incompatibility Factors

The factors described previously not only determine the mating system but also control the formation and maintenance of a fertile heterokaryon. From studies of incompatible mating reactions with *Coprinus cinereus* the functions of the *A* and *B* factors have been established (Swiezynsky & Day, 1960a & 1960b). Nuclear pairing, conjugate nuclear division, and clamp connection formation and septation is controlled by the *A* factors.

Morphogenic sequences controlled by the *B* factors include nuclear migration, clamp cell fusion and septal dissolution (Raper, 1966). In relation to the *B* factor function, A=B≠ (common *A*) heterokaryons of *S. commune* show a ten-fold difference in R-glucanase activity as compared to homokaryons. Thus the enzymatic requirement for septal dissolution functions only when different *B* factors are present, allowing nuclear migration to proceed (Wessels & Niederpruem, 1967).

Clamp connections always occur in a balanced heterokaryon and their role in higher fungi involves the maintenance of the dikaryotic condition (see Fig. 3.1A). Although not present in all basidiomycetes they are characteristic of dikaryotic mycelia (Casselton, 1978), and thus have been used to determine compatibility in numerous species. So called "false" clamp connections occur in several species,

including *L. edodes* (Tokimoto *et al.*, 1973), and are characteristic of A≠B= (common *B*) heterokaryons. False clamps are formed because of the failure of the hook cell to fuse with the sub-terminal cell (see Fig. 3.1B). Takemaru & Ide (1970) were able to observe false clamps at the contact zone of A≠B= crosses only when a minimal medium was used with *Microporus flabeliformis*. Aberrant clamp connections have been found to arise spontaneously in *L. edodes* which was also found to render the dikaryon infertile (Murakami *et al.*, 1987).

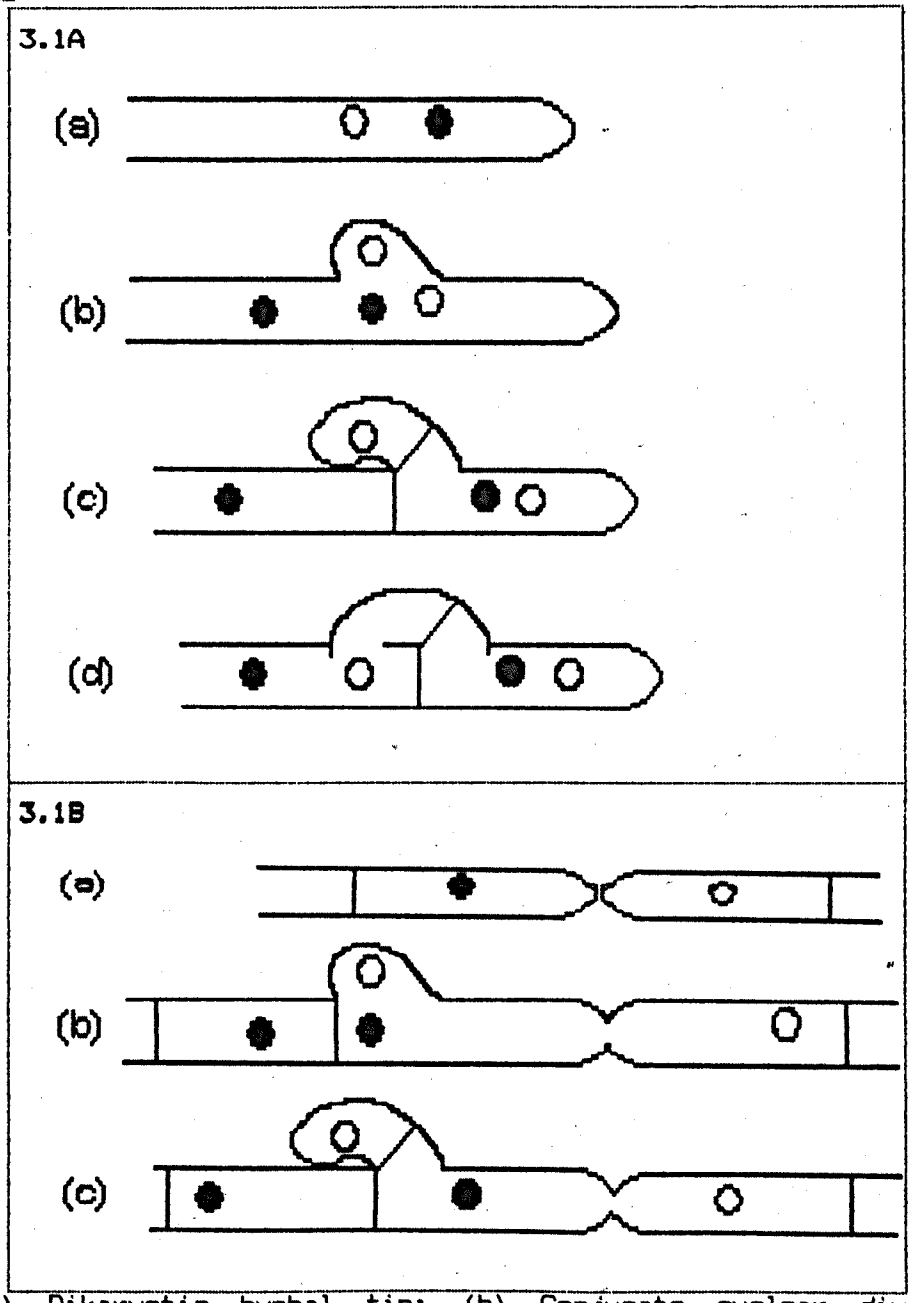
The functions of the *a* and *b* loci of the smut fungus *Ustilago maydis* are quite different from the *A* and *B* factors previously described and are known to control dimorphism and pathogenesis (Rowell & DeVay, 1954). Recent analysis of cloned *b* alleles has show that the products of *b* code for DNA binding polypeptides (Schultz *et al.*, 1990). Interactions between *b* products to form dimers were proposed to regulate development in this fungus.

Several models have been put forward to explain the mechanisms by which the incompatibility factors regulate the complex morphogenic and biochemical changes associated with heterokaryon formation and sexual reproduction (Raper, 1966; Kuhn & Parag, 1972; Ullrich, 1978; Schultz *et al.*, 1990; Metzenberg, 1990). A common feature of these models is the formation of heteromers, of either nucleic acids or proteins, which activate or derepress target genes.

Isolation and characterization of mating-type alleles from several species has been carried out to elucidate the mechanisms by which development is regulated. Sequence divergence between cloned Aα alleles of *S. commune* (Giasson *et al.*, 1989) is also characteristic of *A* and *a* alleles of *Neurospora crassa* (Glass *et al.*, 1988). This dissimilarity between cloned factors has led to the introduction of the term "ideomorph" to replace what has generally been accepted as "allele" (Metzenberg & Glass, 1990). Interestingly, both *A* and *a* ideomorphs of *N. crassa* were found to show homology to several other heterothallic, and one secondary homothallic, species of *Neurospora* in addition to species of the genera *Anixiella*, *Gelasinospora* and *Sordaria* (Glass *et al.*, 1988 and 1990). In *C. cinereus* there is no

cross hybridization between ideomorphs of the Aα and Aß loci (Mutasa *et al.*, 1990).

Fig. 3.1 Clamp Cell Formation in Fully Compatible and Common *B* Matings



A. (a) Dikaryotic hyphal tip; (b) Conjugate nuclear division, formation of a lateral branch; (c) Cross-wall formation separates the migrating nucleus form the apical cell; (d) Lateral branch fuses with the subterminal cell resulting in two dikaryotic cells. B. (a) Confronted monokaryons with common *B* factors; (b) Nuclear exchange and formation of a lateral branch; (c) Lateral branch fails to fuse and a false clamp forms, restricting nuclear migration.

In sharp contrast to the above examples, cloned *b* alleles of *U. maydis* have been found to have a high degree of conservation at the protein level (Schultz *et al.*, 1990). Also, cloned *S* and *s* genes of *Podospora anserina* have revealed a high degree of homology at the both DNA and RNA levels (Turcq *et al.*, 1990.)

Aims of This Study

This study was carried out to identify mating type factors of commercial and wild-type strains which could be adopted as a standard nomenclature for the species *L. edodes*. Characterization of isolates with recombinant factors would be used as a means of elucidating the genetic structure of the mating-type factors. Once strains are characterized novel hybrids could be produced following a strategy proposed in Chapter 1. Fruiting trials would hopefully be carried out to assess the commercial viability of the strains produced.

3.2 Materials and Methods

3.2.1 Media

Strains were maintained on PMA at 25°C (see Chapter 2). For matings, a low cost medium (YPMA) was used which contained per l: malt extract (Boots), 8g; sucrose (FSA), 8g; Yeomans dried potatoes, 1g; agar (Sigma Chemical Co.), 20g. Protoplast regeneration was routinely carried out on RMYG as in Chapter 2.

Media used for the production of fruit bodies included the defined medium of Leatham (1983), a semi-defined medium as used by Tokimoto and Kawai (1975), and the complex substrate developed by Royse (1985).

Leatham's vegetative medium contained, $g.l^{-1}$: glucose, 20g; glucuronic acid, 4g; glutamic acid, 2.5g; $MgSO_4.7H_2O$, 2.0g; KH_2PO_4, 2g; mineral solution, 10ml; trace element solution, 1.0ml; vitamin solution, 1.0ml; salicylic acid solution, 0.1ml; pH adjusted to 4.0 with KOH and sterilized by filtration. Leatham's fruiting medium was similar to the vegetative medium except glucose was increased to $50g.l^{-1}$ and glutamic acid was raised to $5g.l^{-1}$. The mineral solution used contained, $g.l^{-1}$: $CaCl_2.2H_2O$, 3.67g; $MnSO_4.5H_2O$, 4.39g; $ZnSO_4.7H_2O$, 2.20g. The trace element solution contained per l:

$Fe(NH_4)_2(SO_4).6H_2O$, 14.1g; $CuSO_4.6H_2O$, 784mg; $CoCl_2.6H_2O$, 81mg; $NiCl_2.6H_2O$, 81mg; $NaMoO_4.2H_2O$, 51mg; $SnCl_2.2H_2O$, 38mg; concentrated HCl, 2ml. The vitamin solution contained per l: *i*-inositol, 1.0g; thiamine.HCl, 1.0g; *p*-aminobenzoic acid, 0.1g; nicotinic acid, 0.1g; calcium pantothenate, 0.1g; pyridoxine. HCl, 0.1g; riboflavine, 0.1g; biotin, 30mg; cyanocobalamin, 10mg; folic acid, 10mg. Salicylic acid was dissolved in 95% ethanol to give $10g^{l-1}$.

PS medium: fresh potatoes, 200g; sucrose, 20g; pH adjusted to 4.1 and volume to 1l with tap water. Sterilised by autoclaving at 121°C for 30 min.

Spawn medium (for one 500ml flask): yellow millet, 80g; oak sawdust, 6g; $CaCO_3$, 1g; tap water, 105ml. Sterilised by autoclaving at 121°C for 45 min. Spawn substrate: oak sawdust, 158.75g; wheat bran, 170g; tap water, 1l. Sterilised by autoclaving at 121°C for 45 min and repeated sterilization after 24 h.

3.2.2 Strains

Isolates used in this study included all strains listed in appendix A and in addition the strains 30-5002, 30-5009, 30-5025, 30-5051, 30-5068, 30-5079, 30-5085, 30-5099, 30-5103, 30-5104 30-5105, and 30-6073 (see appendix B). Single spore isolates derived from individual strains are denoted by a number in parenthesis, thus isolate number 5 from strain 30-4 would be referred to as 30-4(5). Protoplast regenerants derived from the various strains are denoted by a number after a period placed after the parental strain number, thus regenerant number 5 from strain 30-4 would be referred to as 30-4.5.

Novel dikaryons developed during the course of this work were catalogued according to the origins of the parental homokaryons as summarized in Table 3.2.

TABLE 3.2 A Catalogue System Introduced for Novel Strains of *Lentinula edodes*

GROUP	BDUN SERIES	DETAILS
A	30-1000	Intrastrain crosses of protoplast regenerants.
B	30-2000	Intrastrain crosses of single spore isolates.
C	30-3000	Intrastrain crosses of protoplast regenerants and single spore isolates.
D	30-4000	Interstrain crosses of protoplast regenerants.
E	30-5000	Interstrain crosses of single spore isolates.
F	30-6000	Interstrain crosses of protoplast regenerants and single spore isolates.

3.2.3 Fruit Body Induction and Spore Isolation

Induction of Fruiting Bodies Using a Defined Medium

The method of Leatham (1983) for the production of fruit bodies was carried out as follows: 10 x 4mm plugs of mycelia were inoculated into 25ml of Leatham's vegetative medium in 250ml conical flasks. Two flasks were harvested for each strain after 20 to 30 days growth in static culture at 25°C in the dark. The mycelium was macerated in 200ml (brought to volume with SDW) using a Silverson homogenizer for 20-30 seconds on low speed. 5ml of this was used as an inoculum into 250ml conical flasks containing 20ml of Leatham's fruiting medium at x 1.25 final concentration. Five flasks were inoculated for each of the strains 30-1, 30-2, 30-3, 30-4 and 30-5. The cultures were then incubated statically at 22°C in a Fisons growth cabinet under constant illumination and humidity at 70%.

Induction of Fruiting Bodies by Replacement Culture

The method of Tokimoto and Kawai (1975) was followed and modified where necessary: 150ml of PS medium, contained in 500ml conical flasks, was inoculated with 10 x 4mm plugs and incubated in a Fisons growth cabinet at 25°C in an 8 hour light/ 16 hour dark photoperiod. After 6 weeks growth an established mat of hyphae had developed on the liquid medium. This medium was aseptically removed using a 10ml pipette and replaced with 50ml of an 8% glucose solution, pH 4.1. Flasks were returned to the growth cabinet at 15°C in an 8 hour light 16 hour dark photoperiod.

Induction of Fruiting Bodies Using a Supplemented Sawdust Medium

Spawn medium was prepared in wide neck 500ml conical flasks according to the method of Royse (1985) except rye grain was replaced with yellow millet. 10 x 4mm plugs of mycelia were inoculated into one flask for each strain. Cultures were incubated at 25°C in the dark for 2-3 weeks and flasks were shaken twice weekly. This spawn was then used as an inoculum into spawn substrate medium contained in wide neck glass jars (base diameter, 11cm; neck diameter, 8.5cm; height, 8cm) stoppered with cotton wool. The spawn was allowed to "run" for four weeks at 25°C in an 8 hour light/ 16 hour dark photoperiod, after which time the substrate was well colonized. The temperature was then lowered to 17°C and humidity increased to a maximum 80%. When primodia were observed at the side of the jar or on the undersurface, the glass jars were tilted to one side to allow the fruit bodies to extend freely.

Spore Isolation and Germination

Basidiospores were isolated from fresh fruit bodies by placing caps downwards in sterile crystallizing dishes and left for 24h at room temperature. Spore prints were suspended in 0.01% Tween 80, harvested by centrifugation and washed twice using sterile distilled water (SDW) prior to plating onto PMA containing 50$\mu g ml^{-1}$ ampicillin and 100$\mu g ml^{-1}$ streptomycin. Unused spore suspensions were centrifuged and the spore pellets resuspended in 15%v/v glycerol and stored at -70°C until needed.

3.2.4 Crosses Between Monokaryotic Isolates

Matings were routinely set up between monokaryotic single spore isolates or protoplast regenerants. Organization of large numbers of crosses was aided by sub-dividing matings into groups as determined by the origin of the monokaryotic parents (see Table 3.2). Initial screening was carried out by mating isolates by plating plug inocula onto thick (30-40ml in 9.0cm dishes) PMA, which were incubated at 25°C. After 14-21 days the colonies had merged and small plugs could be easily taken from the contact zone using a tungsten wire needle (0.25mm, Goodfellow, Cambridge, England). A total of 12 plugs were removed and transfered to PMA and incubated for a further 4-7 days at 25°C (see Fig. 3.2A).

The method described was found to be extremely labour intensive, especially when large numbers of crosses were set up. Hence a method was developed which would reduce the time needed to prepare the matings, halved the total number of petri dishes and media required. This method was carried out by placing the two isolates to be crossed 0.5cm apart about 1cm from the edge of the petri dish. After 7-10 days the isolates had made contact and small plugs were removed from where the colonies met, and re-inoculated onto the same dish at the opposite side to where the matings had been initially set up (see Fig. 3.2B).

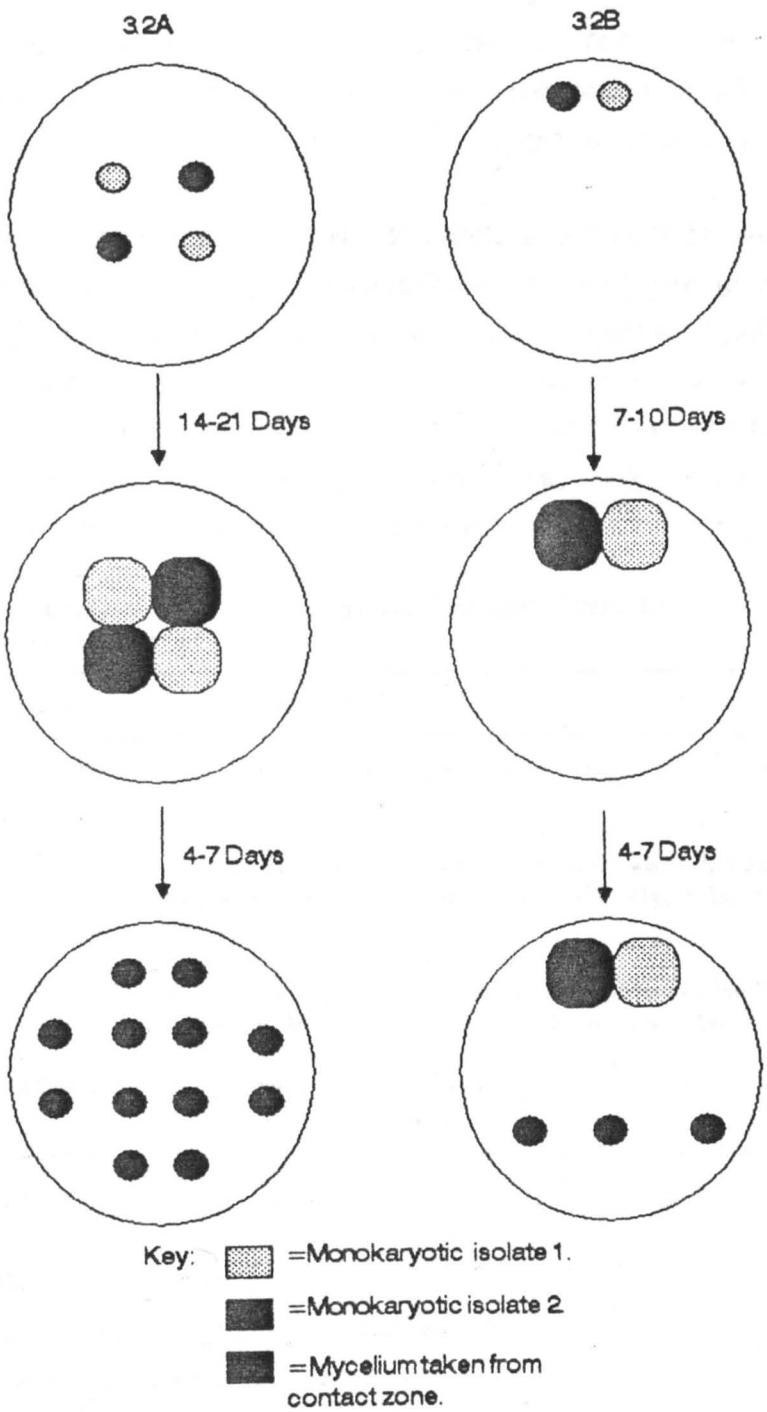
3.2.5 Analysis of Compatibility

Compatibility between two isolates, i.e. the establishment of a balanced heterokaryon, was determined using both macro and microscopic observations. The main points to note were any change in colony morphology or segregation of the morphological types from the sample plugs, and the presence or absence, of true or false clamp connections (see Table 3.3). True clamp connections could be easily observed microscopically, when present, using squash preparation of mycelia from the cross (see Fig. 3.3A). False clamp connections were not commonly observed.

3.2.6 Protoplast Isolation and Regeneration

Routine isolation and regeneration of protoplasts from several strains at any one time was found to be limited by the method developed in Chapter 2, using a regrown hyphal macerate. To obtain suspensions with minimum protoplast clumping, and from a number of isolates at any one time the following procedure was used. 4 x 4mm plugs were inoculated per plate and allowed to grow for 4 days at 25°C. Colonies were then sliced from the cellophane and incubated in 0.6M $MgSO_4$/ 50mm sodium succinate buffer, pH 5.8 containing 10mg.ml^{-1} Novozym 234 (batch PPM1961). One colony was routinely digested with 1ml lytic solution for 1-2 hours depending on the extent of digestion. Mycelial debris was removed by low speed centrifugation in a MSE bench centrifuge at 1,500rpm for 10 min. The supernatant containing protoplasts was then plated directly onto RMYG and incubated at 25°C.

Fig. 3.2 Methods Used for Strain Crosses of *Lentinula edodes*



3.2.7 Growth Rate Analysis

Studies on the rate of colony growth were carried out by inoculating one 4mm plug, taken from the leading edge of an actively growing colony, and placed centrally on plates of PMA. Three plates were inoculated for each strain tested and incubated at 25°C for 8 days. Colony diameter was measured twice, at right angles, for each plate.

Four strains (30-5103, 30-5104, 30-5105, and 30-6073) were chosen for an investigation into the possibility of variable growth rate of heterokaryons with different cytoplasmic parents. From each of the crosses there were three possible combinations of dikaryons: one for each of the two parents and one taken from the contact zone. In addition several protoplast regenerants from strain 30-1 were investigated for variation in growth rate.

TABLE 3.3 Summary of Morphological Changes Associated with Monokaryon x Monokaryon Crosses*

FACTOR COMBINATION	MORPHOLOGICAL CHANGES
A≠B≠ (Uncommon A and B)	Fully compatible cross. Two nuclei per cell and normal clamp cell formation.
A≠B= (Common B)	Incompatible cross. One nucleus per cell. Where fusion has occurred, two nuclei are present in the apical cell only, false clamp cell formation results.
A=B≠ (Common A)	Incompatible cross. Irregular nuclear distribution and no clamp cell formation. Abundant short hyphal branches are often present.
A=B= (Common A and B)	Incompatible cross. One nucleus per cell and no clamp cell formation. No morphological changes occur.

*See Casselton (1978).

3.3 Results

3.3.1 Studies on Single Spore Isolates

Fruit Body Formation

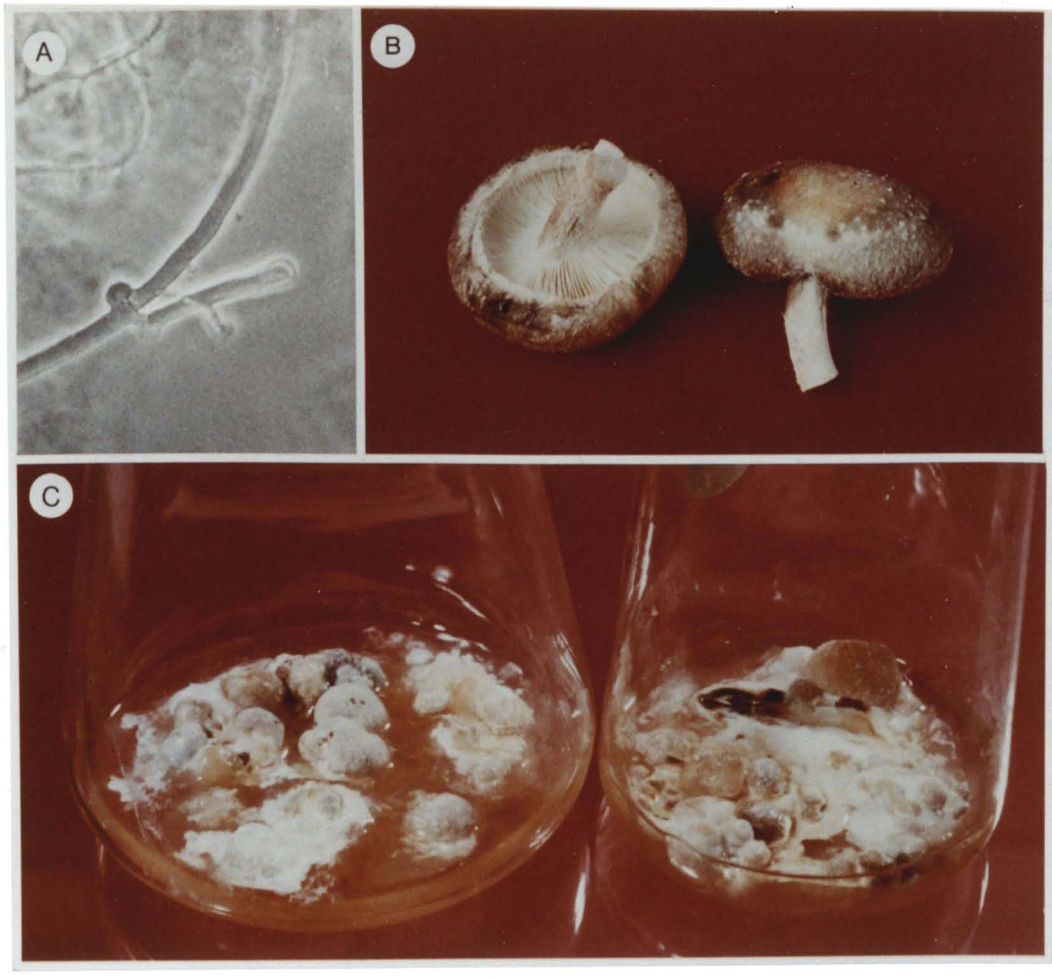
Following the method of Leatham (1983) no fruit bodies were obtained from any of the five strains tested. The resulting mycelial mats were found to turn dark-brown in colour and eventually dried out with no primordia ever formed.

Figure 3.3 Morphological Characteristics of Fertile Dikaryons of *Lentinula edodes*

A. Clamp connection from a *L. edodes* dikaryotic strain (x400).

B. Fruit bodies of *L. edodes* strain 30-2 grown on artificial logs.

C. Primordia and abnormal fruit body formation on liquid medium: left, *L. edodes* strain 30-2; right, *L. edodes* strain 30-4.


A
B
C

Fruit bodies were found to be easily induced using a complex substrate similar to that used routinely for Shiitake cultivation. Strains 30-2, 30-4 and 30-5 were found to fruit readily on this medium, however, strains 30-1 and 30-3 both failed to yield any primordia. Fruit bodies were usually small in size, with a cap diameter of about 3-4cm and occasionally deformed in shape although normal gills were present. Mature mushrooms grown on artificial logs collected from Middlebrook Mushrooms are presented in Fig. 3.3B.

Primordia were formed for strains 30-1, 30-2 and 30-4 using the replacement culture technique (Tokimoto & Kawai, 1975), however, mature fruiting bodies were never observed. In addition, strain 30-3 was never found to produce fruit body initials through repeated experiments (see Fig. 3.3C).

Basidiospore Germination Frequencies

Spores obtained from fruiting artificial logs were commonly found to be contaminated with yeast, *Penicillium* spp. and/or *Trichoderma* spp., although presence of any bacteria was limited by use of antibiotics. Germination frequencies of spores were generally very low, see Table 3.4. Storage at -70°C in glycerol was found to have the effect of reducing viability of several *L. edodes* strains by half but had no effect on the proportion of mating-type factors obtained (data not presented).

Morphological Variation

Single spore isolates obtained from any one strain were found to vary considerably in terms of morphology, and growth rate. It was common for these isolates to form sectors although parental heterokaryons used in this study were not seen to do so. An example of this variation is given in Fig. 3.4.

Mating Type Determination

Homokaryons derived from single spores could be routinely classified into one of four mating type groups according to their mating reaction when paired with siblings. Occasionally non-parental mating-type factors were found (e.g. strain 30-7) which were characterized

by their ability to form heterokaryons with isolates from two mating-type groups from the same strain (see Table 3.5).

To establish a series of test stocks, the following approach was taken: spore progeny from strain 30-2 were designated the alleles A_1B_1, A_1B_2, A_2B_1 and A_2B_2 according to mating reactions. Two testers were taken from each group and mated pairwise with two testers from each of the four groups obtained from strain 30-4. Thus all crosses were repeated in quadruplicate where possible. All testers from strain 30-2 were fully compatible with all testers from strain 30-4, so the new alleles A_3B_3, A_3B_4, A_4B_3 and A_4B_4 were established.

TABLE 3.5 *Lentinula edodes* Basidiospore Germination Frequencies

STRAIN	GERMINATION FREQUENCY	SOURCE OF MATERIAL
30-2	10.30%	Spores obtained from fruit bodies produced at Middlebrook Mushrooms Ltd.
30-2	0.20%	Spores obtained from fruit bodies produced under laboratory conditions.
30-4	3.11%	Spores obtained from fruit bodies produced under laboratory conditions.
30-5	0.10%	Obtained from a very old spore print provided by Prof. S.T. Chang.
30-5	1.13%	Spores obtained from fruit bodies produced under laboratory conditions.
30-7	0.89%	Obtained from a spore print provided by Dr. P.P. Kalberer.
30-8	3.24%	Obtained from a spore print provided by Dr. P.P. Kalberer.
30-9	7.26%	Spores isolated from fresh fruit bodies produced at Oakshire Mushrooms.
30-10	0.60%	Obtained from a spore print provided by Dr. P.P. Kalberer.
30-11	0.36%	Obtained from a spore print provided by Dr. P.P. Kalberer.
30-12	0.07%	Obtained from a spore print provided by Dr. P.P. Kalberer.
30-22	7.92%	Obtained from fresh fruit bodies produced at Saithy Mushrooms Ltd., obtained from J.S. Sainsbury's.
30-23	13.25%	Obtained from fresh fruit bodies produced at Saithy Mushrooms Ltd.

Figure 3.4 Morphological Variation of Single Spore Isolates from *Lentinula edodes*

Six day-old dikaryotic strain 30-2 (top right) and 14 day-old monokaryotic spore progeny derived from strain 30-2.

TABLE 3.5 (cont.)

STRAIN	MATING TYPE	NUMBER OF ISOLATES	ISOLATION FREQUENCY
30-9	A_1B_1	4	25%
	A_2B_2	3	18.75%
	A_1B_2	4	25%
	A_2B_1	5	31.25%
30-10	A_1B_7	22	45.8%
	A_7B_8	9	18.8%
	A_1B_8	12	25%
	A_7B_7	5	10.4%
30-11	A_3B_3	12	60%
	A_5B_5	0	0%
	A_3B_5	7	35%
	A_5B_3	1	5%
30-12	A_5B_3	3	13.63%
	A_6B_6	3	13.63%
	A_5B_6	5	22.7%
	A_6B_3	2	9.1%
	$A_6B_{3/6}$[b]	6	27.3%
	$A_5B_{3/6}$[b]	3	13.63%
30-22	A_5B_3	6	20%
	A_6B_6	8	26.4%
	A_6B_3	9	30%
	A_5B_6	5	16.7%
	$A_6B_{3/6}$[b]	2	6.6%

a. Single spore isolates not in collection.
b. Recombinant B_3/B_6 factor.

Inconsistencies of Mating Type Analysis

During the course of this work several inconsistencies were revealed. Occasionally an isolate would fail to repeat a mating reaction a few weeks or months after initial tests. A list of such inconsistencies is presented in Table 3.7.

Loss of Cultures

One of the most disappointing aspects of this work was the loss of cultures by their inability to grow when further cultured. Death was unavoidable with isolates not in routine use, as stocks stored more than 6 months began to deteriorate. The demise of the following isolates should be noted for the record: 30-2(19), 30-10(19), 30-10(20), 30-10(22).

TABLE 3.6. Mating Types of Several Commercial and Wild-Type Strains of *Lentinula edodes*

Strain	Incompatibility Factors		Parental Mating-Types
30-2	A_1 A_2	B_1 B_2	A_1B_1, A_2B_2
30-3	A_1 $A_{1/2}$[a]	B_1 B_2	A_1B_2, $A_{1/2}B_1$
30-4	A_3 A_4	B_3 B_4	A_3B_3, A_4B_4
30-5	A_3 A_5	B_3 B_5	A_3B_5, A_5B_3
30-6	A_1 A_2	B_1 B_2	N.D.[b]
30-7	A_5 A_6	B_3 B_6	N.D
30-8	A_5 A_6	B_3 B_6	N.D.
30-9	A_1 A_2	B_1 B_2	A_1B_1, A_2B_2
30-10	A_1 A_7	B_7 B_8	N.D.
30-11	A_3 A_5	B_3 B_5	N.D.
30-12	A_5 A_6	B_3 B_6	N.D.
30-13	A_3 A_5	B_3 B_5	N.D.
30-14	A_3 A^c	B_3 B_5	N.D.
30-15	A_1 A_2	B_1 B_2	N.D.
30-16	A_8 A_9	B_9 B_{10}	N.D.
30-17	A_3 A^c	B_5 B^c	N.D.
30-18	A_{10} A_{11}	B_{11} B_{12}	N.D.
30-19	A_2 A_{12}	B_2 B_{13}	N.D.
30-20	A_5 A_{13}	B_7 B_{14}	N.D.
30-21	A_9 A_{14}	B_{15} B_{16}	N.D.
30-22	A_5 A_6	B_3 B_6	N.D.

a. Recombinant A_1/A_2 factor.

b. N.D.= Not determined.

c. Not known.

TABLE 3.7 Inconsistent Mating Reactions Between Paired Monokaryons.

CROSS	REPEATED EXPERIMENT[1]						
	1	2	3	4	5	6	7
30-2(1) x 30-2(6)	+	-	-				
30-4(1) x 30-4(19)	-	+	+				
30-4(3) x 30-4(8)	-	+	+	+	+		
30-4(7) x 30-4(10)	+	-					
30-5(1) x 30-5(9)	-	+	+	-			
30-5(1) x 30-5(25)	+	+					
30-8(1) x 30-8(5)	+	-	-	-	-		
30-9(1) x 30-9(3)	-	-	+				
30-9(1) x 30-9(5)	+	-	-	-	-	+	+
30-9(1) x 30-9(6)	-	-	+				
30-9(1) x 30-9(8)	-	-	+				
30-9(2) x 30-9(4)	-	+	+				

1. - = incompatible mating; + = fully compatible mating.

3.3.2 Studies on Protoplast Regeneration

Neohaplonts obtained through the protoplast isolation technique were characterized in terms of mating type as a means of determining spore progeny from the same strain with recombinant *A* and *B* factors. From initial experiments it was clear that the expected 1:1 ratio of nuclear types was not expressed in the protoplast regenerants screened. Novel dikaryons were further developed by crossing various combinations of *A* and *B* factors and isolating neohaplonts through the method described. The isolation frequency of the two nuclear types from 15 strains is given in Table 3.8.

Timed Regeneration of Protoplasts

Fig. 3.5 shows the result of an experiment carried out with several strains to investigate the possibility of heterokaryon or homokaryon advantage during the regeneration period. After 8 days regeneration small discrete colonies were observed on RMYG. By isolating these colonies as they appeared, the frequency of heterokaryon vs. homokaryon reversion could be determined. The majority of colonies to first appear were found to be dikaryons. The possibility of these dikaryons being the product of hyphal fragments was ruled out by the use of SDW controls. As the regeneration period progressed so did the population of monokaryons began to predominate.

Somaclonal Variation of Protoplast Regenerants

Separation of the component nuclei following the isolation of protoplasts from the dikaryotic strains 30-2, 30-3 and 30-9 resulted in two distinct morphological phenotypes each of which was linked to the mating type of the monokaryons (see Fig 3.6A).

Figure 3.6 Morphological Variation of Protoplast Regenerants Obtained from Dikaryotic Strains of *Lentinula edodes*

A. Neohaplonts from strain 30-2:

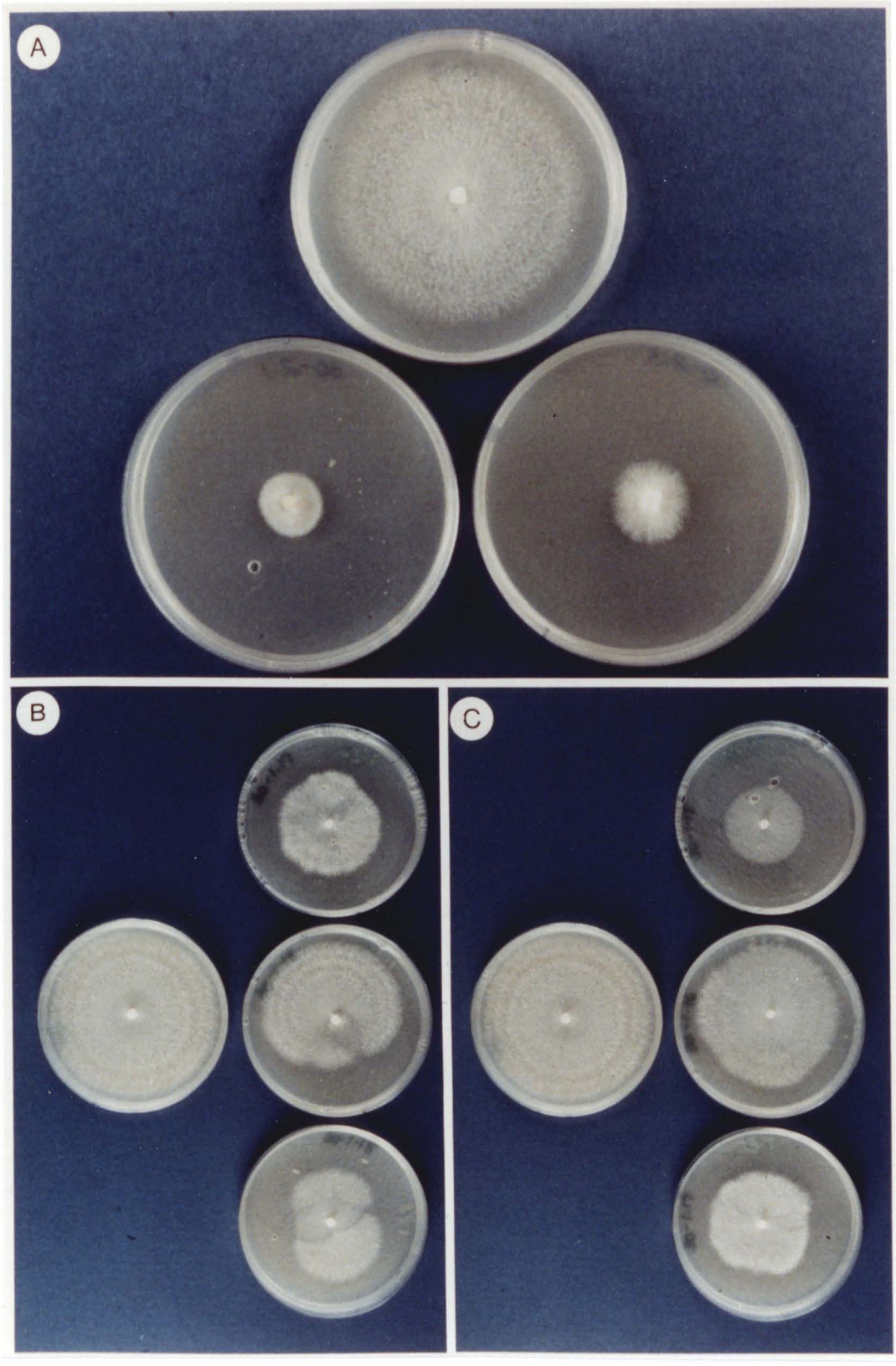
30-2 (top); 30-2.1 (A_1B_1) (bottom left); 30-2.3 (A_2B_2) (bottom right).

B. Parental *L. edodes* strain 30-1 and three sectoring, dikaryotic, protoplast regenerants:

isolates 30-1.18B (top); 30-1.17 (middle); 30-1.15 (bottom).

C. Parental *L. edodes* strain 30-1 and three slow growing dikaryotic protoplast regenerants:

isolates 30-1.18A (top); 30-1.16 (middle); 30-1.15A (bottom).


A
B
C

During routine attempts at de-dikaryotization with strain 30-1, several protoplast regenerants were isolated which were initially thought to be homokaryons because of their relatively slow growth, however, they were later found to be heterokaryons. Established regenerant colonies were then used in growth rate experiments (see Table 3.9). The level of variation detected between replicates was due, in part, to sectoring of the colonies resulting in irregular growth. Colony morphology was found to vary as was growth rate to the parent. Sectoring was seen to occur very frequently amongst the regenerants investigated, a feature that was never observed in nearly 3 years work with the parental strain 30-1 (see Figs. 3.6B & 3.6C). This characteristic was not studied amongst heterokaryotic regenerants of other strains, but was common amongst spore progeny from several strains. Sectors were named by adding the letter A or B after the isolate number: "A" was used to describe fast sectors and "B" used for the slower growing area of the colony (see Table 3.10).

