Studies on Extracellular Polysaccharide-Degrading Enzymes Secreted by *Pleurotus Ostreatus* Grown on Lupin Hull

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Guangyu Chen (BSc)

STUDIES ON EXTRACELLULAR POLYSACCHARIDE-DEGRADING ENZYMES SECRETED BY *PLEUROTUS OSTREATUS* GROWN ON LUPIN HULL

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GUANGYU CHEN (BSc)

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DECLARATION

I certify that unless otherwise stated, the work presented in this thesis is solely my work, and has not been submitted previously, in whole or in part, for any other academic award. The results detailed in Chapter 3 have been previously presented in the following:

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

ADH	Arabitol dehydrogenase
BE	Biological efficiency
BSA	Bovine serum albumin
Cal	Calories
CBFT	Centre for Bioprocessing and Food Technology
CMC	Carboxylmethylcellulose
CMCase	Cellulase degrading carboxyl methyl cellulose
Da	Dalton
DNase	Deoxyribonuclease
DNS	Dinitrosalicylic acid
DP	Degree of polymerisation
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
FPase	Cellulase creating reducing sugars on filter paper
GA	Galacturonic acid
GYE	Glucose yeast extract broth
HMC	Hydroxymethyl cellulose
IEF	Isoelectric focusing
kDa	10^3 Daltons
K_m	Michaelis-Menten constant
LiP	Lignin peroxidase
MCC	Microcrystalline cellulose
MDH	Mannitol dehydrogenase
MEA	Malt extract agar
MG	Methyl glucopyranoside
MnP	Manganese peroxidase
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced
PAGE	Polyacrylamide gel electrophoresis

PALase	Pectate lyase
PDA	Potato dextrose agar
PEase	Pectinesterase
PGA	Polygalacturonic acid
PGase	Polygalacturonase
PLase	Pectin lyase
PMSF	Phenylmethyl sulfonyl flouride
PP	Polypropylene
RH	Relative humidity
RNase	Ribonuclease
RS	Reducing sugars
SCW	Soybean cell wall
SDS	Sodium dodecyl sulfate
SF	Submerged fermentation
SSBM	Basal medium of Shewale and Sadana
SSF	Solid-state fermentation
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
V	Velocity of enzymatic reaction
V_0	Void volume
V_t	Total volume
VUT	Victoria University of Technology
WG	Wheat grain

SUMMARY

Lupin hull is a waste resulting from the processing of the lupin seeds and it contains mainly polysaccharides, which is unusable by human and stock directly. One simple potential approach to utilise this waste is to cultivate mushrooms. Three mushroom species (*Pleurotus ostreatus*, *Lentinus edodes* and *Flammulina velutipes*) were tested for their ability to grow on ground lupin hull and this mixed with sawdust. *Pleurotus ostreatus* was best able to grow and form fruiting bodies under the laboratory conditions used.

During the study of this species grown on lupin hull, it was found that pectic enzymes were the main enzyme type secreted by *Pleurotus ostreatus* during degradation of lupin hull in submerged fermentation and solid-state fermentation although other enzymes, such as cellulase and xylanase, were also present during degradation. In submerged fermentation of lupin hull, polygalacturonase and pectate lyase showed higher activities after 14 days of growth, whereas filter paper cellulase and carboxyl methyl cellulase were higher between the first and second weeks, and xylanase activity was higher at around the 14th day of culture. When grown on a mixture of lupin hull and sawdust in solid-state conditions, all extracellular enzyme activities increased in the first two weeks of growth, then decreased before the fourth week when the mycelium had fully colonised the substrate. An increase in all of the enzyme activities was also observed at the stage corresponding to the development of mushroom fruiting bodies. The activities dropped as the development of the mushroom fruiting bodies declined. Polygalacturonase and pectate lyase activities decreased and disappeared in the basidiocarp with the development of mushroom fruiting bodies, whereas filter paper cellulase, carboxyl methyl cellulase and xylanase activities were detected in most parts of

mushroom fruiting bodies. Polygalacturonase activity was higher than pectate lyase during batch culture in submerged fermentation, whereas pectate lyase activity was higher in solid-state culture conditions.

Polygalacturonase and pectate lyase of *Pleurotus ostreatus* were inducible enzymes as they displayed differential synthesis on different carbon sources. Pectate lyase was produced during growth on most carbon sources, whereas polygalacturonase was only secreted during growth on the polymeric substrates. Polygalacturonic acid, pectin, arabic acid and lupin hull were better inducers for both enzymes, whereas galacturonic acid was a good substrate for fermentation of pectate lyase but not for polygalacturonase.

Biochemical properties of polygalacturonase and pectate lyase were also studied. The optimum pH for polygalacturonase activity was 5.0 and for pectate lyase activity was 8.6. The optimum temperatures for polygalacturonase and pectate lyase were 55°C and 45°C, respectively, as determined by assays based on reducing sugar production. The optimum temperature for pectate lyase was 40°C when using an assay based on product formation as detected by scanning at 235 nm. pI of polygalacturonase was 7.5 (as determined by IEF in the Rotofor cell), whereas IEF of pectate lyase showed two peaks of activity corresponding to pI value of 4.5 and 7.0. The products of pectate lyase degradation of polygalacturonic acid showed an absorbance at 235 nm, whereas products of polygalacturonase did not show a similar absorbance peak. Scanning degradation products of pectin treated with partially-purified enzyme indicated that the lyase was a possible pectate lyase rather than a pectin lyase. Sephacryl S-200 chromatography and polyacrylamide gel electrophoresis analyses suggested that the molecular weights of polygalacturonase and pectate lyase were about

36,500 Da and 66,000 Da. Calcium ions were required for pectate lyase activity after partial purification of the enzyme by Sephacryl S-200 chromatography and DEAE-Sepharose ion-exchange chromatography. The concentration of calcium ions required was optimum between 0.1 mM and 0.2 mM in the assay used. Kinetic studies also showed that the initial velocity of polygalacturonase was linear, whereas a "burst-phase" was observed for pectate lyase using both the reducing sugar method and scanning method for detecting activity. At higher temperatures, polygalacturonase was more stable than pectate lyase. Heavy metal ions (*e.g.* Hg^{++} and Pb^{++}) inhibited both enzyme activities; metabolic inhibitors did not inhibit either enzyme; chelators inhibited pectate lyase more than polygalacturonase, which was consistent with the observed requirement for calcium ion for lyase activity. The K_m value of polygalacturonase for polygalacturonic acid was determined as 0.27 mg/mL.

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CHAPTER 1. INTRODUCTION

1.1 GENERAL INTRODUCTION

The vast majority of energy used by human beings comes directly or indirectly from the sun in the form of light and heat. At the outer edge of our atmosphere, an average of 1.05 X 10^{10} calories (Cal) of solar energy falls per square meter every year. About 4.5% of the solar energy first entering the atmosphere reaches the earth's surface and 0.06% of this (5.83 x 10^6 Cal/m^2/year) is utilised by photosynthetic organisms. Respiration consumes some of this energy so that a total of 4.95 x 10^6 Cal/m^2/year net energy is utilised in production of plant material (Carpenter, 1988). As a result, the net productivity of dry biomass due to photosynthesis by plants is 155.2 billion tonnes per year (Bassham, 1975). Two-thirds of this biomass production occurs on land and about one-third occurs in the oceans. Most terrestrial plant material occurs in forests (about 65%), with slightly more than 15% (about 16 billion tonnes) occurring in grasslands and cultivated lands. However, only about 360 million tonnes or 1.25% of the total plant mass from land is eventually used for human food and 2,000 million tonnes as stock feed. About 13,500 million tonnes of biomass is lost as waste from harvesting or processing operations (McHale, 1970). In many cases, these wastes occur in forms and amounts which make them attractive as raw materials for further utilisation, including bioconversion.

The type and availability of such natural wastes in any particular geographic region depend on a number of factors, such as the climate and environment, use and disuse of these wastes as fuels or/and by-products, culture of the people, as well as the type and nature of technology adopted or developed. For example, rice straw is more prevalent in south-east

Asia countries, while wheat straw and maize by-products are far more common in Europe and the Western countries. It has been estimated that 1,400 million tonnes of cereal straw and corn stalks are produced annually worldwide (see Rajarathnam and Bano, 1989). Wheat straw, soybean stover and corn stover are produced in excess of 100 million tonnes per year, while sorghum stover, oat straw and corn cobs are produced in amounts from 15 to 56 million tonnes per year in the United States of America. Approximately 200 million tonnes of agricultural wastes are produced each year in the USA, including rice hulls and sugarcane bagasse. Within Australia, 15.8 million tonnes of cereal straw, 11 to 12 million tonnes of forestry milling and logging residues and 0.6 to 1.0 million tonnes of sugarcane bagasse are generated *per annum* (Stewart *et al.*, 1979). Much of this unused resource is disposed of by burning, a method which has been increasingly subjected to criticism for the air pollution resulting and the poor economic returns (Rajarathnam and Bano, 1989).

1.2 LUPINS AND WASTES FROM LUPIN PROCESSING

Lupins belong to a genus in the Genisteae tribe of the Leguminosae family. Lupin seeds are a rich source of protein and these have been used as food and animal feed for over three thousand years, where the most important species are *Lupinus albus*, *Lupinus angustifolius*, *Lupinus luteus* and *Lupinus mutabilis* (Kyle *et al.*, 1991). Due to their market value and their ability to fix nitrogen from the air into soil, lupins are grown in rotation with wheat in Australia and they have become an important crop economically in the Australian agricultural system. With an increasing growing area in 1990/1991, it was estimated that annual lupin production would reach one million tonnes in Australia after 1991, which would make Australia a significant factor within the international supply and demand equation (Meyers

Strategy Group, 1993). The value of lupins, however, is limited because its principal usage both overseas and domestically is as stock feed, with only small quantities are used for human consumption. In order to achieve higher economic benefits, many approaches are being explored to process lupins as a protein source for human consumption, including production of bean-milk, bean protein extracts, sauces, beancurd and tempeh. Development of new processed products is being coupled with establishing strains of "sweet" lupins which have a lower alkaloid content and would be more palatable for human consumption (Kyle *et al.*, 1991). Whatever processes are developed for making food products, a certain amount of waste is generated, especially from dehulling the seeds - the first step to process the soybean into meal commercially for the products described above (Snyder and Kwon, 1987).

Like the other natural agricultural and forestry wastes, waste material produced during processing lupins is mainly lignocellulosics, being low in protein, unpalatable and having low digestibility. This limits its use as stock feed or for other purposes. However, these wastes appear in forms and amounts suitable for microbial bioconversion: with suitable pretreatment, it has potential to be used as a substrate for microbial fermentation and it could be used as the substrate for growing mushrooms, which has been reported for similar wastes (Senyah, 1988; Rajarathnam and Bano, 1989; Moyson and Verachtert, 1991). The latter bioconversion has obvious advantages, as the technology involved is low in cost, a protein-rich healthy food is produced from an otherwise unusable source and waste is minimised without environmental burden (Bisaria and Madan, 1983). Furthermore, humus fertiliser or animal feed could be obtained from mushroom compost. In Australia, 30,000 tonnes of straw *per annum* are used currently for growing mushrooms (Australian Mushroom Growers

Association Ltd, 1993). In 1993/1994, the sale of spent compost for nursery fertiliser was estimated at about $3 million for an annual mushroom production valued at more than $120 million in Australia (Anon, 1994a and 1994b).