Fig. 3.5 Timed Regeneration of Monokaryotic Protoplasts Isolated from Dikaryons of *Lentinula edodes*

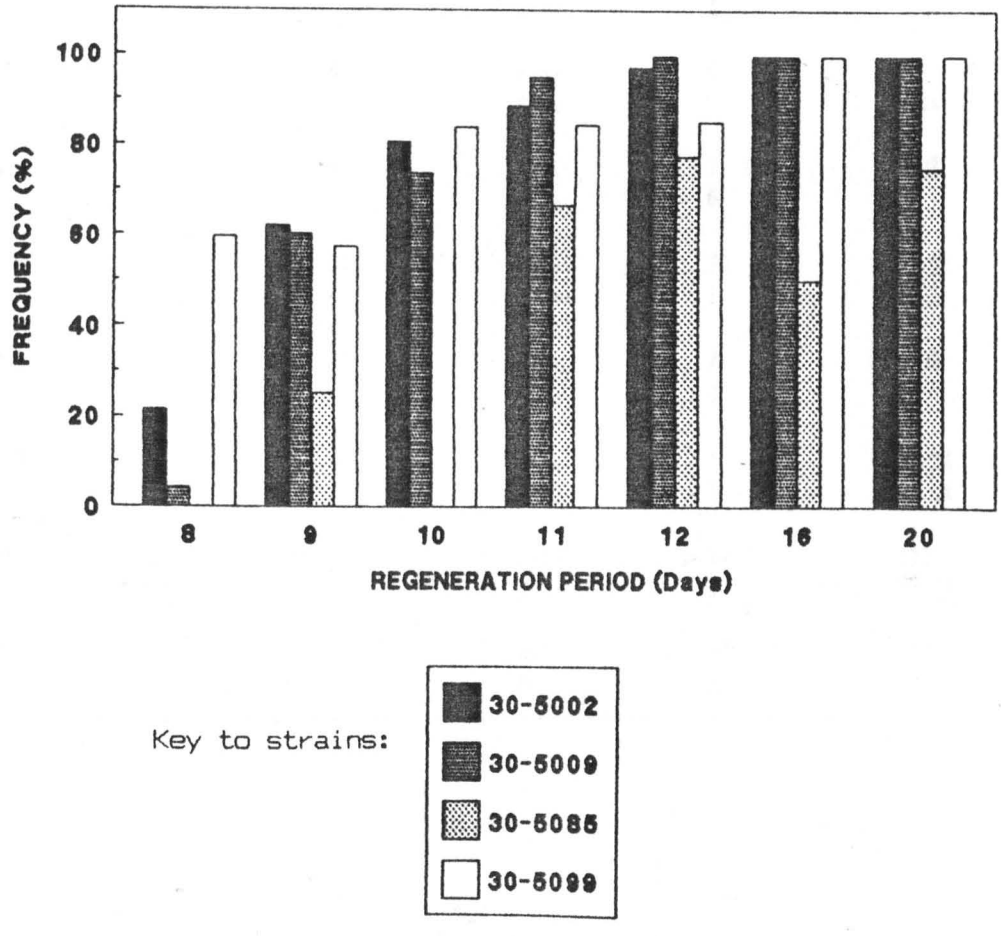


TABLE 3.8 Mating Types of *Lentinula edodes* Protoplast Regenerants

STRAIN	NUMBER OF ISOLATES SCREENED	NUMBER OF HOMOKARYONS ISOLATED	MATING TYPES	NUMBER OF ISOLATES CHARACTERIZED	MATING-TYPE ISOLATION FREQUENCY
30-1	1000	0	-	-	-
30-2	12	5	A_1B_1	1	25%
			A_2B_2	4	75%
30-3	10	8	A_1B_2	7	87.5%
			$A_{1/2}B_1$	1	12.5%
30-4	41	26	A_3B_3	26	100%
			A_4B_4	0	0%
30-5	88	70	A_3B_5	70	100%
			A_5B_3	0	0%
30-9	160	59	A_1B_1	25	42.4%
			A_2B_2	34	57.6%
30-5002	178	151	A_1B_1	88	96.7%
			A_3B_3	3	3.3%
30-5009	172	130	A_1B_1	88	96.7%
			A_3B_5	3	3.4%
30-5019	18	4	A_3B_3	0	0%
			A_4B_4	4	100%
30-5025	16	9	A_2B_1	9	100%
			A_4B_4	0	0%
30-5051	84	31	A_2B_1	30	96.8%
			A_4B_4	1	3.2%
30-5068	110	71	A_2B_1	71	100%
			A_3B_3	0	0%
30-5079	49	3	A_4B_4	1	33.3%
			A_3B_5	2	66.6%
30-5085	36	22	A_1B_2	17	17%
			A_3B_3	5	22.7%
30-5099	300	235	A_3B_3	93	100%
			A_4B_4	0	0%

TABLE 3.9 Colony Diameters of Protoplast Regenerants of *Lentinula edodes* Strain 30-1 After 8 Days Growth

STRAIN	COLONY DIAMETER AFTER 8 DAYS (mm ±S.D.)	PERCENTAGE GROWTH OF PARENT
30-1	76.94 ±0.53	100%
30-1.15	47.26 ±6.56	61.4%
30-1.16	74.05 ±7.19	96.24%
30-1.17	51.62 ±2.20	67.1%
30-1.18	72.80 ±8.14	94.6%
30-1.19	34.79 ±1.74	45.22%
30-1.21	77.63 ±1.44	100%
30-1.22	81.74 ±1.09	106%

TABLE 3.10 Colony Diameters of Sectors from Protoplast Regenerants of *Lentinula edodes* Strain 30-1 After 8 Days Growth

STRAIN	COLONY DIAMETER AFTER 8 DAYS (mm ±S.D.)	PERCENTAGE GROWTH OF PARENT
30-1	79.40 ±0.75	100%
30-1.15A	50.16 ±0.75	63.2%
30-1.15BA	46.50 ±0.83	58.6%
30-1.15BB	35.01 ±6.84	44.1%
30-1.18A	62.97 ±1.68	79.3%
30-1.18BA	62.10 ±1.44	78.2%
30-1.18BB	41.30 ±0.55	52.0%
30-1.19A	34.59 ±4.73	43.6%
30-1.19BA	34.79 ±1.85	43.8%
30-1.19BB	25.33 ±2.04	31.9%

3.3.3 Studies on Strain Development

Growth Rate of Selected Strains

The possibility of cytoplasmic interaction on heterokaryon morphology and growth rate was investigated. Once a heterokaryon was established strains were catalogued as usual but in order to differentiate the cytoplasmic background of the hybrids an appendage was given so that: strains 30-xA and 30-xB were dikaryons with the cytoplasm of the two original parents, and 30-xC the dikaryon from the contact zone and presumed to have mixed cytoplasm. For example, strain 30-6073 was obtained by crossing spore isolate 30-4(1) with protoplast regenerant 30-5025.1; when the dikaryon was isolated from the contact zone it

was designated 30-6063C, and when a dikaryon was formed by the migration of nuclei through one of the monokaryons it was designated 30-6063A (cytoplasm of isolate 30-4(1)0 or 30-6073B (cytoplasm of isolate 30-5025.1. Results of the growth rate analysis are presented in Table 3.11. Morphologies of these isolates can be seen in Fig. 3.7.

TABLE 3.11 Colony Diameters of *Lentinula edodes* Dikaryons with Different Cytoplasmic Parents

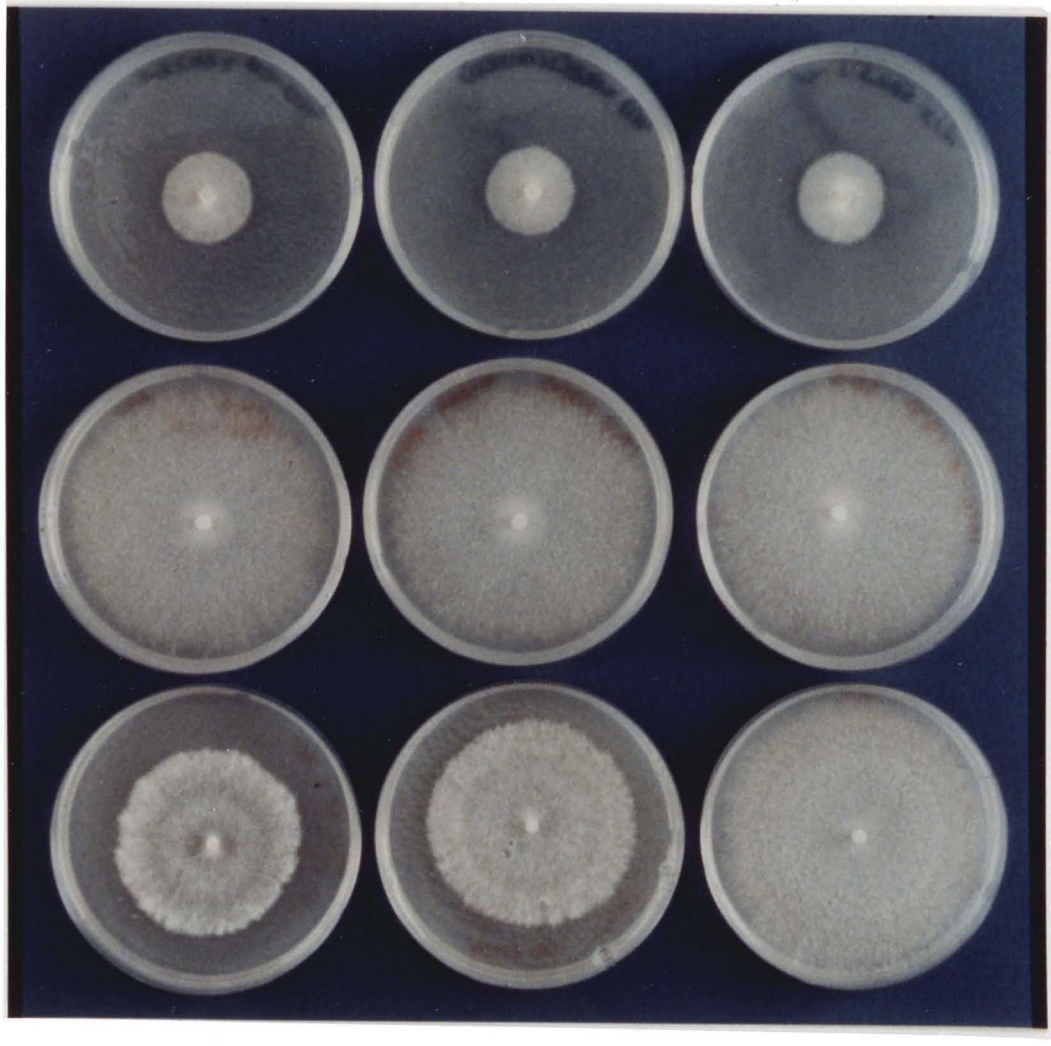
STRAIN	CYTOPLASMIC PARENT	COLONY DIAMETER ATER 8 DAYS (mm ± S.D.)
30-5103A	30-8(6)	72.94 ±2.2
30-5103B	30-19(1)	74.57 ±2.2
30-5103C	mixed	72.22 ±1.63
30-5104A	30-5(3)	59.42 ±5.08
30-5104B	30-18(1)	79.98 ±1.94
30-5104C	mixed	63.15 ±3.27
30-5105A	30-4(3)	67.50 ±0.46
30-5105B	30-16(11)	70.70 ±0.96
30-5105C	mixed	60.27 ±2.36
30-6073A	30-4(1)	28.70 ±2.82
30-6073B	30-5025.1	25.54 ±0.88
30-6073C	mixed	26.23 ±1.50

Morphological Variation of Novel Strains

Some 350 novel heterokaryons were established during the course of this work. A feature of the dikaryons was the morphological variation that was seen amongst them. Pigmentation, branching patterns, aerial mycelia and hyphal morphology were found to differ extensively. An example of such variation is given in Fig. 3.7.

Figure 3.7 Dikaryons of *Lentinula edodes* with Different Cytoplasmic Backgrounds

Top group (left to right): 30-6073A; 30-6073C; 30-6073B.
Middle group (left to right): 30-5106A; 30-5106C: 30-5106B.
Bottom group (left to right): 30-5107A; 30-5107C; 30-5107B.



Assessment of Commercial Value

Strains developed through the strategy proposed in Table 4.2 are currently being screened for commercial potential at Oakshire Mushrooms, Inc., Pennsylvania, U.S.A. Those tested so far have failed to yield any results as it would seem the medium used routinely at Oakshire is too rich for the new strains. Future work is planned using a medium consisting of 80 parts sawdust: 10 parts bran: 10 parts millet. Some of the criteria used to identify valuable strains during fruiting trials at Oakshire includes the number and weight of mushrooms produced during each of six flushes and the weight of substrate before and after fruiting to determine the degree of biological efficiency.

4.4 Discussion

This study has been the largest survey of mating-type factors of commercial strains of *L. edodes* carried out so far, but is limited compared to the survey of 33 strains from the wild by Tokimoto *et al.* (1973). In this latter case 41 *A* and 48 *B* factors were found, predicting 40-65 *A* factors and 63-100 *B* factors occuring in isolates from Japan. It was not considered appropriate to apply Raper's formula (Raper *et al.*, 1958b) to this study as this was for the most part, an artificial population, and relatively little was known about the strains and their origins.

Characterization of mating-type factors of 17 commercial strains revealed 9 different *A* factors and 10 different *B* factors, whilst the four wild-type isolates were shown to have 8 different *A* and 8 different *B* factors. Three *A* factors and two *B* factors were common to the commercial and wild-type isolates. The high degree of similarity of mating-type factors between commercial stocks could be due to several reasons. Extensive breeding between a few isolates with agronomic properties or intrastrain crossing where compatible spore isolates are crossed and progeny selected for desired characteristics would generate such a situation. During the course of this work only one such example has been uncovered, strain 30-3 is the product of intrastrain breeding program with strain 30-9, and so it would be predicted that these strains would have common factors. Alternatively, strains are simply being passed to different sources

and renamed. Other genetic markers such as RFLP mapping or isozyme profiles would be required to determine the relatedness of those strains with all four factors in common. Emphasis on the history or lineage of a strain should be made at this point to ensure diversity within a collection and limit confusion.

Spore progeny from eleven dikaryons were characterized for mating-type factors and a preliminary study on recombinant mating factor loci was carried out. In general, isolates could be classified into one of four mating-type groups, however, the expected 1:1:1:1 ratio was not always found. Severe imbalance was seen in strains 30-5 and 30-11; in addition attempts to isolate the four mating-types from strains 30-14 and 20-17 were unsuccessful, and these strains remain partially characterized. Where determined, these four strains have several factors in common, i.e. $A_5A_6B_3B_6$. Kawasumi *et al.* (1987) reported abnormal recovery of a single mating type from basidiospores of one strain of *L. edodes*, however, no reasons were given for this feature. From results presented in this study, progeny were mostly of two mating-type groups. However, this would have been difficult to discern without the numerous characterized tester isolates.

Day (1959) found eccentric segregation of mating types in dikaryons of *C. cinereus* to be due to the appearance of abnormal cytoplasm in aged cultures. Erratic meiotic divisions were found to reduce the viability of spore progeny because of chromosomal imbalances. In addition, premature mitotic division in the basidium was also detected. These combined effects contributed to tetrad imbalance in these stocks. Meiosis in *C. congregatus* was thought to be inhibited by the presence of a mycoplasma, resulting in the absence of basidiospores (Ross *et al.*, 1976) although further studies found a nuclear gene to be the cause of this disorder (Ross, 1982). Spores were obtained from so-called "difficult" strains of *L. edodes*, and so this explanation would seem unlikely for the effects seen in *L. edodes*. Using both auxotrophic and mating-types as markers in *S. commune*, Raper & Oettinger (1962) found abnormal segregation was not due to any one irregularity although the primary effect was imposed on incompatibility factors. It is possible that a similar effect on meiosis may have appeared in these commercial stocks over numerous

years of continuous sub-culture. Whatever the nature of this lethality, the effect on spore progeny in *L. edodes* is a decrease in intrafertility from a predicted 25% to almost 0%.

Recombination between any pair of factors was not common. It was found that the B_3 and B_6 mating factors recombined in all 4 of the strains with this combination at a frequency ranging from 5-41%. Obviously a larger population of basidiospore progeny would need to be tested if further recombinant factors are to be identified in other strains. This lack of recombinant factors limited work on elucidating the structure of the *A* and *B* factor components of *L. edodes*. It would be worth exploring the effect of recombination on the *B* factors if various isolates carrying the B_3 or B_6 mating type were crossed to other *B* factors in the collection. Spore progeny or neohaplonts could be used to identify the nature of this phenomenon. Recombination between the *A* factors in *L. edodes* has never been reported although non-parental *B* factors have been previously obtained (Takemaru, 1961; Murakami & Takemaru, 1975; Hasebe *et al.*, 1982).

In *Coprinus cinereus* the Aα and Aß loci are very closely linked showing 0.07% recombination (Day, 1963), and in *S. commune* these loci show 27% linkage (Raper *et al.*, 1960). Recombination between *A* factors is found to increase in some strains of *S. commune* if the fruit body is held at higher temperatures prior to spore isolation (Raper *et al.*, 1958a; Stamberg & Simchen, 1970). In addition, extensive work has been carried out with the *B* factors which can be classified into three groups: "recombining", "low-recombining" and "non-recombining" (Koltin & Raper, 1967). Recombination was later found to be due to a gene, *B-rec-1*, which is linked to the Bß allele and strictly controls recombination between the *B* loci (Koltin & Stamberg, 1973). Similar phenomena may occur in other tetrapolar species, but studies have been severely limited.

Protoplasts could be used to generate neohaplonts from dikaryotic mycelia, however, the ratio of the two nuclear types was found to deviate from the expected 1:1 ratio of the dikaryon. In previous reports of de-dikaryotization asymmetric recovery of one nuclear type

was common (e.g. Miles and Raper, 1956; Kinugawa, 1978; Arita, 1979). Using mating-type factors as genetic markers to characterize monokaryons generated by protoplast reversion there seemed to be a dominance effect of one nuclear type over another rendering the other nucleus non-viable. In *S. commune* the *B* factor has been demonstrated to regulate mitotic division in the dikaryon, with the hierarchy of different *B* factors established (Raper, 1983). A more detailed study would be required to further characterize other strains in terms of preferential recovery.

Sectoring variants obtained from *L. edodes* strain 30-1 by protoplast regeneration were found to be highly unstable, rapidly reverting to a growth rate and morphology similar to the original parent. This phenomenon could be due to some instability in the cytoplasm, the nucleus, or both. Alternatively, physiological "incompatibility" between the cytoplasm and the nuclei of the isolates is in force (Fries & Ashan, 1952). Papazian (1955) found sectoring to be a common feature of the vegetative and sexual progeny of certain crosses of *S. commune* and increased temperature was found to promote the frequency of sectors formed. In that study the presence of self duplicating or virus-like particles (VLP) was explored and found to be an unlikely cause. Most strains of *L. edodes* are known to have VLP (Mori *et al.*, 1978; Ushiyama, 1983) and their presence in this strain is possible as studies on DNA have shown (see Chapter 5). If a critical number of VLP are required to bring about physiological and morphological changes, then cytoplasmic variation may be revealed amongst protoplast regenerants. The spontaneous formation of fast growing sectors could thus be related to the exclusion of the VLP population, which is propagated at a rate slower than colony growth. In *L. edodes* dikaryons having high concentrations of VLP have a much slower growth rate than those without (Ushiyama, 1983).

Wessels *et al.* (1976) found pseudoclamp formation occurred in monokaryotic protoplast regenerants of dikaryotic strains of *S. commune* and could continue up to thirty cell divisions after isolation. Production of some cytoplasmic factor by the original dikaryons which is stably passed to regenerating monokaryons, or

transient "activation" of nuclear components were suggested as possible causes of this effect.

The physiological deterioration of the cytoplasm of monokaryons of *L. edodes* (Kinugawa & Inoue, 1977) and of dikaryons of *C. cinereus* (Day, 1959) has been attributed to continuous propagation and subsequent ageing of mycelia. Attempts to isolate monokaryons by protoplasts from strain 30-1 failed, although previous experiments had demonstrated that up to 45% of protoplasts were uninucleate (see chapter 2). It may be that the component nuclei are non-viable due to the presence of recessive lethal mutations which arose through sub-culture.

Somatic or vegetative diploidy had not been investigated in *L. edodes* although in *C. cinereus* (Casselton, 1965) and in *S. commune* diploids form which often result in somatic recombination (Ellingboe & Raper, 1963; Ellingboe, 1964). In *Armillariella mellea* the diplophase is predominant and dikaryons are unstable (Korhonen & Hintikka, 1974). It could be possible that somatic diploids have formed and are isolated by the protoplast procedure. Their relative instability would explain the sectored growth observed as the morphology of diploids and dikaryons can be quite different (Koltin & Raper, 1968). However, in *C. cinereus*, *A. mellea* and *S. commune* the diploid does not form clamp connections, and thus their presence in both slow and fast growing portions of the sectoring colony may limit this as an explanation.

The lack of success experienced with sporophore induction limited the use of basidiospores for mating-type characterization of strains 30-1 and 30-3. Indeed the total failure of the methods of Leatham (1983) and Tokimoto & Kawai (1975) to yield fruitbodies was disappointing. One of the most difficult aspects of this work was the development of a fungal inoculum which could be reproduced qualitatively and quantitatively. In general, work with basidiomycetes involves use of vegetative mycelium, however, very little attention is given to the detail of the preparation of the inoculum. This was the case in the previous chapter on protoplast isolation and was also found to be a

problem when fruit-body induction was attempted. Poor control of humidity and temperature could also explain the failure.

Finally, an attempt to test the proposed breeding strategy for *L. edodes* was severely limited by the lack of resources for fruiting trials and so observations were limited to the laboratory. Variation between monokaryotic isolates was often reflected in novel dikaryons. In addition, the involvement of the cytoplasm in contributing to the morphology and growth rate of several strains was established. Limited "incompatibility" between cytoplasms of different monokaryons was found in one strain 30-5105, where the dikaryon having a mixed cytoplasm had a slower growth rate than the other two dikaryons of the same cross. Kinugawa & Hattori (1974) carried out a similar study with *F. velutipes* and found variation in both growth rate and the fruiting period of dikaryons of reciprocal crosses.

4.1 Introduction

Molecular genetics of filamentous fungi has mainly concentrated on gene cloning and transformation systems, but the application of these approaches to the study of basidiomycetes has been limited to relatively few species. A review of the progress and experiences gained in this growing area is thus called for. The following is a brief review of the literature concerning transformation and gene cloning in basidiomycete species, and recent advances relating to these topics in general.

Transformation

Several excellent reviews on transformation of fungi have been published in the last few years (Mishra, 1985; Rambosek & Leach, 1987; Hynes, 1986; Fincham, 1989), however, only one has focussed attention specifically on basidiomycetes (Ullrich *et al.*, 1985) despite their increasing commercial importance.

Applications

Transformation has been used to demonstrate the feasibility of gene amplification (Mellon & Casselton, 1988; Burrows *et al.*, 1990b) and gene cloning (e.g. Froeliger *et al.*, 1987; Fotheringham & Holloman, 1989) in basidiomycete species. Dominant drug resistant markers have been suggested as being a means of selecting for hybrids during strain crossing and for controlling disease (Elliott, 1979; Challen *et al.*, 1989). DNA mediated transformation could thus be used to by-pass the induction of mutations where spontaneous resistance has not been obtained (e.g. Challen & Elliott, 1987; Challen *et al.*, 1989), and thus provides a direct means of introducing dominant resistance markers into the genome of agronomic crops such as mushrooms.

Considerable attention has also been given to developmental regulation in these fungi *via* the introduction of cloned mating-type idiomorphs DNA (Giasson *et al.*, 1989; Mutasa *et al.*, 1990). As a consequence of these studies, interest in gene structure and genome organization has been aroused.

Methods of Transformation

Numerous protocols are now available for transformation of a wide variety of fungal species. Of those most widely used, protoplast isolation is a prerequisite, followed by treatment with polyethylene glycol (PEG) and the transforming DNA in the presence of calcium ions. PEG has the effect of causing cells to aggregate and one theory is that as protoplasts fuse the DNA is taken into the cell and then becomes incorporated into the genome (Brzbohaty & Kovác, 1985; Hinnen, 1985; Timberlake & Marshall, 1989). DNA encapsulated in synthetic liposomes has been used with some success in *Schizophyllum commune* (Ullrich *et al.*, 1985) as a means of overcoming nuclease destruction of the added DNA.

Several alternative techniques for introducing DNA into cells have been developed. Lithium ion mediated transformation of whole cells was first developed for yeast (Ito *et al.*, 1983) and has since been used with several filamentous fungi (Dhawle *et al.*, 1984; Dickman, 1988; Soliday *et al.*, 1989). Treatment of cells with alkali cations (such as lithium, caesium, potassium, and sodium), PEG and heat shock were found to render cells competent to the incorporation of foreign DNA (Imura *et al.*, 1983). More recently electroporation has been used to introduce DNA into fungal protoplasts (Richey *et al.*, 1989; Thomas & Kennerley, 1989; Goldman *et al.*, 1990) and conidia (Chakraborty & Kapoor, 1990). Introduction of foreign DNA into intact cells of the onion (*Allium cepa*) has been carried out using high velocity microprojectiles (Klein *et al.*, 1987), but this technique is yet to be applied to fungi. These methods can be used to overcome problems of protoplast regeneration encountered amongst certain species, although the frequency of transformation may be lower when alternative methods are used. Binninger *et al.* (1987) reported the inefficiency of lithium acetate for the transformation of intact oidia of *Coprinus cinereus*, where the frequency of transformation was increased 1000 fold using protoplasts. A summary of basidiomycetes transformed is given in Table 4.1 with methods of transformation used.

TABLE 4.1 Reported Transformation of Basidiomycete Species

SPECIES TRANSFORMED	SELECTION SYSTEM	METHOD OF TRANSFORMATION[1] (DNA/ FUNGAL MATERIAL)	TRANSFORMATION FREQUENCY[2]	REFERENCE
Coprinus bilantus	trp$^+$	Pl/ Pp	90	Burrows *et al.* (1990b
Coprinus cinereus	trp$^+$	Pl/ Li	0.5-1	Binninger *et al.* (1987)
	trp$^+$	Pl/ Pp	20-3333	Binninger *et al.* (1987)
	acu$^+$trp$^+$	La & Pl/ Pp	6	Mellon *et al.* (1987)
	acu$^+$trp$^+$	Pl/ Pp	20	Mellon *et al.* (1987)
	acu$^+$trp$^+$	Pl/ Pp	3	Mellon & Casselton (1988)
	trp$^+$	Pl/ Pp	765	Casselton & de la Fuente Herce (1989)
	5FI^R	Not Given	Not Given	Burrows *et al.* (1990)
	ade$^+$paba$^+$A=	Co/ Pp	Not Given	Mutasa *et al.* (1990)
Phanerochaete chrysosporium	ade$^+$	Pl/ Pp	100	Alic *et al.* (1989)
	neo^R	Pl/ Pp	22	Randall *et al.* (1989)
	ade$^+$	Pl/ Pp	350	Alic *et al.* (1990)
	ura$^+$	Not Given	Not Given	Alic *et al.* (1990)
	Kan^R	Pl/ Pp	Not Given	Randall *et al.* (1991)
Pleurotus sp.	Phle^R	Not Given	Not Given	H. Mooibroek (pers. comm.)
Pleurotus florida	leu$^+$	Pl/ Pp	Not Given	Byun *et al.* (1989)
Schizophyllum commune	neo^R	Lpl/ Pp	Not Given	Ullrich *et al.* (1985)
	trp$^+$	Pl/ Pp	30	Muñoz-Rivas *et al.* (1986)
	ade$^+$	La/ Pp	500	Froeliger *et al.* (1987)
	ura$^+$	La/ Pp	500	Froeliger *et al.* (1987)
	paba$^+$	La/ Pp	500	Froeliger *et al.* (1987)
	trp+	Pl/ Pp	200	Specht *et al.* (1988)
	trp+ & A=	Co/ Pp	Not Given	Giasson *et al.* (1989)
	HygB^R	Pl/ Pp		Mooibroek *et al.* (1990)
Ustilago hordei	HygB^R	Pl/ Pp	10-50	Holden *et al.* (1988)
Ustilago maydis	neo^R	Pl/ Pp	10-15	Banks (1983)
	HygB^R	Pl/ Pp	50	Wang *et al.* (1988)
	HygB^R	Rpl/ Pp	1000	Wang *et al.* (1988)
	HygB^R	Co/ Pp	Not Given	Kronstad *et al.* (1989)
	HygB^R	Pl/ Pp	<1	Fotheringham & Holloman (1989)
	HygB^R	Rpl/ Pp	190	Fotheringham & Holloman (1989)
	HygB^R	Pl/ Pp	40,000	Fotheringham & Holloman (1990)
	HygB^R	Rpl/ Pp	900	Fotheringham & Holloman (1990)
	HygB^R	Pl/ Pp	30	Smith *et al.* (1990)

TABLE 4.1 (cont.)

SPECIES TRANSFORMED	SELECTION SYSTEM	METHOD OF TRANSFORMATION[1] (DNA/ FUNGAL MATERIAL)	TRANSFORMATION FREQUENCY[2]	REFERENCE
Ustilago nigra	HygB^R	Pl/ Pp	10-50	Holden *et al.* (1988)
Ustilago violacea	HygB^R	Pl/ Li	80	Bej & Perlin (1989)

1. Co= cosmid DNA, IC= isolated chromosomes, IN= isolated nuclei, La= lambda DNA, Li= lithium acetate and PEG treated fungal cells, Lpl= liposome encapsulated plasmid, Pl= plasmid DNA, Pp= PEG treated protoplasts, Rpl= linearized plasmid DNA (by restriction digest).
2. Transformation Frequency as number of transformants per µg DNA.

Selection Systems

After the fungal material has been through a transformation protocol, cells expressing the transforming DNA have to be selected for. One of the most widely used systems employs reversion of an auxotrophic host to prototrophy, using a wild-type gene cloned usually from the same species, although complementation with heterologous genes has also been used (Alic *et al.*, 1989 & 1990; Casselton & de la Fuente-Herce, 1989; Burrows *et al.*, 1990b). In addition, co-transformation with a nutritional marker has been employed when the gene of interest cannot be directly selected for (Mellon *et al.*, 1987; Casselton & de la Fuente-Herce, 1989; Giasson *et al.*, 1989). Prior to most of these studies isolation and characterization of an appropriate auxotrophic mutant and subsequent isolation of the wild-type gene was required. To overcome such limitations other selection systems have been applied, or developed, particularly to basidiomycetes.

Visual selection has been used for selection of transformants of *Aspergillus nidulans* (Van Gorcom *et al.*, 1985; Yelton *et al.*, 1985) and has been used successfully to clone mating-type genes of *C. cinereus* (Mutasa *et al.*, 1990) and *S. commune* (Giasson *et al.*, 1989) by identifying monokaryotic transformants with false clamp connections. Two-way selection systems may also prove to be useful having the advantage of selecting for resistance to a compound (e.g. 5-fluoroindole, 5FI) which renders the cell auxotrophic for a particular metabolite such as tryptophan (e.g. Dietz *et al.*, 1987; Burrows *et al.*, 1990a).

Several dominant resistance markers have been used successfully with many species in other fungal groups, such as *bml*^{R} (Orbach *et al.*, 1986), *HygB*^{R} (Punt *et al.*, 1984), *neo*^{R} (Suarez & Eslava, 1988), *oligo*^{R} (Ward *et al.*, 1986), *phle*^{R} (Mattern & Punt, 1988), and *su*^{R} (Carramolino *et al.*, 1989). Vectors based on these genes require fungal promoter and terminator sequences fused to the prokaryote structural gene, although regulatory sequences from other sources have proved useful (Bej & Perlin , 1989; Marek *et al.*, 1989; Smith *et al.*, 1990).

These systems have been limited in use in Basidiomycetes other than *Ustilago maydis* (e.g. Banks, 1983; Wang *et al.*, 1988), but this has not been due to a lack of attempts (Ullrich *et al.*, 1985; Casselton & de la Fuente-Herce, 1989; Horgen & Anderson, 1989; N.R. Curtis, pers. comm.; H. Mooibroek, pers. comm). One of the most promising means of achieving transformation in these species is by the cloning of dominant resistance genes from basidiomycetes, such as carboxin resistance (Challen & Elliott, 1990) and a 5FI resistance gene from *C. cinereus* (Burrows *et al.*, 1990a).

Fate of Transforming DNA

Transformation is brought about by DNA entering the cell where autonomous replication or integration into the genome can occur. In filamentous fungi propagation of autonomously replicating plasmids is rare (Buxton & Radford, 1984; Suárez & Eslava, 1988; Gruber *et al.*, 1990) although common in yeast (Fincham, 1989). Integration has been shown to occur in several fungi by one of three types of events: homologous additive integration (type 1), nonhomologous integration (type 2), and gene conversion (type 3). Type 1 and 2 events have been found to be most common although the type of integration which occurs may be governed by the conformation of the plasmid used (Yelton *et al.*, 1984; Kim & Marzluf, 1988; Fotheringham & Holloman, 1990).

Often consequences of incorporation into the genome can be quite dramatic, tandem arrays of the vector (by integrations types 1 and 2) are the result of many copies inserting into the genome (Yelton *et al.*, 1984; Wernars *et al.*, 1985). Gene inactivation by integrative

recombination (Hynes, 1986) or preferential insertion in methylated regions (Mooibroek *et al.*, 1990) can lead to a lack of expression. In *C. cinereus* no examples of type 3 transformants were found, although stability of the transforming phenotype through meiosis has been demonstrated (Binninger *et al.*, 1987).

Factors Limiting Transformation

Although methods for transformation are fundamentally similar, they do vary somewhat in a number of ways. Probably the most extensive study on increasing transformation frequency of a basidiomycete was carried out on *S. commune* by Specht *et al.* (1983). In this study calcium ions and PEG were shown to be essential and their concentrations optimized, in addition, the number of protoplasts and the DNA concentration were important factors governing transformation levels.

Two of the most important factors which affect transformation frequency, arising from several studies, are the physiological status of protoplasts and the DNA vector used. The term 'competence', introduced first in bacterial systems, has been applied to a cell's ability to take up DNA. In fungi 'competence' may be affected simply by the batch of Novozym 234 used (Akins & Lambowitz, 1985; Specht *et al.*, 1988) which may contribute to the presence of nuclease and protease in protoplast preparations (Ullrich *et al.*, 1985; Noel & Labarere, 1989b). Additional factors affecting competence include maintenance at 0°C and storage at -70°C (Specht *et al.*, 1988) and osmotic shock (Akins & Lambowiz, 1985; Specht *et al.*, 1988). Skatrud *et al.* (1986) consider the origin of the protoplasts from the hyphae as an important factor limiting transformation. They propose specific sites in the membrane are essential for protoplasts to be in a competent state for transformation.

Transforming DNA can be a limiting factor in a number of ways, but the most important is the vector sequences involved with expression (Casselton & de la Fuente-Herce, 1989; Mooibroek *et al.*, 1990; Smith *et al.*, 1990). In yeast, selection for prototrophy results in higher transformation frequency when compared to that for drug resistance (Webster & Dickson, 1983; Sreekrishna *et al.*, 1984), although little

is known of this effect in filamentous fungi. The physical nature of the DNA used, such as its purity (Randall *et al.*, 1989) and conformation (Dhawale & Marzluf, 1985; Skatrud *et al.*, 1987; Wang *et al.*, 1988; Fotheringham & Holloman, 1990) can be limiting. However, the differences between linear and circular plasmid DNA on transformation frequency are not universal (Yelton *et al.*, 1984; Oliver *et al.*, 1987).

Other factors such as the site of integration (Mellon & Casselton, 1988; Mooibroek *et al.*, 1990) or failure to integrate can reduce expression, and if integration results in gene disruption it could be potentially lethal (Hynes, 1986). Recently, Goldman *et al.* (1990) found transformants obtained after electroporation had a more stable phenotype than those obtained by traditional PEG methods. It was suggested that differences in the DNA repair system, resulting from the "stress" imposed by the transformation procedure, caused this effect. Unstable transformants have been reported for *Agaricus bisporus* (Horgen & Anderson, 1989; Mooibroek, pers. comm.), *C. cinereus* (Binninger *et al.*, 1987), and *S. commune* (Ullrich *et al.*, 1987). It is thought that abortive colonies result from the failure of DNA to integrate, although this depends very much on the fungal species (Fincham, 1989).

Reduction of nuclease activity by aurintricarboxylic acid (ATA) was found to improve transformation about 500-fold in *Penicillium chrysogenum* (Ramón *et al.*, 1986) but has not been applied to other fungi. Another nuclease inhibitor, heparin, has been used with variable success depending on the species (Randall *et al.*, 1989; Oliver *et al.*, 1987; Soliday *et al.*, 1989). Carrier DNA can also increase transformation frequency, possibly by protecting transforming DNA from nuclease activity (Austin & Tyler, 1990).

Reduction in the level of methylation in the genome by growth with 5-azacytidine (5-AC) was found to enhance expression of foreign genes in *S. commune* (Mooibroek *et al.*, 1990) but had no effect on native gene expression in *Neurospora crassa* (Bull & Wooton, 1984).

Gene Cloning

Isolation, identification and characterization of genes from a wide variety of species are now well established methods in genetic studies. Gene cloning in the filamentous fungi has, until recently, concentrated on a few Ascomycete species and this early work has provided the basis for expanding research with species belonging to other fungal groups. The practicalities involved with, and the experiences gained from, the isolation of genes in fungi will now be discussed.

Methods for Gene Isolation

The general concept of a gene involves understanding its arrangement, i.e. its division into three parts, encoding the promoter, regulatory sequences and the structural portion. One general requirement for studies on gene isolation is the construction of a DNA (or cDNA) library of the strain in question using one of several vector systems. Libraries constructed in plasmid and cosmid vectors have been used for gene isolation by complementation of fungal mutations (e.g. Johnstone *et al.*, 1985; Yelton *et al.*, 1985). Usually auxotrophic mutations are complemented in the host strain by a marker gene present on the vector, facilitating selection for transformants, thus allowing the screening of transformants for functional expression of the gene being sought. For example the plasmid vector pDJB3 has the *A. nidulans pyr-4* gene (Ballance & Turner, 1985) and the cosmid vector pTC20 the *S. commune trp1* gene (Giasson *et al.*, 1989).

Lambda Libraries are used more frequently for probing with heterologous or homologous genes or for cDNA cloning and expression, although lambda clones have been used directly for transformation of fungal species (e.g. Kinnaird *et al.*, 1982; Kinsey & Rambosek, 1984; Froeliger *et al.*, 1987; Mellon *et al.*, 1987). Yeast artificial chromosome vectors (YAC's) are yet to be applied to fungi, although they have been found very useful with higher eukaryotes including plants (Gurzmán & Ecker, 1988) and human cell lines (Anand *et al.*, 1989). General features of these vectors are given in Table 4.2.

TABLE 4.2 General Features of Several Vector Systems

TYPE OF VECTOR	CHARACTERISTICS OF VECTOR
COSMID	Contains the λ*cos* sites to allow for *in vitro* phage packaging, and overcome size selection during transformation of *E. coli*. Able to accommodate additional DNA fragments up to 50Kb in size and has selection systems for *E. coli* (antibiotic resistance) and often for eukaryotic hosts (antibiotic resistance or reversion to prototrophy). Contains the λ bacteriophage origin of replication to allow for propagation in *E. coli*, and an appropriate cloning site. E.g. pWE (Wahl *et al.*, 1987), pTC20 (Giasson *et al.*, 1989).
LAMBDA	
(A) Replacement	Expression in *E. coli* as plaques; DNA fragments from 7 to about 23Kb can be cloned. Recombinants can be selected for by *spi* (sensitive to P2 inhibition) based on inactivation of *red* and *gam* genes, but not in λCharon vectors. Rarely used for functional expression in eukaryotic host, although possible. E.g. λEMBL3 and λEMBL4 (Frischauf *et al.*, 1983).
(B) Expression	Used primarily for screening with antibodies raised against a specific peptide sequence as cDNA is expressed as a β-galactosidase fusion protein. Inserts up to 7.2Kb can be cloned. E.g. λgt11 (Young & Davis, 1983).
PHASMID	Can be propagated as phage or plasmid depending on growth conditions (*E. coli* host, temperature). Has λ*cos* sites for *in vitro* packaging. Insert size ranges from 5-19Kb, recombinants can be selected for, and are easily recloned after identification. E.g. λpGY97 (Vincze & Kiss, 1990).
PLASMID	*E. coli* and eukaryotic selection systems. Maximum size of cloned fragments is (10Kb. Difficult to clone fragments with palindromic sequences and thus representative libraries are not always easily obtained. E.g. pRAL (Akins & Lambowitz, 1985).
YACs	Has an *E. coli* selection system (antibiotic resistance) and yeast selection systems (reversion to prototrophy, and *ade2*). Selection for recombinants is by insertional inactivation giving yeast colonies with a red pigmentation, ade-, and can accept fragments up to 500Kb. Contains at least one ARS, telomeres (forming the ends of linear DNA), a centromere (to ensure normal segregation at mitosis) and origin of replication.

Protocols used for gene isolation vary widely, and a summary with examples emphasizing genes cloned in basidiomycete species is given in Table 4.3. Each of these methods has advantages and disadvantages. Heterologous expression is limited in *E. coli* and *S. cerevisiae* by promoter and intron recognition, often resulting in low levels of expression and incorrectly transcribed proteins. Expression in filamentous fungi has been most successful but depends on an efficient transformation system and use of a vector able to accept large inserts, thus reducing the number of clones that need to be

screened. Probing a library with a heterologous gene or oligonucleotide sequences has also proved to be very rewarding. Sequence divergence between two species can, however, limit the use of heterologous probes as a means of obtaining a particular gene in certain cases (Ullrich *et al.*, 1985; Weiss *et al.*, 1985; Huoponen *et al.*, 1990). Oligonucleotides rely on deriving a nucleotide sequence from a pre-determined protein sequence which is complicated by variation in codon usage for any one amino-acid residue.

Other techniques used to identify genes of interest include 'chromosome walking' and 'chromosome jumping'. Briefly, linkage analysis is used in conjunction with cloning procedures to isolate genes which cannot be cloned by other conventional methods. These methods allow for the selection for library clones with overlapping sequences, and thus the 'ordering' of cloned fragments of genomic DNA can lead to the gene concerned (e.g. Mutasa *et al.*, 1990).

Recovery of the clone from the fungal transformant is carried out by one of several methods. Partial digestion of DNA from transformants, ligation and transformation into *E. coli* followed by selection for antibiotic resistance is known as 'plasmid rescue' and has been used successfully (e.g. Ballance & Turner, 1986; Froeliger *et al.*, 1987; Oakley *et al.*, 1987). Transformed *E. coli* colonies which develop will contain plasmid DNA as the bacterial vector sequence is incorporated into the genome with the fungal insert. However, transformation with other plasmids lacking the sequence being sought may have also occurred, thus hindering the rescue. Selection of the appropriate restriction enzyme for digestion of genomic DNA can also limit this process. Cosmids have the advantage of eliminating the DNA restriction and ligation steps and simply require packaging of undigested DNA to lambda packaging mixtures (available form commercial sources) and transfection of *E. coli* (e.g. Yelton *et al.*, 1985; Weltring *et al.*, 1988).

TABLE 4.3 Methods for Gene Cloning in Fungi

CLONING METHOD	GENES CLONED	ORGANISM	REFERENCE
Oligonucleotide Probing	*aa*	*Neurospora crassa*	Kinnaird *et al.* (1982)
	ligninases	*Phanerochaete chrysosporium*	Zhang *et al.* (1986) and Brown *et al.* (1988)
	GLG3	*P. chrysosporium*	Naidu & Reddy (1990)
Homologous Gene Probing	rRNA	*Schizophyllum commune*	Buckner *et al.* (1988)
	LiP	*P. chrysosporium*	Schalach *et al.* (1989)
Heterologous Gene Probing	*acu-7*	*Coprinus cinereus*	Mellon *et al.* (1987)
	COIII	*S. commune*	Phelps *et al.* (1988)
	CBHI	*P. chrysosporium*	Sims *et al.* (1988)
	AcuA	*Ustilago maydis*	Hargreaves & Turner (1989)
	trp5	*C. cinereus*	Skrzynia *et al.* (1989)
	mnp1	*U. maydis*	Godfrey *et al.* (1990)
	gap	*U. maydis*	Smith & Leong (1990)
	H4	*P. chrysosporium*	Gessner *et al.* (1990)
Heterologous Expression in:			
E. coli	*trp1*	*S. commune*	Muñoz-Rivas *et al.* (1986)
	leu2	*Flammulina velutipes*	Byun *et al.* (1989)
S. cerevisiae	*argB*	*Aspergillus nidulans*	Berse *et al.* (1983)
	pyr6, tpi1, leu2	*U. maydis*	Kronstad *et al.* (1989)
Expression in Fungi:			
Cosmid Libary	*yA*	*A. nidulans*	Yelton *et al.* (1985)
	PDA	*Nectria haematococca*	Weltring *et al.* (1988)
	Aα1, Aα4	*S. commune*	Giasson *et al.* (1989)
	Aα3, Aβ3	*C. cinereus*	Mutasa *et al.* (1990)
Lambda Libary	*ade5, ura1, pab1*	*S. commune*	Froeliger *et al.* (1987)
Plasmid Libary	*acuD*	*A. nidulans*	Ballance & Turner (1986)
	ura1, trp1, ade2, ade5, pab1	*S. commune*	Froeliger *et al.* (1987)
	riboB2	*A. nidulans*	Oakley *et al.* (1987)
	leu1	*U. maydis*	Fotheringham & Holloman (1989)
	REC1	*U. maydis*	Tsukuda *et al.* (1989)
	b1, b2, b3, b4	*U. maydis*	Schulz *et al.* (1990)
	REC2	*U. maydis*	Bauchwitz & Holloman (1990)
Differential Hybridization with cDNA	*IG2*	*S. commune*	Dons *et al.* (1984)
	ligninase	*P. chrysosporium*	Tien & Tu (1987) & Brown *et al.* (1988)
Protein Expression Detected by Antibodies	*lgp3*	*Phlebia radiata*	Saloheimo *et al.* (1989)

The rescue of vector and insert sequences can be eliminated by adopting the method of "sib-selection" (Akins & Lambowitz, 1985) which requires pooling of a number of clones into several groups (e.g. Mutasa *et al.*, 1990). Functional expression is then selected for amongst the groups, which are then subdivided, until the clone in question is identified. Alternatively, putative clones can be isolated by homologous probing of a library with a closely linked gene (e.g. Turgeon *et al.* 1987; Giasson *et al.*, 1989).

Identification and Characterization of Cloned Genes

Initial experiments on the screening of a library for the gene of interest is just the first step to identification, isolation and characterization of that gene. Once putative clones have been identified, screening for functional activity in a fungal host is required as plasmid or cosmid rescue can give a number of different clones which resulted from co-transformation. This can involve sub-cloning into a plasmid vector if a lambda library was used (e.g. Mellon *et al.*, 1987), or direct transformation of the purified clones if a plasmid or cosmid bank was employed.

Transformation can involve selecting for reversion to prototrophy of auxotrophic strains where the complementing gene is being cloned (Froeliger *et al.*, 1987; Mellon *et al.*, 1987), or functional expression in a 'naive' host, i.e. one which would not express the gene product under inducing growth conditions (Weltring *et al.*, 1988).

Now that a clone or number of clones have been identified, nucleotide sequencing and analysis of expression signals can begin. Routine investigations usually involve comparison of the nucleotide and protein sequence to genes cloned in other organisms to assess levels of homology (e.g. Gurr *et al.*, 1987; Gwynne *et al.*, 1987; May *et al.*, 1987).

In summary, success with a wide range of gene cloning and transformation systems have yet to be applied to many basidiomycetes, particularly the edible species. Experience of biotechnology and commercial varieties of edible mushrooms is just beginning.

Aims of This Study

The main objective of this study was to develop a transformation protocol for *P. sajor-caju*. Once this has been achieved and optimized, its application to other *Pleurotus* species was to be explored. Analysis of transformants would be carried out to investigate the integration events which had occurred. Three approaches were taken to try to achieve this: first transformation with previously used fungal vectors, second 'shot-gun' cloning of an efficient promoter from *P. sajor-caju*, and third the cloning of a carboxin resistance gene from an U.V. induced carb^R *P. sajor-caju* with the aim of transforming sensitive *P. sajor-caju* strains.

4.2 Materials and Methods

4.2.1 Strains

Pleurotus spp. used in this study included: *P. cystidiosus* 36-1; *P. florida* 31-1; *P. pulmonarius* 33-1; *P. sajor-caju* 32-1, 32-1[201], 32-1[201(1)], 32-1[R1], 32-1[R2], 32-2; *P. sapidus* 35-1. (See Appendix C for further details.)

Escherichia coli strains used included: DH5α [(r_k^-, m_k^+, F$^-$, *end*A1, *hsd*R17, *sup*E44, *thi*-1, l$^-$, *rec*A$^-$, *gyr*A96, *rel*A1, (*lac*ZYA-*arg*F) U169, ϕ80d*lac*Z m15] (King & Blakesey, 1986); LE392 [(*hsd*R514 (r_k^-, m_k^+) *sup*E44, *sup*F58, *lac*Y1 or D(*lac*IZY) *gal*K2, *gal*T22, *met*B1, *trp*R55; P2392 (*hsd*R514 (r_k^-, m_k^+) *sup*E44, *sup*F58, *lac*Y1 or D(*lac*IZY) *gal*K2, *gal*T22, *met*B1, *trp*R55, (P2)];PLK-17 [*hsd*R$^-$M$^+$, *mcr*A$^-$, *mcr*B$^-$, *sup*E44, *gal*K2, *gal*T22, *met*B1, *hsd*R2] (Kretz *et al.*, 1989); P2PLK-17 [*hsd*R$^-$M$^+$, *mcr*A$^-$, *mcr*B$^-$, *sup*E44, *gal*K2, *gal*T22, *met*B1, *hsd*R2, (P2)] (Kretz *et al.*, 1989). VCS257 (derived from DP50*sup*F).

4.2.2 Media

MMM was used for growth of mycelial homogenates for large scale DNA extractions and contained per 1: 20g glucose (FSA), 0.5g $MgSO_4.7H_2O$, 0.46g KH_2PO_4, 1g K_2HPO_4, 2g asparagine (Sigma), 120µg thiamine, distilled water to 1l.

MYG was used for maintenance of *Pleurotus* spp. and consisted of malt extract (Boots) 4g, yeast extract (Oxoid) 4g, glucose (FSA), agar (Sigma Chemical Co.) 20g, pH to 6.0 and volume to 1l with distilled

water, autoclaved at 121°C for 15 min. Glucose was sterilized separately and added after autoclaving.

Isosensitest medium (Oxoid) was used for testing sensitivity of *Pleurotus* spp. to sulphanilamide, and was made according to manufacturer's instructions and supplemented with 18g.l^{-1} glucose (to give a total of 20g.l^{-1}) which was added after autoclaving.

RMYG was used for protoplast regeneration and contained MYG (as above) supplemented with 0.6M sucrose which was sterilised separately and added after autoclaving. RMYG overlay contained RMYG made with agarose (Sigma Type I) at 0.6% instead of agar.

E. coli was grown on LB medium for maintenance of cultures, plating after transformation and large scale plasmid extraction. LB contained per litre distilled water: Bactotryptone (Difco) 10g, yeast extract (Difco) 5g, NaCl, 10g, pH to 7.5 with NaOH. LB plates were supplemented with 1.5% agar (Difco).

SB medium was used for growth of *E. coli* for plasmid "mini-preps" and contained per litre distilled water: 12g Bactotryptone (Difco), 24g yeast extract (Difco), 90mM phosphate buffer (pH 7.3), 4ml glycerol. Phosphate buffer and glycerol were sterilised separately and added after autoclaving at 121°C for 15 min.

4.2.3 General

Glassware used for all manipulations concerning DNA, was washed with concentrated sulphuric acid, rinsed with water and baked at 150°C for at least 2 hours. This was carried out to destroy nuclease activity. Test-tubes for DNA extraction were siliconized with Sigmacote® (Sigma Chemical Co.) according to manufacturer's instructions.

Agarose gels were cast in 1x TAE or 1x TBE using BRL ultrapure agarose and stained after electrophoresis in ethidium bromide (EtBr; 1µg.ml^{-1}) for 10 min and destained for 10 min with distilled water. Gels were observed on a shortwave U.V. transilluminator (Ultra-violet Products Inc., U.S.A.) and photographed using a Polaroid MP-3 land camera with Polaroid Type 665 film.

Solutions used routinely for manipulations included the following:
x10 Gel Loading Buffer (GLB): 50% (w/v) sucrose, 50mM Na_2EDTA (pH 8.0), 0.1% bromophenol blue; 0.01% SDS.
REact 2 (working concentration): 50mM Tris.HCl (pH 8.0), 50mM NaCl, 10mM $MgCl_2$.
REact 3 (working concentration): 50mM Tris.HCl (pH 8.0), 100mM NaCl, 10mM $MgCl_2$.
REact 4 (working concentration): 20mM Tris.HCl (pH 7.4), 50mM KCl, 5mM $MgCl_2$.
Solution I: 50mM glucose (FSA), 25mM Tris.HCl (pH 8.0), 10mM Na_2EDTA (pH 8.0).
Solution II: 20mM NaOH, 1% SDS (made fresh prior to use).
Solution III: 3M K, 5M acetate made by dissolving
Tris.HCl/ Na_2EDTA Buffer (TE): 10mM Tris.HCl (pH 8.0), 1mM Na_2EDTA (pH8.0). $T_{10}E$: 10mM Tris.HCl (pH 8.0), 0.1mM Na_2EDTA. TE/EtOH: 70% (v/v) ethanol, 30% (v/v) TE.
1x Tris-Acetate Buffer (TAE): 40mM tris base (Sigma), 20mM acetic acid (FSA), 2mM Na_2EDTA (pH 8.0).
1x Tris-Borate Buffer (TBE): 89mM tris base, 89mM boric acid (FSA), 2mM Na_2EDTA.

4.2.4 Determination of Fungicide Sensitivity in *Pleurotus* spp.
Sensitivity of *Pleurotus* spp. to various antifungal agents was tested using MYG except for sulphanilamide which was tested in isosensitest agar. Compounds used included: carboxin, 5-fluroindole (Sigma), G418, hygromycin B (Boehringer Mannheim), phleomycin (CALYA S.A.R.L.), and sulphanilamide (Sigma). Stock solutions of all compounds were made up at 200 times the required concentration. 100µl of this solution could then be added to 20ml of medium to give the highest concentration required, limiting the solute concentration to 0.5%. G418, hygromycin B, and phleomycin were dissolved in sterile distilled water, carboxin was dissolved in methanol, sulphanilamide in acetone and 5-fluroindole in ethanol. Sterilization of these compounds was never found necessary.

Growth response to these inhibitors was carried out by incubating one 4mm diameter inoculum plug, taken from the leading edge of an

actively growing colony, on a petri-dish containing medium plus the inhibitor or the solvent as the control. Plates were incubated for 6-12 days at 25°C; growth was measured across two diameters of the colony. Three replicate plates were used for each strain and each concentration of inhibitor.

4.2.5 Plasmid Preparation

Plasmids Used in This Study

The following plasmids were used: pAN7-1 (Punt *et al.*, 1990); pAN8-1 (Mattern *et al.*, 1987); proH99 (Smith, 1990); proH101 (Smith, 1990). pAN7-1, proH99, and proH101 contain the gene for hygromycin B resistance, although proH99 lacks a promoter. pAN8-1 confers phleomycin resistance. All have ampicillin resistance as a selectable marker to enable use of *E. coli* for plasmid preparation.

'Mini-Prep' Methods

E. coli strain DH5α, transformed with the plasmids being used was grown overnight at 37°C, with shaking, in SB medium supplemented with 50μg.ml^{-1} ampicillin (Sigma). Plasmid DNA was extracted using a Pharmacia plasmid mini-prep kit based on the alkali lysis method of Birnboim & Doly (1979). High yields of DNA were extracted by this method (20-30μg from a 3ml culture) but it was unsuitable for restriction digestions. The protocol provided was thus adapted as follows. After harvesting the bacterial pellet, it was washed with 1ml SDW prior to extraction. In addition, two phenol extractions and back extractions with TE were carried out prior to the ethanol precipitation stage and the final step entailed a wash of the DNA pellet with TE/EtOH. The dried pellet was then resuspended in 20μl $T_{1/10}E$.

'Maxi-Prep' Method

Again the method of Birnboim & Doly (1979) was modified for the larger scale extraction of plasmid from *E. coli* DH5α. 250ml LB supplemented with 50μg.ml^{-1} ampicillin, contained in a 1l conical flask, and seeded with 1ml of an 18 h starter culture, was incubated overnight at 37°C with shaking. For plasmid extraction 2 x 250ml cultures were processed to give one 5ml CsCl gradient (see below). Cells were harvested by centrifugation (6,000rpm, 10min, 4°C) and washed through with SDW. The pellet was resuspended in 10ml solution

I and left on ice for 15min. 20ml of solution II was added and mixed well for 1-2min to ensure complete lysis. 15ml solution III was then added and left on ice for 15min during which time a heavy white flocculent precipitate appeared. This suspension was then centrifuged (13,000x*g*, 15min, 4°C) and the supernatant filtered through 100µm mesh nylon cloth prior to isopropanol precipitation. The precipitate formed was pelleted by centrifugation (13,000x*g*, 10min, 4°C) and resuspended in 15ml TE. 7.5ml 7.5M ammonium acetate was added and left on ice for 20min. This was then centrifuged (10K, 10min, 4°C) and the supernatant recovered. The DNA was precipitated with ethanol at -20°C for 30min, centrifuged as previously, and the white pellet allowed to dry prior to resuspending in 4ml TE.

The large quantity of DNA extracted necessitated purification on a CsCl gradient (4.2g CsCl, BRL *'ultra*pure'; 4.0ml DNA solution; 0.2ml EtBr, 10mg.ml^{-1}; contained in a Beckman 5ml Quickseal disposable centrifuge tube). Samples were spun in a Beckman L565B ultracentrifuge with the VTi65 rotor at 40,00rpm at 15°C for at least 16 hours. After the lower band of plasmid DNA was recovered, the contaminating EtBr was removed by extraction with isopropanol saturated with NaCl until no pigment was visible, then repeated a further two times (Maniatis *et al.*, 1982). CsCl was removed by dialysis against four changes of 2L TE buffer at 4°C for a total period of 21 hours. DNA was then quantified by measuring A_{260} and A_{280} (Maniatis *et al.*, 1982) and also observed by EtBr staining after agarose gel electrophoresis. DNA was stored at 4°C and also in 250µl aliquots at -20°C.

4.2.6 Isolation of Genomic DNA

Two methods for DNA extraction from *P. sajor-caju* were attempted. Cultures were grown for 5-6 days at 25°C on cellophane discs laid on MYG from a 4mm diameter plug of mycelium. The mycelium was scraped off the cellophane (avoiding the original agar plug), placed in a 1oz plastic universal bottle, frozen at -70°C and then freeze-dried. The procedure of Raeder & Broda (1985) for DNA extraction was attempted with this material but with poor results.

As an alternative the CTAB (cetyltrimethyl-ammonium bromide) method (Rogers *et al.*, 1988) was followed. Mycelium was grown as previously and 5 six-day-old colonies harvested from cellophane, and macerated for 30 secs in 15ml MMM using a Silverson homogenizer. 5ml of homogenate was then inoculated into 200ml MMM contained in 1l conical flasks and grown for 3 days on a shaker at 200rpm at 25°C. Mycelium was harvested by filtration through 100µm mesh nylon cloth and washed thoroughly with SDW prior to freezing at -70° and freeze drying. The protocol was followed and an additional chloroform:isoamyl alcohol extraction and centrifugation step was included after the CTAB and chloroform:isoamyl extraction steps.

CsCl gradients for purification of fungal DNA were set up as for plasmid gradients except 4.05g CsCl was dissolved in 4.15ml DNA solution.

4.2.7 Transformation Procedures

Bacterial Transformation

The method of Cameron *et al.* (1975) was followed and competent cells stored in 200µl aliquots with 15% glycerol at -70°C.

Fungal Transformation

Method 1. Protoplasts from *P. sajor-caju* 32-2 were prepared as described in Chapter 2 and diluted to give a suspension of 5×10^7 (or 5×10^8) protoplasts ml^{-1}. Plasmid DNA was added (50µg in 50µl of TE) to 200µl of protoplast suspension followed by 50µl PEG (30% polyethylene glycol, PEG, 6,000, 50mM $CaCl_2$, 10mM Tris. HCl, pH 7.5) The suspension was gently mixed and then held on ice for 20 min. A further 2ml PEG was added and the mixture incubated for 5 min at room temperature. Four ml 0.6M sucrose was then added and the protoplasts pelleted by centrifugation at 700rpm for 10min. The pellet was resuspended in 1ml 0.6M sucrose prior to plating directly on to 20ml osmotically stabilized medium (RMYG). Controls included non-PEG treated protoplasts diluted in 0.6M sucrose and SDW for viability determination, and PEG treated protoplasts without the addition of DNA. After 48h incubation at 25°C, 5ml of overlay medium supplemented with hygromycin B (100µg.ml^{-1}) or phleomycin (75µg.ml^{-1}) was added to a proportion of the plates with DNA-PEG and PEG treated protoplasts.

Method 2. (modified from Smith, 1990) 2 x 10^8 protoplasts in 250µl, stabilised in 0.6M sucrose/ 50mM $CaCl_2$, were mixed gently with 50µg plasmid DNA in 50µl $T_{10}E$, incubated 20 min at room temperature and 250µl PEG solution (50% PEG 8,000, 50mM $CaCl_2$, 50mM glycine, 0.6M sucrose) then added. After a further 20 min incubation at room temperature, 500µl of 0.6M sucrose/ 50mM $CaCl_2$ was added. 100µl of the suspension was then plated directly onto RMYG and selection carried out as above for resistance to hygromycin B.

4.2.8 Construction of Clones of proH99 with *NcoI* Inserts from Genomic *Pleurotus sajor-caju* 32-2 DNA

To obtain *NcoI* fragments ligated into proH99, 1µg DNA from *P. sajor-caju* 32-2 was digested with 5 units *NcoI* (BRL) in REact 3 buffer. After digestion overnight at 37°C the enzyme was heat inactivated at 70°C for 15 min. The digested DNA was separated in agarose by gel electrophoresis followed by staining with EtBr. proH99 was also digested to completion with *NcoI*. The enzyme was heat inactivated as previously and treated with calf intestine phosphatase (CIP) prior to two phenol:chloroform extractions, and ethanol precipitation.

Ligations were set up with proH99 and the *NcoI* 32-2 restricted DNA as follows:

ligation 1= 50ng proH99, ligation buffer, 1mM ATP-10mM DTT, 1 unit T4 DNA ligase, total volume 5µl; ligation 2= 50ng *NcoI* cut proH99, ligation buffer, 1mM ATP-10mM DTT, 1 unit T4 DNA ligase, total volume 5µl; ligation 3= 50ng *NcoI* cut proH99/ CIP treated, ligation buffer, 1mM ATP-10mM DTT, 1 unit T4 DNA ligase, total volume 5µl; ligation 4= 50ng *NcoI* cut proH99/ CIP treated, 25ng 0.6Kb internal fragment of the IPNS gene of *Acremonium chrysogenum* (obtained from A.W. Smith), ligation buffer, 1mM ATP-10mM DTT, 1 unit T4 DNA ligase, total volume 5µl; ligation 5= 50ng *NcoI* cut proH99/ CIP treated, 50ng *NcoI* cut 32-2 genomic DNA, ligation buffer, 1mM ATP-10mM DTT, 1 unit T4 DNA ligase, total volume 5µl. Ligations were carried out at 12°C overnight, after which time the reactions were stopped by heat inactivation. DNA from each ligation was then used to transform competent cells of *E. coli* DH5α with selection for ampicillin resistance.

To identify clones harbouring plasmid with *Pleurotus* *Nco*I fragments ligated into proH99, plasmid mini-preps were carried out for 186 colonies obtained after transformation of *E. coli* with DNA from ligation 5. The plasmid DNA obtained by this method was then cut with *Nco*I and the digests electrophoresed in agarose gels. Clones having fungal DNA *Nco*I inserts were identified by the presence of additional band(s) which ranged in size from ~2-4Kb.

4.2.9 Construction of λEMBL3 Library of *Pleurotus sajor-caju* strain 32-1[201(1)]

Partial digests were carried out with one of the following restriction enzymes in appropriate buffer *Sau*3A (BRL; REact 4), *Mbo*I (Boehringer Mannheim; REact 2), *Bam*HI (BRL; REact 3). Optimum conditions for restriction, giving fragments of an average size of 20Kb, were established for each enzyme by setting up digests for appropriate time intervals (from 0-60 min). Termination of the reaction was carried out by heat inactivation at 70°C for 15 min and placed on ice. Digests were visualized by electrophoresis through 0.8% (w/v) agarose made in TAE buffer.

Once the optimum time for digestion had been determined, dephosphorylation of the terminal 5' phosphoryl groups was carried out by treatment with CIP. Four units CIP was added to 4μg of digested DNA in 200μl, and incubated at 37°C for 30 min. Inactivation of the CIP was carried out by adding 0.5M Na_2EDTA (pH 8.0) to a final concentration of 50mM followed by heating to 70°C for 15 min and two chloroform:isoamyl alcohol extractions. DNA in the aqueous phase was recovered by precipitation with two volumes of ethanol and dissolved in TE.

Problems with the dephosphorylation of restricted 32-2 DNA termini using the above procedure resulted in ligations between 32-2 fragments, as seen after electrophoresis and EtBr staining, which would prevent the packaging of λEMBL3 and reduce subsequent plaque formation. Other methods were attempted to overcome this problem, and improve the efficiency of the phosphatase treatment. To 4 μg of DNA 10 units CIP were added and incubated for 15 min at 37°C after which time the temperature was increased to 65°C and a further 10 units of CIP added. After 15 min incubation the reaction was removed from the

heating block and placed at room temperature and allowed to cool slowly. CIP was inactivated as above. Alternatively CIP treatment was carried out with a CIP buffer containing (final concentration) 50mM Tris.HCl (pH 9.0), 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine (Perbal, 1988).

To overcome the use of CIP, DNA in the 18-22Kb size range was isolated by electrophoresis of 10μg digested DNA through 0.6% Low Melting Point agarose (BRL) in TAE buffer. DNA fragments in the 18-22Kb size range were cut out a block of agarose and either electroeluted (Perbal, 1988) or extracted directly from the agarose according to the method of Perbal (1988) by melting the agarose and extraction with phenol.

Ligations with λEMBL3 (Stratagene, USA) were set up over two days, according to the manufacturer's instructions, once it was established that restricted DNA had ligatable ends (if 18-22Kb fragments were isolated) or were not self-ligatable (if treated with CIP). This was tested by carrying out ligations under manufacturer's conditions but omiting λEMBL3 DNA. Self ligations could be detected as a band of high molecular weight DNA which could be seen after electrophoresis. DNA from each ligation (excluding the cut 32-2 self ligated DNA) was then packaged *in vitro* using Gigapack™II Gold Packaging Extract (Stratagene, USA) according to manufacturer's instructions. To test the ligation and packaging efficiencies of the reactions, aliquots were taken, diluted and transfected to competent *E. coli* strains LE392, P2392, PLK-17, P2PLK-17, and VCS257 (transfected with wild type λc1857Sam7, provided by the manufacturer) according to manufacturer's instructions. After 16 hours incubation at 37°C plaques were visible and efficiencies could be calculated as follows:

$$\frac{(\#\text{ of plaques}) \times (\text{dilution factor}) \times (\text{total packaging volume})}{(\#\text{ of }\mu\text{g DNA packaged}) \times (\#\text{ of }\mu\text{l plated})}$$

Where the total packaging volume= 137.5μl.

4.3 Results

4.3.1 Fungicide Sensitivity of *Pleurotus* spp.

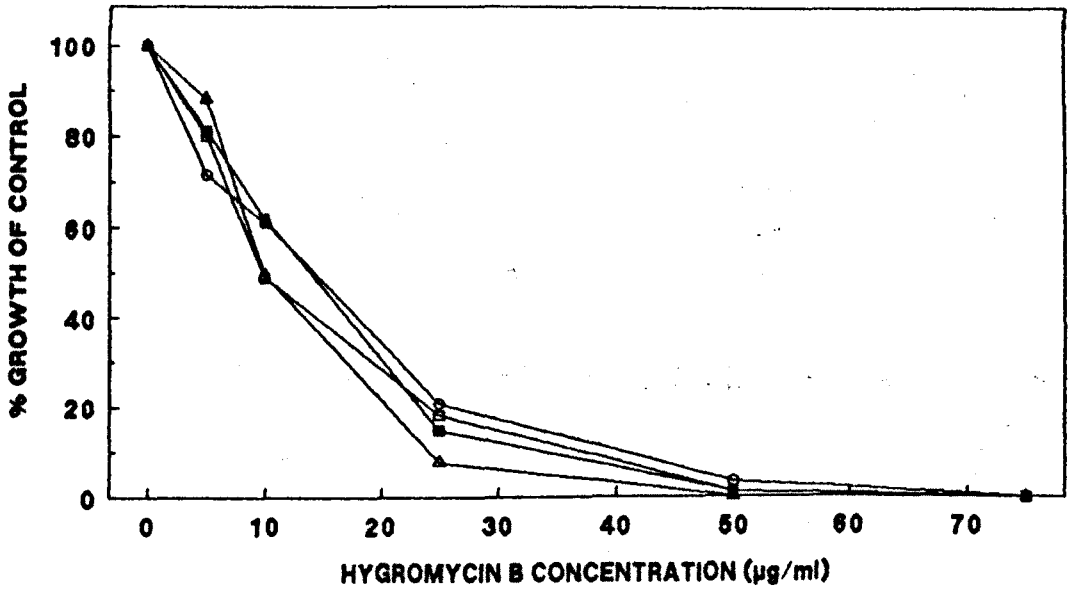
Sensitivity of several dikaryotic strains of *Pleurotus* spp. to 5-fluroindole, G418, hygromycin B, phleomycin, and sulphanilamide was determined (Figs. 4.1-4.5). This was carried out to assess appropriate vector for further work on DNA mediated transformation. Inhibition of growth was incomplete with G418 at 75μg.ml^{-1} or with sulphanilamide at 2.5mg.ml^{-1}, and so these were not used further. Minimum inhibitory concentrations (MICs) for 5-fluroindole, hygromycin and phleomycin for several species and strains of *Pleurotus* are given in Table 4.4.

In addition, the sensitivity of four carboxin resistant mutants (one monokaryon and three dikaryons) derived from *P. sajor-caju* 32-1 is shown in Fig. 4.6.

4.3.2 Transformation of *Pleurotus sajor-caju* 32-2

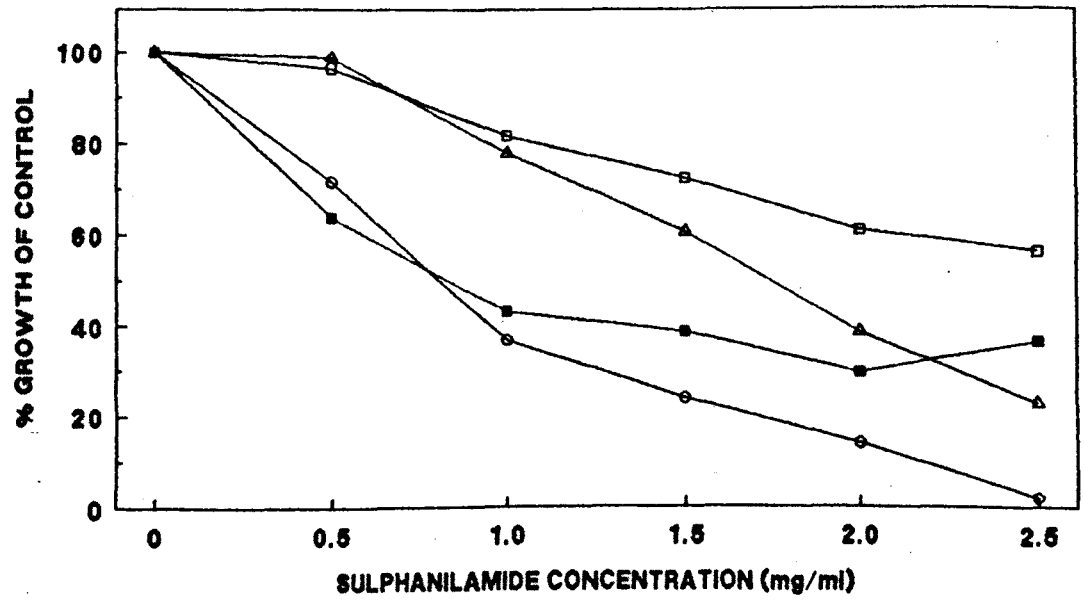
No transformants were obtained for *P. sajor-caju* 32-2 when plasmids pAN7-1, pAN8-1, or proH101 were used. In addition, no transformants were obtained when plasmid constructs of the pPSC series were used in pools of 10 plasmids (5μg of each plasmid used to give a total of 50μg DNA used). No spontaneous resistance mutants were ever obtained (see Table 4.5).

Fig. 4.1 The Effect of Hygromycin B Concentration on the Radial Growth of Four *Pleurotus* Species



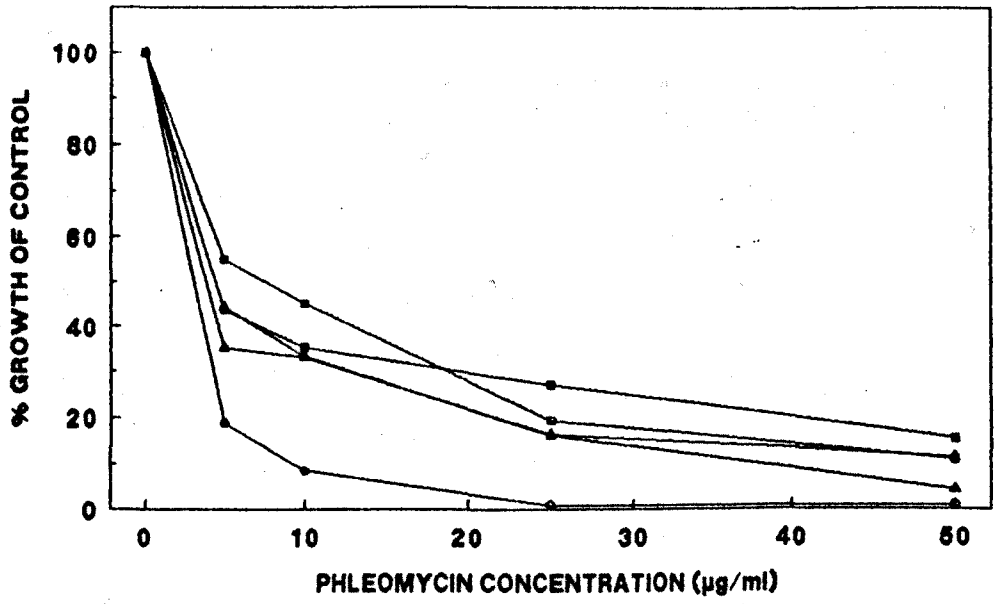
Key: —○— = *P. ostreatus* 7-4; —□— = *P. florida* 31-1; —△— = *P. sajor-caju* 32-2; —■— = *P. pulmonarius* 33-1.

Fig. 4.2 Sensitivity of Four *Pleurotus* Species to Sulphanilamide



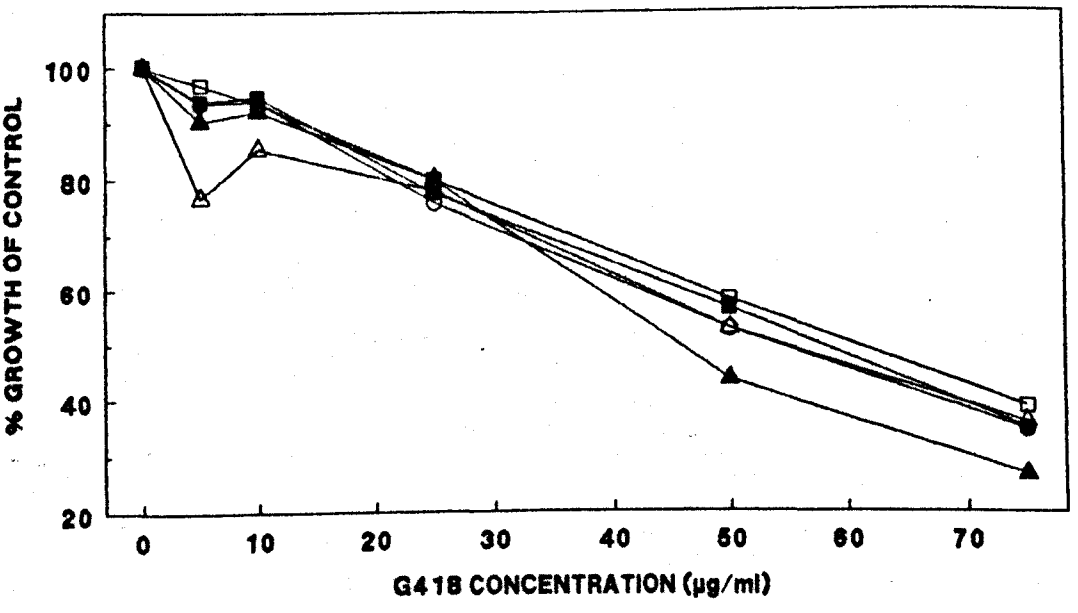
Key: —○— = *P. ostreatus* 7-4; —□— = *P. florida* 31-1; —△— = *P. sajor-caju* 32-2; —■— = *P. pulmonarius* 33-1.

Fig. 4.3 Growth Response Relationships of Five *Pleurotus* spp. to Phleomycin



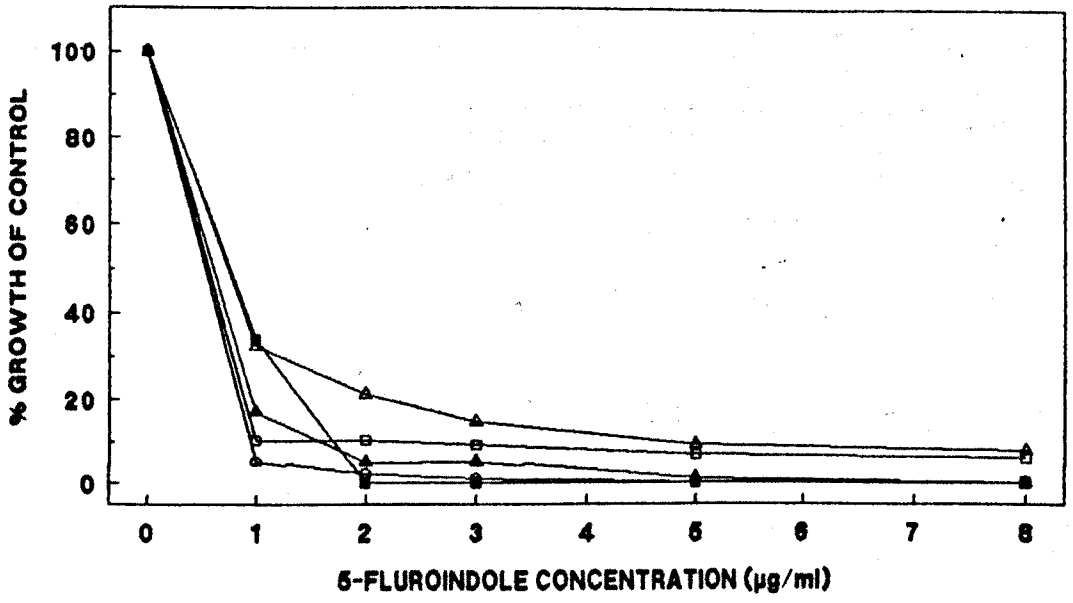
Key: —○— = *P. ostreatus* 7-4; —□— = *P. florida* 31-1; —△— = *P. sajor-caju* 32-2; —■— = *P. pulmonarius* 33-1; —▲— = *P. colombinus* 34-1.

Fig. 4.4 The Effect of G418 on the Radial Growth of Several *Pleurotus* Species



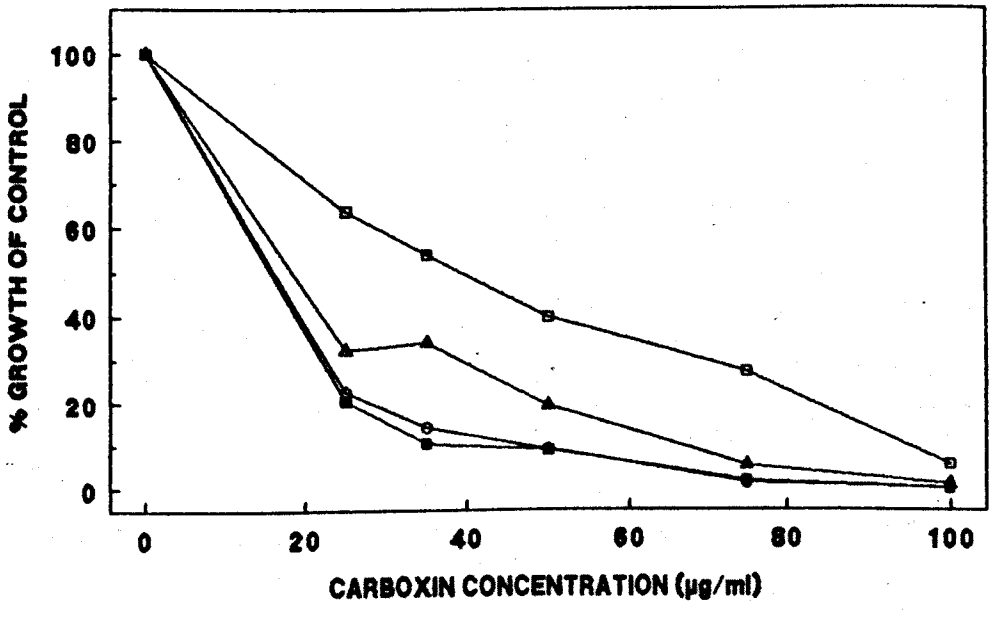
Key: —○— = *P. ostreatus* 7-4; —□— = *P. florida* 31-1; —△— = *P. sajor-caju* 32-2; —■— = *P. pulmonarius* 33-1; —▲— = *P. colombinus* 34-1.

Fig. 4.5 Sensitivity of Several *Pleurotus* Species to 5-Fluoroindole



Key: —○— = *P. ostreatus* 7-4; —□— = *P. florida* 31-1; —△— = *P. sajor-caju* 32-2; —■— = *P. pulmonaris* 33-1; —▲— = *P. colombinus* 34-1; —✳— = *P. sapidus* 35-1.