1.3 MUSHROOMS

1.3.1 OVERVIEW

Mushrooms were at one time collected in the wild, but at present most are cultivated. The records of edible mushroom dated back to at least 1,100 B.C. (see Huang, 1987a) in China, where mushroom sprouting from soil rich in decomposed material was described. The first artificial cultivation of mushrooms - *Auricularia auricula*, (known as "wood-ear", "ear" or "Jew's ear" mushroom) - was estimated to be around 600 A.D. (see Chang, 1993). The mass artificial growth of mushrooms was developed around 1400s in China for various species, where the most successful example was *Lentinus edodes* (Shiitake, "dried mushroom", "black mushroom", "oak mushroom" or "Japanese mushroom") (see Huang, 1987a). Hardwood (oak, maple) logs were laid down and the surfaces damaged slightly using an axe. This was then inoculated with chopped mushroom, covered with leaves and/or earth and watered regularly. Mushrooms were harvested between spring and summer of the second year. This method is still used in some areas today.

Compared to the Eastern countries, Western tastes have favoured the button mushroom (*Agaricus bisporus*, "white mushroom", champignon). The culture of button mushrooms originated in Paris in areas where mushrooms were frequently obtained on used compost resulting from melon crops (Delmas, 1978). Successful cultivation was undertaken around 1810 inside the numerous caves formed during excavation of building stones or gypsum, after

it was observed that this mushroom could grow without light. One hundred years later, a method for growing mushrooms in standard houses was matured and adopted in Europe (Hatch and Finger, 1979). The cultivation of *A. bisporus* involves: preparation of a substrate (the composting or Phase 1); pasteurisation of the compost (peak-heating or Phase 2); addition of spawn (pure fungal culture grown on cereal grains as an inoculum) to the compost (spawning); covering the compost surface after it has been fully colonised by the mushroom mycelium with a layer of casing soil, or casing peat moss (casing); sporophore (fruiting body) initiation and then harvest. Many raw materials (for example, cereal straw, animal manure) and other agricultural and industrial waste products (such as cotton seed hull, cotton seed meal, grass, bagasse etc.) can served as a substrate for button mushroom cultivation (Anon, 1994a and 1994b; Teuber *et al.*, 1987).

Nowadays, mushrooms are becoming increasingly important in our diet, especially for people who have recently become much more aware of nutrition. Chemical analysis reveals that about 5 to 15% of fresh mushroom is dried material (organic and mineral), and the moisture content averages 90% (Crisan and Sands, 1978), which is comparable to the common vegetables (*e.g.* cabbage - 90%) and fruit (*e.g.* apple - 85%; grape - 89%) (Holland *et al.*, 1992). Data generated from 112 species in Japan (see Huang, 1987b) showed that in the dried mushroom, the average protein content accounted for 25%, carbohydrates 60% (sugar 52% and fibre 8%), lipid 8% and ash 7%. It is now well-established that mushrooms contain reasonable amounts of proteins, carbohydrates, minerals and vitamins: nutritionally they rank between vegetables and meats (Garcha *et al.*, 1993). Although most of the world's population lives on grains, tubers, meats and small quantities of pulses and vegetables, some human health problems have been attributed to animal products, such as obesity and

cardiovascular disease. In contrast, vegetables provide hardly any dietary proteins and proteins from cereals are generally deficient in essential amino acids (especially lysine and sulphur-containing amino acid), whereas pulses are deficient in other essential amino acid such as tryptophan (see Garcha *et al.*, 1993). Mushrooms could be a good source of protein for human consumption because they contain all of the essential amino acids as well as the most commonly occurring non-essential amino acids and amides (Crisan and Sands, 1978). In addition, mushrooms are cholesterol-free and contain virtually no fat or sodium; they supply dietary fibre and provide a good source of several important B-group vitamins, especially niacin and riboflavin. Furthermore, most mushrooms have medical attributes (Cochran, 1978). For example, many edible mushrooms have been used traditionally in China and Japan for their medicinal and tonic properties. Mushrooms were prescribed in China for lowering blood pressure, enhancing the human immune system and minimising asthma. A well-known observation that oyster mushrooms reduce serum cholesterol was recently found to be related to a mushroom component which increases activity of a serum enzyme (cholesterol 7-α-hydroxylase) which lowers the serum and liver cholesterol (Bobek *et al.*, 1994). Other studies showed that some components of basidiomycetes have anti-cancer effects, where most of the active components were β-glucans, PSK (a protein-bound polysaccharide preparation) and PSP (a peptide-bound polysaccharide preparation) (see Chang, 1993). In Japan, PSK is reported as the top-selling anti-cancer drug and its annual sale in 1987 reached $US358 million and accounted for 25.2% of the total sales of anti-cancer drugs (Fukushima, 1989). Lentinan, an extract from the mushroom *L. edodes*, was listed eighth in annual sales at $US31 million (Fukushima, 1989). Anti-tumour polysaccharides were also isolated and studied recently from mushroom *Pleurotus* species (Zhang *et al.*, 1994).

Increased awareness of the benefits of mushrooms has resulted in a dramatic increase in world demands for mushrooms in recent years. World production of cultivated edible mushrooms increased from 2.176 million tonnes in 1986 to 4.273 million tonnes in 1991 (Chang, 1993), representing an increase in mushroom production of 96.4% or an annual increase of 32.1%. Although production of all cultivated mushroom species increased during this period, *Agaricus* species were still the dominate species under cultivation and production increased from 1.215 million tonnes in 1986 to 1.590 million tonnes in 1991, representing an increase of 30.9%. Other species, such as members of *Auricularia*, *Tremella* ("silver ear", "jelly mushroom"), *Flammulina* ("winter mushroom") and *Lentinus*, also increased in their production. The most promising species are members of *Pleurotus*, with a total production of 0.169 million tonnes in 1986 which ranked it fourth in production in 1986 and second (to *Agaricus* species) in 1991, with a production of 0.917 million tonnes. This represented an increase of 442.6% in production. This tremendous jump reflected not only its characteristic pleasant flavour and acceptance by consumers, but its biological ability to grow over a wide range of temperatures on various natural lignocellulosic wastes without the need of pre-fermentation of raw materials (equivalent of Phase I of button mushroom cultivation). *P. ostreatus* is the commonest species cultivated artificially (Wood and Smith, 1987).

Mushroom cultivation began in Australia in the 1930's. With the improvement of techniques and strong demand of the market, the Australian mushroom industry has become an intensive horticultural industry with an annual production valued at more than $120 million in 1993/1994 at farm gate. Consumption of cultivated mushrooms in Australia has increased steadily in the last two decades. The average consumption of all mushrooms in 1992/1993

was 2.16 kg/person per year, up from an estimated 1.48 kg in 1984/1985. This compares very favourably to USA figures of 1.77 kg *per capita* in 1992. Mushrooms are the third most valuable vegetable crop in Australia, after potatoes and tomatoes. A recent government study of new horticultural industries identified the mushroom industry as one of the three most successful new industries in Australia. The industry has been growing at a much faster rate than the national economy as a whole, and domestic mushroom production has expanded at an average annual rate of about 10% since 1974. In 1992/1993, more than 30,000 tonnes of mushrooms were grown by 80 commercial mushroom producers compared to 6,000 tonnes in 1974/1975. Industry figures show that in excess of 1,800 people are employed directly by the mushroom industry. Most of the mushrooms produced in Australia are common white mushrooms (*A. bisporus*). It is estimated that common white mushrooms comprise 98% of the market. However, there is a growing demand for specialty mushrooms (*e.g.* Shiitake, oyster, straw mushroom *etc.*) and niche markets will continue to develop for these products over the next few years (Australian Mushroom Growers Association Ltd, 1993; Anon, 1994a and 1994b; Cho and Nair, 1987).

1.3.2 *PLEUROTUS OSTREATUS*

P. ostreatus belongs to the Amastigomycota division, Basidiomycetes class, Agaricales order and Tricholomataceae family. The genus is taxonomically difficult because of variability in fruiting body morphology in several species, resulting in species being described under more than one name, especially from different regions of the world. The taxonomic disputation has also created ambiguous name. For example, *Pleurotus florida* was considered as *P. ostreatus* or *Pleurotus pulmonarius* (Hilber, 1989); several commercial strains labelled *Pleurotus sajor-caju* have proven to be incorrectly named as they are inter-compatible with

P. pulmonarius (Hilber, 1989), whereas it has been suggested that other strains of *P. sajor-caju* are based on either *Pleurotus cystidiosus* or *Pleurotus abalonus* or a related species (Nair and Kaul, 1980). It is not my attention to discuss the taxonomy here, but the following information related to *Pleurotus* species may contain some names from the original literature where nomenclative was inappropriately applied.

Notwithstanding the taxonomic confusion surrounding *Pleurotus*, *P. ostreatus* was undoubtedly regarded as an independent species and is the commonest species cultivated within the genus (Zadrazil, 1978; Wood and Smith, 1987). It is commonly known as "oyster mushroom" (Wood and Smith, 1987), however, some authors regarded "oyster mushroom" as a collective term for mushrooms in the genus *Pleurotus* (Hay, 1985). In nature, *P. ostreatus* is a wood-destroying saprophytic fungus and is widespread in the temperate zones. It fruits in autumn and winter at temperatures up to 15°C to produce a pileus which is stemmed at the side, is shell-, spatula-, or tongue-shaped and later depressed. It grows to 5 to 15 cm across and is of grey, grey-brown, or slate-grey colour (Zadrazil, 1978). However, the fruiting bodies can be varied in colour and the shape depends on the strain plus growth conditions in artificial cultivation (Wood and Smith, 1987).

Like other members of basidiomycete, *P. ostreatus* reproduce sexually by the fusion of hyphae of different mating types to form uninucleate and haploid spores. The spores form single mating types of hyphae when the conditions are favourable; hyphae fuse again when two different mating types meet each other (at this stage, two nuclei present in special hyphal cell called dikaryons), then a visible fruiting body (basidiocarp) appears to form new spores.

The sexual characteristics of mushrooms and the application of this in breeding have been well reviewed (Eger, 1978; Raper, 1978; Wu, 1987).

The word "mushroom" refers to its basidiocarp: a complex fruiting structure involved in producing its spore. The typical basidiocarp consists of a stipe (stalk), lamellate hymenophore (lamellae or gills) and a pileus (cap). The stipe is a sterile structure which elevates the hymenophore to a degree sufficient to allow most of the spores to fall free of the basidiocarp and be carried to new locations by air currents. The stipe is basically hyphae. The pileus is a structure bearing lamellae. The lamellae are the place where basidiospore produced on the outside of a specialised, spore-producing structure (basidium). Each basidium bears four basidiospores and each basidiospore is haploid and results from meiotic divisions of a diploid cell at the tip of the basidium (Alexopoulos and Mims, 1979; Wu, 1987).

Cultivation of *Pleurotus* species was first described by Falck in 1917 where the mushrooms were grown on tree stumps and logs (see Zadrazil, 1978). An important innovation in cultivation was achieved in late 1950s on sawdust (Block *et al.*, 1958) and on mushroom compost (Block *et al.*, 1959). Mass production of *Pleurotus* substratum on a straw base was first carried out in 1960s (Bano and Srivastava, 1962). The foundation for the industrial production of substratum and fruiting bodies was developed in 1970s (see Zadrazil, 1978).