Fig. 4.6 Dose Responses of Four Carboxin Resistant *P. sajor-caju* Mutants



Key: —○— = strain 32-1[201]; —□— = 32-1[201(1)]; —△— = 32-1[R1]; —■— = 32-1[R2].

TABLE 4.4 Minimium Inhibitory Concentrations of Three Antifungal Agents on Several *Pleurotus* Species

		MIC ($\mu g.ml^{-1}$)*		
SPECIES	STRAIN	HYGROMYCIN B	PHLEOMYCIN	5-FLUROINDOLE
P. colombinus	34-1	-	40	-
P. cystidiosus	36-1	-	-	5
P. florida	31-1	65	80	-
	31-2	-	80	8
P. ostreatus	7-4	65	-	-
	7-10	65	-	>8
	7-11	-	-	>8
	7-12	-	40	5
P. pulmonarius	33-1	60	>80	-
	33-2	-	80	>8
	33-3	-	70	>8
P. sapidus	35-2	-	-	5
P. sajor-caju	32-1	55	55	8
	32-2	45	55	8
	32-3	-	-	>8
	32-4	-	-	>8
Hybrid	H7	-	40	-

*-= not attempted.

TABLE 4.5 Results of Transformation Experiments with *Pleurotus sajor-caju* 32-2

EXPT. NUMBER	PLASMID USED	TRANSFORMATION METHOD USED[1]	TOTAL NUMBER OF PROTOPLASTS TRANSFORMED	REGENERATION FREQUENCY(%) -PEG -DNA	+PEG -DNA	+PEG +DNA[2,3]	NUMBER OF TRANSFORMANTS OBTAINED
1	pAN7-1	1	1×10^7	2.30	0.04	0.04	0
2	pAN7-1	1	1×10^7	1.62	0.02	0.02	0
3	proH101	1	1×10^7	3.29	1.60	ND	0
4	pAN8-1	1	1×10^8	2.63	0.04	ND	0
5	pAN8-1	1	1×10^8	4.15	0.62	ND	0
6	pPSC lots 1-5	2	2×10^8	3.30	1.74	ND	0
7	pPSC lots 6-10	2	2×10^8	2.53	0.08	ND	0

1. See section 4.2.7 for fungal transformation procedures.
2. 50µg plasmid DNA was used for each transformation experiment.
3. ND= not determined.

4.3.3 Analysis of Plasmid Series pPSC

Transformation of *E. coli* DH5α with plasmid constructs containing random fragments of *Pleurotus* DNA was partially successful (see Table 4.6). Although a low level of transformants obtained with ligation 3 indicated incomplete dephosphorylation of *Nco*I cut proH99, plasmid mini-preps were carried out with random amp^R *E. coli* colonies obtained with DNA from ligation 5. The plasmids isolated were named pPSC1 to pPSC186 in order of isolation.

From the results of the *E. coli* transformation about 26% of bacterial colonies would be expected to harbour the religated proH99 with no 32-2 *Nco*I insert. However, 100 of the 186 plasmids were found to have inserts, i.e. 46% of clones lacked an insert. During these experiments several clones were found to have several *Nco*I fragments, i.e. more than two bands resulted after restriction with *Nco*I (see Fig. 4.7). The size frequency of fragments obtained is given in Fig. 4.8, and where several fragments were obtained the total size of the fragments is given.

TABLE 4.6 Transformation Frequency of *E. coli* DH5α with proH99 and Ligation Constructs

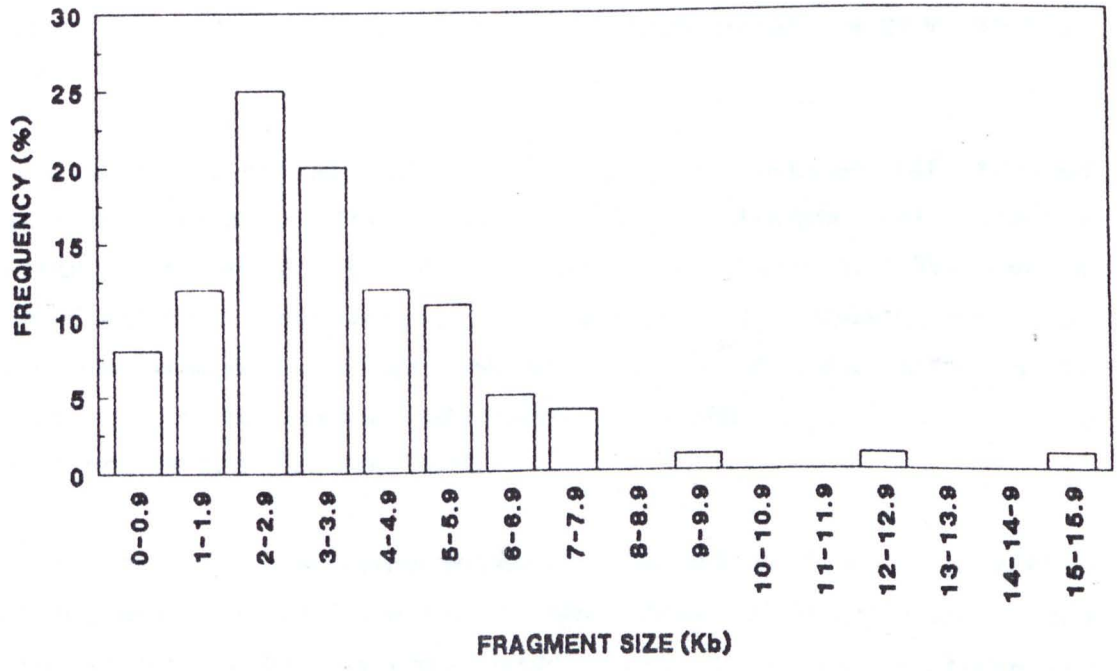
LIGATION NUMBER	LIGATION CONTENTS	TRANSFORMATION FREQUENCY*
1	proH99	7.30×10^4
2	*Nco*I-proH99	5.00×10^3
3	*Nco*I-proH99/ CIP treated	3.90×10^3
4	*Nco*I-proH99/ CIP + 0.6Kb fragment	1.01×10^4
5	*Nco*I-proH99/ CIP + *Nco*I 32-2 DNA	1.52×10^4

*As transformants per μg DNA. No amp^R colonies were obtained for untransformed cells.

Fig. 4.7 *Nco*I Restriction Digests of Various pPSC Plasmids

Top lanes 1-11: 1. Lambda *Eco*RI/ *Hin*dIII; 2. *Nco*I/ pPSC148; 3. *Nco*I/ pPSC149; 4. *Nco*I/ pPSC150; 5. *Nco*I/ pPSC151; 6. *Nco*I/ pPSC152; 7. *Nco*I/ pPSC153; 8. *Nco*I/ pPSC154; 9. *Nco*I/ pPSC155; 10. *Nco*I/ pPSC156; 11. *Nco*I/ proH99. Bottom lanes 1-11: 1. Lambda *Eco*RI/ *Hin*dIII; 2. *Nco*I/ proH99; 3. *Nco*I/ pPSC157; 4. *Nco*I/ pPSC158; 5. *Nco*I/ pPSC159; 6. *Nco*I/ pPSC160; 7. *Nco*I/ pPSC161; 8. *Nco*I/ pPSC162; 9. *Nco*I/ pPSC163; 10. *Nco*I/ pPSC164; 11. *Nco*I/ pPSC165.

Fig. 4.8 Size Frequency of *Pleurotus sajor-caju* 32-2 *Nco*I Fragments Cloned into Vector proH99



4.3.4 Towards the Cloning of a Carboxin Resistant Gene from *Pleurotus sajor-caju*

Initial screening of several carboxin resistant mutants demonstrated that these isolates varied in their sensitivity to carboxin (see Fig. 4.6). Strain 32-1[201] was chosen for further study and fruit bodies obtained to isolate single spore progeny and to show that this resistance was stable through meiosis. A carboxin resistant monokaryon, 32-1[201(1)], was recovered as a single spore isolate, which showed a high level of resistance to carboxin compared to the other isolates. The MIC of the parental strain 32-1 was determined at $2\mu g ml^{-1}$ and thus 32-1[201(1)] showed a marked decrease in sensitivity by growing at $100\mu g ml^{-1}$, albeit more slowly. Attempts to make a λEMBL3 library with this strain were made.

Optimum conditions for digestion with the different enzymes used involved restricting 4μg DNA with 10 units enzyme in a total volume of 200μl at 37°C. The length of digestion time required varied with the three enzymes used: 12 min with *Sau*3A, 25 min with *Mbo*I (or *Nde*I), and 25 min with *Bam*HI. These times were chosen by identifying the digest time which gave the greatest intensity (after EtBr staining) in the range 18-21Kb and choosing the time before this. Thus the maximum *number* of fragments in this size range were obtained, and selection was not based on the *mass* of DNA in that region. An example of a timed restriction digest is given in Fig. 4.9A.

Considerable problems with self-ligations between CIP treated restricted genomic DNA limited early attempts of library construction. An example of this can be seen in Fig. 4.9B. Once it was established that phosphatase treatment was complete, and that non-phosphatased restricted DNA had "ligatable" ends, attempts to clone into λEMBL3 vector were made. An example of the resulting ligations is given in Fig. 4.9C.

Results of the three experiments on the construction of a λEMBL3 library are given in Table 4.7. *E. coli* strain LE392 was found in the first experiment to give the greatest number of plaques and hence was used in subsequent studies. The packaging reaction was tested in experiments 1 and 3 to demonstrate the packaging efficiencies of

wild-type λDNA (λc1857*Sam7*). This ruled out possible problems with variables not linked to the ligation as being limiting factors to the library construction.

Comparison of the plaque forming units (pfus) obtained for the λ-self ligations and the 32-1[201(1)] library shows that in the first experiment the pfu for the two were almost the same, indicating that no λEMBL3-restricted genomic DNA ligations had either occurred or had not been packaged. Subsequent experiments were more successful in that the number of pfu obtained for the λEMBL3 library ligation was slightly higher than the λEMBL3 self ligation. According to Maniatis *et al.* (1989) 10^6-10^7 recombinants per μg bacteriophage arms should be expected, however, considerably less were obtained in these experiments and for this reason construction of the λEMBL3 library was thought to have failed. From experiments described in Chapter 5 the total size of chromosomes from the parental strain *P. sajor-caju* 32-1 was about 20.3Mb (=2.03 x 10^4Kb =1.34x10^{10}daltons). Using this figure a total of 4,672 λEMBL3 recombinant clones would be required for complete representation of the *P. sajor-caju* genome, at a probability of 0.99, and the average fragment size equal to 20Kb, according to the formula derived by Clarke & Carbon (1976):

$$N = \frac{\ln (1-P)}{\ln (1-f)}$$

Where: N= number of clones; P= probability that all DNA sequences are represented; f= fragment size divided by total genome size).

No attempt was made to clone the carboxin resistance gene in *C. cinereus* by complementation of a carboxin sensitive strain with λDNA from the partial library constructed.

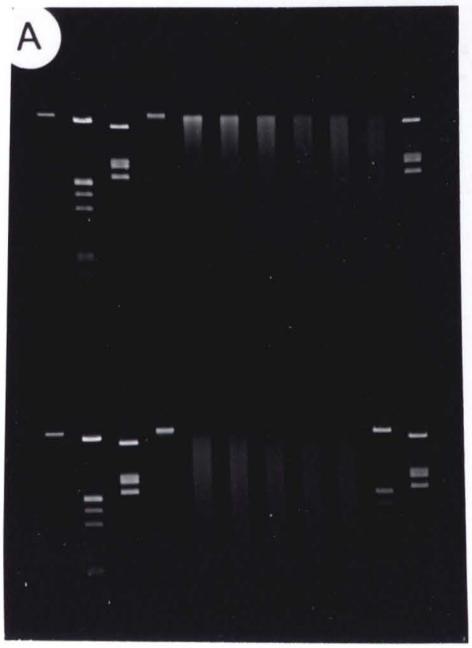
Figure 4.9 Electrophoresis of Size-fractionated Genomic DNA from *Pleurotus sajor-caju* 32-1[201(1)]

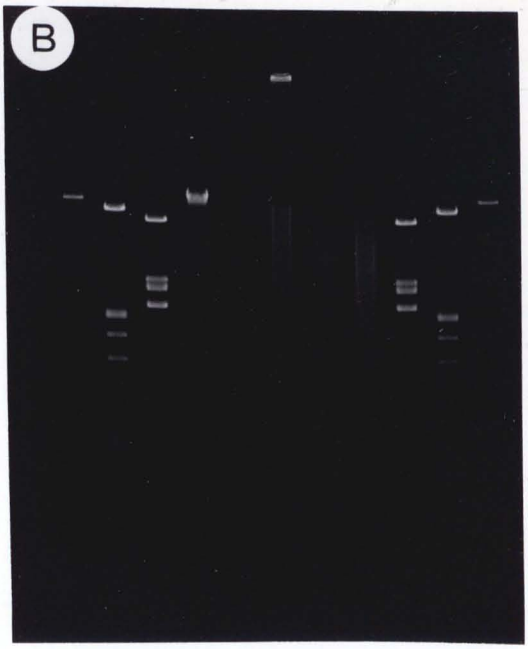
A. Partial digests with *Mbo*I:
top lanes (left to right)-1, undigested λ DNA; 2, *Eco*RI/ *Hin*dIII λ DNA; 3, *Hin*dIII λ DNA; 4, undigested 32-1[201(1)] genomic DNA; tracks 5 to 10 genomic DNA digested for 5, 7, 10, 13, 15 and 20 min with *Mbo*I; 11, *Hin*dIII λ DNA.

bottom lanes (left to right)-1, undigested λDNA; 2, *Eco*RI/ *Hin*dIII λ DNA; 3, *Hin*dIII λ DNA; 4, undigested 32-1[201(1)] genomic DNA; tracks 5 to 9 genomic DNA digested for 25, 30, 35, 40, and 45 min; 10, *Eco*RI/ *Hin*dIII λ DNA; 11, *Hin*dIII λ DNA.

B. Test ligations of restricted genomic DNA:
lanes- 1, undigested λ DNA; 2, *Eco*RI/ *Hin*dIII λ DNA; 3, *Hin*dIII λ DNA; 4, uncut genomic DNA; 5, *Mbo*I digested genomic DNA; 6, *Mbo*I digested genomic DNA ligated overnight; 7, *Mbo*I digested genomic DNA CIP treated; 8, *Mbo*I digested genomic DNA CIP treated, ligated overnight; 9, *Hin*dIII λ DNA; 10, *Eco*RI/ *Hin*dIII λ DNA; 11, undigested λ DNA.

C. λEMBL3 ligations:
lanes- 1, *Eco*RI/ *Hin*dIII λ DNA; 2, *Hin*dIII λ DNA; 3, uncut genomic DNA; 4, *Mbo*I digested DNA, CIP treated, self ligated; 5, λEMBL3 self ligated; 6, λEMBL3 ligated to *Mbo*I restricted, and CIP treated genomic DNA (2.5:1); 7, λEMBL3 ligated to *Mbo*I restricted, CIP treated genomic DNA (1.25:1); 8, (blank); 9, λEMBL3 ligated to PME insert; 10, PME insert; 11, *Hin*dIII λDNA.


A


B

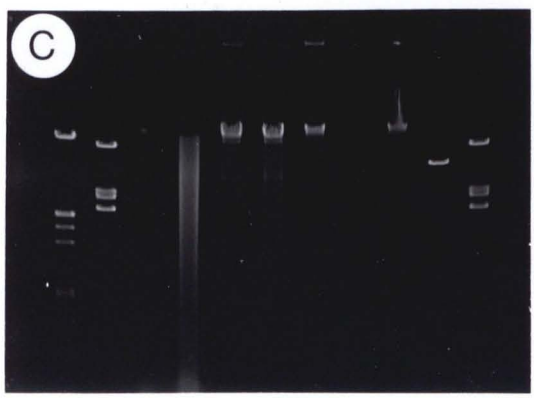

C

TABLE 4.7 Packaging Frequencies for λEMBL3 Library Ligations

EXPT. NUMBER	METHOD USED	BACTERIAL STRAIN	pfu[1]/ LIGATION λ-SELF	λ-PME	λ-*Pleurotus* DNA
1[2]	*Sau*3A digested DNA, CIP treated, ligated into λEMBL3 arms.	LE392	5.56×10^{6}	1.39×10^{7}	5.6×10^{4}
		P2392	3.54×10^{6}	1.4×10^{7}	4.47×10^{4}
		PLK-17	2.78×10^{6}	1.28×10^{7}	3.76×10^{4}
		P2PLK-17	3.18×10^{6}	1.13×10^{7}	4.3×10^{4}
2	*Mbo*I digested fragments sized 18-22Kb electroeluted, ligated into λEMBL3 arms.	LE392	3.63×10^{6}	4.05×10^{6}	8.75×10^{4}
3[2]	*Bam*HI digested fragments sized 18-22Kb phenol extracted from LMP agarose, ligated into λEMBL3 arms.	LE392	1.03×10^{8}	1.41×10^{6}	3.89×10^{5}

1. Plaque forming units per μg DNA.
2. pfu for wild type λc1857*Sam*7: Expt. 1. 1.05×10^{8}; Expt. 3= 5.8×10^{7}.

4.4 Discussion

Increasing interest in several edible fungal species as food resources high in protein and as micro-organisms able to convert lignocellulosic waste to a commercially viable product (Bisara & Madan, 1983) has concentrated on exploiting their biological efficiency *via* standard strain breeding methods (e.g. Eugenio & Anderson, 1968; Prillinger & Molitoris, 1979). Application of more directed approaches, using DNA mediated transformation, could prove to be useful in strain improvement programmes in several ways. It was the aim of this study to obtain transformants from *P. sajor-caju* as the first step in the application of this methodology to the breeding of *Pleurotus* spp. with potential applications to other edible fungi. To date, stable and efficient transformation of an edible basidiomycete species remains an elusive goal.

The approach of using a dominant resistance marker as the selection system was taken for the simple reason that no well characterized auxotrophic mutants were available and hence reversion to prototrophy could not be attempted. The three compounds carboxin, hygromycin B, and phleomycin were chosen because they were found to be highly toxic to several species of *Pleurotus*. In addition, H. Mooibroek (pers. comm.) has reported transformation of a *Pleurotus* sp. with the vector pAN8-1, conferring phleomycin resistance. The antimetabolites G418 and sulphanil amide were shown to be less effective, although it is

possible that sensitivity to sulphanilamide could be increased by changing the medium used (Carramolino *et al.*, 1989). Such nutrient changes may have subsequent effects on the regeneration of protoplasts, and stability of transformants when transferred to more complex growth substrates where selection pressure cannot be maintained. Sulphanilamide has several advantages over other antibiotics in that it is stable at room temperature, is not hazardous to humans, and is inexpensive.

Attempts to transform *P. sajor-caju* to $HygB^R$ with pAN7-1 and proH101 (carrying the *Acremonium chrysogenum* high efficiency *pcbC* promoter and the *pcbC* 3' terminator regions) and to $phleo^R$ with pAN8-1 by a routine protoplast transformation procedure failed. Of the numerous variables affecting transformation, the vector DNA was thought to be the most limiting. proH101 was used because it has been shown to give relatively high transformation frequency and a lower rate of abortive transformants in *A. chrysogenum* (Smith, 1990), but this also was unable to generate hygromycin B resistant colonies with *P. sajor-caju* 32-2.

Several workers had hinted at the possible inefficiency or lack of function of ascomycete promoters in basidiomycete species (Ullrich *et al.*, 1985; Casselton & de la Fuente Herce, 1989; Saunders *et al.*, 1989; Skrzynia *et al.*, 1989). Thus, overcoming limitations in the regulatory sequences controlling gene expression was assumed to be the most obvious method for obtaining transformants. 'Shot-gun' cloning has been used successfully to obtain promoter regions from *Cochliobolus heterostrphus* (Turgeon *et al.*, 1987) and *Saccharomyces cerevisiae* (Santangelo *et al.*, 1988), but results from such an approach with *Agaricus bitorquis* are incomplete (Horgen & Anderson, 1989). It was felt that a similar strategy would prove to be successful with *P. sajor-caju*.

Fragments of DNA from strain 32-2 were obtained by restriction with *Nco*I which recognizes the sequence 5'C↓CATGG3', and ligated into the *Nco*I site of proH99. It was expected that *Nco*I would increase the chances of obtaining a promoter sequence as in the vast majority of eukaryotic genes translation is initiated at the proximal ATG site

(Kozak, 1984; Gurr *et al.*, 1987). Although this assumption precedes extensive characterization of regulatory sequences in cloned genes from basidiomycetes, the two previously mentioned reports by Turgeon *et al.* (1987) and Santangelo *et al.* (1988) used enzymes *Mbo*I (5'↓GATC3') and DNAaseI respectively with no obvious selective advantage. Whilst this enzyme, i.e. *Nco*I, has not been used for such a purpose, it would have no known disadvantages.

Of the plasmids isolated and characterized, there was a higher than expected frequency of plasmids lacking an insert. This was due in part to transformation with self-ligated *Nco*I cut proH99 vector which was inefficiently de-phosphorylated. As no control was carried out with *Nco*I-cut proH99 transformed into *E. coli* it is possible that incomplete digestion of the vector (not visibly detected by gel electrophoresis and EtBr staining) had occurred and this added to the discrepancy. Transformation could have been limited if the foreign DNA confers a selective disadvantage on *E. coli* as has been found with *Neurospora crassa* DNA (Akins & Lambowitz, 1985) and several clones were found to have slow growth rate and/or abnormal colony morphology. Palindromic sequences can cause plasmid instability and may lead to the inability to clone certain DNA fragments (Collins, 1981; Hagen & Warren, 1983). Turgeon *et al.* (1987) also found that 50% of their promoter-plasmid constructs lacked an insert.

In general the low transformation frequency in *E. coli* was not found to be limiting as only 100 recombinants were deemed necessary for the experiment here. Transformation frequency could possibly be increased by size selection for fragments 2-3Kb prior to ligation, as ligations between restricted DNA and subsequent ligation to the vector could lead to large DNA molecules which will not transfect *E. coli* (Little, 1987). Of the 100 recombinant plasmids retrieved, 13 were found to have 2 or more *Nco*I 32-2 fragments and it is assumed that this was due to ligation between fragments as complete digestion of the genomic DNA had been carried out.

From these attempts, no transformants were obtained and possible reasons for such need to be explored. If the vector used is assumed to be a key factor limiting transformation then it is possible that

screening of more recombinant clones may lead to the isolation of promoters recognized by this species. Alternatively, the failure of the approach may be due to some sort of lethal effect associated with the expression of the bacterial *hph* gene in a heterologous system, such as proteolysis during physiological stress (Saunders *et al.*, 1989). Possible consequences of integration into regions of methylated, or *de novo* methylation could not be studied here as has been investigated with *S. commune* (Mooibroek *et al.*, 1990), because no transformants were ever obtained for analysis and no co-transformation system exists for this, or any other *Pleurotus* spp. Methylation effects on gene expression in *S. commune* have previously been described (Buckner *et al.*, 1988), although little is known about its importance in other basidiomycete species. The expense related to 5-AC did not allow for tests on its effect on transformation frequency and gene expression.

Selection pressure for transformants was carried out by adding the appropriate inhibitor 24 hours after the transformation manipulations had been carried out. Little is known about the timing associated with the application of selection in filamentous fungi, but in *S. cerevisiae* it can have serious consequences on the number of transformants obtained (Webster & Dickson, 1983). In addition, the high density of protoplasts used for plating may have been detrimental to the selection of transformants (Misra, 1985).

Other limiting factors could include the competence of the protoplasts themselves, caused by the batch of Novozym 234 used and heterogeneity in the nuclear status of the protoplast population. Lytic enzymes used for transformation have already been discussed as possible limitations on protoplast competence (e.g. Rambosek & Leach, 1987). It had previously been found with *P. sajor-caju* 32-2 that up to 60% of protoplasts were anucleate (see Chapter 2) and thus unable to revert to hyphal form and express resistance, also the strain 32-2 is a dikaryon and further heterogeneity in the nucleated protoplast population would be expected. As the majority of nucleated protoplasts have one nucleus, subsequent reversion could lead to the development of homokaryons which may have variable sensitivity to the fungal inhibitor. Although this was not investigated it would seem

appropriate in these studies to limit variation as much as possible, and so future investigations should take this into account. Viability controls showed that PEG greatly reduced the number of regenerating colonies, but it is difficult to assess the level of toxicity on protoplasts because of clumping which results.

Carboxin resistance was also chosen for study as several *Pleurotus* spp. were found to be highly sensitive to this inhibitor (M. Nisik, pers. comm.). With the aim of cloning a carb^R gene from *P. sajor-caju*, U.V.-induced carb^R strains in the Nottingham collection were characterized for their level of resistance. Growth on selective media varied between 35-50 times the MIC of the wild type strain, and the most resistant isolate tested was found to be a homokaryotic single spore isolate obtained from a fruiting body which developed from a carboxin resistant dikaryon. It is not known if increased resistance was a consequence of de-dikaryotization, meiotic recombination, or spontaneous mutations which can result during the sexual cycle, but it is interesting to note that this monokaryon has roughly twice the resistance level of the parental dikaryon.

1,4 oxathiins such as carboxin are inhibitors of succinate oxidation in mitochondria (Georgopoulos *et al.*, 1972). Resistant mutants have been found to have an altered succinate dehydrogenase (Georgopoulos *et al.*, 1972), although reduced sensitivity to the fungicide in some fungi could be due to differential uptake of the compound (Mathre, 1968), and in *Verticillium albo-atrum* carboxin is detoxified by riboflavin or riboflavin phosphate in light (Ragsdale & Sisler, 1970). Carboxin resistance has been obtained in a number of basidiomycete species including *C. bilantus* (Challen & Elliott, 1990), *A. bisporus* (Challen & Elliott, 1987), and *U. maydis* (Georgopoulos *et al.*, 1972), however, the nature of resistance in most of these species has not been investigated.

Cloning of the *P. sajor-caju* carb^R gene by complementation in *C. cinereus* was not possible as only a partial library was constructed and time did not allow for further attempts. In this laboratory several DNA libraries from a variety of species have been successfully made using the protocol used here (e.g. Blakemore, 1990;

Vanstein, 1990, C. Ulhoa, pers. comm.), however, this method failed to produce any recombinants in the first instance with *P. sajor-caju* 32-1[201(1)] DNA. This could possibly be due to excessive treatment of the restriction fragments with CIP resulting in DNAase action to give non-ligatable ends (Little, 1987). Ligation and packaging conditions appeared to be quite suitable as the controls gave a high number of pfus, but it is also possible that some contaminating factor in the restricted DNA was limiting ligation to the λEMBL3 arms. Digests were optimized to produce fragments 18-22Kb in length, however, the vector used here can accept fragments from 9-23Kb and so it is unlikely that the limitations were a result of inadequate size selection. After prolonged storage (six months) at -70°C the packaging extracts were found to be reduced in efficiency by half as results from packaged and transfected wild-type lambda DNA showed.

During the first attempt at producing a library, four strains of *E. coli* were used for plating of the library. Strains P2392 and P2PLK-17 will propagate recombinant phage only, and so are used in early stages to asses the proportion of non-recombinant vs. recombinant phage produced. Host strains PLK-17 and P2PLK-17 are used to increase the yield of phage with methylated DNA inserts, specifically 5-methylcytosine (Kretz *et al.*, 1989). However, no difference in the plating efficiencies was detected in this first experiment because no ligations to the lambda arms had occurred. For matters of convenience the two subsequent attempts used only strain LE392 to test phage yield, and not to determine the exact proportion of recombinants in each instance.

Size selection of fragments 18-22Kb prior to ligation and packaging was useful in that it bypassed the need to phosphatase the restricted DNA, as inadequate CIP-treatment was found to be a constant problem. Modifying the method of Lu (1989) using size selected DNA gave 2-3 times more pfus with lambda-*Pleurotus* DNA than the lambda self ligation did, but in no experiment did the yield approach the 10^6-10^7 recombinants expected.

Reports on the failure to construct DNA libraries with lambda replacement vectors are few. Vincze & Kiss (1990) were unable to

produce a library for alfalfa (*Medicago sativa*) in λEMBL4 which was attributed to *cos-cos* ligations giving low packing efficiencies. It is not clear why this should give difficulties with DNA from this species only.

Recently a resistance gene to 5FI has been cloned in *C. cinereus* and this may be very useful for transformation of basidiomycetes sensitive to this compound. Several *Pleurotus* spp. and strains of *Lentinula edodes* (see Appendix D) have been shown to be highly sensitive to this compound which also confers tryptophan auxotrophy in resistant isolates. In addition, attempts to isolate a carboxin resistance gene in *C. cinereus* are currently underway (Challen & Elliott, 1990), and so the advent of such novel vectors for basidiomycetes may prove to be the most promising step towards transformation of edible species.

5.1 Introduction

Electrophoretic analysis of DNA fragments by conventional techniques is limited by the resolution achieved by molecules greater than about 20 kilobases (Kb: Smith *et al.*, 1986; Birren *et al.*, 1989). This size limitation was first overcome by use of low percentage agarose gels (Fangman, 1978; Sewer, 1981) and later by adopting an electrode configuration which periodically altered the direction of the electric field (Schwartz *et al.*, 1982).

Since demonstrating the feasibility of pulsed-field gel electrophoresis (or PFG) for separating megabase (Mb) sized DNA molecules, several areas of research have benefited (Barlow & Lehrach, 1987). Numerous adaptations and improvements have since been made to the original system (Anand, 1986; Eby, 1990; Knight, 1989) and these are listed in Table 5.1. The following is a brief account of PFG technology and its potential application to the study of *Pleurotus* spp.

Theoretical Basis of PFG

During conventional electrophoresis resolution of small DNA molecules (0.2-20Kb) is achieved by gel filtration (Cantor & Schimmel, 1980), and mobility is found to vary logarithmically with size. The sieving properties of the agarose gel are, however, not applicable to large molecules as they are larger than the pores present in the matrix and are therefore not separated effectively. Large DNA molecules can still migrate through the gel by a process known as "reptation", but size dependent fractionation is lost (Smith *et al.*, 1986).

To overcome limited separation of large DNA molecules the concept of PFG was introduced by Schwartz *et al.* (1982). This takes advantage of those factors which limit conventional electrophoresis, namely the orientation and elongation of DNA when under an electric field (Cantor *et al.*, 1988b).

TABLE 5.1 Pulsed Field Gel Electrophoresis Systems

ACRONYM	TRANSLATION	MANUFACTURER	REFERENCE
AFIGE	Assymetric Field inversion Gel Electrophoresis		Denko *et al.* (1989)
CHEF	Contour-clamped Homogeneous Electric Field	Bio-Rad CHEF-DRII™/ BRL Hex-A-Field™	Chu *et al.* (1986)
CUFE	Constrained Uniform Field Electrophoresis	Owl Scientific Inc.	
ED	Electrical Device		Schwartz *et al.* (1989)
FIGE	Field Inversion Gel Electrophoresis	Hoefer SuperSub™ [1]	Carle *et al.* (1986)
OFAGE	Orthogonal Field Alternation Gel Electrophoresis		Carle & Olson (1984)
PACE	Programmable Autonomously Controlled Gel Electrode	Bio-Rad	Clark *et al.* (1988)
PFGE	Pulsed Field Gradient Electrophresis	Pharmacia Pulsaphor™	Schwartz & Cantor (1984)
PHOGE	Pulsed Homogeneous Orthogonal Field Gel Electrophoresis		Bancroft & Wolk (1988)
RAGE™	Rotating Agarose Gel Electrophoresis	Stratagene RAGE™	Eby (1990)
RFE	Rotating Field Electrophoresis		Ziegler *et al.* (1987)
RGE	Rotating Gel Electrophoresis	Biometra Rotaphor™/ Appligene Pulsar™	Southern *et al.* (1987) Ziegler (1987) Gekeler *et al.* (1989) Sewer & Hayes (1989)
ST/RIDE	Simultaneous tangential/ rectangular inversion decussate electrophoresis		Kölble & Sin (1991)
TAFE	Transverse Alternating Field Electrophoresis	Beckman GeneLine™	Gardiner & Patterson (1988)
TFAGE	Transverse Field Alternation Gel Electrophoresis		Olson (1989)
ZIFE	Zero Integrated Field Electrophoresis		Turmel *et al.* (1990)

1. Numerous controllers are also commercially available.

During PFG alternating electrical fields are applied for a length of time known as the "pulse time", whilst the time taken to change field direction is less than 0.1 second. Separation is achieved because the time taken to re-orientate along the field and move forward is size dependent. Numerous factors are known to affect separation under PFG and some of these are discussed below.

Variables Affecting PFG

Systematic studies into PFG have been extensive with several variables known to critically affect mobility and band sharpening. These key variables are briefly discussed below.

Pulse Time It is well established that separation of megabase sized DNA is linearly dependent on pulse time (Birren *et al.*, 1988; Cantor *et al.*, 1988b; Mathew *et al.*, 1988c; Smith *et al.*, 1987; Southern *et al.*, 1987). Migration during PFG relies on the size dependent relationship of DNA molecules and their ability to reorientate to an alternating electric field. The time required to change direction increases with size and so the pulse time is a critical variable affecting resolution. If a pulse time is too short or too long then DNA migrates by reptation and no resolution will be obtained.

Pulse time can remain at a fixed period for the duration of the run or it can be steadily decreased or increase for a period of time during electrophoresis, which is known as a pulse-time "ramp". Ramping of this type has the advantage of separating a wide size range of DNA molecules during a single run.

As mentioned previously the time taken for the field direction to change is less than 0.1 second, however, field intermittency (or pulse delay) can be used to improve electrophoresis results (Jamil & Lerman, 1985). Noolandi *et al.* (1987) first investigated "self-trapping" of DNA molecules during conventional electrophoresis, and Smith *et al.* (1989) have since observed DNA adopting U-shaped structures around obstructions during electrophoresis. By turning the field off periodically, DNA molecules relax and adopt a random conformation which has low mobility (Olson, 1989). This helps to reduce trapping of large molecules resulting in greater migration

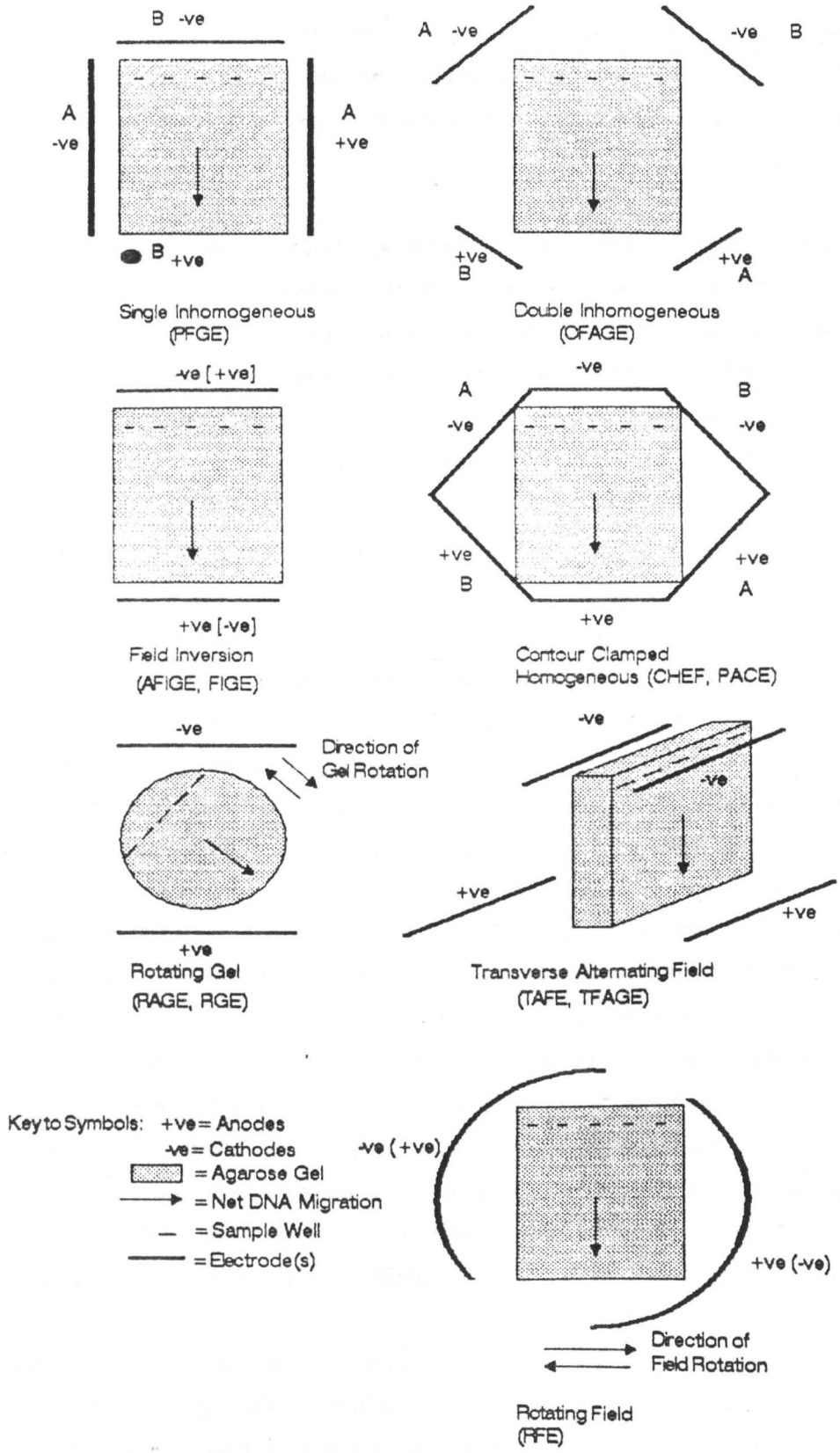
(Lalande *et al.*, 1988; Turmel *et al.*, 1990). Relaxation time has been shown to increase with size and decrease as the field strength increases (Holzwart *et al.*, 1987; Kalyaniwalla & Bean, 1985; Sutherland *et al.*, 1987). Lai *et al.* (1988) used zero field intervals of up to 180 seconds to give greater mobility of the larger *S. cerevisiae* chromosomes with no effect on migration of the smaller chromosomes.

Electric Field Shape Electrode configuration, and the resulting electric field generated has, a great influence on the separation of PFG. There are two main designs used for PFG, namely field inversion and transverse-field alternation (see Fig. 5.1). During field inversion the direction of the electric field is periodically reversed at an angle of 180°, with the "forward time" longer than the "reverse time" resulting in net forward movement. Larger DNA molecules migrate ahead of the smaller ones (Heller & Pohl, 1989) and has so far been limited to resolving DNA of up to 1.6Mb.

Numerous transverse-field alternation type PFG systems have been developed which have electrodes separated at a variable angle depending on the apparatus used. An angle of 90° was used in early studies but ideally an obtuse angle greater than 110° should be employed (Cantor *et al.*, 1988a). Here, the electric field alternates between the sets of electrodes for a suitable switch time and DNA migrates forward. Early PFG apparatus used non-homogeneous electric fields which resulted in field gradients giving lane distortion. Homogeneous electric fields such as the CHEF system of Chu *et al.* (1986) give excellent separation and resolution of chromosomes without lane distortion. Interestingly, studies with filamentous fungi have tended to used CHEF.

Electric Field Strength Low fields have been shown to give the best resolution of megabase-sized DNA (Smith *et al.*, 1987), and high voltages can cause band smearing possibly caused by shearing (Mathew *et al.*, 1988c).There is an inverse relationship between field strength and pulse time which should also be taken into consideration (Mathew *et al.*, 1988c).

Fig. 5.1 Examples of Field Geometries Used for Pulsed Field Gel Electrophoresis



Gel Matrix Agarose is used routinely for PFG studies and the concentration of agarose used can greatly affect resolution. Increasing gel concentration gives better separation but requires a corresponding increase in the run time (Cantor *et al.*, 1988; Mathew *et al.*, 1988b). The grade of agarose used for PFG analysis is also known to affect migration (Mathew *et al.*, 1988b; Upcroft *et al.*, 1989).

Temperature Effects In general, migration increases with temperature but with poorer resolution (Birren *et al.*, 1988). The reasons for temperature dependence on DNA mobility are not clear, and several theories have been proposed (Cantor *et al.*, 1988; Mathew *et al.*, 1988b). Olschwang & Thomas (1989) used temperature gradients in a FIGE system to obtain greater resolution of larger DNA molecules, however, most PFG equipment maintains constant temperature through a cooling system and buffer circulation.

DNA: Preparation and Topology To prevent mechanical shearing of intact chromosomes several DNA extraction methods have been further developed which vary with the species being investigated (Smith *et al.*, 1988). The most widely adopted method involves embedding cells in a high quality, low melting point agarose which facilitates lysis *in situ* (Olson, 1989). Enzymatic treatment is usually included to remove residual protein or RNA present in the sample. With filamentous fungi this involves isolating protoplasts as the cell wall acts as a barrier to DNA migration and also helps to concentrate DNA, although McCluskey *et al.* (1990) have developed a technique which avoids the use of protoplasts. Enzymatic lysis of cell walls *in situ* has been attempted with condiospores (Orbach *et al.*, 1988) and with mycelium (Rollo *et al.*, 1989). The concentration of cells used should give 0.5-20µg DNA in a 10x5x2mm insert (Smith *et al.*, 1988). Very high DNA concentrations have the effect of causing band broadening (Cantor *et al.*, 1988b).

Other features such as DNA methylation, protein binding, and regions of oligo(dA)-oligo(dT) result in DNA bending and reduced mobility during standard electrophoresis (Marini *et al.*, 1982; Wu & Crothers, 1984; Hagerman, 1986; Kimura *et al.*, 1989; Levene & Zimm, 1989).

However, Neimark & Lange (1990) were unable to correlate migration distance with the G+C content of mycoplasma chromosomes which had previously been suggested to be responsible for anomalous mobility (Maniloff, 1989).

DNA configuration can affect migration as work with plasmids up to 97Kb in size has shown (Hightower *et al.*, 1987; Mathew *et al.*, 1988b; Sobral & Atherly, 1989; Simske & Scherer, 1989; Sewer & Hayes, 1989). These studies have demonstrated that small supercoiled DNA molecules are relatively insensitive to pulse time and open circular forms migrate slower than linear DNAs of the same size.

Self-trapping of DNA during conventional electrophoresis has been predicted by Noolandi *et al.* (1987) to be a consequence of DNA configuration during migration under a constant electric field. With variable frequency that is related to DNA size, looplike (or compact) or extended structures are adopted. Looplike conformations, having ends close to each other, are more stable for longer DNA fragments although more infrequent.

DNA Length Standards To determine the size of DNA molecules resolved by PFG the use of standards whose molecular weights are known is required. Lambda DNA concatamers are the most widely accepted markers for separation in the range 10-200Kb (Cooney *et al.*, 1989) or between 40-1,500Kb (Smith *et al.*, 1986; Waterbury & Lane, 1989). Ligated plasmid DNA has also been used to provide standards in the 10-300Kb range (Hanlon *et al.*, 1989) whilst ligated concatamers of mycoplasma DNA give accurate sizes in the range 0.48-3.36Mb (Whitley & Finch, 1990). Yeast chromosomal DNA is commercially available as standards ranging from 0.245-2.2Mb for *S. cerevisiae* and 3.5-5.7Mb for *Sch. pombe*. Alternatively, *Hansenula wingei* chromosomes are of the range 1-3.3Mb and may prove to be useful size markers (Jones *et al.*, 1990). It is important to use a strain that has been previously characterized for use as size markers as several yeast isolates are known to show differences in chromosome size.

Applications of PFG

Classical approaches of karyotyping involve microscopic investigations of condensed chromosomes. This is widely used for studies on human and plant chromosomes, but is limited in use if a species has small chromosomes which do not condense (Anand, 1986; Smith *et al.*, 1986). This is where PFG has had one of its main applications.

Separation of megabase (Mb) sized DNA through an agarose matrix by PFG allows studies on genome organization to be made and has been applied to numerous species (summarised in Table 5.2). Subsequent assignment of specific genes to chromsomes can be carried out to complete karyological studies. This type of linkage analysis has been done for species without any classical genetic analysis, including *Colletotrichum gloesporoides* (Masel *et al.*, 1990) and *Histoplasma capsulatum* (Steele *et al.*, 1989). Numerous fungal species with established genetics have also been investigated including *Aspergillus nidulans* (Brody & Carbon, 1989), *Acremonium chrysogenum* (Skatrud & Queener, 1989), *Coprinus cinereus* (Pukkila, 1990) and *Neurospora crassa* (Orbach *et al.*, 1988; Lai, 1989) and several yeast species (Carle & Olson, 1985; Kinscherf & Leong, 1988; Magee *et al.*, 1988; Pretorius & Marmur, 1988; Smith *et al.*, 1987).

Differences in chromosome size and numbers detected by PFG have been used for taxonomic studies of yeasts (Johnston & Mortimer, 1986; Steensma *et al.*, 1988; Coetzee *et al.*, 1987; Zimmermann *et al.*, 1988). Identification of interspecific hybrids of *Kluyveromyces* spp. (Witte *et al.*, 1989) and *Saccharomyces* spp. (Hoffmann *et al.*, 1987) and intergeneric hybrids of *Candida* and *Kluyveromyces* (Zimmermann *et al.*, 1988) and *Kluyveromyces* and *Saccharomyces* (Witte *et al.*, 1989) have also been verified by means of PFG.

DNA molecules up to 500Kb in size, have been cloned using PFG technology to isolate fragments obtained by use of restriction enzymes with infrequent recognition sites (Levine & Cech, 1989). Isolated fragments can be cloned in yeast artificial chromosomes (YACs) and subsequently used for transforming *S. cerevisiae* (e.g. Guzman & Ecker, 1888; Anand *et al.*, 1989).

TABLE 5.2 Reported Separation of Intact Chromosomal DNA from a Variety of Species

SPECIES	PFG SYSTEM(S)	DNA SIZE RANGE (Mb)	NUMBER OF BANDS RESOLVED	REFERENCE
MYXOMYCETES				
Dictyostelium discoideum	CHEF	<3.6-9.0	6-7	Cox *et al.* (1990)
YEASTS				
Candida albicans	OFAGE	1.1-1.6	4	Johnston & Mortimer (1986)
	PFGE	1.2-10.0	6	Snell & Wilkins (1986)
	CHEF	1.2->2.2	10	Vollrath & Davis (1987)
	OFAGE	1.0->3.5	8	Lasker *et al.* (1989)
	FIGE	1.0->3.5	6	Lasker *et al.* (1989)
	FIGE	Not Given	5	Lott *et al.* (1990)
	ST/RIDE	0.44-3.4	8	Kölble & Sia (1991)
Candida macedoniensis	OFAGE	Not Given	9	Zimmermann *et al.* (1988)
Candida tropicalis	PFGE	1.0-2.8	8	Suzuki *et al.* (1991)
Candida utilis	OFAGE	1.2	1	Johnston & Mortimer (1986)
Cryptococcus neoformans	OFAGE	0.26->1.6	8-9	Polacheck & Lebens (1989)
Hansenula wrakii	OFAGE	Not Given	10	Sor (1988)
Hansenula wingei	PFGE	1.03-3.3	7	Jones *et al.* (1990)
Histoplasma capsulatum	CHEF FIGE	0.5-~8.7	7	Steele *et al.* (1989)
Kluyveromyces dobzanski	OFAGE	1.0-1.75	5	Sor (1988)
Kluyveromyces fragilis	OFAGE	1.0-1.75	5	Sor (1988)
Kluyveromyces lactis	OFAGE	1.2-1.6	3	Johnston & Mortimer (1986)
	OFAGE	1.25-3.0	5	Sor (1988)
	OFAGE	1.0-4.0	5	Steensma *et al.* (1988)
Kluyveromyces marxianus	OFAGE	1.0-3.0	5-7	Steensma *et al.* (1988)
	OFAGE	Not Given	10	Zimmermann *et al.* (1988)
	TAFE	Not Given	11	Witte *et al.* (1989)

TABLE 5.2 (cont.)

SPECIES	PFG SYSTEM(S)	DNA SIZE RANGE (Mb)	NUMBER OF BANDS RESOLVED	REFERENCE
Kluyveromyces vanudeni	OFAGE	1.25-3.0	5	Sor (1988)
Kluyveromyces wickerhamii	OFAGE	0.75-1.75	5	Sor (1988)
Ophiostoma ulmi	CHEF	<3.5->5.7	6	Royer *et al.* (1991)
Pichia canadensis	OFAGE	1.0-1.5	2-3	Johnston & Mortimer (1986)
Saccharomyces cerevisiae	OFAGE	0.2-2.2	11	Carle & Olson (1984)
	OFAGE	0.2-?	12	Carle & Olson (1985)
	TAFE	Not Given	15	Rank & Casey (1988)
Saccharomyces exigus	OFAGE	0.25->2.0	7-9	Coetzee *et al.* (1987)
Saccharomyces kluyveri	OFAGE	1.0-1.5	3	Johnston & Mortimer (1986)
	OFAGE	>1.0	3	Coetzee *et al.* (1987)
Saccharomyces unisporus	OFAGE	0.5->2.0	8-10	Coetzee *et al.* (1987)
Schizosaccharomyces pombe	PFGE	3.0-9.0	3	Smith *et al.* (1987)
	PACE	3.0-7.0	3	Clarke *et al.* (1988)
	OFAGE	3.0-9.0	3	Sor (1986)
Schwanniomyces occidentalis	OFAGE	1.1-1.6	4	Johnston & Mortimer (1986)
Ustilago hordei	PFGE	0.25->2.2	~13	McCluskey *et al.* (1990)
	CHEF	0.17-3.15	15-20	McCluskey & Mills (1990)
Ustilago maydis	OFAGE	0.3->1.0	~20	Kinscherf & Leong (1988)
	CHEF	<0.45->1.6	~14	Fotheringham & Holloman (1990)
FILAMENTOUS FUNGI				
Acremonium chrysogenum	TAFE	~1.7->4.0	8	Humphrey *et al.* (1988) & Skatrud & Queener (1989)
Agaricus bisporus	CHEF	1.1-~5.0	10	Horgen (pers. comm.)
Aspergillus nidulans	CHEF	2.9-5.0	6	Brody & Carbon (1989)
Aspergillus niger	CHEF	3.5-6.6	4	Debets *et al.* (1990)

TABLE 5.2 (cont.)

SPECIES	PFG SYSTEM(S)	DNA SIZE RANGE (Mb)	NUMBER OF BANDS RESOLVED	REFERENCE
Cladosporium fulvum	PFGE	2.0-5.0	~9	Oliver (1990)
Claviceps purpurea	CHEF	0.5-6.0	Not Given	Pazoutava (1990)
Claviceps paspali	CHEF	0.4->7.0	Not Given	Pazoutava (1990)
Colletotrichum gloeosporiodies	CHEF	0.27->6.0	6-15	Masel *et al.* (1990)
Coprinus cinereus	CHEF/OFAGE	1.0-5.0	9-10	Putkila (1990)
Magnaporthe grisea	CHEF	0.5->10.0	6-9	Orbach *et al.* (1990)
Neurospora crassa	CHEF	4.0-12.6	5	Orbach *et al.* (1988)
Phoma tracheiphila	OFAGE	0.78->1.6	12	Rollo *et al.* (1989)
Phanerochaete chrysosporium	CHEF	2.0-4.8	7	Gaskell *et al.* (1991)
Phytophthora megasperma	CHEF	1.4->3.0	9	Howlett (1989)
Podospora anserina	CHEF	3.9-6.3	5	Osiewacz *et al.* (1990)
Septoria nodorum	CHEF	1.0-4.0	~16	Cooley (1990)
Tilletia caries	CHEF	1.0->2.2	8	McCluskey *et al.* (1990)

These restriction enzymes have also been applied to macrorestriction mapping of numerous species including prokaryotes with relatively small chromosomes to the higher eukaryotes (e.g. Fan *et al.*, 1988; Cocks *et al.*, 1989; Ferdows & Barbour, 1989; Frutos *et al.*, 1989; Leblond *et al.*, 1990). Gamma-ray induced DNA cleavage was used by Neimark & Lange (1990) with several mycoplasma species to determine chromosome size by PFG.

Other applications include restriction mapping of lambda DNA clones (Daniels *et al.*, 1990) and DNA sequencing (Birren *et al.*, 1990; Goode & Feinstein, 1990), where greater resolution has been achieved by use of PFG. Isolation of mitochondrial DNA from yeast cells lysed in agarose has also been possible by use of PFG (Skelley & Maleszka, 1989). Potential applications that have not been investigated are the construction of single chromosome libraries (Smith & Cantor, 1987), and applications to protein electrophoresis (Lai *et al.*, 1989).

Taxonomic Approaches to *Pleurotus* spp.

Pleurotus species are the second most commonly cultivated mushrooms in Europe with a variety of genotypes that make them widely adaptable for agronomic purposes (Zadrazil, 1978; Farr, 1983). Understanding natural breeding relationships is of importance for studies on speciation in this genus and how to manipulate such information for the improvement of existing cultivars. Speciation can occur by two processes, each involving population differentiation and reproductive isolation. Allopatric speciation characterizes the formation of discrete species by geographic isolation, whereas sympatric speciation occurs without this insularity (Reiger *et al.*, 1976). Amongst the basidiomycetes, the genus *Coprinus* has been most widely studied in terms of speciation (Kemp, 1983 & 1985) and much attention has been focused on *Pleurotus* (Bresinsky *et al.*, 1987).

The genus Pleurotus represents a widely variable group of species whose taxonomic relationships are often less than clear (Rajarathnam & Bano, 1987). Confusion originates, in part, from the influence of environmental factors on morphological criteria used for identification (Bresinsky *et al.*, 1987; Eger *et al.*, 1979). In addition, reproductive barriers allowing interbreeding to occur between species are not fully understood and create further confusion.

Features used for classification often include: pileus and spore colour, host substrate, temperature range for fruiting, and mating reactions with other isolates (Anderson *et al.*, 1973; Eugenio & Anderson, 1968). More recently isozyme analysis has been used to distinguish species of the genus *Pleurotus* to aid taxonomic studies

(Magae *et al.*, 1988; May *et al.*, 1988; May & Royse, 1988). Reports of chromosome numbers have however been limited (Bresinsky *et al.*, 1987; May *et al.*, 1988; Slézec, 1984), for summary see Table 5.3, and no attempt to characterize *Pleurotus* spp. by PFG has been made previously.

TABLE 5.3 Reported Chromosome Numbers and Linkage Groups in *Pleurotus* Species

SPECIES	*n*=	METHOD OF DETECTION	REFERENCE
P. calyptratus	8 to 10	DAPI Fluorescence	Bresinsky *et al.* (1987)
P. cornucopiae	8 to 10	DAPI Fluorescence	Bresinsky *et al.* (1987)
P. cystidiosus	6	DAPI Fluorescence	Bresinsky *et al.* (1987)
P. dryinus	8 to 10	DAPI Fluorescence	Bresinsky *et al.* (1987)
P. erygnii	12 or 14 12 to 14	Light Microscopy DAPI Fluorescence	Slézec (1984) Bresinsky *et al.* (1987)
P. ferulae	12	Light Microscopy	Slézec (1984)
P. florida	≥6	Linkage Analysis	May *et al.* (1988)
P. nebrodensis	13	Light Microscopy	Slézec (1984)
P. ostreatus	8 to 10 ≥6	DAPI Fluorescence Linkage Analysis	Bresinsky *et al.* (1987) May *et al.* (1988)
P. pulmonaris	8 to 10	DAPI Fluorescence	Bresinsky *et al.* (1987)
P. salmoneo-straineus	18	DAPI Fluorescence	Bresinsky *et al.* (1987)
P. sapidus	≥6	Linkage Analysis	May *et al.* (1988)

Aims of This Study

The purpose of this investigation was to apply PFG to aid chromosome separation and enumeration of several species, strains and hybrids of *Pleurotus*. Information gathered from characterized isolates would then be used to investigate taxonomic relationships within the genus *Pleurotus*.

5.2 Materials and Methods

5.2.1 General

As in Chapter 4 glassware used for all manipulations was acid washed and baked to destroy potential nuclease contamination. Agarose gels stained with ethidium bromide were observed using a short wavelength U.V. transilluminator (Ultra-violet Products Inc., U.S.A.) and photographed using a Polaroid MP-3 land camera with Polaroid Type 665 film.

Solutions

Solutions used for gel electrophoresis, hybridization etc. included the following:

50x Denhardt's (Maniatis *et al.*, 1982): Ficoll (Sigma Chemical Co.), 0.5g; polyvinylpyrrolidone (Sigma Chemical Co.), 0.5g; BSA (Sigma Chemical Co.), 0.5g; volume to 50ml with AnalaR water and filter sterilized through 0.22µm disposable Millipore filter.

Sodium Dodecylsulphate (NDS) Lysis buffer (Orbach *et al.*, 1988): 1% SDS; 0.5M Na_2EDTA (pH 8.0); 10mM Tris.HCl (pH 8.5), sterilized at 121°C for 20 min.

20x SSC (Maniatis *et al.*, 1982): NaCl, 175.3g; trisodium citrate, 88.2g; pH to 7.0 with NaOH and volume to 1l with AnalaR water, sterilized at 121°C for 20 min.

10x Tris-Acetate buffer (TAE) (Maniatis *et al.*, 1982): Trizma base (Sigma Chemical Co.), 48.4g; glacial acetic acid (BDH), 11.42ml; 0.5M Na_2EDTA (pH 8.0), 20ml; volume to 1l with distilled water.

10x Tris-Borate buffer (TBE) (Maniatis *et al.*, 1982): Trizma base (Sigma Chemical Co.), 108g; boric acid (FSA), 55g; 0.5M Na_2EDTA, 40ml; volume to 1l with distilled water.

Tris-EDTA buffer (TE) (Maniatis *et al.*, 1982): 10mM Tris.HCl (pH 8.0); 1mM Na_2EDTA (pH 8.0), sterilized at 121°C for 20 min.

5.2.2 Preparation of DNA from *Lentinula edodes* and *Pleurotus* spp.

Extraction of Genomic DNA

DNA from *L. edodes* 30-1 and *P. sajor-caju* 32-2 was extracted using the "mini-prep" method of Raeder & Broda (1985), however, further purification was required before restriction digests could be carried out. Additional extraction with re-distilled phenol equilibrated in TE or ammonium acetate precipitation was required to remove residual

proteins in the DNA. Use of the commercial product Vitrogene (Calbio, Cambridge) based on the glass-milk method of Vogelstein & Gillespie (1979) either resulted in losses of DNA or failed to improve restriction digests.

Ammonium acetate precipitation was carried out by adding 5M NH_4Ac to a solution of DNA to give a final concentration of 2M. This was followed by 20 min incubation on ice, and then centrifugation at 13,000x *g* in an MSE microcentrifuge which resulted in the formation of a pellet of protein. DNA remaining in the supernatant was precipitated with 0.6 volumes ice cold isopropanol and centrifuged for 10 min at 4°C. The pellet of DNA was washed twice with 70% EtOH/ 30% TE (pH 8.0), allowed to air dry and resuspended in TE.

1µg DNA from both *L. edodes* 30-1 and *P. sajor-caju* 32-2 were digested with 10 units *Hind*III in buffer containing final concentrations 50mM NaCl, 50mM Tris, (pH 7.5), 10mM $MgCl_2$, at 37°C and incubated overnight.

Large gels for Southern Blotting were cast with 1x TAE containing 1% agarose (BRL Ultrapure electrophresis grade). 1µg of restricted DNA was loaded per lane and electrophoresed for at least 15 hours at 40V. Gels were stained for 20 min in EtBr solution ($1\mu g ml^{-1}$) with gentle shaking and washed twice with distilled water for 10 min each, prior to observing with shortwave U.V. light.

Isolation of Intact Chromosomal DNA

Protoplasts were isolated from *Pleurotus* spp. dikaryons (see Appendix C) following the method developed in Chapter 2. Chromosomal DNA was routinely prepared from protoplasts following a modified method of Orbach *et al.* (1988). Protoplasts stabilised in 0.6M sucrose, at a concentration of 0.5-1 x 10^9 per ml, were embedded in low melting point agarose to give a final concentration of 0.6% SeaPlaque low gelling temperature agarose (LGT) (FMC BioProducts Ltd.), 60mM Na_2EDTA (pH 8.0) and 0.6M sucrose, and allowed to set in the insert mould (BioRad Ltd.) on ice, for 10 min.

Protoplast lysis was carried out *in situ* by removing the plugs from the mould and transferring to sterile petri dishes containing NDS lysis buffer (prewarmed to 50°C, prior to gentle mixing) and, when used, Proteinase K (Sigma Chemical Co.) at a final concentration of $2mg.ml^{-1}$. Plugs were incubated overnight at 50°C and washed thoroughly with 50mM Na_2EDTA at 50°C, usually 5 washes over a period of 8h. Samples were stored in 0.5M Na_2EDTA (pH 8.0) at 4°C in 1oz plastic universals or 5ml plastic bijou bottles.

Samples prepared from lysed protoplasts during early experiments were found to have very little fragmented DNA present. However, degradation was found to be a constant problem during isolation from several species of *Pleurotus* in later experiments. Degradation appeared as a smear at the gel front after staining with ethidium bromide. In an attempt to reduce degradation, $CaCl_2$ was eliminated from the lytic mixture and the nuclease inhibitor aurintricarboxilylic acid (ATA, Aldrich) was added to reduce potential nuclease activity during protoplast release. When used, ATA was dissolved in sterile distilled water at a concentration of 100mM by warming in a water bath at 48°C prior to adding to the lytic enzyme mixture at a final concentration of 1mM. The lytic enzyme was then sterilized by centrifugation as described in Chapter 2. However, ATA was found to reduce protoplast yields considerably affecting the number of protoplasts harvested and use for sample preparation.

When used, ATA was found to reduce the level of degradation encountered (see Table 5.4). The appearance of degraded DNA was found to coincide with the use of one particular batch of Novozym 234 (PPM2934). However, this was absent when a different batch (PPM1961) was used (see Table 5.4).

5.2.3 Conditions for C.H.E.F. Analysis of DNA

14cm x 12.7cm gels (100ml) were cast in running buffer (0.5x TBE or 0.5x TAE), initially with BRL ultrapure agarose, however, chromosomal grade agarose (BioRad) was later adopted and prepared according to manufacturer's instructions. The agarose concentration used ranged, between runs, from 0.5% to 0.8%, but 0.7% was later adopted as

standard. CHEF-DR™II (Bio-Rad) equipment was used routinely in a cold room at 4°C and electrophoresis carried out at a constant 8°C.

During optimization of electrophoresis conditions, pulse times varied between runs usually involving "ramping" from long pulse times (from 3,000 to 4,800 seconds) to short pulse times (350 to 2,200 seconds). Exact conditions for each run are given in figure legends and in Table 5.6.

After electrophoresis, gels were stained in 200ml EtBr (2μg.ml^{-1}) for 30 min with gentle shaking at room temperature, then destained twice for 30 min each in 200ml distilled water prior to observing on a U.V. transilluminator.

5.2.4 Southern Blotting

Alkali blotting was carried out onto Hybond-N nylon filter (Amersham) according to the manufacturer's instructions. Solutions for blotting were made fresh on the day required. After transfer, filters were cross-linked to the nylon by exposing to shortwave U.V. light on a transilluminator for 5 min.

5.2.5 α^{32}P DNA Labelling

10-50ng DNA was labelled with [α-^{32}P]d-CTP (Amersham) using a nick translation kit (BRL) or random priming kit (Pharmacia LKB) according to manufacturer's instructions. Unincorporated nucleotides were removed by gel filtration through nick columns (Sephadex G-50, Pharmacia LKB). The purified probe was denatured by heating to 100°C for 2 min and cooled on ice prior to adding to the pre-hybridization solution.

Plasmids used for hybridization studies included: pBT6 (containing the *Neurospora crassa* ß-tubulin gene; Orbach *et al.*, 1986), pY1rG12 (1.8Kb *EcoR*I fragment containing a *S. cerevisiae* 18s rRNA gene; Petes *et al.*, 1978), pHDB102 (*A. nidulans* ornithine carbanmyl transferase gene, *argB*; courtesy of Delta Biotechnology, Nottingham), pYR7ΔR (*S. cerevisiae* N(5'-phosphoribosyl) anthranilate isomerase, *trp1*; courtesy of Dr. M.J. Dobson).

TABLE 5.4 Sample Preparation Methods for *Pleurotus* spp.

SPECIES/ STRAIN		SAMPLE PREPARATION				
		$CaCl_2$ USED?	ATA USED?	NOVOZYM 234 BATCH USED	FINAL PROTOPLAST CONCENTRATION[1]	DEGRADATION PRESENT[2]
P. columbinus	34-1	+	-	2934	8.5×10^7 (-)	n.d.
	34-1	+	-	2934	1.0×10^9 (+)	+
	34-1	-	-	2934	6.7×10^7 (-)	n.d.
	34-1	-	+	2934	3.1×10^8 (+)	-
	34-1	+	-	2934	4.0×10^8 (+)	-
P. cystidiosus	36-1	-	+	2934	8.1×10^8 (+)	-
	36-1	-	-	1961	3.1×10^9 (+)	-
P. florida	31-1	+	-	1961	1.8×10^8 (+)	-
	31-1	+	-	1961	4.3×10^8 (+)	-
	31-1	+	-	1961	5.7×10^8 (+)	+
	31-1	+	-	2934	1.9×10^9 (+)	+
	31-1	+	-	1961	5.0×10^8 (+)	-
	31-1	-	+	2934	8.5×10^8 (+)	-
	31-2	-	+	2934	1.0×10^8 (-)	n.d.
	31-2	+	-	1961	3.1×10^8 (+)	-
P. ostreatus	7-4	+	-	1961	3.2×10^8 (+)	-
	7-4	+	-	1961	6.3×10^8 (+)	±
	7-12	-	-	2934	7.8×10^7 (-)	n.d.
	7-12	-	-	2934	1.1×10^9 (+)	+
P. pulmonaris	33-1	+	-	1961	1.8×10^8 (-)	n.d.
	33-1	+	-	1961	3.5×10^8 (+)	+
	33-1	+	-	2934	2.5×10^8 (+)	+
	33-1	-	+	2934	3.3×10^8(+)	-
	33-3	-	-	2934	7.7×10^8 (+)	+
P. sajor-caju	32-1	+	-	1961	2.0×10^8 (+/-)	-
	32-2	+	-	1961	4.0×10^8 (+)	-
	32-2	+	-	1961	2.3×10^8 (+)	-
	32-2	+	-	1961	8.0×10^8(+)	-
	32-3	+	-	1961	3.0×10^8(+/-)	-
	32-4	+	-	1961	2.0×10^8(+/-)	-
P. sapidus	35-1	-	+	2934	3.2×10^8 (+)	±
	35-2	+	-	1961	4.0×10^8 (+)	±
Hybrid H_7		+	-	1961	1.2×10^8 (-)	n.d
		+	-	2934	1.3×10^9 (+)	+

1. As protoplasts per ml final concentration. DNA concentrations sufficient (+) or too low (-) to be visualized on a gel.
2. + = present; - = absent; n.d.= not determined as DNA concentration too low.

5.2.6 Hybridization of Heterologous Probes to ^{32}P-Labelled DNA

Filters were pre-hybridized for 2-4 hours in 25ml 6x SSC, 5x Denhardt's, 0.5% SDS according to manufacturer's instructions. Denatured α-^{32}P labelled DNA probe was added to the pre-hybridization solution and incubated on a shaking platform in a heated water bath. Hybridization was carried out between 48°C to 65°C overnight, depending on the probe being used.

Filters were washed twice with 2x SSC for 15 min followed by a final wash with 2x SSC and 0.1% SDS at 48-65°C and sealed in plastic bags. Autoradiography was carried out using Fuji RX or Amersham Hyperfilm-MP x-ray film with intensifying screens in a Kodak X-ray cassette. Filters were developed after 2-14 days exposure at -70°C.

5.3 Results

5.3.1 DNA Extraction

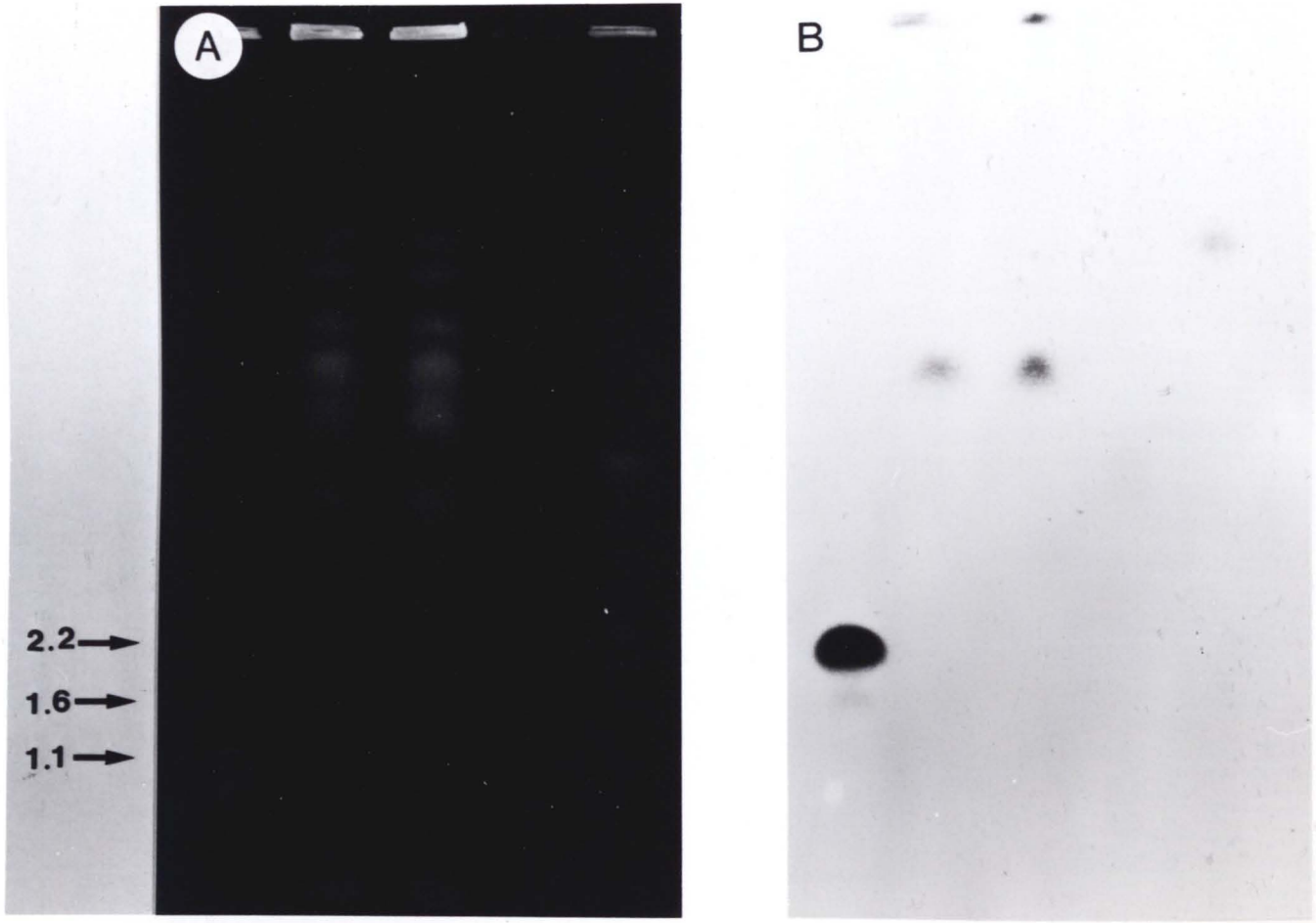
This was found to be the most important step for PFG investigations. Problems of degradation were overcome by one of two means: changing the batch of Novozym 234 used for protoplast release, or incorporation of a nuclease inhibitor (ATA) during lysis. ATA was found to reduce the appearance of degraded DNA, although the yields of protoplasts obtained were reduced. Proteinase K treatment was eliminated from sample preparation as it was found to have no effect on DNA migration nor hybridization pattern with samples of *A. nidulans* (Fig. 5.2), and had a considerable effect on reducing the expense of sample preparation. Optimum protoplast concentrations were determined to be above 1 x 10^8 protoplasts per ml, and ideally between 0.5-1 x 10^9 protoplasts per ml.

Figure 5.2 CHEF Gel Electrophoresis and Southern Blot Hybridization

A. CHEF gel electrophoresis carried out with a pulse time ramp from 3000-900secs over 160hrs, at 50V, in 0.7% agarose and 0.5 x TAE. Lane 1, *S. cerevisiae*; 2, *A. nidulans* 2-124 (not treated with proteinase K); 3, *A. nidulans* 2-124 (treated with proteinase K); 4, *Penicillium chrysogenum*; 5, *P. sajor-caju* 32-2.

B. Southern blot of chromosomal DNA hybridized with [^{32}Pα]dCTP oligolabelled 1.8Kb fragment from pY1rG12 (yeast 18s rRNA gene).

Sizes of yeast standards are given in megabases.


A
B
2.2
1.6
1.1

Attempts to isolate genomic DNA from *L. edodes* 30-1 revealed an artefact present in the extracts when large amounts (100-200ng) of undigested DNA were loaded onto mini-gels. Two high-molecular weight species were found, with sizes greater than the 50Kb lambda DNA standard, with the heavier of the two found to be present in smaller quantities. The nature of these artefacts was investigated by digestion with DNAse free RNAase (RNAase A) or RNAase free DNAse (DNAase 1) for four hours. Digestion with RNAase A did cause the disappearance of the two bands, whereas the treatment with the DNAase 1 did not.

5.3.2 Separation of Megabase DNA From *Pleurotus* spp.

To establish optimum conditions for separation of *Pleurotus* spp. samples were electrophoresed using conditions for the separation of the molecular weight markers. *S. cerevisiae* (0.245-2.2Mb) and *Sch. pombe* (3.5-5.7Mb), both from BioRad Ltd., or lambda DNA concatamers (0.05-1.2Mb, Pharmacia LKB). These early experiments quickly established the pulse time range which was required for chromosome separation of *P. sajor-caju* 32-2. Chromosomal DNA from *P. pulmonaris*, *P. florida*, and *P. ostreatus* were found to migrate between 1.6Mb to 5.7Mb as determined by the molecular weight standards (Figs. 5.8A & 5.9). Increasing the voltage was found to result in further migration through the gel with no corresponding increase in resolution (gel no. 14; not presented).

Increasing the agarose concentration to 0.7% resulted in resolution of up to 7 bands, representing possibly 8 chromosomes (one doublet based on fluorescence intensity) with *P. sajor-caju* 32-2 (gel no. 6; Fig. 5.4). Steeper ramps were used to try to obtain greater resolution of the smaller chromosomes of *P. sajor-caju* 32-2, however, despite greater separation only six bands were visible (gel no. 7; Fig. 5.4). In comparison eight bands were observed for the steepest ramp used (gel no. 8; Fig. 5.4).

Increasing the agarose to 0.8% gave no improvement (gel no. 4; Fig. 5.3) and lower concentrations (i.e. 0.5%) with a higher voltage resulted in smearing at the end of the gel, indicating that samples had migrated too far (gel no. 15; not presented).

A steep ramp gave four distinct bands for *P. ostreatus*, and conditions for gel no. 6 were modified using a slightly lower voltage to separate chromosomes from *P. ostreatus* 7-4 (gel no. 16; Fig. 5.8A). This resulted in 10 bands with a lack of resolution in the lower molecular weight range.

The buffer used routinely for all runs was found to precipitate in the electrophoresis chamber which was found to be awkward for cleaning. At this time conditions for separation of *Aspergillus nidulans* chromosomes were published which used 0.5x Tris-acetate buffer, TAE (Carbon & Brody, 1989). This was used instead of the previously used tris-borate buffer (TBE) in a repeat of the conditions used for gel no. 7. Samples of *P. sajor-caju* were used for comparison resulting in no increase in the number of bands observed, although migration was further down the gel and separation of the bands was found to be increased slightly (gel no. 9). Lower voltages resulted in less separation between the bands (gel no. 10).

Longer pulse times and lower voltages were used in an attempt to resolve the two largest *P. sajor-caju* chromosomes (gel no. 13). Also, smaller chromosomes of *P. sajor-caju* were separated to a limited degree using optimum conditions for separation of *Candida albicans* (BioRad CHEF-DR^{II} manual) (gel no. 2).

After establishing optimum agarose concentrations, buffer, voltage and pulse ramp conditions, the effect of the run time was tested. An increased run time of 210 hours was found to be much too long for the separation of *P. sajor-caju* and *A. nidulans* (gel no. 19). However, run times of 160 hours (gels no. 12, 18) or 138 hours (gel no. 11) gave good separation for these two species. Shorter run times of 113 hours were finally found to be very convenient and gave extremely good resolution of several *Pleurotus* spp. including, *P. columbinus*, *P. cystidiosis*, *P. florida*, *P. sajor-caju*, *and P. sapidus* (gel nos. 20-22).

5.3.3 Hybridization of Heterologous Probes

Strips cut from Southern blots of digested DNA were used to select for DNA probes which showed hybridization to both *L. edodes* and *P. sajor-caju*, and to determine optimum conditions for hybridization. A summary of the conditions used for hybridization and the results obtained are given in Table 5.5. The ß-tubulin gene of *N. crassa* (pBT6) and the yeast 18s rRNA (1.8Kb *EcoRI* fragment from pY1rG12) gene were found to hybridize strongly (see Fig. 5.6). However, the *arg*B (pHOB102) and the *trp*1 (pYR7ΔR) genes failed to hybridize to *P. sajor-caju* DNA.

TABLE 5.5 Conditions used for Hybridization of Heterologous DNA Probes to Restricted DNA from *Lentinula edodes* 30-1 and *Pleurotus sajor-caju* 32-2

DNA PROBE	HYBRIDIZATION TEMPERATURE	WASH TEMPERATURE	HYBRIDIZATION TO GENOMIC DNA[1]	
			L. edodes	*P. sajor-caju*
pBT6	48°C	48°C	+	+
pHOB102	48°C	56°C	+	-
pY1rG12	57°C	65°C	+	+
pY1rG12	65°C	65°C	+	+
pYR7ΔR	48°C	48°C	-	-

1. += homology detected; -= nonhomologous.

TABLE 5.6 Conditions Employed for Chromosome Separation of *Pleurotus* spp. with CHEF

GEL NO.	AGAROSE CONCN.	BUFFER	VOLTAGE	PULSE TIMES (SECONDS)	TOTAL RUN TIME (HOURS)	FIGURE NUMBER
1	0.8%	0.5x TBE	150V	180 (12 h) 120 (24 h)	36	5.3
2	0.8%	0.5x TAE	150V	120 (24 h) 180 (12 h)	36	5.3
3	0.5%	0.5x TBE	50V	3600-350 (r)	156	5.3
4	0.8%	0.5x TBE	50V	3,600 to 900 (r)	156	5.3
5	0.7%	0.5x TBE	45V	3,000 to 2,200 (r)[1]	156	5.4
6	0.7%	0.5x TBE	50V	3,000 to 2,200 (r)	156	5.4
7	0.7%	0.5x TBE	50V	3,000 to 900 (r)	156	5.4
8	0.7%	0.5x TBE	50V	3,600 to 350 (r)	156	5.4
9	0.7%	0.5x TAE	50V	3,000 to 900 (r)	156	5.5
10	0.7%	0.5x TAE	35V	3,000 to 900 (r)	156	5.5
11	0.7%	0.5x TAE	50V	3,000 to 900 (r)	138	5.5, 5.8B
12	0.7%	0.5x TAE	50V	3,000 to 900 (r)	160	5.5, 5.7A
13	0.7%	0.5x TAE	35V	4,800 to 2,000 (r)	168	5.5
14	0.6%	0.5x TBE	55V	3,000 to 2,200 (r)	156	N.P
15	0.5%	0.5x TBE	100V	3,600 to 900 (r)	66	N.P.
16	0.7%	0.5x TBE	50V	3,000 to 900 (r)	156	5.8A
17	0.6%	0.5x TBE	45V	3,000 to 2,000 (r)	156	5.9B
18	0.7%	0.5x TBE	50V	3,000 to 900 (r)	160	5.10A
19	0.7%	0.5x TAE	50V	4,800 to 2,200 (r)	210	N.P.
20	0.7%	0.5x TAE	50V	3,000 to 1,300 (r)	113	5.7B
21	0.7%	0.5x TAE	50V	3,000 to 1,300 (r)	113	5.9A
22	0.7%	0.5x TAE	50V	3,000 to 1,300 (r)	113	5.10B

1. (r)= Ramped run.
N.P.= Gel not presented.

Figure 5.3 CHEF Gel Electrophoresis of *Pleurotus sajor-caju* 32-2.

Gel 1: lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. sajor-caju* 32-2.

Gel 2: lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. sajor-caju* 32-2.

Gel 3: lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. sajor-caju* 32-2.

Gel 4: lane 1, *S. cerevisiae*; 2, *Sch. pombe*; 3, *P. sajor-caju* 32-2.

VARIABLE FACTORS	GEL NUMBER			
	1	2	3	4
VOLTAGE (V):	150	150	50	50
PULSE (s):	180 (12hrs) 120 (24hrs)	120 (24hrs) 180 (12hrs)	3600-350 ramped	3600-900 ramped
RUN TIME (hrs):	36	36	156	156
BUFFER:	0.5x TBE	0.5x TAE	0.5x TBE	0.5x TBE
AGAROSE:	0.8%	0.8%	0.5%	0.8%

RESULT:

Figure 5.4 CHEF Gel Electrophoresis of *Pleurotus sajor-caju* 32-2.

Gel 5: lane 1, *Sch. pombe*; 2, *P. sajor-caju* 32-2.

Gel 6: lane 1, *S. cerevisiae*; 2, *Sch. pombe*; 3, *P. sajor-caju* 32-2.

Gel 7: lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. sajor-caju* 32-2 (sample 1); 4, *P. sajor-caju* 32-2 (sample 2).

Gel 8: lane 1, *S. cerevisiae*; 2, *Sch. pombe*; 3, *P. sajor-caju* 32-2.

VARIABLE FACTORS	GEL NUMBER			
	5	6	7	8
VOLTAGE (V):	45	50	50	50
PULSE (s):	3000-2200 ramped	3000-2200 ramped	3000-900 ramped	3600-900 ramped
RUN TIME (hrs):	156	156	156	156
BUFFER:	0.5x TBE	0.5x TBE	0.5x TBE	0.5x TBE
AGAROSE:	0.7%	0.7%	0.7%	0.7%

RESULT:

Figure 5.5 CHEF Gel Electrophoresis of *Pleurotus sajor-caju* 32-2.