When compared with the methods applied to cultivation of *Agaricus* species, the requirements are simpler and less costly. Although techniques involved in growing *Pleurotus* might be

various, for most systems a pasteurisation of substrate ingredients is necessary so that competitor organisms are controlled. Low cost systems are applicable to this species, but in recent years some advances have been made in production methods. The safest and most common system is a variation of the Till method used in mushroom cultivation (Zadrazil, 1978; Hayes, 1985; Pettipher, 1987). This method is based on the use of polythene sacks, arranged in layers in an environmentally controlled growing room, in which temperature, aeration and humidity can be controlled. When incubated at the optimum temperature (about 25°C) for mycelial growth, colonisation of the substrate is rapid, around two to three weeks. When the substrate is fully colonised, the plastic containers are opened, exposed to light, the growing room aerated and temperatures adjusted to the optimum for fruit formation (about 20°C). Relative humidity in the growing atmosphere is also important and is maintained between 80 and 95% from the initiation of mushroom fruiting body formation until being harvested before maturity.

Although straw is the most popular substrate, various other wastes are also commercially used for growing *Pleurotus* species depending on availability. A number of publications have reported screening different wastes (Jandaik and Kapoor, 1976; Chang *et al.*, 1981; Bisaria *et al.*, 1987a and b; Calzada *et al.*, 1987; Madan *et al.*, 1987; Pettipher, 1987; Senyah, 1988; Silanikove *et al.*, 1988; Biacs *et al.*, 1989; Rajarathnam and Bano, 1989; Azizi *et al.*, 1990; Moyson and Verachtert, 1991). Successful growth and yield of *Pleurotus* have been demonstrated on the following substrates: rice straw and bran, wheat straw, banana pseudostems and leaves, cotton waste, cotton stalk and seed hull, wood, pulpmill sludges, cocoa shell, apple pomace, wastepaper, tea leaves, sawdust, maizecobs, coffee pulp and

bagasse. Cultivation on a commercial scale has been proven to be economical using rice straw, wheat straw, maizecobs, cotton waste, sawdust and bagasse. To my knowledge, growing *Pleurotus* on beanhull or pulse hull has not been reported yet.

Like other fungi known as decomposers in nature, *Pleurotus* species are devoid of chlorophyll. It derives nutrients by breaking down the waste products. This capacity is attributed to secretion of a range of degradatory enzymes. Once the wet substrate is available for it, the existing mycelial cells of the mushroom start degrading the substrate by secreting hydrolysing and oxidising enzymes. The cells then biotransform the degraded materials and multiply in number, resulting in growth culminating in the production of fruiting bodies.

In order to stimulate higher bioconversion and to increase the efficiency of turnover as measured by net protein yield in the form of biomass or fruiting bodies from the waste, it is desirable to have a thorough understanding of these enzymes.

1.4 STUDIES OF MUSHROOM ENZYMES

The enzymology of mushrooms has been studied intensively because this is involved in the various metabolic activities of fungi. Enzyme production by mushrooms has been used as a means of genetic characterisation for species identification (Hseu and Wang, 1986) and an indication of strain improvement (Babasaki and Ohmasa, 1991; Iracabal *et al.*, 1991; Wood *et al.*, 1991). Most of the enzymes studied can be classified into four categories, which are described briefly in the following sections.

1.4.1 ENZYMES WITH COMMERCIAL POTENTIAL AND ENZYMES IN INDUSTRIAL PROCESSES

A number of enzymes from mushrooms have been tested for their potential use. These enzymes include: cellulase, xylanase and phenol oxidase (Shieh *et al.*, 1979; Wood and Smith, 1988). They are of industrial importance and are commonly found in the mushroom compost (Schmitz and Eger, 1981; Wood and Smith, 1987; Khowala *et al.*, 1988; Mishra *et al.*, 1990). Cellulose is the most abundant potential source of utilisable sugar, however, is not easy to hydrolyse completely. Cellulase is a group of enzymes which can degrade cellulose into soluble sugar so that further processes would be possible. Although direct application of cellulases in industry for glucose production from cellulose hydrolysis is still under development, the use of degradatory enzymes from *Pleurotus* species has been advocated by many researchers. Some applications are described in the following.

After colonisation by the fungi and mushroom harvesting, the spent substrate is more soluble, having lower lignin content and relatively higher amounts of free sugars and amino acids. The free sugars are three to five times higher compared with the undegraded substrate due to the activities of *Pleurotus* cellulase and hemicellulase; free amino acids are two to three times higher due to the activity of mushroom proteases. Lignin content is decreased by 35%-45% after degradation by lignin-degradatory enzymes of *Pleurotus* (Rajarathnam and Bano, 1989). Due to the activity of all these enzymes, the spent substrate has higher *in vivo* digestibility and it was strongly suggested by Zadrazil (1978) and Rajarathnam and Bano (1989) that spent mushroom compost would be better than the untreated substrate as an upgraded ruminant feed. Spent compost for soil amelioration has been commercially

practiced around the world, including the use of spent compost for nursery fertiliser (Anon, 1994a).

Other investigations on the potential applications of mushroom enzymes have been concerned with the hydrolysis of lignocellulosic substrates for their further utilisation in industrial processes. The possibility of utilising straw degraded by *Pleurotus* for production of biogas was studied by Muller and Trosch (1986), who found that *Pleurotus*-treated straw released high levels of sugars due to enzymatic hydrolysis. Bisaria *et al.* (1983, 1990) also found biological pretreatment by *P. sajor-caju* enhanced biogas yields by 54% using rice straw and by 38.8% using paddy straw. Another study (Hatakka, 1983) showed that wheat straw biodegraded by *Pleurotus* species over 14 days doubled the yield of free sugars compared with the undegraded substrate, which suggested that straw degraded by *Pleurotus* species could be used for the further production of fermentation products such as single cell proteins or ethanol. *Pleurotus* species have been used also for pretreatment of lignocellulosic substrates prior to further degradation by other fungi. Azizi *et al.* (1990) found that production of cellulase and xylanase by *Trichoderma reesei* during solid-state fermentation (SSF) of bagasse as substrate was greater if the bagasse had been degraded by a *Pleurotus* species first. Similarly, cultivation of *P. ostreatus* on a substrate consisting of used tea leaves and waste paper was found to provide the correct nutritional conditions for the subsequent growth of *A. bisporus* due to decomposition of cellulose, hemicellulose and lignin by this previous cultivation (Harsh and Bisht, 1984; Mayes, 1978). Polysaccharide-degrading enzymes of *Pleurotus* species have also been tested for treatment of dew-retted flax fibres (Sharma, 1987). Xylanase, polygalacturonase (PGase) and laccase were present at high activities along with minor amounts of pectin lyase (PLase) and cellulase in extracts of

Pleurotus, compared with other enzyme sources. The enzyme-treated roves produced high quality yarns compared with the yarns spun from the untreated roves. Polysaccharide-degrading enzymes of mushrooms will be reviewed in Sections 1.5.2, 1.5.3 and 1.6.3.

Delignifying enzymes of *Pleurotus* has been applied in the cardboard/paper industry. A patent lodged by Eisentan (1982) described a method to delignify lignocellulosic materials using *P. ostreatus*, where the product was suitable for paper or board manufacture. This process was claimed to be less expensive to operate than a chemical pulping process and avoids pollution problems. Decolourisation of spent Kraft liquor by *Pleurotus* was studied by Livernoche *et al.* (1983), who showed that *P. ostreatus* and *Pleurotus versicolor* removed 40% of the colour (which arose mainly from lignin-degradation products) after eight days of growth. Application of ligninolytic fungi and enzymes to bioremediation of environments polluted by industrial wastes was suggested because they have potent activity to degrade organopollutants, including chlorinated phenols from lignin found in pulp-bleaching plant effluent, heterocyclic dyes, polycyclic aromatic hydrocarbon and others (Hammel, 1989). Other mushroom polyphenol oxidases have also been investigated for application in degrading phenolic substances (Aitken *et al.*, 1994). *Agaricus* species tyrosinase immobilised on controlled-pore glass was tested for measuring the concentration of phenolic substance in polluted water and drinking water (Zachariah and Mottola, 1989).

In some commercial processes, the presence of mushroom enzymes is not desirable. Studies on mushroom tyrosinase showed that some phenolic antioxidants (such as 2,4,5-trihydroxybutyrophenone and 2,3,4-trihydroxyacetophenone) used as food additives could react with this enzyme resulting in reduced antioxidant efficiency and unfavoured

colour development. It was recommended that these chemicals should not be used as antioxidants in foods with high tyrosinase activity (Kahn, 1990; Kahn *et al.*, 1991). In the commercial preparation of cooked fish meat gel (kamaboko, a traditional Japanese food), small amounts of mushroom *A. auricula* are often added to the gel to mask the fish odour. However, it was observed that the addition of uncooked mushroom to the gel decreased the gel strength, lowering its commercial value. It was shown that with casein as substrate, *A. auricula* has a proteinase and this acted on fish myofibrillar proteins, including myosin, actin and tropomysin. In kamaboko containing uncooked *A. auricula* mushroom, fish proteins were hydrolysed and gel strength decreased. When the mushroom was added at 90°C, neither the lowering of gel strength nor the hydrolysis of proteins occurred (Makinodan and Hujita, 1990).

1.4.2 ENZYMES RELATED TO THE QUALITY OF MUSHROOMS POST-HARVEST

At the post-harvest stage, mushrooms may undergo tissue browning, textural changes and nutrition loss, including protein or polynucleotide degradation and carbohydrate conversion. Enzymes of mushrooms are directly involved in these changes. Enzyme fractions which decompose nucleic acids and related substances were isolated and studied from the fruiting bodies of *L. edodes*, *Tricholoma matsutake*, *Flammulina velutipes* and *A. bisporus* (Mori *et al.*, 1970; Min *et al.*, 1992). Effects of γ-irradiation on the stability and activity of nucleic acid-related substances and enzymes in *Psalliota bisporus* and *L. edodes* were also examined (Mouri *et al.*, 1968). Their ribonuclease, phosphodiesterase and phosphomonoesterase activities were little affected even at the high dose of 5×10^6 rad. Trehalose was found to be the only simple sugar in the cultivated mushroom *A. bisporus* and the level of trehalose

decreased markedly on ripening. The activity of the corresponding enzyme trehalase was found to increase at the same stage (Hashimoto, 1970). Soluble carbohydrates, arabitol dehydrogenase (ADH) and mannitol dehydrogenase (MDH) were measured in tissues of fruiting bodies of *L. edodes* at different stages of development. Arabitol and mannitol accumulated in growing fruiting bodies, and arabitol increased in the stipe tissue (Minamide and Iwata, 1987). During storage at 15°C after removing the fruiting body from logs, fruiting body growth stopped and arabitol content decreased rapidly. Mannitol content, on the other hand, increased in fruiting body tissues. ADH activity was higher in pileus tissue than in the gill and stipe. ADH activity decreased, accompanying the decline in arabitol after harvest. MDH activity of the fruiting body increased sharply in gill tissue during storage. Lipoxygenase-hydroperoxide lyase is an enzyme directly responsible for the formation of the mushroom flavour 1-octen-3-ol in the *A. bisporus* fruiting body. During post-harvest storage at 12°C, this enzyme activity and 1-octen-3-ol content decreased dramatically over time (Mau *et al.*, 1992). Enzymes involved in flavour development are described in Section 1.4.3.

Activities of five enzymes involved in carbohydrate catabolism were measured in mushrooms stored for up to four days at 18°C. Glucose-6-phosphate dehydrogenase decreased rapidly during storage. Phosphofructokinase, glucose phosphate isomerase and MDH showed smaller decreases. NADPH oxidase activity showed an overall increase in storage. Glucose phosphate isomerase showed a peak in activity two to three days after harvest (Hammond 1978).