Gel 9: lane 1, *Sch. pombe*; 2, *P. sajor-caju* 32-2.

Gel 10: lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. sajor-caju* 32-2.

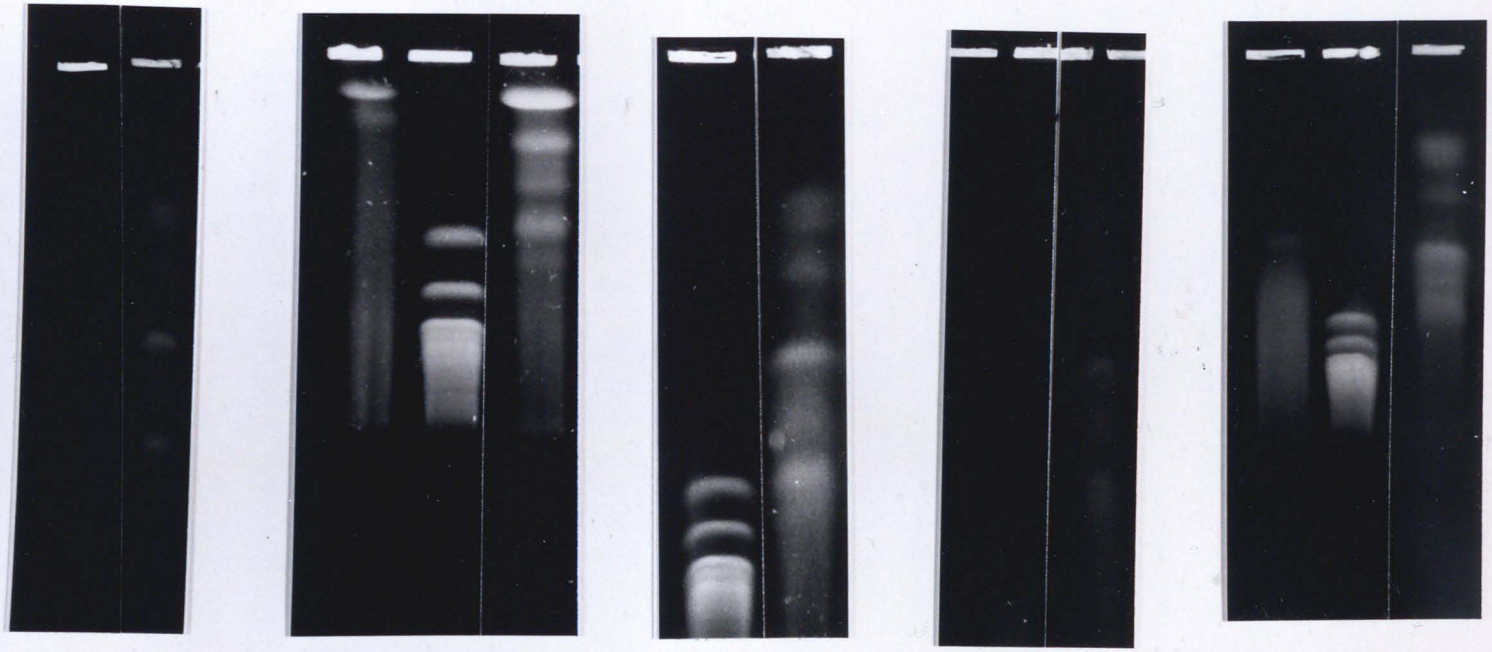
Gel 11: lane 1, *S. cerevisiae*; 2, *P. sajor-caju* 32-2.

Gel 12: lane 1, *S. cerevisiae*; 2, *P. sajor-caju* 32-2.

Gel 13: lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. sajor-caju* 32-2.

VARIABLE FACTORS	GEL NUMBER				
	9	10	11	12	13
VOLTAGE (V):	50	35	50	50	35
PULSE (s):	3000-900 ramped	3000-900 ramped	3000-900 ramped	3000-900 ramped	4800-2200 ramped
RUN TIME (hrs):	156	156	138	160	168
BUFFER:	0.5x TAE	0.5x TAE	0.5x TAE	0.5x TAE	0.5x TAE
AGAROSE:	0.7%	0.7%	0.7%	0.7%	0.7%

RESULT:



5.3.4 Molecular Karyotype Analysis of *Pleurotus* spp.

Banding patterns obtained from several *Pleurotus* spp. by PFE were used to estimate chromosome numbers and sizes. The results obtained from several gels are presented in Figs. 5.7 -5.10 and summarized in Table 5.7. Sizes were calculated by plotting DNA size vs. distance migrated of the yeast size standards, and numbered in order of decreasing size. By using migration distance of the bands obtained size could thus be extrapolated (see Fig. 5.11). When one of the yeast standards was not present on the gel (usually *S. pombe*, as the supplier was unable to provide it for several months) size was estimated by use of previously estimated chromosome sizes of other species.

TABLE 5.7 Chromosome Numbers and Estimated Sizes for Six Species of *Pleurotus* as Determined by CHEF Electrophoresis

SPECIES/ STRAIN	BAND NUMBER AND ESTIMATED SIZE (Mb) 1	2	3	4	5	6	7	8	9	10	GENOME SIZE (Mb)
P. colombinus											
34-1	3.8	3.4	3.05	2.7	2.3	1.8					17.05
P. cystidiosus											
36-1	4.5	3.9	3.4	3	2.7	2.5					20.00
P. florida											
32-1	4.2	3.65	3.4	2.95	2.05	1.7	1.1				19.05
32-2	4.2	3.65	3.4	2.95	2.05	1.7	1.1				19.05
P. ostreatus											
7-4	>6	4.19	3.8	3.4	3.25	3	2.75	2.6	2.49	2.29	>39.49
P. pulmonaris											
33-1	5.5	3.9	3.2	2.7	2.3						17.6
P. sajor-caju											
32-1	5	4.05	3.1	2.9	2.75	2.5					20.3
32-2	>6	4.75*	4.19	3.5*	3.2	2.75	2.35				>34.99
32-3	>6	4	3	2.8	2.4						>18.20
32-4	4.6	4.1	3.5	3.2	2.9	2.7	2.5	2.4			25.9
P. sapidus											
35-1	>5	4.8	4.4	4	3.8	3.35	>1.7	1.7			>28.75

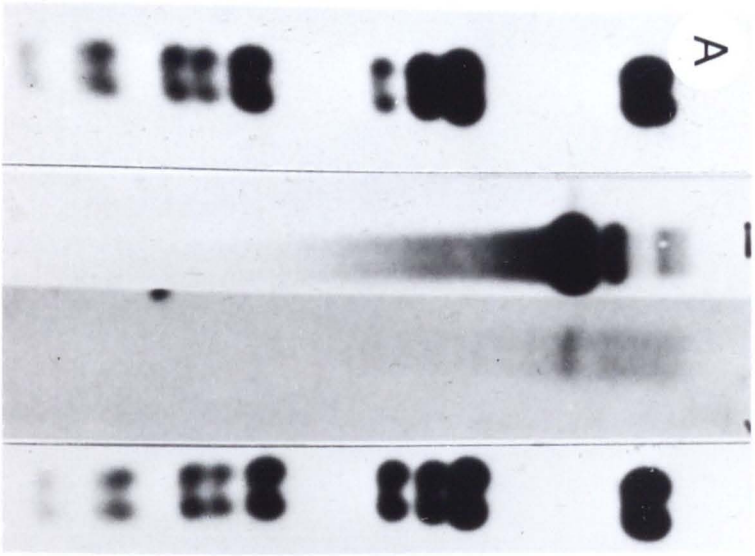
*Possible doublet as estimated from flouresence intensity.

Figure 5.6 Southern Blot Hybridizations of *Lentinula edodes* and *Pleurotus sajor-caju* DNA

A. *P. sajor-caju* 32-2: lanes 1 and 4 lambda DNA restricted with *Eco*RI and *Hin*dIII; 2 and 3, *Hin*dII restricted genomic DNA.

B. *L. edodes* 30-1: lane 1, lambda DNA restricted with *Eco*RI and *Hin*dIII; 2 to 4, *Hin*dIII restricted genomic DNA.

See text for hybridization conditions and details of DNA probes used.


A
λEcoRI/Hind III
pY1rG12
pBT6
λEcoRI/Hind III

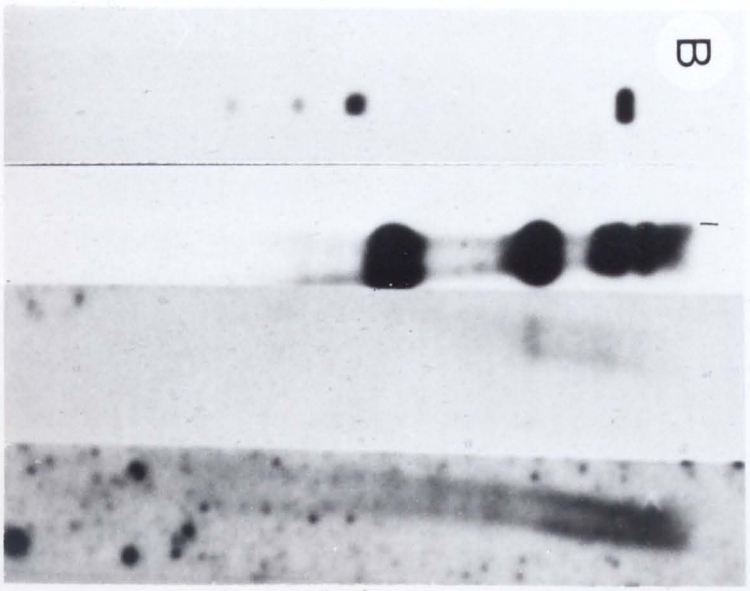
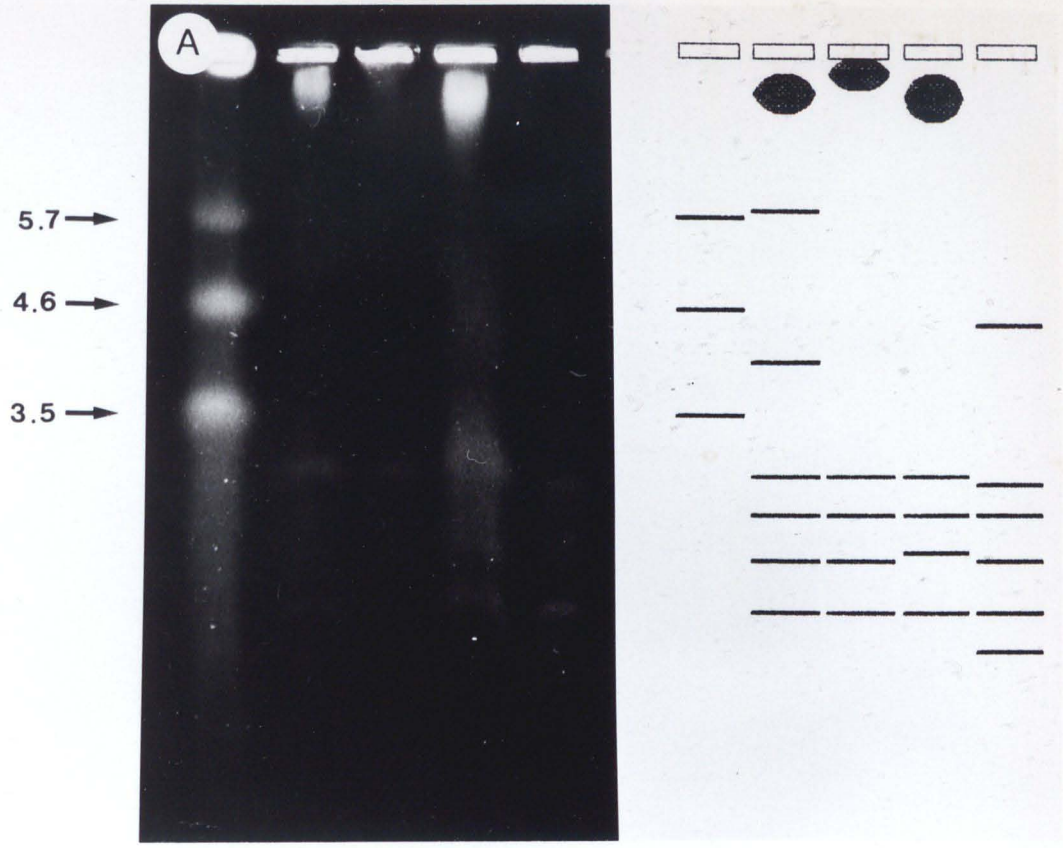

B
λEcoRI/Hind III
pY1rG12
pBT6
pHOB102

Figure 5.7 Molecular Karyotype Analysis of *Pleurotus sajor-caju* Strains

A. Lane 1, *Sch. pombe*; 2, *P. sajor-caju* 32-1; 3, *P. sajor-caju* 32-2; 4, *P. sajor-caju* 32-3; 5, *P. sajor-caju* 32-4. Electrophoresis carried out for 160 h with a pulse-time ramp of 3000-900secs, at 50V, in 0.7% agarose and 0.5x TAE.

B. Lane 1, *Sch. pombe*; 2, *P. sajor-caju* 32-1; 3, *P. sajor-caju* 32-2; 4, *P. sajor-caju* 32-3; 5, *P. sajor-caju* 32-4. Electrophoresis carried out for 113 h with a pulse-time ramp of 3000-1300secs, at 50V, in 0.7% agarose and 0.5x TAE.

Sizes of yeast standards are given in megabases. Diagramatic representation of banding patterns is given on the left.


A
5.7
4.6
3.5

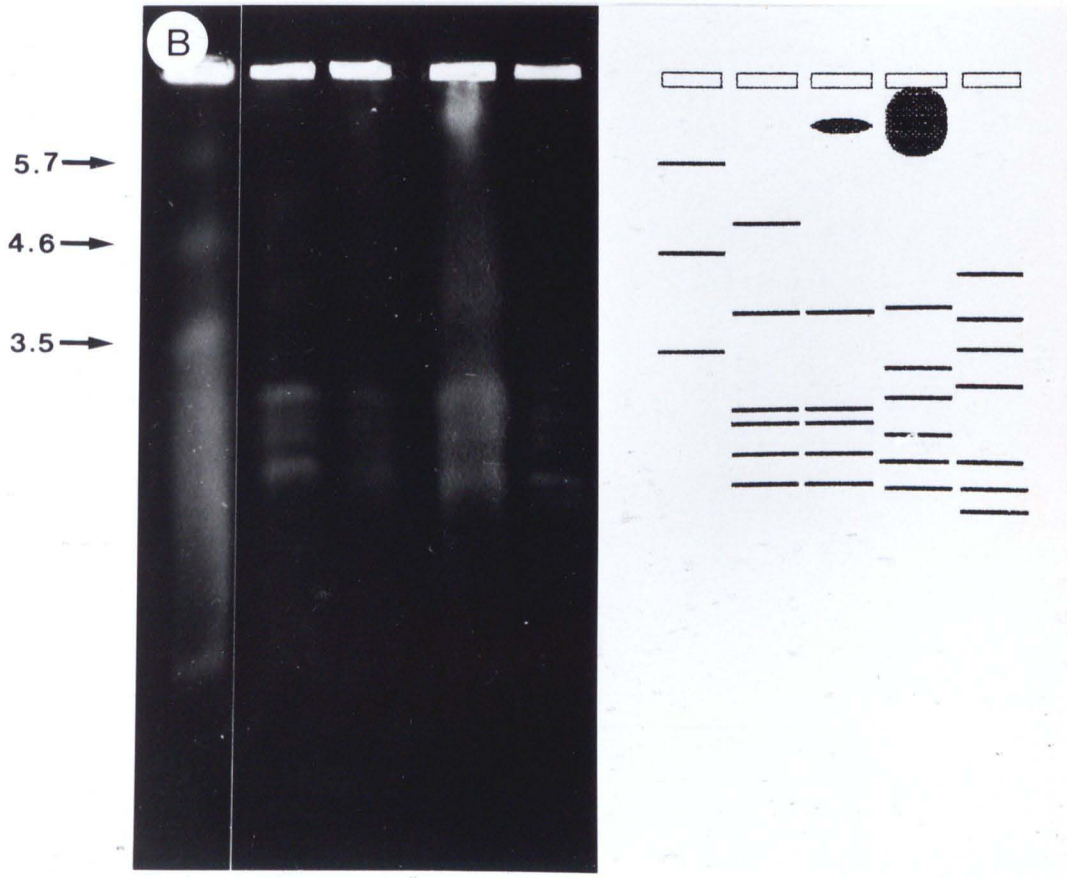
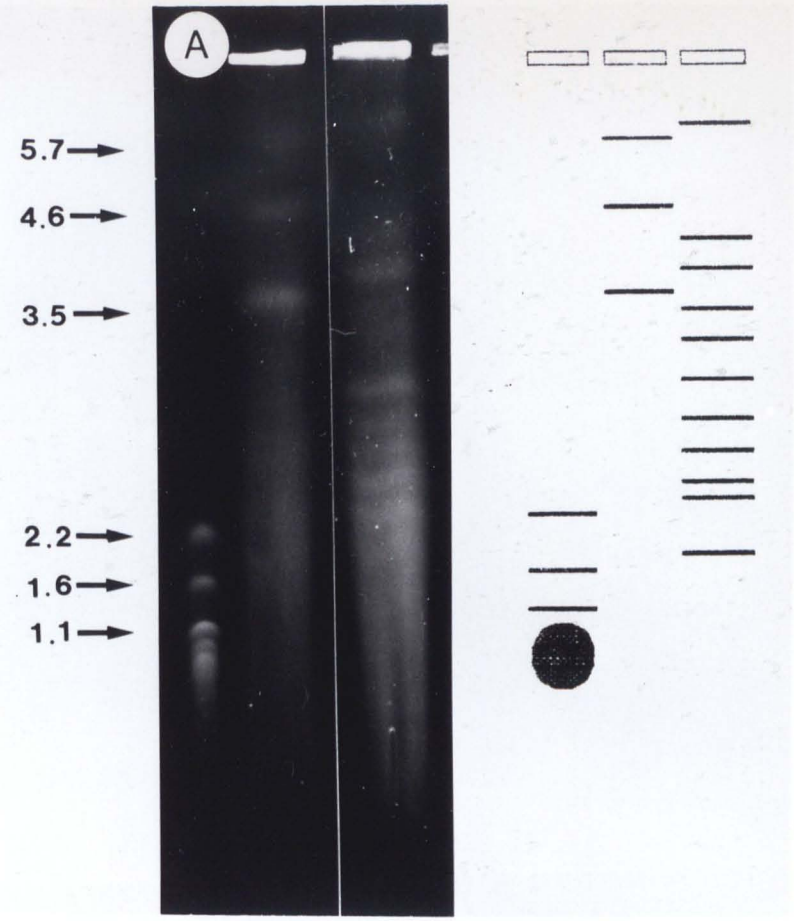

B
5.7
4.6
3.5

Figure 5.8 Molecular Karyotype Analysis of *Pleurotus colombinus* and *Pleurotus ostreatus*

A. Lane 1, *S. cerevisiae*; 2, *Sch. pombe*; 3, *P. ostreatus* 7-4. Electrophoresis carried out for 156 h with a pulse-time ramp of 3000-900secs, at 50V, in 0.7% agarose and 0.5x TBE.

B. Lane 1, *S. cerevisiae*; 2, *P. colombinus* 34-1. Electrophoresis carried out for 138 h with a pulse-time ramp of 3000-900secs, at 50V, in 0.7% agarose and 0.5x TAE.

Sizes of yeast standards are given in megabases. Diagramatic representation of banding patterns is given on the left.


A
5.7
4.6
3.5
2.2
1.6
1.1

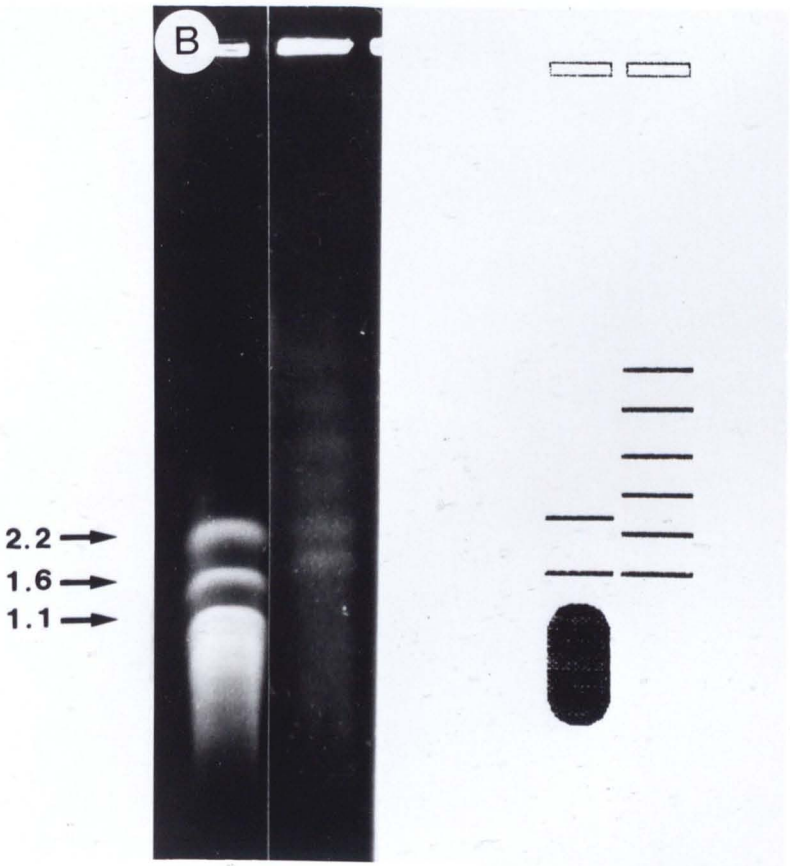
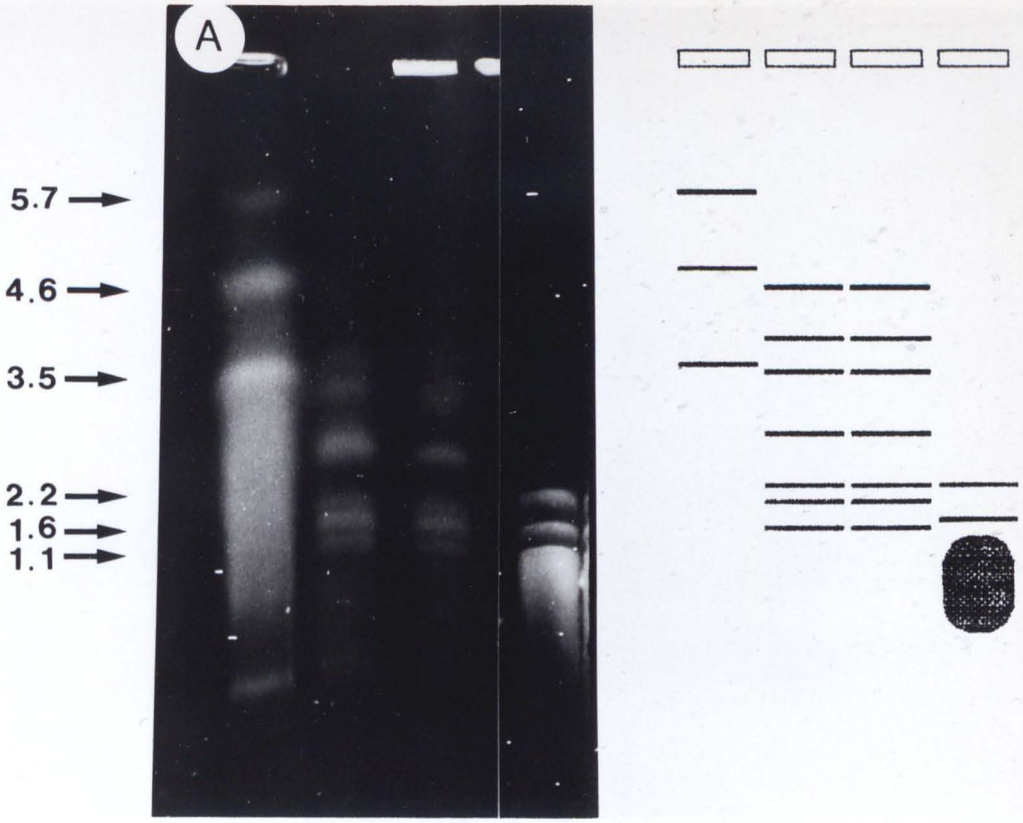

B
2.2
1.6
1.1

Figure 5.9 Molecular Karyotype Analysis of *Pleurotus florida* and *Pleurotus pulmonaris*

A. Lane 1, *Sch. pombe*; 2, *P. florida* 31-1; 3, *P. florida* 31-2; 4, *S. cerevisiae*. Electrophoresis carried out for 113 h with a pulse-time ramp of 3000-1300secs, at 50V, in 0.7% agarose and 0.5x TAE.

B. Lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *P. pulmonaris* 33-1. Electrophoresis carried out for 156 h with a pulse-time ramp of 3000-2200secs, at 45V, in 0.6% agarose and 0.5x TBE.


A
5.7
4.6
3.5
2.2
1.6
1.1

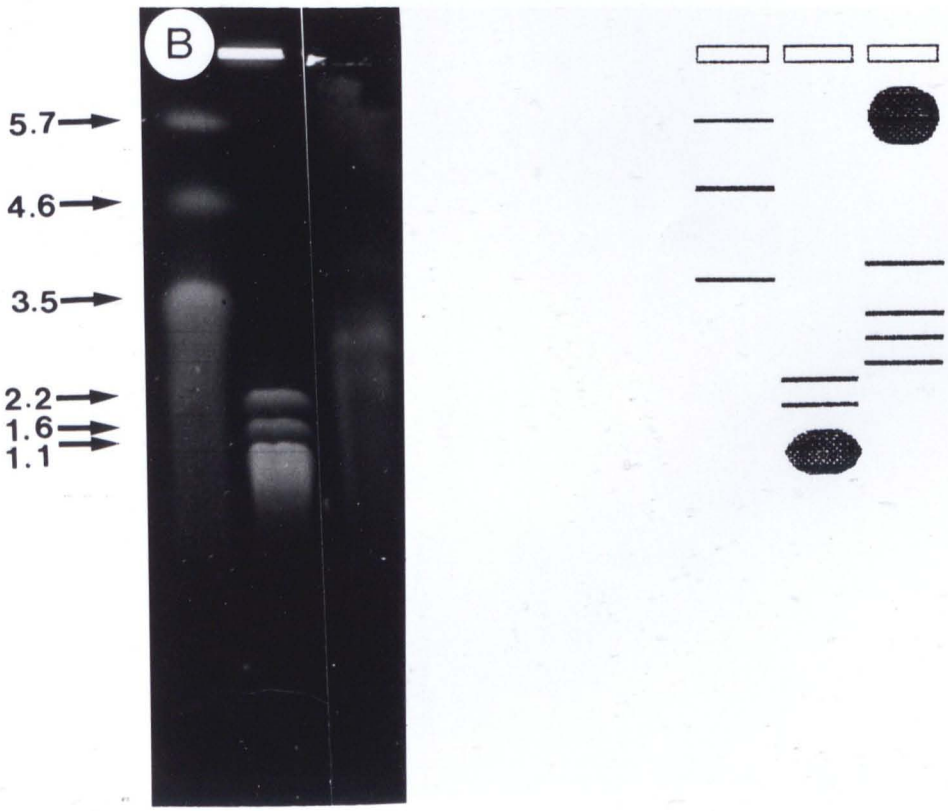
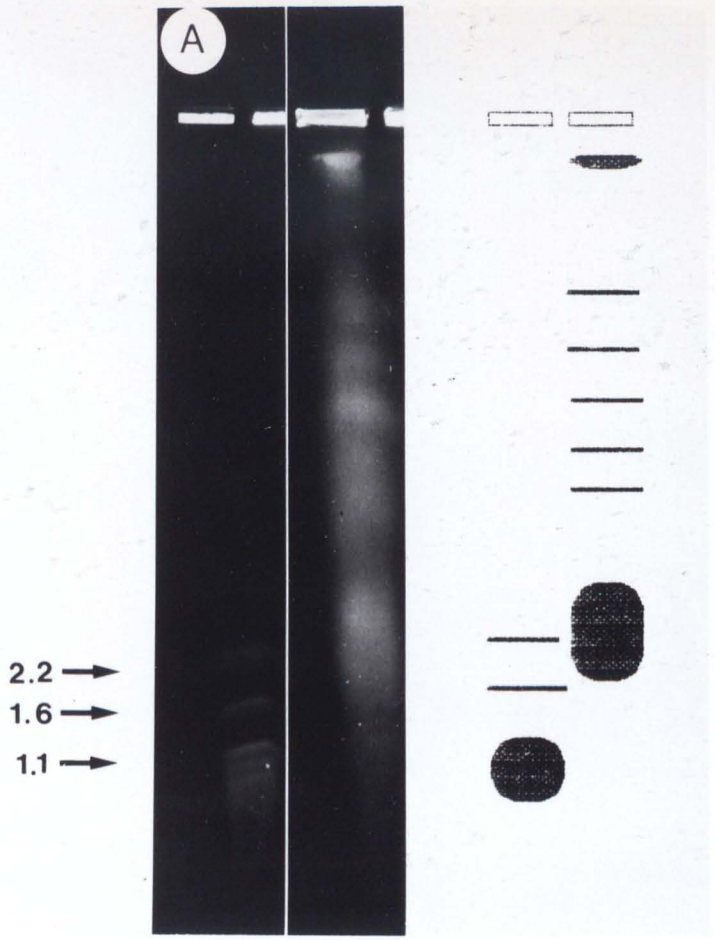

B
5.7
4.6
3.5
2.2
1.6
1.1

Figure 5.10 Molecular Karyotype Analysis of *Pleurotus cystidiosis* and *Pleurotus sapidus*

A. Lane 1, *Sch. pombe*; 2, *P. sapidus* 35-1. Electrophoresis carried out at 160 h with a pulse time ramp of 3000-900secs, at 50V, in 0.7% agarose and 0.5x TBE.

B. Lane 1, *Sch. pombe*; 2, *P. cystidiosis* 36-1. Electrophoresis carried out at 113 h with a pulse time ramp of 3000-1300secs, at 50V, in 0.7% agarose and 0.5x TAE.

Sizes of yeast standards are given in megabases. Diagramatic representation of banding patterns is given on the left.


A
2.2
1.6
1.1

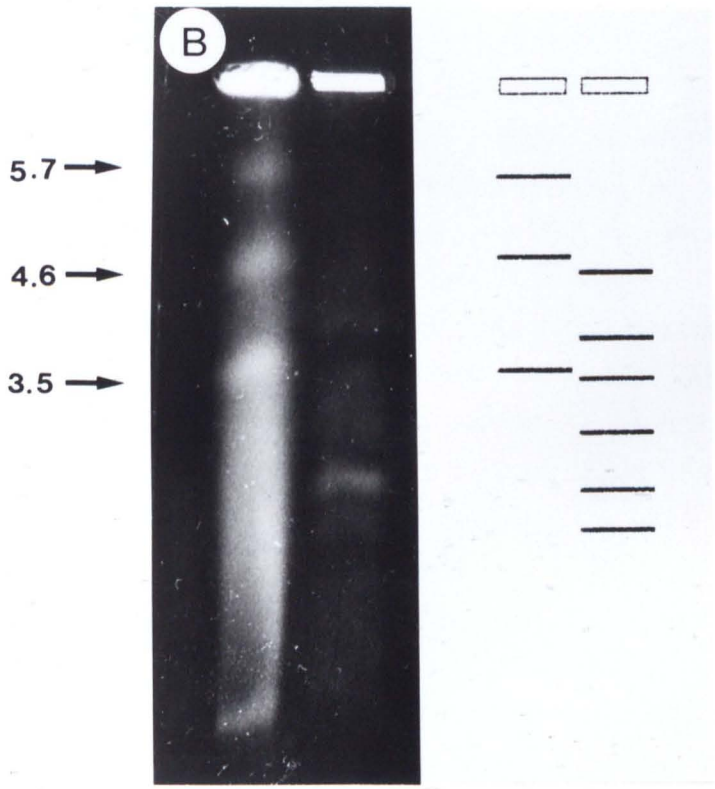
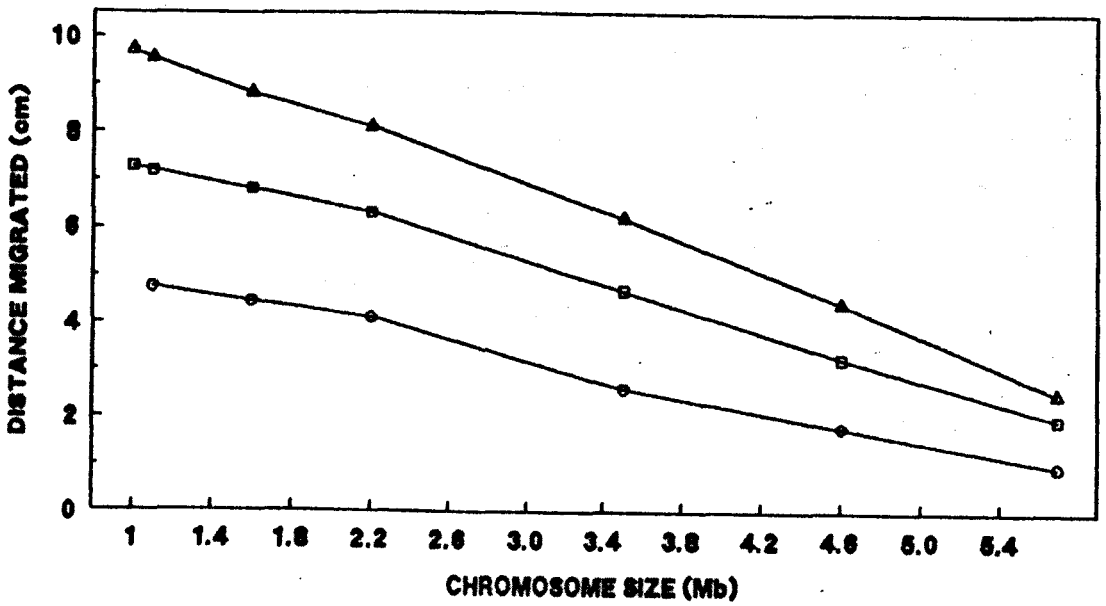

B
5.7
4.6
3.5

Fig. 5.11 Migration of Yeast Size Standards During CHEF Gel Electrophoresis



Key: —⊖— = 4,800-2,200sec pulse time ramped over 168 h, at 35V, in 07% agarose and 0.5 x TAE; —⊟— = 3,000-1,300sec pulse time ramped over 113 h, at 50V, in 0.7% agarose and 0.5 x TAE; —△— = 3,000-900 sec pulse time ramped over 160 h, at 50V, in 0.7% agarose and 0.5 x TAE.

6.4 Discussion

The argument for use of monokaryotic isolates during PFE analysis is strong and the main criticism of this study would be the use of dikaryons for these investigations. Chromosome length polymorphisms (CLPS) between component nuclei of a dikaryon would confuse estimates of chromosome numbers between strains and species being studied. Pukkila (1990) and Horgen (pers. comm.) have both found evidence for extensive CLPS in *Coprinus cinereus* and *Agaricus bisporus* respectively. However, the number of chromosomes separated for the various *Pleurotus* species by CHEF was in close agreement to previous reports determined by other methods. Haploid chromosome numbers of *P. cystidiosus* and *P. ostreatus* have been estimated as n=6 and n=8 to 10 respectively (Bresinsky *et al.*, 1987) and both *P. florida* and *P. sapidus* have been shown to have at least six linkage groups each. In addition, no variation was found for the two *P. florida* strains although CLPS were present for the four *P. sajor-caju* isolates investigated. These arguments make it difficult to validate estimates of chromosome numbers, and appreciation of the importance in the use of monokaryons should not be overlooked in future investigations.

Size estimation of the bands obtained was difficult because of the lack of accurate size standards in the range 2.2-3.7Mb. Migration of the standards was not perfectly linear with molecular weight. Different zones of resolution are known to develop during PFG (Vollrath & Davis, 1987; Cantor *et al.*, 1988b) with zones of compression most obvious for the *S. cerevisiae* chromosomes from 0.245-1.125Mb for the conditions used in this study. The extent of compression at higher molecular weights is difficult to estimate, again because of the lack of accurate size standards. For this reason considerable attention was given to separation of chromosomes of *Acremonium chrysogenum* and *Candida albicans* species which have been previously characterized by PFG and found to have chromosome sizes in the range of the *Pleurotus* spp. studied here. It was difficult to use these species as size determination is again dependent on the use of known standards and the *A. chrysogenum* strain used was found to differ in size from previously published results (Smith *et al.*, 1991). Limited resolution of the *C. albicans* DNA also restricted its use.

Conditions used for band separation were established by altering pulse time and run time after agarose concentration, run temperature and buffer had been established. It was often difficult to achieve optimum separation for particular isolates if attempts to separate other fungal species were being made concurrently. DNA was prepared for CHEF analysis by adapting previously established methods (Orbach *et al.*, 1988), however, proteinase K treatment was eliminated from the lysis solution. This was found to have no effect on migration of *P. sajor-caju* nor *Aspergillus nidulans* chromosomes. In addition, this had no effect on the hybridization pattern obtained from the radiolabelled 18s rDNA gene to *A. nidulans* DNA. Protein present in the samples was most likely removed by the sodium dodecyl sulphate present in the lysis solution. Jackson & Cook (1985) demonstrated that up to 99% of protein and 72% of RNA was removed from cells embedded in agarose beads and subsequently treated with lithium dodecyl sulphate.

DNA degradation was found to be the greatest set back in this study, and so consideration should be give to its occurrence. Nuclease

activity is prevented after protoplast isolation by the use of high concentrations of Na_2EDTA, and so it was assumed degradation occurred during mycelial digestion or by physical shearing during the embedding procedure. This later point seemed unlikely as intact chromosomal DNA was readily obtained by the author from *Pleurotus* and *Aspergillus* spp. (see Appendix H) during the early part of this study. Nuclease was either released from intracellular location(s) or entered the cells from an external source, namely the lytic enzyme. Nucleases have been shown to be located in mitochrondria, vacoules and to a lesser extent in the nucleus (Fraser & Cohen, 1983; von Tigerstrom & Stelmaschuk, 1985).

DNA degradation has been mentioned in other reports on basidiomycete species, however, no further discussion was given (Ullrich *et al.*, 1985; de Jonge *et al.*, 1986). Howlett (1989) reported DNA degradation was prevented during sample preparation of *Phytophthora megasperma* if Na_2EDTA was used with calcium chloride in the lytic solution used for protoplast isolation. Jackson & Cook (1985) also found that intact chromatin was "nicked" by high concentrations of Mg^{2+} ions. However, neither calcium chloride nor the relatively high concentrations of Mg^{2+} used during protoplast isolation were thought to be the cause of the degradation. Samples prepared earlier on in the study had little or no degradation, and it was only after the exhaustion of one particular batch of Novozym 234 that problems began. Genomic DNA obtained from protoplasts of *Agrocybe aegerita* was found to be sheared and was suggested as resulting from nuclease action of Novozym 234 used for mycelial digestion (Noel & Labarere, 1989).

Degradation was prevented by the addition of ATA, which had previously been shown to increase transformation frequency of *Penicillium chrysogenum* by 400-fold (Ramon *et al.*, 1986). ATA was shown to enter protoplasts of *P. chrysogenum* and *S. cerevisiae* and inhibit both endogenous and exogenous sources of DNAases. *P. chrysogenum* was found to have greater internal nuclease activity than *S. cerevisiae*, although different lytic enzymes were used for protoplast isolation, Novozym 234 and cellulase CP being used for *P. chrysogenum* and Glusulase used for *S. cerevisiae*, which may have contributed to the effect. ATA is a strong inhibitor of nucleic acid

binding proteins and its mechanism has been characterized (Blumenthal & Landers, 1973; González *et al.*, 1980). ATA was found to reduce protoplast yields (results not presented) and may have other activities resulting in the reduced action of lytic enzymes or decreased protoplast stability, although the former was more likely as the extent of mycelial digestion was noticeably reduced.

Continuing the discussion on sample preparation, DNA levels were found to be related to the protoplast concentration in the sample plugs. The number and intensity of bands present in *P. sajor-caju* 32-2 samples was found to be reduced if the DNA content was low. Protoplasts from *P. sajor-caju* 32-2 had previously been investigated for nuclear content and found to have up to 70% anucleate protoplasts (see Chapter 2). This low proportion of nulceated protoplasts severely limited studies as efforts focussed on maximizing yields.

In general, difficulties in classifying this genus arise because of conflicting reports on strain compatibility. The use of PFG as a taxonomic aid relies on obtaining distinguishable and reproducible banding patterns for each species. Although straight forward in principle complications as described above hindered progress. The *P. ostreatus* complex appears to be the most variable species studied in this genus. Studies on isozyme variability by May & Royse (1988) and Kulkarni *et al.* (1987) and the breeding relationships with other species (Anderson *et al.*, 1973; Rajaratham & Bano, 1987; May *et al.*, 1988; Magae *et al.*, 1990) demonstrate how variable this species is. The taxonomic significance of karyological studies is limited in view of the relatively few species and strains studied here, although the foundations for further investigations have been laid. Several species had chromosomes similar in size , e.g. 3.4Mb band which was present in all of the species studied except *P. sapidus* and *P. pulmonaris*, but it is impossible to say they are homologous without the use of radiolabelled probes.

As mentioned previously, CLPS can be attributed to deletion, translocation, duplication and insertion of DNA into a chromosome causing a change in migration during electrophoresis (Ono & Ishino-Arao, 1988). Masel *et al.* (1990) have proposed that extensive CLPS

may add to genetic variation within a population and allow it to adapt more readily. CLPS in *Plasmodium falciparum* are due to gene deletions (Cocoran *et al.*, 1986) and gene duplication and transposition have been shown to occur in *Trypanosoma brucei* (van der Ploeg *et al.*, 1984). CLPS have been found in a number of species including strains and oidial siblings of *C. cinereus* and were found to be due, in part, to changes in the copy number of rDNA genes (Pukkila, 1990). Homologous chromosomes have been recently found to be dimorphic in terms of electrophoretic mobility in a dikaryon of *Phanerochaete chrysosporium* (Gaskell *et al.*, 1991). Chromosomal rearrangements between repeated rDNA genes has also been suggested as contributing to CLPS in morphological mutants of *Candida tropicalis* (Suzuki *et al.*, 1991).

Attempts were also made to identify genes cloned from other micro-organisms which would show homology to *L. edodes* and *P. sajor-caju* DNA. This was intended for use on the molecular karyotyping of the genomes of these micro-organisms. However, not all of the probes used hybridized, notably the *S. cerevisiae trp1* gene. Skrzynia *et al.* (1989) were able to clone *trp1* from *C. cinereus* by relatively low stringency heterologous hybridization with this same gene. Greatest homology was detected with the 18s rRNA gene of *S. cerevisiae*. Structural organization of rDNA has been studied in *S. commune* (Buckner *et al.*, 1988a & b) and in *C. cinereus* (Cassidy *et al.*, 1984), with the rDNA unit repeats found on one chromosome in haploid nuclei of *C. cinereus*. The rDNA and ß-tubulin genes are both highly conserved in a wide range of organisms and would be expected to shown homology. It is possible that genes from other basidiomycete species may show greater homology and could be used in further investigations (e.g. Muñoz-Rivas *et al.*, 1986; Mellon *et al.*, 1987).

Recent interest in the cultivation of Shiitake and oyster mushrooms in Britain prompted this investigation on strain improvement of these fungi at the University of Nottingham. Protoplasts have been applied to several aspects of breeding in these edible species including more classical methods of mating, and the more direct approach of DNA mediated transformation. As previously discussed, protoplast isolation from basidiomycetes has become a relatively large field of research because of the numerous applications to strain improvement including fusion, transformation and de-dikaryotization. There are, however, several barriers limiting the use of protoplasts in many basidiomycete species. These include poor yields of protoplasts with relatively low frequencies of reversion as compared to well studied organisms such as *Aspergillus* and *Penicillium* spp. Possible reasons for this have been considered earlier (Chapter 2), and there is no doubt that improvements can be made to both aspects. By modifying the lytic enzyme used, pre-treatment of the mycelium, and the use of physiological stabilizers to improve yields, and a further study to optimize conditions for regeneration these barriers could be overcome.

Alternatively, other approaches to strain improvement should be investigated which by-pass the need for protoplast isolation and reversion. Electroporation, lithium acetate, or high velocity microprojectiles have already been used to successfully obtain transformants without protoplast isolation (Dhawle *et al.*, 1984; Klein *et al.*, 1987; Chakraborty & Kapoor, 1990). Although lithium acetate treatment of oidia was found to be less effective than transforming protoplasts of *Coprinus cinereus* (Binninger *et al.*, 1987), it is worth investigating this technique with other basidiomycete species.

Transformation has proved to be a direct means of introducing specific structural genes under the regulation of sequences controlling expression in fungal species (Ballance, 1991). Possible applications of this approach include the introduction of genes encoding for mating-type idiomorphs to overcome problems of incompatibility between isolates of commercial value or enzymes

required for substrate utilization, such as xylanases, lignin peroxidases, and exo/endo-glucanases. Purification of a xylanase and a manganese peroxidase from *L. edodes* (Forrester *et al.*, 1990; Mishra *et al.*, 1990) could lead to the isolation of the genes encoding for these degradative proteins. Enhanced production of such enzymes may be obtained simply by increasing copy number of a particular gene or in more sophisticated ways involving the manipulation of promoter and other control sequences. An interesting aspect that needs further consideration concerns the barriers to expression of genes from other groups of fungi in the basidiomycetes. As yet such studies have been limited (Casselton & de la Fuente Herce, 1989; Hynes, 1989) but indicate that genes from basidiomycete species can be expressed and function in ascomycetes, although the reverse has not yet been possible. If this general conclusion holds true, then future investigations may need to concentrate on understanding differences in the control of expression in these groups. The cloning of additional genes from basidiomycetes can only add to the information available.

Now the second most commonly cultivated mushroom in Europe, the popularity of the oyster mushroom is most likely due to its ease of production as well as much wider consumer acceptability. It would obviously be of some interest to expand on the existing variation in the fungus by attempting studies on protoplast fusion between species of this genus. Interspecific protoplast fusion has been used with a number of *Pleurotus* spp. by selecting for prototrophic colonies after fusion between complementing auxotrophic isolates, which were verified by isozyme analysis (Yoo *et al.*, 1984; Toyomasu *et al.*, 1986; Toyomasu & Mori, 1987a). However, several *Pleurotus* hybrids produced by protoplast fusion have since been found to be sterile and unable to produce fruitbodies (Toyomasu & Mori, 1897b). Thus, limitations may be due to sexual incompatibility which would be difficult to overcome, or may be due to limited fruiting ability of the auxotrophs obtained by mutagenesis. One potential means of selecting for fusion products without inducing mutations is by utilizing differential sensitivity of *Pleurotus* species to antifungal agents. Species were found to have different sensitivities to several antifungal agents (Chapter 4), however, a preliminary attempt to

isolate fusion products from *Pleurotus* spp. by this means was unsuccessful (results not presented) and would require further refinement to obtain hybrids. Alternatively, screening of fusion products by isozyme patterns may not be totally reliable, and hence a more stable genotypic characteristic should be used such as RFLPs. RFLP mapping has been applied to *C. cinereus* (Wu *et al.*, 1983), *Agaricus bisporus* and *A. bitorquis* (Castle *et al.*, 1988a & b; Loftus *et al.*, 1988; Hintz *et al.*, 1989) where polymorphisms between isolates were detected. However, such a tool is yet to be applied to other edible species for discriminating between stocks, and as a means of identifying fusion products.

There are thought to be nearly 39 species of *Pleurotus*, although there is much confusion as to their taxonomic relatedness (Edger, 1978b). For this reason attempts were made to characterize strains by molecular karyotyping utilizing PFG technology. However, this study was quite limited in the number of species and isolates screened but nevertheless provides the basis for further study. PFG has demonstrated its accessibility to species with limited genetic characterization and could be applied to other species, such as *L. edodes* where abnormalities in mating type segregation, low spore viability and extensive morphological variation have been found (Chapter 3). The nature of these characteristics could be investigated by PFG to explore the possible involvement of DLPS affecting meiosis and chromosome segregation. Reduced ascospore viability in *S. cerevisiae* has been attributed to deletions and or translocations occurring during meiosis (Ono & Ishino-Arao, 1988).

Although time did not allow for karyotype analysis of *L. edodes* studies were initiated on the screening of DNA probes for hybridization and mapping to chromosomal DNA. During this work two high molecular weight RNA species were isolated from the strain 30-1 (Chapter 5). Three sizes of polyhedral virus-like particles have been characterized in mycelia of *L. edodes* and the largest found to contain dsRNA about 5 x 10^6 daltons although no study was made for the other two sizes (Ushiyama, 1983). Further investigation would possibly show the presence of the virus in other strains. Protoplast regenerants could also be included in any study to investigate

protoplast isolation and regeneration as a means of 'curing' strains of the virus. Variation in the nature of these dsRNA species could possibly reveal differences between fungal species. If such variation is found to exist, the matter of their virulence and fungal resistance then arises.

As mentioned previously, there have been numerous reports on the isolation and regeneration of protoplasts from edible species, but as yet the application of the various techniques mentioned here have been restricted to a few studies. It is hoped that the work presented here will aid the understanding of how these limitations are imposed, and possible means of overcoming them.

Appendix A

Lentinula edodes Strain List

The following table lists details of the various strains of *Lentinula edodes* in stock at the University of Nottingham. Strains will be referred to by their BDUN accession code number. Single spore isolates derived from individual strains are denoted by a number contained in brackets.

BDUN ACCESSION NUMBER	OTHER NAMES	DETAILS
30-1	Les Miz	Commercially used strain, French origin[1]. Has very vigorous growth.
30-2	MS20	Commercially used strain, Korean origin[1]. Fruits well. Standard used against all other strains at Oakshire Mushrooms Ltd. From Mark Miller who obtained the culture in 1986 from a grower in Virginia. Originally used for natural log cultivation and found to adapt very well to sawdust.
30-3	237	Commercially used strain[1], developed by Mark Miller, Lambert Spawn, PA., U.S.A. Product of crossing single spore isolates numbers 2 and 37 from strain MC305 (BDUN 30-9). Successful crosses with isolate number 2 were found to have good pinning ability, however, both isolates have since been lost. Once had commercial use but has since been abandoned due to progressive strain breakdown. Grows slowly, but yields high quality caps.
30-4	STCL54/cr-02	Strain isolated from Fukien, China[2]. Very vigorous growth and fruits readily. Cultivation on sawdust requires one month vegetative phase and yields small fruit bodies. Spore isolates[2] characterized in terms of mating type as follows: STCL54(21) A_1B_1; STCL54(22) A_1B_2; isolates STCL54(23), STCL54(24) and STCL54(25) failed to yield fertile heterokaryons with either the A_1B_1 or the A_1B_2 testers.
30-5	STCL13/TMI830	Japanese strain isolated by Professor Miles, yields good quality mushrooms[2].
30-6	STCL34	Strain isolated from Chekiang, China. Originally from Zhejiang Agricultural University, China. Spore isolates characterized in terms of mating type as follows: STCL34(11) A_1B_1; STCL34(21) A_2B_1; STCL34(22) A_2B_2; STCL354(12) A_1B_2[2,4].

(cont.)

BDUN ACCESSION NUMBER	OTHER NAMES	DETAILS
30-7	LEM	Originally from Dr. K. Mori, The Mushroom Institute of Japan[3,4].
30-8	LE5	Isolated from Kiryu Province, Japan in 1974[3,4].
30-9	ATCC48861/ WC305/PSU305	Strain originally from S. Wong, Royal Mushrooms. Parent strain to 237 (BDUN 30-3)[4].
30-10	4055	Commercial strain from Sonycel, isolated from grain spawn 1989[3,4].
30-11	L30	Commercial strain from Hauser Champigon, isolated from grain spawn 1989[3,4].
30-12	S600	Commercial strain from Royal Mushrooms, isolated from grain spawn 1988[3,4].
30-13	STCL35	From Shanghai, China. Spore isolate STCL35(1) compatible with STCL35(8) and STCL35(15)[2,4].
30-14	STCL38	Originally from Dr. Bononi, Brazil. Twenty random spore isolates in collection[2,4].
30-15	STCL40	Tissue culture isolate, origin not known. Spore isolate STCL40(1) compatible with STCL40(2) and STCL40(4)[2,4].
30-16	STCL46	From Shanghai, China. Spore isolate STCL46(1) compatible with STCL46(11) and STCL46(13)[2,4].
30-17	STCL60	From Peking, China. Twenty random spore isolates in collection[2,4].
30-18	STCL65/ 8704	Wild-type isolate from Kwangtung, China. Spore isolate STCL65(1) compatible with STCL65(3) and STCL65(7)[2,4].
30-19	STCL67/ 8707	Wild-type isolate from Kwangtung, China. Spore isolates STCL67(1) compatible with STCL67(6) and STCL67(7)[2,4].
30-20	STCL68/ 8708	From Kwangtung, China. Spore isolate STCL68(1) compatible with STCL68(11) and STCL68(12)[2,4].
30-21	STCL70/ 8801	Wild-type isolate from Kwangtung, China. Spore isolate STCL70(1) A_1B_1 compatible with STCL70(8) A_2B_2; STCL70(3) A_1B_2 compatible with STCL70(8) A_2B_1[2,4].
30-22	Le2	Originally from K.K. Tan, Singapore. High temperature tolerant. Fruit bodies obtained from J.S. Sainsbury's, dikaryon isolated from stipe culture[5].

(cont.)

BDUN ACCESSION NUMBER	OTHER NAMES	DETAILS
30-23	866	Originally from K.K. Tan, Singapore. High quality winter strain. Dikaryon isolated from stipe culture[5].
30-24	Not Known	Stipe culture from commercially sold fruit bodies obtained from Keihan Hall, Osaka, Japan in September 1990.

1. Strain obtained from Dr. J.L. Burden, Middlebrook Mushrooms Ltd., Selby, U.K. 2. Strain obtained from Professor S.T. Chang, The Chinese University of Hong Kong. 3. Spore prints isolated and obtained from Dr. P.P. Kalberer, Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture, Switzerland. 4. Strain from G. Schroeder, Oakshire Mushrooms, Avondale, PA., U.S.A. 5. Obtained from Jim Heale, Smithy Mushrooms Ltd, Ormskirk, Lancashire, England. 6. Parental heterokaryon not in Nottingham collection.

Appendix B

Lentinula edodes Extended Strain List

BDUN	PARENT 1	PARENT 2	BDUN	PARENT 1	PARENT 2
30-1000	30-3.1(A_1B_2)	30-3.10($A_2B_{1/2}$)	30-2031	30-2(2)(A_1B_1)	30-2(24)(A_2B_2)
30-1001	30-3.3(A_1B_2)	30-3.10($A_2B_{1/2}$)	30-2032	30-2(14)(A_1B_1)	30-2(24)(A_2B_2)
30-1002	30-3.8(A_1B_2)	30-3.10($A_2B_{1/2}$)	30-2033	30-2(20)(A_1B_1)	30-2(24)(A_2B_2)
30-1003	30-3.9(A_1B_2)	30-3.10($A_2B_{1/2}$)	30-2034	30-2(22)(A_1B_1)	30-2(24)(A_2B_2)
30-1004	30-9.1(A_2B_2)	30-9.11(A_1B_1)	30-2035	30-2(24)(A_2B_2)	30-2(28)(A_1B_1)
30-1005	30-9.1(A_2B_2)	30-9.24(A_1B_1)	30-2036	30-2(24)(A_2B_2)	30-2(33)(A_1B_1)
30-1006	30-9.1(A_2B_2)	30-9.32(A_1B_1)	30-2037	30-9(2)(A_2B_1)	30-9(4)(A_2B_2)
30-1007	30-9.1(A_2B_2)	30-9.43(A_1B_1)	30-2038	30-9(2)(A_2B_1)	30-9(11)(A_1B_2)
30-1008	30-9.1(A_2B_2)	30-9.51(A_1B_1)	30-2039	30-9(2)(A_2B_1)	30-9(9)(A_2B_2)
30-1009	30-2.1(A_1B_1)	30-2.3(A_2B_2)	30-2040	30-9(2)(A_2B_1)	30-9(16)(A_1B_2)
30-1010	30-2.1(A_1B_1)	30-2.4(A_2B_2)	30-2041	30-4(2)(A_4B_3)	30-4(4)(A_3B_4)
30-1011	30-2.1(A_1B_1)	30-2.5(A_2B_2)	30-2042	30-9(7)(A_2B_1)	30-9(9)(A_2B_2)
30-1012	30-2.1(A_1B_1)	30-2.6(A_2B_2)	30-2043	30-9(7)(A_2B_1)	30-9(11)(A_1B_2)
30-1013	30-5051.1(A2B1)	30-5051.26(A4B4)	30-2044	30-9(7)(A_2B_1)	30-9(16)(A_1B_2)
30-2000	30-4(1)(A_4B_4)	30-4(3)(A_3B_3)	30-2045	30-4(1)(A_4B_4)	30-4(7)(A_3B_3)
30-2001	30-9(4)(A_2B_2)	30-9(7)(A_2B_1)	30-2046	30-4(2)(A_4B_3)	30-4(13)(A_3B_4)
30-2002	30-9(4)(A_2B_2)	30-9(13)(A_2B_1)	30-2047	30-4(3)(A_3B_3)	30-4(5)(A_4B_4)
30-2003			30-2048	30-4(3)(A_3B_3)	30-4(12)(A_4B_4)
30-2004	30-2(1)(A_1B_2)	30-2(12)(A_2B_1)	30-2049	30-4(3)(A_3B_3)	30-4(14)(A_4B_4)
30-2005	30-2(1)(A_1B_2)	30-2(13)(A_2B_1)	30-2050	30-4(3)(A_3B_3)	30-4(16)(A_4B_4)
30-2006	30-2(1)(A_1B_2)	30-2(21)(A_2B_1)	30-2051	30-4(3)(A_3B_3)	30-4(17)(A_4B_4)
30-2007	30-2(1)(A_1B_2)	30-2(23)(A_2B_1)	30-2052	30-4(3)(A_3B_3)	30-4(18)(A_4B_4)
30-2008	30-2(1)(A_1B_2)	30-2(26)(A_2B_1)	30-2053	30-4(2)(A_4B_3)	30-4(6)(A_3B_4)
30-2009	30-2(1)(A_1B_2)	30-2(35)(A_2B_1)	30-2054	30-4(3)(A_3B_3)	30-4(8)(A_4B_4)
30-2010	30-2(1)(A_1B_2)	30-2(203)(A_2B_1)	30-2055	30-4(4)(A_3B_4)	30-4(10)(A_4B_3)
30-2011	30-2(1)(A_1B_2)	30-2(204)(A_2B_1)	30-2056	30-4(4)(A_3B_4)	30-4(15)(A_4B_3)
30-2012	30-2(1)(A_1B_2)	30-2(206)(A_2B_1)	30-2057	30-4(7)(A_3B_3)	30-4(8)(A_4B_4)
30-2013	30-2(10)(A_1B_2)	30-2(12)(A_2B_1)	30-2058	30-4(6)(A_3B_4)	30-4(10)(A_4B_3)
30-2014	30-2(11)(A_1B_2)	30-2(12)(A_2B_1)	30-2059	30-4(7)(A_3B_3)	30-4(10)(A_4B_4)
30-2015	30-2(12)(A_2B_1)	30-2(15)(A_1B_2)	30-2060	30-4(10)(A_4B_3)	30-4(20)(A_3B_4)
30-2016	30-2(12)(A_2B_1)	30-2(16)(A_1B_2)	30-2061	30-5(3)(A_3B_5)	30-5(9)(A_5B_3)
30-2017	30-2(12)(A_2B_1)	30-2(25)(A_1B_2)	30-2062	30-9(5)(A_1B_1)	30-9(10)(A_2B_2)
30-2018	30-2(12)(A_2B_1)	30-2(27)(A_1B_2)	30-2063	30-9(5)(A_1B_1)	30-9(12)(A_2B_2)
30-2019	30-2(12)(A_2B_1)	30-2(34)(A_1B_2)	30-2064	30-9(1)(A_2B_2)	30-9(5)(A_1B_1)
30-2020	30-2(12)(A_2B_1)	30-2(205)(A_1B_2)	30-2065	30-4(10)(A_4B_3)	30-4(13)(A_3B_4)
30-2021	30-2(3)(A_2B_2)	30-2(19)(A_1B_1)	30-2066	30-4(1)(A_4B_4)	30-4(19)(A_3B_3)
30-2022	30-2(5a)(A_2B_2)	30-2(19)(A_1B_1)	30-2067	30-4(2)(A_4B_3)	30-4(20)(A_3B_4)
30-2023	30-2(9)(A_2B_2)	30-2(19)(A_1B_1)	30-2068	30-4(4)(A_3B_4)	30-4(11)(A_4B_3)
30-2024	30-2(17)(A_2B_2)	30-2(19)(A_1B_1)	30-2069	30-5(1)(A_3B_5)	30-5(9)(A_5B_3)
30-2025	30-2(18)(A_2B_2)	30-2(19)(A_1B_1)	30-2070	30-5(5)(A_3B_5)	30-5(9)(A_5B_3)
30-2026	30-2(19)(A_1B_1)	30-2(24)(A_2B_2)	30-2071	30-5(6)(A_3B_5)	30-5(9)(A_5B_3)
30-2027	30-2(19)(A_1B_1)	30-2(29)(A_2B_2)	30-2072	30-5(8)(A_3B_5)	30-5(9)(A_5B_3)
30-2028	30-2(19)(A_1B_1)	30-2(35)(A_2B_1)	30-2073	30-5(9)(A_5B_3)	30-5(11)(A_3B_5)
30-2029	30-2(19)(A_1B_1)	30-2(36)(A_2B_2)	30-2074	30-5(9)(A_5B_3)	30-5(12)(A_3B_5)
30-2030	30-2(19)(A_1B_1)	30-2(39)(A_2B_2)	30-2075	30-9(4)(A_2B_2)	30-9(14)(A_2B_1)

(cont.)

BDUN	PARENT 1	PARENT 2
30-2076	30-9(4)(A_2B_2)	30-9(15)(A_2B_1)
30-2077	30-4(1)(A_4B_4)	30-4(21)(A_3B_3)
30-2078	30-4(5)(A_4B_4)	30-4(21)(A_3B_3)
30-2079	30-4(8)(A_4B_4)	30-4(21)(A_3B_3)
30-2080	30-4(2)(A_4B_3)	30-4(22)(A_3B_4)
30-2081	30-4(10)(A_4B_3)	30-4(22)(A_3B_4)
30-2082	30-4(2)(A_4B_3)	30-4(24)(A_3B_4)
30-2083	30-4(10)(A_4B_3)	30-4(24)(A_3B_4)
30-2084	30-4(2)(A_4B_3)	30-4(25)(A_3B_4)
30-2085	30-4(10)(A_4B_3)	30-4(25)(A_3B_4)
30-2086	30-6(2)(A_2B_1)	30-6(1)(A_1B_1)
30-2087	30-7(1)(A_5B_3)	30-7(3)(A_6B_4)
30-2088	30-7(1)(A_5B_3)	30-7(16)(A_6B_4)
30-2089	30-8(1)(A_4B_3)	30-8(6)(A_5B_4)
30-2090	30-10(1)(A_1B_7)	30-10(20)(A_7B_8)
30-2091	30-10(1)(A_1B_7)	30-10(22)(A_7B_8)
30-2092	30-12(3)(A_2B_2)	30-12(6)(A_1B_1)
30-2093	30-12(3)(A_2B_2)	30-12(15)(A_1B_1)
30-2094	30-10(5)(A_1B_7)	30-10(20)(A_7B_8)
30-2095	30-12(1)(A_5B_3)	30-12(4)(A_6B_4)
30-2096	30-12(3)(A_5B_3)	30-12(4)(A_6B_4)
30-2097	30-8(5)(A_6B_3)	30-8(6)(A_5B_4)
30-2098	30-5(7)(A_3B_3)	30-5(25)(A_5B_5)
30-3000	30-2.1(A_1B_1)	30-2(201)(A_2B_2)
30-3001	30-2.1(A_1B_1)	30-2(3)(A_2B_2)
30-3002	30-2.1(A_1B_1)	30-2(5)(A_2B_2)
30-3003	30-2.1(A_1B_1)	30-2(9)(A_2B_2)
30-3004	30-2.1(A_1B_1)	30-2(13)(A_2B_1)
30-3005	30-4.1(A_3B_3)	30-4(1)(A_4B_4)
30-3006	30-4.1(A_3B_3)	30-4(5)(A_4B_4)
30-3007	30-4.1(A_3B_3)	30-4(7)(A_3B_3)
30-3008	30-4.1(A_3B_3)	30-4(12)(A_4B_4)
30-3009	30-4.1(A_3B_3)	30-4(14)(A_4B_4)
30-3010	30-4.1(A_3B_3)	30-4(16)(A_4B_4)
30-3011	30-4.1(A_3B_3)	30-4(17)(A_4B_4)
30-3012	30-4.5(A_3B_3)	30-4(1)(A_4B_4)
30-3013	30-4.5(A_3B_3)	30-4(5)(A_4B_4)
30-3014	30-4.5(A_3B_3)	30-4(8)(A_4B_4)
30-3015	30-4.5(A_3B_3)	30-4(12)(A_4B_4)
30-3016	30-4.5(A_3B_3)	30-4(14)(A_4B_4)
30-3017	30-4.5(A_3B_3)	30-4(16)(A_4B_4)
30-3018	30-4.5(A_3B_3)	30-4(17)(A_4B_4)
30-3019	30-4.5(A_3B_3)	30-4(18)(A_4B_4)
30-3020	30-5.12(A_5B_5)	30-5(9)(A_5B_3)
30-3021	30-5.14(A_3B_5)	30-5(9)(A_5B_3)
30-3022	30-5.15(A_3B_5)	30-5(9)(A_5B_3)
30-3023	30-5.16(A_3B_5)	30-5(9)(A_5B_3)
30-3024	30-9.1(A_2B_2)	30-9(5)(A_1B_1)
30-3025	30-9.11(A_1B_1)	30-9(1)(A_2B_2)
30-3026	30-9.11(A_1B_1)	30-9(10)(A_2B_2)
30-3027	30-2.3(A_2B_2)	30-2(7)(A_1B_1)
30-3028	30-2.3(A_2B_2)	30-2(2)(A_1B_1)
30-3029	30-2.4(A_2B_2)	30-2(2)(A_1B_1)
30-3030	30-2.4(A_2B_2)	30-2(7)(A_1B_1)
30-3031	30-2.5(A_2B_2)	30-2(7)(A_1B_1)
30-3032	30-2.5(A_2B_2)	30-2(2)(A_1B_1)
30-3033	30-2.6(A_2B_2)	30-2(2)(A_1B_1)
30-3034	30-2.6(A_2B_2)	30-2(7)(A_1B_1)
30-4000	30-2.1(A_1B_1)	30-4.1(A_3B_3)
30-4001	30-2.1(A_1B_1)	30-5.12(A_3B_5)
30-4002	30-3.3(A_1B_2)	30-4.1(A_3B_3)
30-4003	30-3.3(A_1B_2)	30-5.12(A_3B_5)
30-4004	30-2.1(A_1B_1)	30-3.10($A_2B_{1/2}$)
30-4005		
30-4006	30-3.10($A_2B_{1/2}$)	30-4.1(A_3B_3)
30-4007	30-3.10($A_2B_{1/2}$)	30-5.12(A_3B_5)
30-5000	30-2(2)(A_1B_1)	30-4(1)(A_4B_4)
30-5001	30-2(2)(A_1B_1)	30-4(2)(A_4B_3)
30-5002	30-2(2)(A_1B_1)	30-4(3)(A_3B_3)
30-5003	30-2(2)(A_1B_1)	30-4(4)(A_3B_4)
30-5004	30-2(2)(A_1B_1)	30-4(5)(A_4B_4)
30-5005	30-2(2)(A_1B_1)	30-4(6)(A_3B_4)
30-5006	30-2(2)(A_1B_1)	30-4(7)(A_3B_3)
30-5007	30-2(2)(A_1B_1)	30-4(8)(A_4B_4)
30-5008	30-2(2)(A_1B_1)	30-4(13)(A_3B_4)
30-5009	30-2(2)(A_1B_1)	30-5(1)(A_3B_5)
30-5010	30-2(2)(A_1B_1)	30-5(2)(A_3B_3)
30-5011	30-2(2)(A_1B_1)	30-5(3)(A_3B_5)
30-5012	30-2(2)(A_1B_1)	30-5(4)(A_3B_5)
30-5013	30-2(2)(A_1B_1)	30-9(1)(A_2B_2)
30-5014	30-2(3)(A_2B_2)	30-(A_4B_4)4(1)
30-5015	30-2(4)(A_1B_2)	30-4(1)(A_4B_4)
30-5016	30-2(6)(A_2B_2)	30-4(1)(A_4B_4)
30-5017	30-2(9)(A_2B_2)	30-4(1)(A_4B_4)
30-5108	30-2(12)(A_2B_1)	30-4(1)(A_4B_4)
30-5019	30-4(1)(A_4B_4)	30-5(4)(A_3B_5)
30-5020	30-4(1)(A_4B_4)	30-9(1)(A_2B_2)
30-5021	30-4(1)(A_4B_4)	30-9(2)(A_2B_1)

(cont.)

BDUN	PARENT 1	PARENT 2	BDUN	PARENT 1	PARENT 2
30-5022	30-4(1)(A_4B_4)	30-9(3)(A_1B_1)	30-5066	30-2(1)(A_1B_2)	30-4(1)(A_4B_4)
30-5023	30-4(1)(A_4B_4)	30-9(4)(A_2B_2)	30-5067	30-4(2)(A_4B_3)	30-9(7)(A_2B_1)
30-5024	30-4(1)(A_4B_4)	30-9(5)(A_1B_1)	30-5068	30-4(3)(A_3B_3)	30-9(7)(A_2B_1)
30-5025	30-4(1)(A_4B_4)	30-9(7)(A_2B_1)	30-5069	30-4(4)(A_3B_4)	30-9(7)(A_2B_1)
30-5026	30-4(1)(A_4B_4)	30-9(9)(A_2B_2)	30-5070	30-4(5)(A_4B_4)	30-9(7)(A_2B_1)
30-5027	30-2(1)(A_1B_2)	30-5(3)(A_3B_5)	30-5071	30-4(6)(A_3B_4)	30-9(7)(A_2B_1)
30-5028	30-6(1)(A_1B_1)	30-2(3)(A_2B_2)	30-5072	30-4(7)(A_3B_3)	30-9(7)(A_2B_1)
30-5029	30-2(3)(A_2B_2)	30-5(3)(A_3B_5)	30-5073	30-4(8)(A_4B_4)	30-9(7)(A_2B_1)
30-5030	30-2(4)(A_1B_2)	30-5(3)(A_3B_5)	30-5074	30-4(13)(A_3B_4)	30-9(7)(A_2B_1)
30-5031	30-4(1)(A_4B_4)	30-5(3)(A_3B_5)	30-5075	30-5(1)(A_3B_5)	30-9(7)(A_2B_1)
30-5032	30-2(6)(A_2B_2)	30-5(3)(A_3B_5)	30-5076	30-5(2)(A_3B_3)	30-9(7)(A_2B_1)
30-5033	30-2(7)(A_1B_1)	30-5(3)(A_3B_5)	30-5077	30-4(1)(A_4B_4)	30-5(2)(A_3B_3)
30-5034	30-2(9)(A_2B_2)	30-5(3)(A_3B_5)	30-5078	30-5(4)(A_3B_5)	30-9(7)(A_2B_1)
30-5035	30-2(12)(A_2B_1)	30-5(3)(A_3B_5)	30-579	30-4(1)(A_4B_4)	30-5(1)(A_3B_5)
30-5036	30-4(8)(A_4B_4)	30-5(3)(A_3B_5)	30-5080	30-4(2)(A_4B_3)	30-5(3)(A_3B_5)
30-5037	30-5(3)(A_3B_5)	30-9(1)(A_2B_2)	30-5081	30-4(3)(A_3B_3)	30-5(5)(A_3B_5)
30-5038	30-5(3)(A_3B_5)	30-9(2)(A_2B_1)	30-5082	30-2(1)(A_1B_2)	30-4(2)(A_4B_3)
30-5039	30-5(3)(A_3B_5)	30-9(3)(A_1B_1)	30-5083	30-2(12)(A_2B_1)	30-4(2)(A_4B_3)
30-5040	30-5(3)(A_3B_5)	30-9(4)(A_2B_2)	30-5084	30-2(6)(A_2B_2)	30-4(2)(A_4B_3)
30-5041	30-5(3)(A_3B_5)	30-9(5)(A_1B_1)	30-5085	30-2(1)(A_1B_2)	30-4(3)(A_3B_3)
30-5042	30-5(3)(A_3B_5)	30-9(7)(A_2B_1)	30-5086	30-2(4)(A_1B_2)	30-4(3)(A_3B_3)
30-5043	30-5(3)(A_3B_5)	30-9(9)(A_2B_2)	30-5087	30-2(7)(A_1B_1)	30-4(3)(A_3B_3)
30-5044	30-2(1)(A_1B_2)	30-6(2)(A_2B_1)	30-5088	30-2(9)(A_2B_2)	30-4(3)(A_3B_3)
30-5045	30-2(4)(A_1B_2)	30-6(2)(A_2B_1)	30-5089	30-2(1)(A_1B_2)	30-4(4)(A_3B_4)
30-5046	30-2(6)(A_2B_2)	30-6(2)(A_2B_1)	30-5090	30-2(4)(A_1B_2)	30-4(4)(A_3B_4)
30-5047	30-4(1)(A_4B_4)	30-6(2)(A_2B_1)	30-5091	30-2(6)(A_2B_2)	30-4(4)(A_3B_4)
30-5048	30-4(2)(A_4B_3)	30-6(2)(A_2B_1)	30-5092	30-2(1)(A_1B_2)	30-4(7)(A_3B_3)
30-5049	30-4(3)(A_3B_3)	30-6(2)(A_2B_1)	30-5093	30-2(9)(A_2B_2)	30-4(7)(A_3B_3)
30-5050	30-4(4)(A_3B_4)	30-6(2)(A_2B_1)	30-5094	30-2(3)(A_2B_2)	30-9(5)(A_1B_1)
30-5051	30-4(5)(A_4B_4)	30-6(2)(A_2B_1)	30-5095	30-2(12)(A_2B_1)	30-9(9)(A_2B_2)
30-5052	30-4(6)(A_3B_4)	30-6(2)(A_2B_1)	30-5096	30-2(7)(A_1B_1)	30-9(10)(A_2B_2)
30-5053	30-4(7)(A_3B_3)	30-6(2)(A_2B_1)	30-5097	30-2(1)(A_1B_2)	30-9(13)(A_2B_1)
30-5054	30-4(8)(A_4B_4)	30-6(2)(A_2B_1)	30-5098	30-2(2)(A_1B_1)	30-6(1)(A_1B_1)
30-5055	30-4(13)(A_3B_4)	30-6(2)(A_2B_1)	30-5099	30-4(1)(A_4B_4)	30-5(2)(A_3B_3)
30-5056	30-5(1)(A_3B_5)	30-6(2)(A_2B_1)	30-5100	30-2(1)(A_1B_2)	30-5(2)(A_3B_3)
30-5057	30-5(2)(A_3B_3)	30-6(2)(A_2B_1)	30-5101	30-2(3)(A_2B_2)	30-5(2)(A_3B_3)
30-5058	30-5(3)(A_3B_5)	30-6(2)(A_2B_1)	30-5102	30-2(12)(A_2B_1)	30-5(2)(A_3B_3)
30-5059	30-5(4)(A_3B_5)	30-6(2)(A_2B_1)	30-6000	30-2(12)(A_2B_1)	30-3.3(A_1B_2)
30-5060	30-5(5)(A_3B_5)	30-6(2)(A_2B_1)	30-6001	30-2(19)(A_1B_1)	30-3.10($A_2B_{1/2}$)
30-5061	30-6(2)(A_2B_1)	30-9(4)(A_2B_2)	30-6002	30-3.10($A_2B_{1/2}$)	30-4(1)(A_4B_4)
30-5062	30-6(2)(A_2B_1)	30-9(9)(A_2B_2)	30-6003	30-3.10($A_2B_{1/2}$)	30-4(2)(A_4B_3)
30-5063	30-2(6)(A_2B_2)	30-9(7)(A_2B_1)	30-6004	30-3.10($A_2B_{1/2}$)	30-4(3)(A_3B_3)
30-5064	30-2(4)(A_1B_2)	30-9(7)(A_2B_1)	30-6005	30-3.10($A_2B_{1/2}$)	30-9(4)(A_2B_2)
30-5065	30-2(1)(A_1B_2)	30-9(7)(A_2B_1)	30-6006	30-2(1)(A_1B_2)	30-4.1(A_3B_3)

(cont.)

BDUN	PARENT 1	PARENT 2
30-6007	30-2(3)(A_2B_2)	30-4.1(A_3B_3)
30-6008	30-2(12)(A_2B_1)	30-4.1(A_3B_3)
30-6009	30-2(19)(A_1B_1)	30-4.1(A_3B_3)
30-6010	30-2(1)(A_1B_2)	30-5.12(A_3B_5)
30-6011	30-5(3)(A_3B_5)	30-5.12(A_3B_5)
30-6012	30-2(12)(A_2B_1)	30-5.12(A_3B_5)
30-6013	30-2(19)(A_1B_1)	30-5.12(A_3B_5)
30-6014	30-3.3(A_1B_2)	30-9(2)(A_2B_1)
30-6015	30-4.1(A_3B_3)	30-9(2)(A_2B_1)
30-6016	30-5.12(A_3B_5)	30-9(2)(A_2B_1)
30-6017	30-4.1(A_3B_3)	30-9(4)(A_2B_2)
30-6018	30-3.3(A_1B_2)	30-9(7)(A_2B_1)
30-6019	30-4.1(A_3B_3)	30-9(7)(A_2B_1)
30-6020	30-5.12(A_3B_5)	30-9(7)(A_2B_1)
30-6021	30-3.3(A_1B_2)	30-6(2)(A_2B_1)
30-6022	30-4.1(A_3B_3)	30-6(2)(A_2B_1)
30-6023	30-4.5(A_3B_3)	30-6(2)(A_2B_1)
30-6024	30-5.12(A_3B_5)	30-6(2)(A_2B_1)
30-6025	30-5.14(A_3B_5)	30-6(2)(A_2B_1)
30-6026	30-3.3(A_1B_2)	30-4(1)(A_4B_4)
30-6027	30-5.12(A_3B_5)	30-6(1)(A_1B_1)
30-6028	30-4.1(A_3B_3)	30-6(1)(A_1B_1)
30-6029	30-2.1(A_1B_1)	30-6(1)(A_1B_1)
30-6030	30-5.12(A_3B_5)	30-9(1)(A_2B_2)
30-6031	30-4.1(A_3B_3)	30-9(1)(A_2B_2)
30-6032	30-2.1(A_1B_2)	30-9(1)(A_2B_2)
30-6033	30-5.12(A_3B_5)	30-9(5)(A_1B_1)
30-6034	30-4.1(A_3B_3)	30-9(5)(A_1B_1)
30-6035	30-5.12(A_3B_5)	30-9(4)(A_2B_2)
30-6036	30-5.12(A_3B_5)	30-6(1)(A_1B_1)
30-6037	30-4.1(A_3B_3)	30-6(1)(A_1B_1)
30-6038	30-3.3(A_1B_2)	30-4(2)(A_4B_3)
30-6039	30-3.3(A_1B_2)	30-4(3)(A_3B_3)
30-6040	30-3.3(A_1B_2)	30-4(4)(A_3B_4)
30-6041	30-2(2)(A_1B_1)	30-3.10($A_2B_{1/2}$)
30-6042	30-2(6)(A_2B_2)	30-3.10($A_2B_{1/2}$)
30-6043	30-2(7)(A_1B_1)	30-3.10($A_2B_{1/2}$)
30-6044	30-2(12)(A_2B_1)	30-3.1(A_2B_2)
30-6045	30-2(13)(A_2B_1)	30-3.1(A_2B_2)
30-6046	30-2(12)(A_2B_1)	30-3.2(A_2B_2)
30-6047	30-2(21)(A_2B_1)	30-2.3(A_1B_2)
30-6048	30-2(13)(A_2B_1)	30-3.4(A_1B_2)
30-6049	30-2(21)(A_2B_1)	30-3.4(A_1B_2)
30-6050	30-2(12)(A_2B_1)	30-3.5(A_1B_2)
30-6051	30-2(13)(A_2B_1)	30-3.5(A_1B_2)
30-6052	30-2(21)(A_2B_1)	30-3.5(A_1B_2)
30-6053	30-2(21)(A_2B_1)	30-3.6(A_1B_2)
30-6054	30-5(4)(A_3B_5)	30-5019.1(A4B4)
30-6055	30-5(4)(A_3B_5)	30-5019.3(A4B4)
30-6056	30-4(1)(A_4B_4)	30-5025.2(A2B1)
30-6057	30-4(10)(A_4B_3)	30-5025.6(A2B1)
30-6058	30-4(5)(A_4B_4)	30-5051.1(A2B1)
30-6059	30-4(4)(A_3B_4)	30-5068.1(A2B1)
30-6060	30-4(5)(A_4B_4)	30-5068.1(A2B1)
30-6061	30-4(6)(A_3B_4)	30-5068.1(A2B1)
30-6062	30-4(7)(A_3B_3)	30-5068.1(A2B1)
30-6063	30-4(10)(A_4B_3)	30-5068.1(A2B1)
30-6064	30-9(4)(A1B2)	30-5068.1(A2B1)
30-6065	30-9(9)(A1B2)	30-5068.1(A B1)
30-6066	30-5(25)(A_5B_5)	30-4.1(A_3B_3)
30-6067	30-9(4)(A B)	30-3.10($A_2B_{1/2}$)
30-6068	30-9(5)(A_1B_1)	30-3.10($A_2B_{1/2}$)
30-6069	30-9(8)(A_1B_1)	30-3.10($A_2B_{1/2}$)
30-6070	30-9(7)(A_2B_1)	30-3.1(A_2B_2)
30-6071	30-9(2)(A_2B_1)	30-3.1(A_2B_2)
30-6072	30-4(5)(A_4B_4)	30-5051.29(A2B1)

Appendix C

Pleurotus spp. Strain List

The following table lists details of the various species and strains of *Pleurotus* in stock at the University of Nottingham. Strains will be referred to by their BDUN accession code number.