It was first reported in late 1960s that mushrooms contain large quantities of tyrosinase (polyphenol oxidase, Enzyme Commission [EC] 1.10.3.1 *ortho*-diphenol oxidoreductase)

which is responsible for brown pigment formation (Long and Alben, 1969). Tyrosinases were characterised as two types of activity responsible for: (i) the oxidation of monophenols to the corresponding *ortho*-quinones; and (ii) the oxidation of *ortho*-diphenols to the corresponding *ortho*-quinones (Long and Alben, 1969). Subsequently, tyrosinase activity was studied intensively in *Agaricus* and the other species (Fujimoto *et al.*, 1970a and 1970b). Other enzymes, such as amylase, lipase, proteinase, peroxidase, catalase and polyphenol oxidase are suspected of being involved in changes in the fruiting body flavour, food nutrients and browning of *Pleurotus*, *Lentinus* and other mushroom species (Yoshimoto *et al.*, 1988; Kadiri and Fasidi 1990; Fasidi and Kadiri, 1991).

Different methods have been tried to prevent enzymatic changes after harvest, including irradiation, chemical inhibition and blanching (Fujimoto *et al.*, 1970a and 1970b; Fang *et al.*, 1976; Wedzicha *et al.*, 1987; Albisu *et al.*, 1989). Blanching (two minutes in boiling water) inhibited peroxidase and diphenol oxidase activities, but had adverse effects on texture (Prestano and Fuster, 1982). Irradiation reduced polyphenol oxidase activity and improved the quality of mushrooms in the laboratory (Nayga-Mercado and Alabastro, 1989). However, it is impractical because mushrooms will still deteriorate rapidly at temperatures of 15°C-20°C even if they have been irradiated (Blackholly, 1989).

1.4.3 ENZYMES INVOLVED IN MUSHROOM FLAVOUR FORMATION

Mushroom odour attracted people's attention possibly as early as when they were adopted as food. In 1934, some odour properties of fungal fruiting bodies were reported (see Jong and Birmingham, 1993). However, enzymatic studies on mushroom flavour did not start until the 1970s (Yasumoto *et al.*, 1971; Fujimoto *et al.*, 1976; Iwami and Yasumoto, 1982;

Iwami *et al.*, 1974a and b), possibly due to the lack of availability of suitable instrumentation for research. The presence of enzyme activity capable of converting lentinic acid to lenthionine, the principal aroma compound of Shiitake mushrooms, was firstly demonstrated in the protein fraction prepared from fresh fruiting bodies in the early 1970s (Yasumoto *et al.*, 1971). The pathway of enzymatic formation of lenthionine in *L. edodes* from the non-volatile precursor (lentinic acid) was established by Yasumoto *et al.* (1976). Lentinic acid is first converted into glutamyl-lentinic acid by the action of γ-glutamyl transpeptidase, which catalyses a rate-limiting step in the sequence of aroma formation. The resultant cysteine sulphoxide is decomposed to pyruvic acid, ammonia and thiolsulphinate by the catalysis of a specific C-S lyase in *L. edodes*. The thiolsulphinate may spontaneously degrade into cyclic polythiepanes including lenthionine, with concomitant release of formaldehyde and acetaldehyde. C-S lyase I and II were further purified and characterised from Shiitake mushroom. Isoelectric points of lyase I and II were 4.15 and 4.65, respectively, with pH optima both at approximately 8.7. The inhibitors and enzyme substrates were also studied (Iwami and Yasumoto, 1980).

The other main mushroom flavours belong to the C-8 volatiles group commonly known as mushroom alcohols, including 1-octen-3-ol. Early research showed that artificially synthesised 1-octen-3-one was converted into 1-octen-3-ol during incubation with an extract from the fruiting body of *A. bisporus* (Chen and Wu, 1984a and b). Recent studies demonstrated the enzymatic formation of 1-octen-3-ol in the cultivated mushroom, *A. bisporus* (see Hadar and Dosoretz, 1991). The metabolic pathway involves three major steps regulated by two key enzymes: lipoxygenase and hydroperoxide-lyase. The first step is the hydrolysis of lipids to free fatty acids (mainly linoleic and linolenic acids) by acyl hydrolase.

The second step involves the hydroperoxidation of polyunsaturated fatty acids by lipoxygenase, which catalyses oxidation of fatty acids to produce conjugated hydroperoxidiene derivatives. The third step is postulated to involve lipoxygenase in combination with hydroperoxide lyase. Optimal activity of lipoxygenase-hydroperoxide lyase enzymes for 1-octen-3-ol formation occurred over the pH range 5.0 to 7.0 and the highest amount of 1-octen-3-ol was produced at pH 6.0 in the reaction. During the crop cycle, 1-octen-3-ol content of mushrooms varied from 19.3 to 37.2 p.p.m. More 1-octen-3-ol was produced in the gills than other morphological tissues (Mau *et al.*, 1992).

1.4.4 EXTRACELLULAR DEGRADATORY ENZYMES OF MUSHROOMS

This category includes various kinds of enzymes which are related to the reaction of the fungi with their environment. As stated before, fungi are known as decomposers in nature. Their ability to survive in nature depends on the presence of other dead or living organisms, where fungi obtain energy and nutrition by degradation and utilisation of those organisms. They must have a strong capacity to secrete the extracellular enzymes to break down macromolecules into smaller units which can penetrate the tough cell walls of fungi for further utilisation inside the fungal cell to support growth.

Mushrooms have been long known to produce proteases, lipases, nucleases and polysaccharidases to degrade the proteins, lipids, nucleotides and polysaccharides, respectively. DNase (EC 3.2.21.1) and RNase (EC 3.1.27.5) were detected qualitatively in the supernatant of *A. bisporus* cultured in phosphate-free medium (Fermor and Wood, 1981). Three types of proteinases (acid, neutral and alkaline) were found in *A. bisporus* (Fermor and Wood, 1981; Kalisz *et al.*, 1987). It was also demonstrated that *A. bisporus* can produce

enzymes (β-N-acetylmuramidase, β-acetylglucosaminidase and laminarinase) to lyse the cell wall of bacteria, fungi and actinomycete mycelium (Fermor, 1983; Grant *et al.*, 1984). Lysis of fungal cell walls was also reported in relationship to chitinase and glucanase of *P. ostreatus* (Schmitz and Eger, 1981).

Despite the numerous reports on extracellular enzymes as exemplified above, more attention has been given to mushroom enzymes related to lignocellulosics degradation because of their role in utilisation of substrate. This subject is therefore reviewed separately in the following section.

1.5 LIGNOCELLULOSE-DEGRADING ENZYMES OF MUSHROOMS

In nature, cellulose microfibrils are bound mostly through a hemicellulose and lignin (a complex phenylpropanoid polymer) network into a semi-rigid matrix, called lignocellulose (Rajarathnam and Bano, 1989). The complex structure of lignocellulose limits the use of plant polysaccharides as chemical feedstocks or ruminant feed (Crawford, 1981; Platt *et al.*, 1984). Although a wide range of microorganisms can degrade cellulose, far fewer have affect on natural lignocellulose substrate because lignin limits access to cellulose and hemicellulose (Rajarathnam and Bano, 1989). Thus, degradation of lignocellulose requires not only enzymes which digest polysaccharides (including cellulose and hemicellulose), but also enzymes which remove lignin.

1.5.1 LIGNIN DEGRADATION

Lignin is a polyphenolic macromolecule which constitutes up to 30% of plant fibres in nature (Detroy *et al.*, 1981). It is relatively higher in carbon and hydrogen and lower in oxygen

than cellulose and hemicellulose and has the highest heat value of the three components (Detroy *et al.*, 1981).

Pleurotus, *Agaricus* and other mushroom species preferentially degrade lignin during growth on a lignocellulosic waste so that the degraded substance becomes more bright (white) in colour due to the exposed cellulose, where this is known as white-rot fungal decay. The lignolytic system of the white rot fungus *Phanerochaete chrysosporium* has been particularly well characterised (Gold and Alic, 1993). It is presently known to consist of two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP). The important difference between these peroxidases is in the nature of the substrate. LiP catalyses the oxidation of non-phenolic lignin model compounds such as veratryl alcohol to veratryl aldehyde. MnP catalyses the oxidation of lignin and lignin derivatives and a variety of phenolic lignin model compounds; oxidation of lignin and other phenols by MnP is dependent on free manganous ion. The other enzyme, commonly found in many mushroom species, is laccase (a polyphenol oxidase), which catalyses the one-electron oxidation of diphenols to phenoxy radicals. Other mushrooms, such as *Poria cocos*, prefer to degrade polysaccharide, or cannot degrade lignin, resulting in the residue being brown in colour: this is known as brown-rot fungal decay.

Lignin degradation by *P. ostreatus*, *L. edodes* and *A. bisporus* has been investigated by several workers (Durrant *et al.*, 1991; Kerem *et al.*, 1992). Using ^{14}C-lignin in culture media, $^{14}CO_2$ accumulated in the head space, which indicated the degradation of radioactively-labelled lignin and synthetic lignin polymers by mushrooms. MnP has been demonstrated in SSF of *A. bisporus*. In contrast, reports on the LiP and MnP produced by

P. ostreatus have been contradictory. By using cotton stalk as carbon source, Kerem *et al.* (1992) claimed that no LiP activity was detected in either the water extract of SSF or liquid cultures of *P. ostreatus*. Masaphy and Levanon (1992) also reported that no LiP activity was found in submerged fermentation (SF) of *P. ostreatus* with various carbon sources. However, both LiP and MnP were found in other studies of *Pleurotus* species under commercial cultivation (Buswell *et al.*, 1993). Furthermore, MnP from *P. ostreatus* was isolated and purified by Becker and Sinitsyn (1993).

The commonly-recognised phenol oxidase produced by *P. ostreatus*, *L. edodes* and *A. bisporus* is laccase (polyphenol oxidase). It catalyses the oxidation of *o*- and *p*-diphenols and of diaminoaromatic compounds. Laccase was first identified in several mushroom varieties by Bertrand in 1896 (see Zawistowski *et al.*, 1991) and the activity of laccase has been found in SSF and SF of *A. bisporus*, *P. ostreatus*, *L. edodes* and other species (Turner, 1974; Turner *et al.*, 1975; Iwahara *et al.*, 1981; Jablonsky, 1981). Although some of them have been purified and their properties well characterised (Wood *et al.*, 1990), the roles of laccase in lignin degradation is still not clear (Rajarathnam and Bano, 1989). It has been suggested that laccase is involved with the release of recalcitrant or bound nutrients linked to phenolic polymers and thus it contributes to the "selectivity" of mushroom compost as a growth medium for the fungus (Wood, 1993). The evidence that suggests laccase has a role in the degradation of lignin is that laccase-deficient strains of *Sporotrichum pulverulentum* are unable to degrade polymeric dyes, which are used as substrates for the assay of fungal lignin-degradation systems and studying the involvement of laccase in demethoxylation processes (Ander *et al.*, 1983).