SPECIES	BDUN ACCESSION NUMBER	OTHER NAMES	DETAILS
P. columbinus	34-1	3030	Commercial strain[1].
P. cystidiosus	36-1	DAOM 22543/ FPRL 212A	Isolated from Princes Risborough, England[2].
P. florida	31-1	STCPL10	No further information available[3].
P. florida	31-2	3025	Not used commercially[1].
P. ostreatus	7-1	Not Known	Lost in collection.
P. ostreatus	7-2	Not Known	Lost in collection.
P. ostreatus	7-3	Not Known	Isolated from Darnycel straw culture, January 1989.
P. ostreatus	7-4	1014	Commercially used production strain[4].
P. ostreatus	7-5	4-34	*ade*$^-$ auxotroph, A_1B_1 mating type[4].
P. ostreatus	7-6	7-92	*paba*$^-$ auxotroph, A_2B_2 mating type[4].
P. ostreatus	7-7	4B	Single spore isolate, A_2B_2 mating type[4].
P. ostreatus	7-8	2δ	Single spore isolate, A_2B_1 mating type[4].
P. ostreatus	7-9	2τ	Single spore isolate, A_1B_2 mating type[4].
P. ostreatus	7-10	3200/ R250 (T. Elliott)	Sporeless strain obtained from T. Elliott.
P. ostreatus	7-11	3001	Commercially used strain[1].
P. ostreatus	7-12	3004	Commercially used strain[1].

(cont.)

SPECIES	BDUN ACCESSION NUMBER	OTHER NAMES	DETAILS
P. pulmonaris	33-1	HVZ	Obtained from the Vietnam Jungle, has a fast vegetative phase, producing small fruit bodies[4].
P. pulmonaris	33-2	Q107	Not used commercially[1].
P. pulmonaris	33-3	3014B	Hybrid of *P. pulmonaris* and *P. sajor-caju*, used commercially[1].
P. sajor-caju	32-1	STCPL27	No further information available[3].
P. sajor-caju	32-2	Not Known	No further information available.
P. sajor-caju	32-3	3014-14	Not used commercially[1].
P. sajor-caju	32-4	3015	Used commercially[1].
P. sapidus	35-1	DAOM 21473/ M153	Found growing on *Acer saccharum* and collected by V.J. Nordin in Ontario, Canada[2].
P. sapidus	35-2	DAOM R-621-4	Found growing on *Picea glauca* at Willow River, Victoria, British Columbia, Canada[2].

1. Obtained from Somycel, Langeais, France, via L. Miller. 2. Obtained from Biosystematics Research Centre, Ottowa, Ontario, Canada, via C. Babcock. 3. Obtained from Professor S.T. Chang, The Chinese University of Hong Kong, Hong Kong. 4. Obtained from or via M. Nisik, Belgrade, Yugoslavia.

Appendix D

Fungicide Sensitivity in *Lentinula edodes*

Introduction

Lentinula edodes has been the topic of many studies, which have so far concentrated on the physiological and ecological aspects of vegetative and reproductive growth. So far, there has been no investigations into drug sensitivity of this species and subsequent isolation of drug resistance mutants. Development of resistance strains may have a number of commercial applications. Resistance may be used as an additional genetic property for the registration of shiitake strains which occurs in Japan (Anonymous, 1980; Royse *et al.*, 1985). In terms of disease control where application of routine fungicides has proved to be no longer of any use, other compounds cold be employed for which resistance has been bred into commercial varieties (Elliott & Langton, 1981; Challen *et al.*, 1989). Alternatively, screening may reveal strains with enhanced tolerance as has been found with *Agaricus bisporus* (Gandy, 1981) or compounds to which *L. edodes* is not naturally sensitive. Resistance mutants can also be used to select for hybrids produced by breeding techniques including protoplast fusion or hyphal crosses (Elliott, 1978; Miles & Chang, 1980).

Materials and Methods

Fungicide sensitivity was tested as described in Chapter 4, however, the media used included PMA and LMM (see Chapter 2), and plates incubated for 9 to 15 days at 25°C. Strains used included *L. edodes* 30-1, 30-2, 30-3, 30-4 and 30-5 (see Appendix A).

Results

Preliminary results on the sensitivity of *L. edodes* to sodium selenate had shown that five strains (30-1 to 30-5) tested varied in relation to their sensitivity to this compound. Strain 30-3 was found to be most sensitivive and strain 30-4 slightly less sensitive. For this reason these isolates were used throughout in tests to determine dose response to several fungal inhibitors. Work was completed by growing the same five strains on media containing a much narrower range of fungicide concentrations, allowing the MIC for particular strains to be defined.

Results of the dose: response of strains 30-3 and 30-4 allowed the ED_{50} to be determined for these compounds (see table 1D). Minimum inhibitory concentrations for five strains to the compounds acriflavine, carboxin, cycloheximide, benodanil, hygromycin B, malachite green and propiconazole are presented in Table 2D. A concentration conversion table is also given (Table 3D).

TABLE 1D ED_{50} Concentrations for Several Antifungal Compounds on the Growth of *Lentinula edodes*

COMPOUND	MEDIUM USED[1]	ED_{50} CONCENTRATIONS STRAIN 30-3	STRAIN 30-4
Acriflavine	MM	0.7µg.ml^{-1}	3.38µg.ml^{-1}
Acriflavine	CM	2.42µg.ml^{-1}	2.42µg.ml^{-1}
Benodanil	CM	3.30µM	3.80µM
$KClO_3$	MM+NO_3	1.26%	ND[2]
$KClO_3$	MM+Asp	1.44%	ND
Carboxin	MM	0.38µM	0.38µM
Carboxin	CM	0.15µM	0.19µM
Crystal violet	MM	7.96µg.ml^{-1}	6.85µgml^{-1}
Crystal violet	CM	11.15µg.ml^{-1}	16.92µgml^{-1}
Cycloheximide	CM	4.30µM	6.3µM
5-Fluroindole	CM	4.5µM	5.3µM
Hygromycin B	CM	7.5µM	7.0µM
Imazalil	CM	143.14µM	176.47µM
Iprodione	CM	16.1µM	17.5µM
Malachite Green	MM	0.83µg.ml^{-1}	5µg.ml^{-1}
Malachite Green	CM	3.92µg.ml^{-1}	8.8µg.ml^{-1}
MDPC	CM	46.7µg.ml^{-1}	46.7µg.ml^{-1}
Nystatin	CM	1.28units.ml^{-1}	4.72units.ml^{-1}
Propiconazole	CM	14.2µM	13µM
$NaSeO_4.12H_2O$	MM+SO_4	0.07%	0.09%
$NaSeO_4.12H_2O$	MM+Cys	2.06%	2.94%

1. MM= Minimal medium; CM= Complete medium; Cys= cysteine; Asp= asparagine; NO_3= nitrate; SO_4= sulphate.
2. ND= Not Determined.

Discussion

Sensitivity of *L. edodes* to several antifungal compounds revealed slight differences between commercial strains as measured by radial growth. Initially two strains 30-3 and 30-4 were used for extensive dose:response tests.

TABLE 2D Minimum Inhibitory Concentrations of Selected Compounds on the Growth of *Lentinula edodes*

COMPOUND	MIC/ Strain 30-1	30-2	30-3	30-4	30-5
Acriflavine	5μg.ml^{-1}	6μg.ml^{-1}	3μg.ml^{-1}	9μg.ml^{-1}	7μg.ml^{-1}
Benodanil	25μM	45μM	35μM	25μM	40μM
Carboxin	3μM	4μM	5μM	4μM	4μM
Cycloheximide	40μM	30μM	30μM	30μM	30μM
Hygromycin B	35μM	30μM	25μM	35μM	30μM
Malachite Green	50μg.ml^{-1}	45μgml^{-1}	45μg.ml^{-1}	50μg.ml^{-1}	50μg.ml^{-1}
Propiconazole	100μM	80μM	80μM	100μM	100μM

TABLE 3D Concentration Conversion Table for Fungal Inhibitors

COMPOUND	MOLECULAR WEIGHT	10μM EQUIVALENT	10μgml^{-1} EQUIVALENT
Benodanil	323.10	3.23μg.ml^{-1}	30.95μM
Carbendazim	191.20	1.91μg.ml^{-1}	52.3μM
Carboxin	235.30	2.35μg.ml^{-1}	42.5μM
Cycloheximide	281.40	2.81μg.ml^{-1}	30.95μM
Fluroacetate	100.04	1.00μg.ml^{-1}	100.00μM
Flurilazole	315.38	3.15μg.ml^{-1}	31.71μM
5-Fluroindole	135.10	1.35μg.ml^{-1}	74.2μM
Hygromycin B	527.50	5.28μg.ml^{-1}	18.96μM
Imazalil	297.20	2.97μg.ml^{-1}	33.65μM
Iprodione	330.20	3.30μg.ml^{-1}	30.30μM
Malachite Green	364.90	3.65μg.ml^{-1}	27.40μM
MDPC	220.00	2.20μg.ml^{-1}	45.50μM
Propiconazole	342.20	3.42μg.ml^{-1}	29.20μM
S-32165	282.30	2.82μg.ml^{-1}	5.50μM
Sulphanilamide	172.20	172 μg.ml^{-1}	58.14μM
Triademenol	295.18	2.95μg.ml^{-1}	33.88μM

Of the two strains, 30-3 was found to be generally more sensitive to the compounds tested, with the exception of the fungicide carboxin. Sensitivity to the antimetabolites crystal violet and malachite green were shown to be dependent on the medium used, both compounds being highly inhibitory when tested in minimal medium.

Further tests to determine MIC's of five strains revealed similarities in response to 7 different compounds. This contrasts with variation found amongst commercial strains of *A. bisporus* and *A. bitorquis* (Gandy, 1981; Challen & Elliott, 1985). Further strains may need to be tested before any variation can be detected.

Appendix E

Cellulase Activities in *Lentinula edodes*

Introduction

Degradation of the various components of the wood medium on which Shiitake is commercially produced is of great importance for improving the biological efficiency of this fungus. White rot fungi such as *Lentinula edodes* and *Pleurotus ostreatus* can degrade this substrate by production of cellulases, ligninases and xylanases (Hong *et al*, 1986; Leatham & Kirk, 1983; Oriaran *et al*, 1989). Recently, a xylanase (Mishra *et al.*, 1990) and a manganese peroxidase (Forrester *et al.*, 1990) from *L. edodes* have been purified and characterized as a means of understanding substrate utilization.

Considerable work has been carried out on both the biochemical and genetic background of these enzymes and the comparable ease of the assay system for cellulases as opposed to ligninases (Mandels *et al*, 1976; Leatham & Kirk, 1983) prompted the task of comparing cellulase activities of several strains of *L. edodes* in stock.

Methods and Materials

Several strains of *L. edodes* were assayed for their ability to produce cellulase over a period of 28 days.

A macerate was produced for each strain by growing twelve, 4mm plugs of mycelia taken from the leading edge of an actively growing colony grown on LMM. Mycelium was grown at 25°C on 8.0cm cellophane discs on LMM for 6 to 7 days depending on the strain. Growth from six plates was harvested by scraping the mycelium and placing into 15ml of salt solution containing: KH_2PO_4, 2g; $MgSO_4.7H_2O$, 2g; Leatham's mineral solution, 10ml; volume to 1l with distilled water. Mycelia was homogenized by vigorous shaking with glass beads. The macerate was then collected by centrifugation and washed once with 10ml salt solution prior to resuspending in 15ml cellulose medium (Avicel, 10g; glutamic acid, 2.5g; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 2.0; Leatham's mineral solution, 10ml; trace element solution, 1.0ml; vitamin solution 1.0ml, pH to 5.5, volume to 1l with distilled water). 1ml of macerate was inoculated into 25ml cellulose medium in 9.0cm plastic petri dishes. A total of twelve plates were inoculated for each strain and

incubated as static cultures at 25°C within a propagator containing small dishes of water to maintain humidity.

Culture supernatants were harvested by centrifugation and assayed for carboxymethylcellulase (CMCase) activity after 7, 14, 21 and 28 days. Three replicates were used for each strain for each time point.

Each strain time sample was assayed for CMCase activity in triplicate for each replicate as follows: 0.25ml assay substrate (2% CMC in 50mM sodium acetate buffer, pH 5.0) was mixed with 0.25ml culture filtrate in glass test tubes and covered with glass marbles and incubated in a water bath at 50°C for 60 min. The reaction was terminated by placing the tubes in a boiling water bath for 15 min followed by immediate cooling in a bath of cold water.

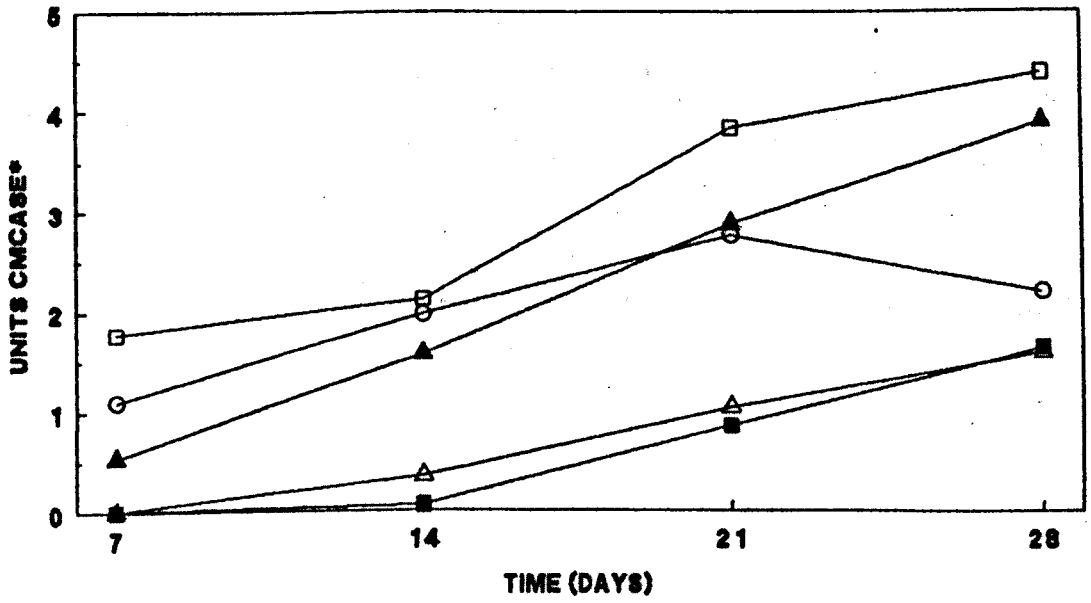
The release of reducing sugar from the CMC substrate was determined using the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). This was carried out as follows: 0.5ml of copper reagent ($CuSO_4.5H_2O$, 4g; Na_2SO_4 (anhydrous), 36g; volume to 200ml with distilled water) was mixed with 0.5ml of sample, placed in a boiling water bath for 20 minutes, followed by cooling in a water bath. 1ml of colour reagent (KNa tartrate, 12g; $NaHCO_3$, 16g; Na_2CO_3 (anhydrous), 144g; volume to 800ml with distilled water) is added, vortexed and 5ml distilled water added and the solution mixed by inversion. The CMC precipitate was allowed to settle for at least 2 hours to overnight prior to reading OD_{500}.

Controls included a distilled water blank and boiled test samples. Standard curves for absorbance versus reducing sugar were carried out by assaying dilutions of a standard glucose solution (Sigma Chemical Co.).

Results

Results of this experiment are presented in Fig. E.

Fig. E Cellulase Activity of Several Strains of *Lentinula edodes*



Key to strains: —□— = strain 30-1; —△— = strain 30-2; —■— = strain 30-3; —▲— = strain 30-4; —○— = strain 30-5.

*µmol reducing sugar (as glucose) produced per ml sample min^{-1}.

Discussion

Fungal cellulases can be classified into three major groups according to their mode of action: cellobiohydrolases, cleaving ß-1,4-glycosidic bonds to yield cellobiose; endoglucanases, cleaving internal glucosidic bonds; ß-glucosidases, which split short cello-oligosaccharides to glucose. Each of these groups contains several isoenzymes which makes the task of assaying any one component complex (Knowles *et al*, 1987)

Complexity in terms of cellulase production may be related in part to the substrate itself. The natural substrate of *L. edodes* is mainly hardwood species where efficient utilization of the lignocellulose complex is dependent on the production of lignanases, xylanases and cellulases as studies by Hong *et al* (1985) have demonstrated. Xylanase and cellulase activity were found by Ishikawa *et al.* (1983) to increase from the start of primordium formation to the end of fruiting and thus have an important role in the conversion of sawdust to a commercially viable product, that is, edible mushrooms.

During the course of this experiment, production of cellulases was induced by growing the fungal strains on Avicel, a crystalline form of cellulose. This allowed different commercial strains to be assessed *in vitro* for their ability to produce cellulolytic enzymes. Strains were found to differ in the quantitative amount of CMCase produced, with strains 30-2 and 30-3 slow to reach high CMCase activity although strains 30-1, 30-4 and 30-5 were found to produce detectable levels of activity after the first week of growth. Strains 30-1 and 30-5 were seen to level off in activity on the fourth week of growth and strain 30-4 continued increasing in CMCase activity, with no plateau detected at the end of the experimental period.

These results could be related to the slow growth of strains 30-2 and 30-3 as compared to 30-1, 30-4 and 30-5 as found on solid medium. Correlating CMCase activity with an increase in dry weight of the strain is a difficult procedure as the mycelium, when harvested, becomes "contaminated" by the remaining undigested avicel. The total protein content of the spent culture solution would yield little information in terms of specific activity of CMCase because of the long time period over which this experiment was conducted. Cell lysis may be occurring and the release of proteases may affect the stability of proteins in the suspension.

The levels of CMCase activity detected in *L. edodes* are considered very low compared to *Trichoderma* spp. which can produce cellulases in great abundance (Knowles *et al*, 1987). Increased cellulase production in strains of *L. edodes* by breeding and strain selection may prove to be useful in the cultivation of Shiitake. Although techniques of recombinant-DNA technology applied to basidiomycetes may in future play a role in strain development.

Appendix F

Fermenter Growth of *Lentinula edodes*

Introduction

Growth kinetics of edible fungi in submerged culture have been largely studied for their application to the food industry. Mycelium grown in fermenters may have applications as a food or flavour additive (Litchfield, 1967; Moustafa, 1960). There has been interest in the growth of large amounts of inoculum for spawn production for which a fermenter vessel, where growth can be easily controlled and monitored, is ideally suited. There are, however, problems associated with physiological adaptation to the sawdust substrate due to the presence of inhibitory substances (Leatham & Griffin, 1984).

This study was carried out as a means of simply producing large amounts of *L. edodes* mycelia as a substrate for the induction of cell wall lytic enzymes from *Streptomyces* spp. (see Chapter 2).

Methods and Materials

Ten 4mm plugs of mycelia of strain 30-1 grown on PMA were grown in 50ml of liquid medium in 250ml conical flasks for 14 days. The growth of ten flasks were harvested in a sterile 500ml centrifuge bucket. The volume was brought up to 500ml with sterile distilled water prior to maceration with a Silverson homogenizer.

This suspension was transferred to 1.5l of liquid medium (at 1.25 normal concentration) in a 3l fermenter vessel. Thus the inoculum level was 1:4. Sterile air was fed through the fermenter at a rate of 2 $lmin^{-1}$, temperature was maintained at 25°C and the speed of stirring was a constant 100rpm.

Samples were taken after 0, 3, 6, 10, 11 and 12 days by bleeding 20ml prior to removing a sample for dry weight estimation. Two 10ml samples were taken for each time point and placed into centrifuge tubes. Samples were spun for 10 minutes at 4,000rpm in a MSE bench centrifuge and the supernatant removed. Samples were stored at -70°C prior to freeze drying and dry weight determination.

Strain 30-1 was grown in a 10l fermenter following the above procedure in a 3 l vessel. Ten days after inoculation the contents of the smaller vessel were aseptically transferred to 6 l of medium in a 10 l fermenter. This gave an inoculum level of 1:4 and a working volume of 8 l. Air was fed at a rate of 6 $lmin^{-1}$, temperature maintained at 25°C and stirring was a constant 100rpm. Samples were taken after 0, 2, 4, 7, 8, 9, 10, 11, 14 and 15 days by blowing sterile air through the sampling port prior to bleeding.

Results

Dry weight analysis of the growth rate of *L. edodes* strain 30-1 in 3 l and 10l fermenters are presented in Figs. 1F and 2F respectively.

Discussion

Mycelial dry weight increased steadily in the 3 l fermenter reaching a peak after 9 days. The decrease in dry weight experienced after 10 days could be due to autolysis or the development of large growth points which were difficult to sample.

Growth in the 10l fermenter followed a lag phase of 6-7 days before a rapid increase in biomass. The cause for the delay in weight increase may be due to the physiological adaptation to the new medium or the interval for new growth points to be formed by fragmentation.

Optimized conditions for the growth of *L. edodes* in fermenter vessels has been developed by Song *et al* (1987) with up to 16.5mg dry weight mycelia ml^{-1} obtained. Several factors were found to affect biomass, including temperature, pH, and the use of air lift fermenter rather than a shaking vessels. Studies on several basidiomycetes including *P. ostreatus* have found that dry weight yield can be increased considerably by use of polymeric additives such as Junlon PW110 and Hostacerin (Jones *et al*, 1988). These compounds prevent mycelial clumping and allow the growth of dispersed hyphal filaments so that exponential growth can be maintained for longer. Use of these compounds may have some application to the growth of *L. edodes* in submerged culture.

Fig. 1F Growth of *Lentinula edodes* 30-1 in a 3 l Fermenter Vessel

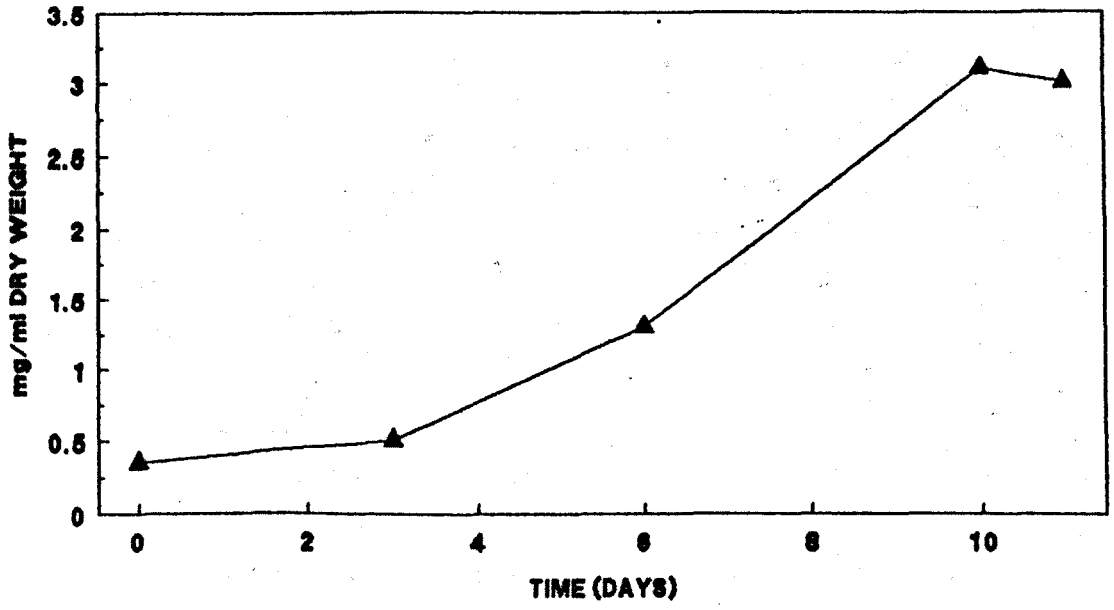
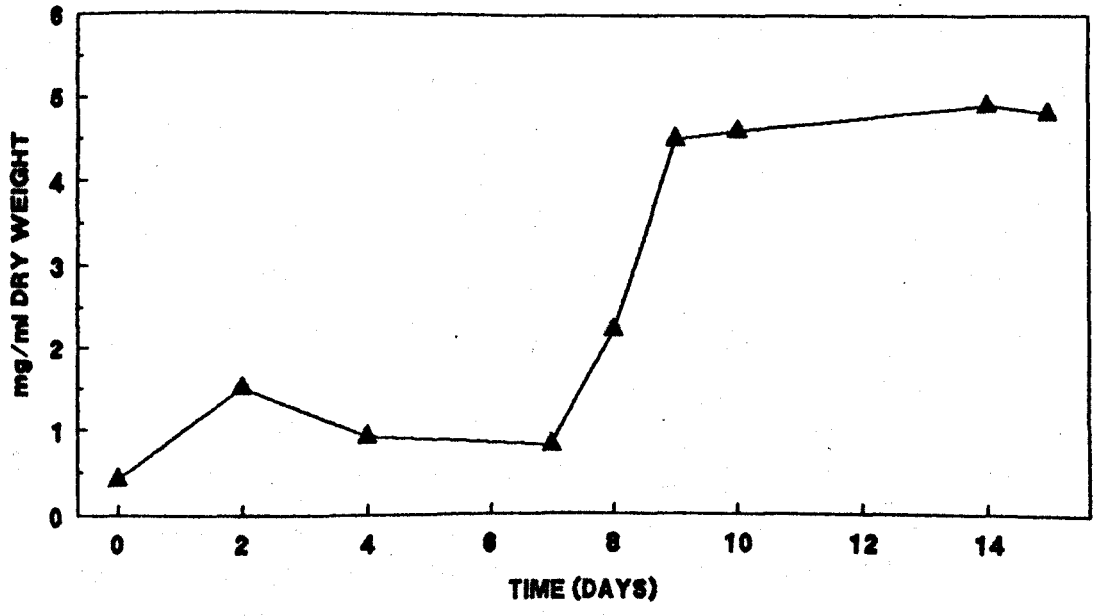


Fig. 2F Growth of *Lentinula edodes* 30-1 in a 10l Fermenter Vessel



Appendix G
Protoplast Release and Regeneration from *Penicillium chrysogenum*

Introduction

Fungal protoplasts are commonly isolated using the commercially produced enzyme Novozym 234 (see Chapter 2). Batch variation on the yield and regeneration of *P. chrysogenum* strain NRRL1951 was investigated as a continuation of the original study on *L. edodes* and *P. sajor-caju*.

Methods and Materials

Mycelium for protoplast isolation was prepared as follows: 4 day old spores were isolated from plate cultures on MYG in 0.01% Tween 80 and washed twice with SDW. Spores were then inoculated into *Penicillium* complete medium, PCM (sucrose, 30g; corn steeped liquor, 10g; casamino acids, 2g; yeast extract; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; KCl, 0.5g; $FeSO_4.7H_2O$, 0.01g; pH to 6.0-6.5 with Na_2HPO_4 and volume to 1 l with distilled water) to give a final concentration of 1 x 10^7 spores ml^{-1} and incubated, with shaking, for 20 hours at 28°C.

Mycelium was harvested and thoroughly washed with 0.7M KCl and damp pressed onto filter paper. Wet weight was determined and the mycelium incubated with lytic enzyme dissolved in 0.7M KCl/ 0.2M phosphate buffer, pH 5.8 at a ratio of 1g mycelium: 10ml lytic solution. Novozym 234 and Cellulase CP [2697/5] were each used at a concentration of $5mg.ml^{-1}$. Enzyme solutions were sterilized by centrifugation (30,000 x *g*, 4°C for 30 min.).

Protoplasts were harvested after 90 minutes although release was monitored for up to 2.5 hours. Protoplasts were harvested through 30µm nylon mesh filters, spun at 1,500rpm for 15 min. and washed once. Protoplasts were resuspended and diluted in 0.7M KCl and regenerated on PCM stabilized with 0.7M KCl. Regeneration was determined after 2 days incubation at 28°C. As a control, protoplasts were also diluted in SDW and plated onto stabilized medium to determine the proportion of viable hyphal fragments and ungerminated spores present in the suspension.

Results

Release of protoplasts from *P. chrysogenum* with time are presented in Fig. G. Regeneration frequencies of protoplasts isolated with different batches of Novozym 234 are in Table G.

TABLE G. The Effect of Novozym 234 Batch Variation on the Yield and Regeneration of Protoplasts from *Penicillium chrysogenum* Strain NRRL1951.

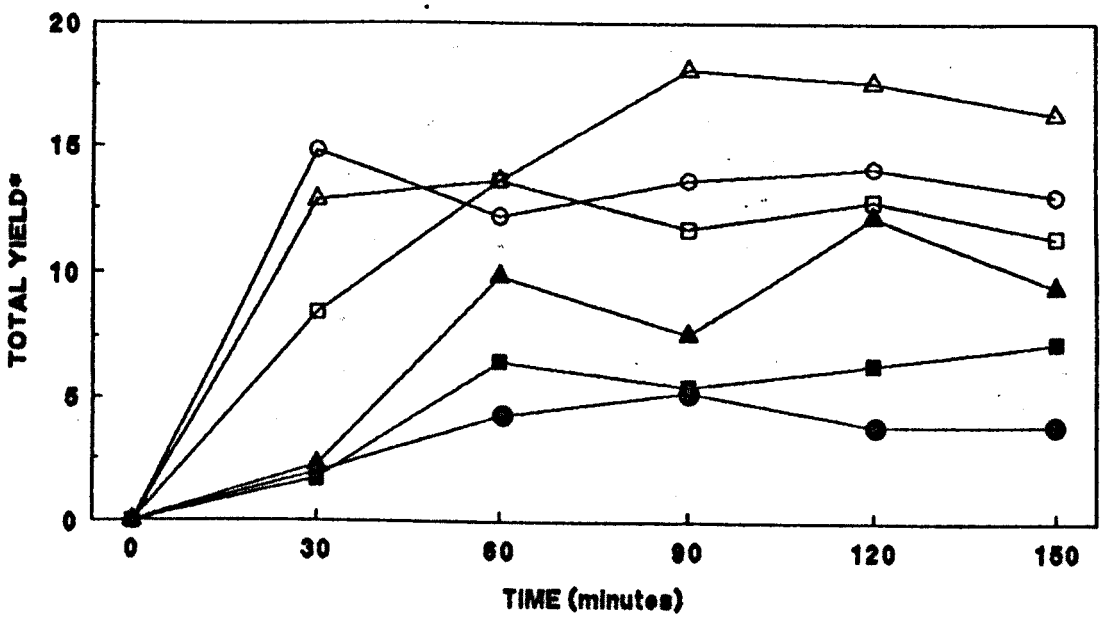
LYTIC ENZYMES USED (5mg.ml^{-1})	PROTOPLAST YIELD*	PROTOPLAST REGNERATION FREQUENCY (%)
Novozym 234 [PPM1523] & Cellulase CP [2697/5]	5.20 x 10^{8}	7.26%
Novozym 234 [PPM1906] & Cellulase CP [2697/5]	1.36 x 10^{9}	5.13%
Novozym 234 [PPM1961] & Cellulase CP [2697/5]	1.17 x 10^{9}	4.55%
Novozym 234 [PPM2934] & Cellulase CP [2697/5]	1.81 x 10^{9}	4.4%
Novozym 234 [SP-C25-BRT0789] & Cellulase CP [2697/5]	5.44 x 10^{8}	9.99%
Novozym 234 [SP-C25-BRT1289] & Cellulase CP [2697/5]	7.57 x 10^{8}	10.74%

*Protoplasts/ gram mycelium/ 90 minutes digestion.

Discussion

Release of protoplasts from *P. chrysogenum* strain NRRL1951 was found to occur rapidly and steadily for all of the batches of Novozym 234 tested. However, of the six enzymes PPM1906, PPM1961 and PPM2934 were found to be the most efficient in terms of digestion of the mycelia, although protoplast regeneration was lowest after use of these batches. The two purified enzymes SP-C25-BRT0789 and SP-C25-BRT1289 gave slower release and less efficient lysis. However, regeneration of protoplasts isolated using these enzymes was nearly twice that found for batches PPM1906, PPM1961 and PPM2934. This may be due to the low protease activity detected in these highly purified batches. Protease may affect the membrane surface of protoplasts once isolated and thus affect regeneration. Alternatively, more efficient lysis of mycelium may cause a greater proportion of anucleate protoplasts to be released, which would in turn affect viability. Nuclear staining was not carried out to verify this.

Fig. G Timed Release of Protoplasts from *Penicillium chrysogenum* NRRL 1351



*Protoplasts per gram fresh weight mycelia x10^{6}.

Key to Novozym 234 batches: —●— = batch PPM1523; —○— = batch PPM1906; —□— = batch PPM1961; —△— = batch PPM2934; —■— = batch SP-C25-BRT0789; —▲— = batch SP-C25-BRT1289.

Appendix H
Electrophoretic Karyotyping of *Aspergillus* spp. and *Candida albicans*

Introduction

Pulsed-field gel electrophoresis (PFG) has found numerous uses since first shown to separate intact yeast chromosomes (Schwartz *et al.*, 1982). Since then, several improvements have been made to the original system to give greater resolution of DNA banding patterns in the megabase (Mb) size range (Anand, 1986; Knight, 1989). One of the most widely used applications of PFG has been molecular karyotyping of fungal species (see Chapter 5), although size estimation of chromosomes is greatly limited due to a lack of accurate molecular weight standards between 2.2-3.5Mb and greater than 5.7Mb. As a means of improving size estimation of chromosomes from *Plerutous* spp. (Chapter 5) it was the aim of this study to use two previously characterized species, *Aspergillus nidulans* and *Candida albicans* as size standards. This, however, necessitated extensive characterization of these species. In addition, another *Aspergillus* sp., *A. alliaceus*, was used in these studies to compare chromosome size differences between the two species.

Methods and Materials

Protoplasts from *Aspergillus nidulans* strains 2-35 (*paba*, *y*) and 2-124 (*argB*) and *A. alliaceus* were diluted to give 2 x 10^8 protoplasts per ml osmotic stabilizer. An equal volume of agarose (1.2% SeaPlaque LGT, 50mM Na_2EDTA, 0.6M KCl) was then added to the protoplasts and mixed gently prior to allowing to set in a BioRad sample mould on ice. Samples were lysed overnight at 50°C in NDS buffer (see Chapter 5), and washed with 50mM Na_2EDTA at least 5 times over 8 hours, and stored at 4°C in 0.5M Na_2EDTA.

An attenuated strain of *Candida albicans* was grown in shake flasks following the method of Snell & Wilkins (1986). Plugs were prepared by lysis *in situ* of intact yeast cells (Snell & Wilkins, 1986) and by embedding protoplasts according to the method of Vollrath & Davis (1987).

Electrophoresis was carried out at 8°C using a BioRad CHEF-DR^{II} system. Gels were cast using BioRad chromosomal grade agarose in 0.5x

TAE or TBE (see Chapter 5). After electrophoresis was completed, gels were stained and destained as in Chapter 5.

Results

Banding patterns obtained by CHEF gel electrophoresis, and conditions employed for *Aspergillus* species and *C. albicans* are given in Fig. H. Estimated chromosome sizes are given in Table H (details for size estimations are given in Chapter 5).

TABLE H. Chromosome Numbers and Estimated Sizes for *Aspergillus* spp. and *Candida albicans*

SPECIES/STRAIN	BAND NUMBER AND ESTIMATED SIZE (Mb)									GENOME
	1	2	3	4	5	6	7	8	9	SIZE (Mb)
A. alliaceus	>6*	>6	>6	5.8	3.9					>27.70
A. nidulans 2-35	>6	>6	>6	5.4	4.7	3.8	1.3			>33.20
A. nidulans 2-124	>6	>6	5.8	5.3	4.0	3.5				>30.60
C. albicans (cells)	3.8	3.1	2.1	1.85	1.2	1.1	1.0			14.15
C. albicans (protoplasts)	4.5	3.8	2.9*	1.95*	1.85*	1.3	1.2	1.1	1.0	26.30

*Possible doublet as estimated from fluorescence intensity.

Discussion

Difficulties which arise during molecular karyotyping often involve size determination of the bands separated, especially if greater than 2.2Mb, the length of the largest *Saccharomyces cerevisiae* chromosome. *C. albicans* and *A. nidulans* were thought to be quite suitable as size markers as these species have previously been characterized to have chromosomes in the range 1-10Mb (Snell & Wilkins, 1986) and 2.9-5Mb (Brody & Carbon, 1989) respectively.

However, problems with this approach were soon apparent as slight chromosome length polymorphisms were seen between strains of *A. nidulans* and the size estimations here differed to those previously reported (Brody & Carbon, 1989). In addition, variability between samples of *C. albicans* prepared from cells lysed *in situ* and the protoplast method gave great differences in banding patterns although

prepared from the same strain at the same time. Sample plugs made from protoplasts had a higher DNA content which resulted in band broadening (Cantor *et al.*, 1988b) and gave an additional band of high molecular weight not present in the sample made from intact yeast cells.

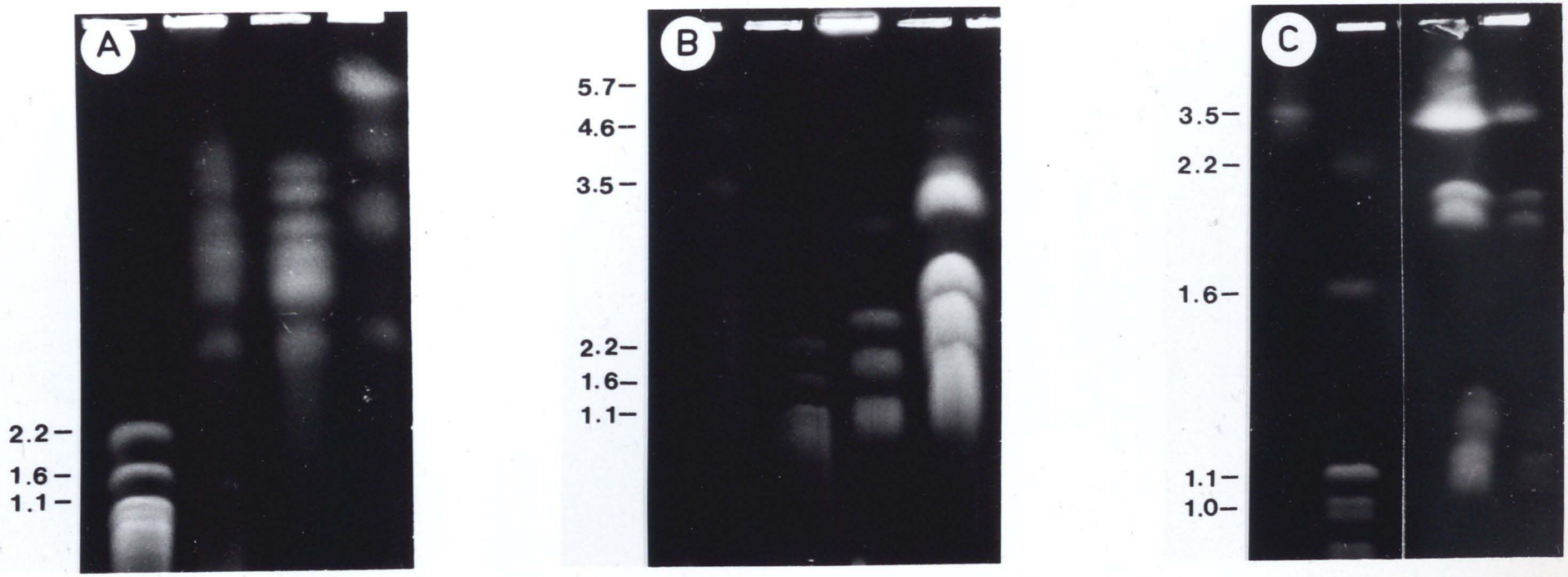
Another result of this study was the possible application of PFG to taxonomic relationships in the Aspergilli, as the two species investigated here had clearly different chromosome numbers and sizes. The only other *Aspergillus* spp. so far used in such a study has been *A. niger* which was found to have 4 chromosomes although no details were given on their size (Debets *et al.*, 1990). Also structural differences between heterokaryon compatibility groups which limit hyphal anastomosis of any one species (Croft, 1985) may be investigated. Extensive taxonomic studies by this means has until recently been limited to several yeast species (Johnston & Mortimer, 1986; Steensma *et al.*, 1988; Zimmermann *et al.*, 1988) and filamentous fungi are clearly under represented in such studies.

Figure H Molecular Karyotyping of *Aspergillus* spp. and *Candida albicans*

A. Lane 1, *Saccharomyces cerevisiae*; 2, *A. nidulans* 2-124; 3, 2-35; 4, *A. alliaceus*. Electrophoresis was carried out for 138 h with a pulse-time ramp of 3000-900secs, at 50V, in 0.7% agarose and 0.5x TAE.

B. Lane 1, *Schizosaccharomyces pombe*; 2, *S. cerevisiae*; 3, *C. albicans* (cells lysed *in situ*); 4, *C. albicans* (protoplasts lysed *in situ*). Electrophoresis was carried out for 156 h with a pulse-time ramp of 3000-900secs, at 50V, in 0.7% agarose and 0.5x TBE.

C. Lane 1, *Sch. pombe*; 2, *S. cerevisiae*; 3, *C. albicans* (protoplasts); 4, *C. albicans* (cells). Electrophoresis was carried out with two phase pulse conditions (120secs pulse-time for 24 h followed by 180secs pulse-time for 12 h), at 150V, in 0.8% agarose and 0.5x TAE.


A
2.2
1.6
1.1
B
5.7
4.6
3.5
2.2
1.6
1.1
C
3.5
2.2
1.6
1.1
1.0

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