The laccase of *A. bisporus* is possibly the most studied one compared to the other mushrooms. The molecular properties of laccase protein from both liquid and compost cultures have been extensively analysed. The enzyme is abundant, forming over 2% of cell protein and its various molecular forms have been purified from malt extract medium and from compost cultures at maximal activity (high activity form) and low activity (low activity form). The purified liquid culture form is a laccase of 100 kDa molecular weight (MW), with substrate specificities, kinetic properties and inhibitor profile similar to other fungal laccase but with lower copper content and is yellow. The high activity form of laccase from compost has considerable enzyme and structural similarities to the liquid culture form of the enzyme, but has more copper and consequently is blue. The low activity form of laccase differs considerably: it is nearly copper deficient and has a very different amino acid profile. Studies on biosynthesis in liquid culture show that a single major excreted protein of 68 kDa is made. The higher MW of the purified enzyme may be due to dimerisation. Poly(A) containing mRNA from these cultures translates an immunoprecipitable product of 57 kDa, consistent with a carbohydrate (glycosylation) level of 15% for the final form of the exported enzyme (Wood, 1993). Two laccase genes in *A. bisporus* were also identified. A cDNA library was constructed using mRNA from 11-day-old mycelium of *A. bisporus*. Comparison of the deduced amino acid sequences with that of other cloned fungal laccases, and with the ascorbate oxidases from higher plants which have similar sequences, shows that whilst some sequence is absolutely conserved at and around the amino acid residues involved in copper binding, the overall sequence similarities are low (Perry *et al.*, 1993).

1.5.2 CELLULOSE DEGRADATION

Cellulose is the most abundant organic compound in our biophere, constituting about 50% or more of the carbon in vegetation. It is a homogeneous polymer of glucose units linked by β-1,4 glucosidic bonds. Reactive -OH moieties projecting from both sides of the cellulose molecule allow it to link up to adjacent molecules, thereby forming stable, tough fibres, known as fibrous crystal (Goksoyr and Eriksen, 1980).

Many microorganisms have the enzymes necessary to break the glucosidic bonds in cellulose. Early theory on the degradation of microcrystalline cellulose (MCC) is based on the C_1, C_x concept, where C_1 was an enzyme component capable of forming shorter, linear cellulose chains from native cellulose, then these chains could be further hydrolysed by C_x (Reese *et al.*, 1950). However, C_1 and C_x have not been purified, whereas various individual enzymes degrading β-1,4 glucosidic bonds have been purified and studied and aspects of the biochemistry of cellulases have been reviewed by several authors (*e.g.* see Goksoyr and Eriksen, 1980; Klyosov, 1990; Wood, 1991). The degradation of crystalline cellulose is a complex process, requiring the participation of several enzymes. It is now well established that there are at least three different types of cellulolytic activity (Goksoyr and Eriksen, 1980), according to their mode of action and substrate specificity:

a. Endoglucanases (EC 3.2.1.4, endocellulase, Carboxyl Methyl Cellulase [CMCase]), which attack cellulose molecules at random, combining at multiple sites within the molecule;

b. Exoglucanases (EC 3.2.1.91, exocellulase), which attack cellulose chains from the non-reducing end liberating cellobiose or glucose residue at each step;

c. β-glucosidases, which hydrolyse cellobiose and low-molecular mass cellulosic oligosaccharides to D-glucose.

Many cellulases have been evaluated for their ability to hydrolyse cellulose to glucose. Early experiments carried out in one mL scale showed that 30% (w/v) of glucose was produced by using concentrated enzyme from *Trichoderma viride*. However, in larger scale experiment the yields were considerably lower (Katz and Reese, 1968; Goksoyr and Eriksen, 1980). Industrial enzymatic saccharification of cellulose to produce glucose or other chemicals is under development. At present, use of cellulases has been limited to a few specific applications, where cellulases are mainly used as a component of mixed hydrolytic enzymes. Currently, cellulases are mainly used in the food, juice, brewing, stock feed, textile industries and in detergents. In brewing and cereal extraction, cellulase activity speeds up mash filtration and increases extract yield (Ward, 1985). In juice processing or plant extraction, cellulase activity supplements pectinases to increase the yield of juice extraction and speed up processing (Pilnik and Voragen, 1991). Cellulases are also used for extraction or refining, of cereal proteins, and in starch production (Hidaka *et al.*, 1993). In the textile industry, cellulase was used to remove the cotton from silk in traditional Japanese garment processing and is used for blue jean "stone-washing" to achieve the fashionable "worn" look with a soft feel and to prevent damage caused by true stone-washing, a process so-called "bio-stoning" (Hidaka *et al.*, 1993; Sato *et al.*, 1993). Nevertheless, cellulase was added to cattle feed in order to achieve higher digestibility of feed in Australia (see Goksoyr and Eriksen, 1980).

The cellulases currently used commercially are produced by strains of *Aspergillus niger*, *T. reesei*, *Penicillium funiculosum* and *Rhizopus* species (Ward, 1985). The *Trichoderma* enzyme is most effective on native and crystalline cellulose, however, the cellobiase activity in *Trichoderma spp.* is less effective due to inhibition of glucose accumulated during the saccharification. Cellobiase produced by *A. niger* is particularly suitable as a supplement to *Trichoderma* cellulases for cellulose saccharification, as it is less susceptible to end-product inhibition than the *Trichoderma* enzyme. The *A. niger* cellulase is less effective on crystalline cellulose but it has high activity of cellobiase or β-glucosidase to produce glucose as end product. The endoglucanase of *P. funiculosum* is less sensitive to inhibition by cellobiose than the *Trichoderma* enzyme.

Practically all reports on cellulase production are based on small-scale experiments, either with batch, continuous or semicontinuous cultures. Batch culture can be performed stationary or submerged with aeration by shaking or by sparges (Goksoyr and Eriksen, 1980). Cellulase is an inducible enzyme system and cellulose is generally considered to be the best inducer of the complete cellulase enzyme complex. Glucose causes catabolite repression of cellulase synthesis. With *T. reesei* QM9414, the final enzyme yields in the fermentor were found to be proportional to the initial cellulose concentration up to 8% cellulose, when cellulose was supplied as sole carbon source (Sternbert and Dorval, 1979).

Mushrooms have been known to produce cellulases for a long time and many of the enzymes have been characterised: the endocellulase of *A. bisporus* is possibly the most studied enzyme. Experiments carried out to examine the regulation of endocellulase production by *A. bisporus* on defined minimal medium showed that this was very similar to that seen for

other fungi (Manning and Wood, 1983; Wood, 1991). Extracellular endocellulase production by *A. bisporus* closely paralleled mycelial growth in cultures containing MCC. The enzyme was induced by various celluloses and cellobiose. In the presence of a cellulose inducer, glucose and cellobiose repressed production of the enzyme. Endocellulase activity in culture filtrates was inversely related to cellulose concentration in the cultures. Low activity of free enzyme in high concentrations of cellulose indicated the absorption of enzyme by cellulose (Manning and Wood, 1983). Purification of endocellulase produced by *A. bisporus* from bulk liquid cultures grown on cellulose as sole carbon source was achieved by successive ultrafiltration, ion exchange and preparative gel electrophoresis steps (Wood, 1993). The characterisation and regulation of *A. bisporus* cellulase genes have been reported. For example, a 52 kDa protein, CEL3, has been separated from the culture filtrate of *A. bisporus* during growth on cellulose. Two allelic CEL3 cDNAs have been isolated by using PCR-derived probes which showed 98.8% homology in their nucleotide sequences. The deduced amino acid sequence and domain architecture of CEL3 showed a high degree of similarity to those of cellobiohydrolase II of *T. reesei*. Recombinant CEL3 secreted by yeast showed enzymatic activity towards crystalline cellulose. *Cel3* gene expression was induced by cellulose and repressed by glucose, fructose, 2-deoxyglucose and lactose (Raguz *et al.*, 1992; Chow *et al.*, 1994; Yague *et al.*, 1994).

By comparison with *Trichoderma*, the cellulase activity of *Pleurotus* species and other mushrooms (*e.g. Volvariella volvacea*) is lower (Rajarathnam and Bano, 1989; Chang and Steinkraus, 1982). Possibly for this reason, the enzymology studies performed to date have been associated with mushroom physiology rather than for enzyme production: to my knowledge, the purification of cellulase in *Pleurotus* species has not been reported.

However, other biochemical properties have been studied. The optimal conditions for cellulase production of *Pleurotus* species were studied in SSF (Danilyak, 1981) and SF (Hong *et al.*, 1985). In SF, cellulose powder was the most effective substrate for the production of exocellulase and β-glucosidase, while CMC was good for the production of CMCase; 1% glucose completely depressed the production of cellulase. After seven days of culturing *Pleurotus* on cellulose medium in shake flasks, endocellulase showed maximum activity. When *P. sajor-caju* was grown on cellulosic material, the optimal initial pH for cellulase production was 5.0 (Madan and Bisaria, 1983). In SF of *P. pulmonarius*, addition of cotton-wheat straw to the media increased consumption of glucose and NH_4^+ by the fungal mycelium and induced CMCase plus increased β-glucosidase activity (Masaphy and Levanon, 1992).

The optimum pH values for assay of *P. sajor-caju* Avicelase, CMCase and β-glucosidase activities were reported to be 5.5, 4.5 and 6.0, respectively. The optimal temperature for the three enzymes was 40°C, and the three are stable below the optimum temperature, but unstable at temperatures higher than 50°C. The K_m values of Avicelase and CMCase were 30.77 mg / mL and 14.64 mg / mL, respectively. The K_m of β-glucosidase was 5.13 mg salicin/mL. The activities of the three enzymes were increased by Ca^{++} (10^{-2} M) and inhibited by Hg^{++} and Ag^{++} (Hong *et al.*, 1984).

1.5.3 HEMICELLULOSE DEGRADATION

Hemicellulose is the polysaccharide fraction which is soluble in alkali and is associated with cellulose of the plant cell wall. It includes the non-cellulosic β-D-glucans, the pectic substances and several complex heteropolysaccharides such as those enriched in galactose,

mannose and xylose (Dekker, 1985). Hemicellulose accounts for up to 40% of some plant materials and constitutes the next most abundant fraction of plant material (Gong *et al.*, 1981). Enzymes attacking hemicellulose are hydrolytic by nature and are referred to as hemicellulolytic enzymes or hemicellulases. Hemicellulases (EC 3.2.1) specifically degrade those glycans that make up the backbone chain of the hemicelluloses (Dekker, 1985). Typical hemicellulases are β-D-galactanases, β-D-mannanses and β-D-xylanases. Hemicellulases, like most other polysaccharide-degrading enzymes (glycan hydrolase), degrade their substrates using exo- or endo- hydrolytic attack. An exo- enzyme degrades the polysaccharide by successive removal of terminal glycose or oligosaccharide units usually from the non-reducing end of the polysaccharide chain. Endo- enzyme attacks polysaccharide in a random manner, causing multiple scission that is accompanied by a marked decrease in the degree of polymerisation (DP) of the substrate. The polymer is progressively degraded into shorter fragments until undegradable products (usually monosaccharides or disaccharides) are formed. Hemicellulases of the endo- type constitute the most common group of the hemicellulases (Dekker, 1985).

Xylanase is possibly the most studied hemicellulase type in mushrooms. It has been demonstrated in *Agaricus*, *Pleurotus*, *Lentinus*, *Volvariella*, *Flammulina* and other species (Hong, 1976; Mishra *et al.*, 1990; Cai *et al.*, 1994). Compared with cellulase activity, xylanase activity is relatively higher in *P. ostreatus* (Rajarathnam and Bano, 1989). When *P. ostreatus* was grown on rice straw medium, the optimum pH for xylanase secretion was found to be between five to six (Danilyak, 1981). The optimum pH for xylanase action was 5.0 and the enzyme was stable at pHs between 4.5 and 6.0. The optimum temperature for activity was 50°C and the activity was completely lost after 10 minutes at 70°C. Xylanase

activity was inhibited by Mn^{++}, but was stimulated by other metal ions, especial K^{+}, Mg^{++} and Ca^{++} (Hong, 1976). Purification of *L. edodes* xylanase was achieved for enzyme made during growth on a commercial oak wood medium. The major enzyme was a non-debranching endo-β-D-xylanase (EC 3.2.1.8) which was highly specific for xylans; it had a MW of 41,000 Da and a pI of 3.6. With aspen glucuronoxylan as substrate, the enzyme showed optimal activity at pH 4.5 to 5.0 at 60°C, with a K_m of 0.66 mg/mL and specific activity of 310 μmoles reducing sugars (RS)/minute/mg protein at 40°C (Mishra *et al.*, 1990).

Other enzyme activities were also demonstrated in *L. edodes* during growth on a lignocellulosic medium, these included α-L-arabinosidase (EC 3.2.1.55), β-D-xylosidase (EC 3.2.1.37 or EC 3.2.1.32), β-D-galactosidase (EC 3.2.1.23), α-D-galactosidase (EC 3.2.1.22), β-D-mannosidase (EC 3.2.1.25), α-D-mannosidase (EC 3.2.1.24), PGase (EC 3.2.1.67 or EC 3.2.1.15) and enzymes degrading glucuronoxylan, arabinoglucuroxylan or glucomannan (Leatham, 1985).

Some biochemical properties of mushroom laccase, xylanase and cellulase are summarised in Table 1.1.

1.5.4 ROLE OF DEGRADATORY ENZYMES DURING THE MUSHROOM LIFE CYCLE

A feature of mushrooms which is different from the other fungi is the formation of a basidiocarp and their life cycle. Changes in enzyme activity during the course of fruiting body formation have been regarded to distinguish differences typical of the various stages of development, from spawning until the end of cropping of basidiomycetous fruiting fungi.

Table 1.1 Some biochemical properties of mushroom lignocellulose-degrading enzyme

Enzyme and Sources of Enzyme	MW (Da)	Optimum Temp. (°C)	pI	pH Optimum	K_m (mg/mL)	Reference
Laccase						
Pleurotus ostreatus			5.94		3.0209mM	Lee, 1987.
Lentinus edodes	100,000			4.0		Leatham and Stahann, 1981.
Agaricus bisporus	100,000					Wood, 1993.
Xylanase						
Pleurotus ostreatus		50		4.5-6.0		Hong, 1976.
Lentinus edodes	41,000	60	3.6	4.5-6.0	0.66	Mishra *et al.*, 1990
Cellulase (Avicel)						
Pleurotus sajor-caju		40	5.5		30.77	Hong *et al.*, 1984.
Cellulase (CMC)						
Pleurotus sajor-caju		40	4.5		14.64	Hong *et al.*, 1984.
β-glucosidase						
Pleurotus sajor-caju		40		6.0	5.13	Hong *et al.*, 1984.
Termitomyces clypeatus	110,000	65	4.5	5.0	0.5mM	Sengupta *et al.*, 1991.

This has been the subject of investigation for more than 25 years, where the first report on an increase of enzymes (α-D-galactosidase and β-D-glucosidase) during maturation of *P. ostreatus* basidiocarps was made by Michalski and Beneke (1969). Turner (1974) found that *A. bisporus* extracellular laccase activity increased during the spawn run and reached a maximum at the beginning of fructification (fruiting body formation); similar results were obtained by Rajarathnam *et al.* (1979) for *Pleurotus flabellatus*. A large increase in cellulase activity at the time of *A. bisporus* fruiting was also reported (Turner *et al.*, 1975). By assaying several enzymes in extracts from mycelium-colonising wood meal-rice bran mixtures during growth and fruiting of *P. ostreatus*, Iwahara *et al.* (1981) reported that laccase increased during mycelial growth and then declined rapidly at the start of fruiting. Cellulase could not be detected throughout mycelial growth but rapidly increased at the fruiting stage, whereas xylanase was detected throughout mycelial growth and increased at fruiting. The rapid increase of cellulase at the start of fruiting was hindered by the addition of glucose (Iwahara *et al.*, 1981). Rajarathnam *et al.* (1979) showed that cellulase, β-D-glucosidase and xylanase increase over the first few days of *P. flabellatus* culture. Furthermore, the levels of polysaccharide-degrading enzymes gradually increased during the spawn run and showed a steep increase during fructification, where the latter were correlated with a parallel decrease in the amount of polysaccharides and an increase in free sugars (Rajarathnam and Bano, 1989). These changes implied strongly that the monosaccharides were directly related to the construction of the fruiting body. It is still unknown whether degradation of lignin enables access to polysaccharides or whether this provides energy for growth (Rajarathnam and Bano, 1989), since lignin is a high-energy-containing substrate (Detroy *et al.*, 1981).

Regulation of endocellulase activity associated with *A. bisporus* fruiting body development has also been studied by Claydon *et al.* (1988). By using different harvesting techniques, such as continuous picking or allowing large fruiting bodies to reach senescence on the production beds, it was shown that fruiting body biomass positively regulates the extracellular endocellulase activity in the substrate. It is assumed that the physiological role of endocellulase is to act on cellulose in the compost. As a result of its action, soluble carbohydrates are produced which can be assimilated directly, or after further degradation by other cellulases. When fruiting bodies subsequently form on the mycelium, the levels of intracellular mycelial carbon compounds are depleted due to translocation of these to the developing fruits. This depletion activates a putative signalling system which eventually results in more cellulase being produced to replenish carbohydrate levels in the mycelium. When fruiting bodies are harvested, there is no requirement for increased cellulase levels and enzyme activity is down regulated. Biosynthesis of endocellulase protein in compost cultures parallels expression of enzyme activity, with maximum biosynthesis occurring at the senescent stage of maximal fruiting body biomass and synthesis declines rapidly on harvesting fruiting bodies. Successive "flushes" are associated with new rounds of enzyme biosynthesis (Wood, 1993).

Although considerable attention has been given to the study of hemicellulases of mushrooms, relatively little work has been done on the pectinases of mushrooms during the life cycle. Literature in this area is reviewed in the following section.

1.6 PECTINASES

1.6.1 THE SUBSTRATE

Pectic substances are structural polysaccharides, which occur mainly in the middle lamella and primary cell wall of higher plants. Although they are present in quantities generally less than 1% of the weight of fresh plant material (see Rombouts and Pilnik, 1980), they are largely responsible for the integrity and coherence of plant tissues. The middle lamella is the cementing layer between cells in plant tissues, consisting principally of pectic substances and hemicelluloses. In the primary cell wall, cellulose microfibrils are bound through H-bonding forces through a hemicellulose-pectic substances network into a semi-rigid matrix. Therefore, degradation of natural pectic substances requires mainly pectinases, with assistance from cellulases and other related enzymes (Pilnik and Voragen, 1991).

Chemically, pectic substances are molecules with a α-1,4-D-polygalacturonide polymer, complexed with other polymers, such as highly branched L-araban, β-1,4-D-galactan and rhamnogalacturonases. The carboxyl groups of the galacturonic acid (GA) units are partially esterified with methanol. Side chains are composed of other sugars, such as D-galactose, L-arabinose, L-rhamnose, D-xylose and D-glucose (Pilnik and Voragen, 1991; Redgwell *et al.*, 1992).

1.6.2 THE ENZYMES

1.6.2.1 General properties of pectinases

Pectic enzymes occur in higher plants and are also produced by microorganisms. The endogenous pectic enzymes of plants can produce important biochemical changes which may be involved in cell elongation, plant growth, ripening of fruits and abscission of leaves (Rombouts and Pilnik, 1980; Crookes and Grierson, 1983). Microbial pectolyses play a key

role in plant pathogenesis, symbiosis, decomposition of plant deposits, digestion of plant foods, rotting processes, certain fermentations and fruit and vegetable spoilage (Rexova-Benkova and Markovic, 1976; Rombouts and Pilnik, 1980; Sharma, 1987).

Pectic enzymes are classified into two main groups, according to their mode of attack on the galacturonan part of the pectic molecule: de-esterifying enzymes (pectinesterases) and chain splitting enzymes (deploymerases). Pectinesterase (PEase, also known as pectin methylesterase) is classified as a carboxyl ester hydrolase and is systematically named as pectin pectyl-hydrolase, EC 3.1.1.11; it de-esterifies pectin, producing methanol and pectic acid. The pectic deploymerases, on the other hand, split the glycosidic bonds of their substrates either by hydrolysis (hydrolases) or by β-eliminatation (lyases). PGases (polygalacturonase) split galacturonyl linkages next to free carboxyl groups on C-6 by hydrolysis; pectate lyases (pectic acid lyases, PALase, EC 4.2.2.2 and 4.2.2.9) split glycosidic linkages next to free carboxyl groups by β-eliminatation, producing unsaturated products named 4,5-dehydrogalacturonates (Figure 1.1). Both endo- and exo-types of PGase and PALase have been found in higher plants, bacteria and fungi: the endo-types (EC 3.2.1.15 and 4.2.2.2) split the pectin chain at random; exo-PGases (EC 3.2.1.67) split off galacturonate monomers or dimers from the non-reducing end of the chain; while exo-PALases (EC 4.2.2.9) split off unsaturated dimers from the reducing end. For the PGases and PALases, the preferred substrates are pectates and low-methoxyl pectins; both of these two types of enzymes have very low activity on highly methylated pectins. Such pectins are, however, degraded by PLase (EC 4.2.2.10) or a combination of PEase with PGase or PALase. Only endo-type PLase has been found to have degradative activity against highly esterified pectins (Pilnik and Voragen, 1991; Rombouts and Pilnik, 1980).

Pectinases and their mode of action were summarised in Fig. 1.1.

All PGases so far studied from fungi have acidic pH optima ranging from 3.8-6.5, mostly around 4.5-5.0 (Sreekantiah *et al.*, 1975; Cervone *et al.*, 1978; Rombouts and Pilnik, 1980). Previous work also indicated that the optimum pH for PALase action was in the alkali range (Castelein and Pilnik, 1976; Chesson and Codner 1978; Rombouts *et al.*, 1978; Karbassi and Luh, 1979; George *et al.*, 1991; Stutzenberger, 1991), from pH 8.3 for *Bacillus polymyxa* (Nagel and Wilson, 1970) to pH 10 for *Erwinia carotovora* enzymes (Yoshida *et al.*, 1991). Only one PLase from *Aureobasidium pullulans* LV10 was reported having an acidic optimum pH value at 5.0 for its action (Manachini *et al.*, 1988).

As reviewed previously (Rombouts and Pilnik, 1980), PGases might be produced by many microorganisms in a multiple molecular form with PEase or as isoenzymes. Most of these endo-PGases have a MW of 30,000 to 35,000 Da, although a higher MW for PGase (68,000 Da by SDS gel electrophoresis and thin layer gel electrofocusing) was reported recently for the *Colletotrichum gloeosporioides* enzyme (Prusky *et al.*, 1989).

Reported pI values for PGase vary between microorganisms, from pH 3.2 for an endo-PGase isoenzyme of the commercially-available enzyme Pectinex Ultra SP-L to pH 7.1 for the *Rhizopus fragariae* enzyme and to a pI of 10.7 for the PGase from *E. carotovora* (Rombouts and Pilnik, 1980; George *et al.*, 1991; Okai and Gierschner, 1991). Most PALase have a higher pI, ranging from the reported values for *E. carotovora* of 8.9 and 10.3 for *Pseudomonas fluorescens* (Rombouts *et al.*, 1978; Rombouts and Pilnik, 1980; George *et al.*, 1991).

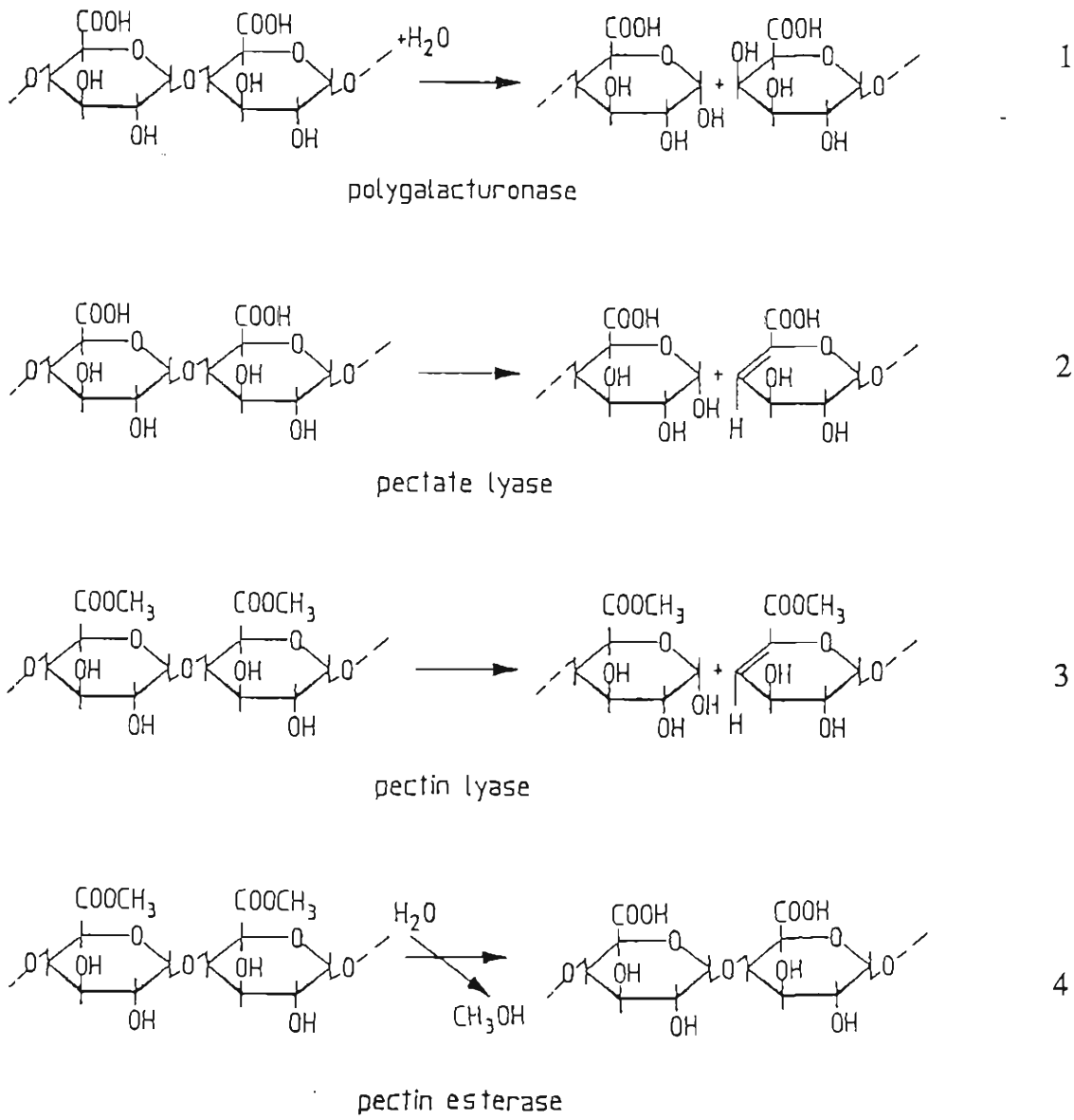


Figure 1.1 Pectinases and their mode of action (Rombouts and Pilnik, 1980)

1 and 2: Polygalacturonic acid degraded by PGase and PALase;

3 and 4: Pectin degraded by PLase and PEase.

Ca^{++} requirement for PALase and PLase has been observed by several authors (Gardner and Kado, 1976; Rombouts *et al.*, 1978; Stutzenberger, 1991; Yoshida *et al.*, 1991). Early work suggested that the true substrate of PALase was calcium polygalacturonate (Atallah and Nagel, 1977). Recent research on the structure of *Bacillus subtilis* PALase revealed that the enzyme complexes with calcium (Pickersgill *et al.*, 1994). A Ca^{++} requirement for the bacterial PALase of *E. carotovora* was optimal at 0.2 mM (Yoshida *et al.*, 1991); the optimal concentrations of Ca^{++} reported for other PALase from *Erwinia rubrifaciens* were 0.5 to 1.0 mM (Gardner and Kado, 1976).

Previous research showed that most of the polyphenolic compounds found in apple juice inhibit PGase and PEase found in commercial pectinase preparations (Mitek and Drzazga, 1988). Inhibition of pectinase by ions seem to vary with the species. Endo-PGase of *Rhizopus stolonifer* is stimulated by Fe^{+++}, Mg^{++}, Co^{++} and inhibited by Mn^{++} and Zn^{++} (Manachini *et al.*, 1987). Inhibition of *Byssochlamys fulva* PGase by Zn^{++} or Fe^{++} at 0.25 to 1.25 mM and Ca^{++} at 0.75 to 1.25 mM was also reported (Kotzekidou, 1990). Activity of PGase from *Rhizopus oryzae* was inhibited completely by Cu^{++}, Pb^{++}, Zn^{++} and Mn^{++} at concentration of 1 mM (Chung *et al.* 1992). In contrast to PALase, calcium has been reported to inhibit PGase activity of *Penicillium expansum* and *Fusarium oxysporum* (Conway *et al.*, 1988; Vazquez *et al.*, 1993).

Most fungal PGases have a K_m value from 0.54 mg/mL to 1.7 mg/mL against polygalacturonic acid (PGA), although the K_m was found to be as low as 0.09 mg/mL for exo-PGase from *Geotrichum lactis* (Pardo *et al.*, 1991) and as high as 20 mg/mL for exo-PGase of *A. niger* (Hara *et al.*, 1984).

Some biochemical properties of PGase and PALase are summarised in Table 1.2.

1.6.2.2 Fungal pectinases and their commercial application

Early studies (Phaff, 1947) revealed that PGase and PEase are strongly induced in *Penicillium chrysogenum* by pectin, pectic acid, gum tragacanth, D-galacturonate, mucate and L-galacturonate. The majority of studies thereafter concentrated on various fungus species and evaluated the regulation of enzyme synthesis. *P. expansum* produces PGase and PLase in apple tissue (Swinburne and Corden, 1969; Spalding and Abdul-baki, 1973). Formation of both enzymes is repressed by a variety of sugars. Dialysis of apple medium results in greatly increased levels of enzyme production (Spalding and Abdul-baki, 1973). Similar repression of the synthesis of inducible PGase by *A. niger* was reported by Maldonado *et al.* (1989). The synthesis of PGase was repressed by glucose, even in the presence of the inducer. The production of PGase started again once the free sugar was used up, or when the mycelium was washed free of glucose and incubated in a glucose-free medium containing the inducer. This proved the reversibility of the repression mechanism. *Penicillium digitatum* produces PLase constitutively at a relatively constant rate, independent of the culture conditions (Lobanok *et al.*, 1977). Studies by Riou *et al.* (1991) on the production of cell wall-degrading enzymes by the phytopathogenic fungus *Sclerotinia sclerotiorum* showed that PGase activities were produced constitutively on all of the substrates tested (glucose, β-methylglucoside, xylose, sorbose, sophorose, cellobiose, Avicel, CMC, hydroxymethyl cellulose (HMC), citrus pectin, apple pectin, PGA (Na^+-salts), galactan, arabinogalactan, laminarin and xylan) where citrus pectin, apple pectin, PGA, galactan and arabinogalactan showed higher induction over the other carbon sources. Two different

Table 1.2 Some biochemical properties of PGase and PALase

Enzyme and Sources of Enzyme	MW (Da)	Optimum Temp. (°C)	pI	pH Optimum	K_m (mg/mL)	Reference
PGase						
Trichoderma koningii I[a]	32,000		6.41	5.0	0.80	Fanelli *et al.*, 1978
II[a]	32,000		6.57	5.0	0.85	
Geotrichum lactis	53,000	40		5.0	0.09	Pardo *et al.*, 1991.
Rhizopus arrhizus	30,300			5.0	0.54	Liu and Luh, 1978.
Fusarium oxysporum	68,000	60		5.6	0.64mM	Vazquez *et al.*, 1993.
PALase						
Thermomonospora curvata I[a]	35,000	60			5.4	Stutzenberger, 1991.
II[a]	56,000				0.6	
Erwinia carotovora	39,500	60		10.0		Yoshida *et al.*, 1991.
Erwinia rubrifaciens	41,000	37	6.25	9.5	5.0	Gardner and Kado, 1976.

[a] Enzyme exists in multiple forms.

PALase forms (MW, K_m) were found with the different carbon sources used for cultivating *Thermomonospora curvata* (Stutzenberger, 1991).

Pectic enzymes from fungi are of considerable importance commercially, as their catalytic action is used in the food and beverage industry, as processing aids for the extraction and clarification of fruit juices, for the maceration of fruits and vegetables, for the extraction of vegetable oil and other uses, such as coffee processing (Colton, 1991; Pilnik and Voragen, 1991). Fruit juice clarification is the oldest and still the largest use of pectinases, which are applied mainly to juice from deciduous fruits and to grape juice. The raw pressed juice is viscous due to the presence of dissolved pectin and has a persistent cloudiness caused by cell wall fragments and complexes of cell wall fragments with cytoplasmic proteins. Consequently, pectinase treatment is essential for lowering the viscosity and clarifying fruit juices. Extraction of fruit juices generally involves pressing. For pulping soft fruits with a high content of soluble pectin, *e.g.* pear or peach, addition of pectinase results in faster processing and a higher yield of extraction (Rombouts and Pilnik, 1980). Vegetable and fruit maceration results from the hydrolysis of the middle lamella pectin in parenchyma cells. In enzymatic maceration, suspensions of loose cells are produced from fruit and vegetable tissue, a process which is especially applicable to firm-flesh fruits such as apples (Rombouts and Pilnik, 1980). Because of the nature of the plant cell wall structure, pectinases are used in the other applications in fruit and vegetable processing, such as olive oil, coffee processing and in the wine industry (Manachini *et al.*, 1988; Colton, 1991; Servili *et al.*, 1992).

Commercial pectinases are produced from *A. niger* using both surface and submerged cultivation techniques (Ward, 1985). The major enzyme components of these preparations

are PEase, endo-PLase and PGase. Different ratios of activity for the three major enzyme components, and of other contaminating enzymes such as cellulases and hemicellulase, are observed in surface and submerged pectinase fermentations. Surface culture is generally considered more suitable, as high yields of endo-PLase are produced under these conditions. Pure pectin is not used to any extent for inducing enzyme production due to economic reasons, although pectinase is efficiently induced by pectin. For commercial production of pectinases by *A. niger*, dried sugar-beet cossettes, citrus peel or apple pomace together with wheat bran are the main carbon and energy sources in fermentation, as these by-products from other industries are easier to access and provide a cheaper source of natural pectin than pure pectin (Rombouts and Pilnik, 1980).

1.6.2.3 Molecular studies of microbial pectinases

Molecular studies on the amino acid sequence of pectinase proteins revealed that the same enzyme produced by different species may share similarities possibly in regions associated with the substrate binding and/or catalytic sites. PGase protein of *A. niger* shares significant homology with PGase proteins from the tomato and *E. carotovora*. Comparison of these three enzymes revealed a highly conserved region in their C-terminus (Ruttkowski *et al.*, 1991). Studies on PALase from an *Amycolata spp.* also showed that the N-terminal sequence shared high similarity to the N-terminal sequences of PALaseA and PALaseE from *Erwinia chrysanthemi* (Bruhlmann, 1995).

Genetic approaches have been applied to obtain DNA sequence information for fruit and microbial PGases and PALases (Liao, 1991; Ruttkowski *et al.*, 1991; Nikaidou *et al.*, 1993). Genes of the same enzymes from different species were also found to show some DNA

sequence similarities. Recent studies found that two PGase genes (*pecA* and *pecB*) of *Aspergillus flavus* share a high degree of sequence identity with PGase genes from *Aspergillus parasiticus* and *Aspergillus oryzae*. The sequence similarity between the *A. flavus pecA* gene and the *A. oryzae* gene encoding PGase and *A. parasiticus pec-1* gene was 99 and 96%, respectively. Comparison of nucleotide sequences of *pecA* and *pecB* with genes for other fungal endo-PGases (*A. niger* PGaseII, *Aspergillus tubigensis*, *A. niger* PGaseI, *S. sclerotiorum*, *Cochliobolus carbonum*, *A. niger* PGaseC, *Fusarium moniliforme*) showed that the degree of sequence similarity ranged from 55 to 67% (Whitehead *et al.*, 1995). In *E. chrysanthemi*, five PALases are specified by five genes (*pelA*, *pelB*, *pelC*, *pelD* and *pelE*). After analysing the relationship between the three genes of the *pelADE* cluster, it was found that there was homology between each of the three *pel* genes. A 3,560 base pair DNA fragment containing the *pelE* and *pelD* genes was sequenced. Two genes show extensive homology in the structural gene coding regions but only low homology in the 5'- and 3'-non-coding regions. Both genes exhibit sequences homologous to the *Escherichia coli* CAP-binding site consensus sequence upstream of the start codon and an inverted repeat sequence which may act as a *rho*-independent transcriptional terminator after the translational stop (Gijsegem, 1989).

Genetic technology has been applied to improving microbial degradation of pectin. A PALase-encoding gene (*pelE*) from *E. chrysanthemi* and a PGase-encoding gene (*peh1*) from *E. carotovora* were inserted between a novel yeast expression-secretion cassette and a yeast gene terminator, then cloned separately into a yeast-centromeric shuttle vector (YCp50), generating recombinant plasmids *p*AMS12 and *p*AMS13. Transcription initiation signals present in the expression-secretion cassette were derived from the yeast alcohol

dehydrogenase gene promoter (ADC1P), whereas the transcription termination signals were derived from the yeast tryptophan synthase gene terminator (TRP5T). Secretion of PALase and PGase was directed by the signal sequence of the yeast mating pheromone alpha-factor (MFalpha1s). A pectinase cassette comprising ADC1P-MFalpha1s-pelE-TRP5T and ADC1P-MFalpha1s-peh1-TRP5T was subcloned into YCp50, generating plasmid pAMS14. Subsequently, the dominant selectable geneticin G418-resistance (Gt-R) marker, APH1, inserted between the yeast uridine diphosphoglucose 4-epimerase gene promoter (GAL10P) and yeast orotidine-5'-phosphate carboxylase gene terminator (URA3T), was cloned into pAMS14, resulting in plasmid pAMS15. Plasmids pAMS12, pAMS13 and pAMS14 were transformed into a laboratory strain of *Saccharomyces cerevisiae*. pAMS15 was stably introduced into two commercial wine yeast strains. DNA-DNA and DNA-RNA hybridization analyses revealed the presence of these plasmids and the *pelE* and *peh1* transcripts in yeast transformants, respectively. A polypectate agarose assay revealed the extracellular production of biologically active PALase and PGase by *S. cerevisiae* transformants and confirmed that co-expression of the *pelE* and *peh1* genes synergistically enhanced pectate degradation. These results can be applied in the wine industry for introduction of more efficient yeast strains which not only perform the normal fermentation, but clarify the grape extracts due to its ability to secrete enzymes to degrade pectin (Laing and Pretorius, 1993).

1.6.3 MUSHROOM PECTINASES

Despite the extensive studies on the pectinase produced by fungi, relatively little is known about pectinases produced by mushrooms. PGase and β-D-galactosidase activities were found in oak sawdust colonised by *L. edodes* together with the other polysaccharide-

degrading enzyme activities. When cultivating *L. edodes* on oak sawdust, levels of extracellular PGase activity were comparable to the activity of enzymes which degraded amylopectin, amylose and xylanogalacturonan. Extracellular PGase activity was lower than the activity of the enzymes which created RS from glucuronoxylan, arabinoglucuronoxylan and glucomannan. This activity was, however, higher than the other hemicellulases which degraded arabinogalactan, galactomannan, galactoarabinan, glucurronorhamnogalacturonan and yeast cell wall α-mannan. While most of these enzymes increased their activity after 12 days of cool treatment at 5°C followed by three days at 22°C, β-D-galactosidase significantly decreased in activity (Leatham, 1985).

PGase and PALase were also detected in cultures of a hybrid strain of *P. ostreatus* and *P. florida* (Sharma, 1987). When grown on the flax shive, the culture fluid exhibited laccase, cellulase, xylanase, PGase and PALase activities. After 25 days of growth at 20°C in near total darkness, the activities of PGase and PALase were found to be 475 and 175 μg/mL/hour GA equivalents at 30°C. These enzyme activities were not further characterised and culture conditions for their induction by different carbon sources, besides flax shive, were not defined. No further information on the characterisation of pectinases of any mushroom species has been reported.

1.7 AIMS OF THIS THESIS

The work described in this thesis is part of a broader project which aims at developing new products from lupins for human consumption. As part of this project, it was desirable to use all of the lupin seed in food products or in the formation of parallel products, such as using lupin wastes as a fermentation substrate. This lead to evaluating the suitability of lupin

wastes as substrates for mushroom production. After screening several mushroom species for their ability to grow on lupin hulls, *P. ostreatus* was selected for study of mushroom formation, including determining the types of enzymes produced during the life cycle so that mushroom formation could be optimised if the substrate did not provide the best nutritional base for this. When measuring the level of degradatory enzymes, it became apparent that pectinase occurred during vegetative growth and mushroom formation. The aims of this thesis then became:

a. mapping enzyme production in liquid fermentation using different substrates, to obtain some information on factors which regulate production of degradatory enzymes, in particular pectinases;

b. determining the fluxes in degradatory enzymes levels during SSF and mushroom development, to obtain information on the role of these enzymes during fruiting body production;

c. characterising the pectinase activity observed, to provide some fundamental biochemical information on enzymes from *P. ostreatus* which have not been characterised previously.

CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 FUNGAL STRAINS AND THEIR MAINTENANCE

All three fungal species, *P. ostreatus* (originally from China), *L. edodes* (originally from a Tokyo supermarket) and *F. velutipes* (American Type Culture Collection 34552) were purchased from Dr. C. Y. Cho, Department of Agriculture, University of Sydney. Strains were routinely maintained on Potato Dextrose Agar (PDA) (Oxoid) slants, grown at 26°C for one to two weeks in MacCartney bottles (25 mL capacity) and then kept refrigerated at 5°C and subculturing at least monthly according to Jong (1978). PDA plate cultures were incubated similarly for six to seven days prior to use as inocula for SFs or in preparation of inocula for SSFs.

2.1.2 CHEMICALS

The sources of all chemicals are shown in Appendix, abbreviations used for these are also listed on p.iv. All reagents, where not specified, were analytical grade and supplied by BDH (UK). Distilled water was prepared using the Milli-RO Water Purification System or deionised water using the Milli-Q Ultrapure Water System throughout.

Hardwood sawdust was purchased from A. J. Hofert Company, Richmond, Victoria, Australia. Lupin hull was from the Gungurru Farm, West Australia, provided by Prof W. S. A. Kyle (Department of Food Technology, VUT). Okara, the fibrous material obtained from lupin seeds after extraction of liquids by pressing, was provided by Ms. Ivana Radojevic, Centre for Bioprocessing and Food Technology (CBFT), VUT. Sources of other materials and media are otherwise given in the text describing their use or preparation.

2.1.3 BUFFERS

All buffers were made up as previously described by Dawson *et al.* (1986) except ammonium acetate-acetic acid. The pH of each buffer was checked on a Linbrook pH meter and adjusted with appropriate concentrated solutions of salts, acids or bases before use.

Clark-Lubs solutions, pH 8.0-10.2, for routine use were made by dissolving 0.373 g potassium chloride and 0.309 g boric acid in 50 mL, then diluting to 100 mL with distilled water after adding appropriate volumes of 0.1 M sodium hydroxide. Double strength pH 8.6 Clark-Lubs solution was made from dissolved 3.728 g potassium chloride, 3.092 g boric acid and 0.472 g sodium hydroxide in 500 mL distilled water; the buffer was stored at room temperature for up to one week. This pH 8.6 stock solution was double the concentration of that described by Dawson *et al.* (1986) and was used for routine assay of PALase activity (see Section 2.4.5). The pH was checked in the reaction mixture when it was used.

McIlvaine buffer solutions, pH 3.0-7.5, were prepared from 0.1 M citric acid and 0.2 M di-sodium hydrogen phosphate according to Dawson *et al.* (1986). Double strength pH 5.0 McIlvaine buffer solution was prepared by dissolving 5.095 g citric acid monohydrate and 7.313 g $Na_2HPO_4.2H_2O$ in 250 mL distilled water. This buffer was stored at 5°C for up to two weeks and used after two-fold dilution; the pHs of the diluted buffer and of the reaction mixture were checked before each use.

Other buffer solutions (Dawson *et al.*, 1986) were made freshly before use and included: citric acid-sodium citrate buffer solution, pH 3.0-6.0, prepared directly from 0.1 M citric acid and 0.1 M tri-sodium citrate; sodium acetate-acetic acid buffer solutions, pH 4.0-5.5,

prepared directly from 0.2 M sodium acetate and 0.2 M acetic acid; succinic acid-NaOH buffer solution, pH 4.0-6.0, prepared from 0.2 M succinic acid and 0.2 M NaOH, with a concentration of 0.05 M succinic acid in final buffer solutions; tris(hydroxymethyl)aminomethane-maleate buffer solutions, pH 5.5-8.0, prepared from 0.2 M Tris-maleate (24.20 g Tris + 23.20 g maleic acid in 25 mL) and 0.2 M NaOH, with a final concentration of 0.05 Tris-maleate in the buffer solutions; Na_2HPO_4-NaH_2PO_4 buffer solutions, pH 6.0-8.0, prepared from 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, with a final concentration of 0.1 M in the buffer solutions; Clark-Lubs solution, pH 6.0-8.0, prepared from 0.1 M KH_2PO_4 and 0.1 M NaOH, with a final concentration of 0.05 M KH_2PO_4 in the buffer solutions; tris(hydroxymethyl)aminomethane (Tris-HCl) buffer solutions, pH 8.0, prepared from 0.1 M Tris base and 0.1 M HCl, then appropriately diluted for defined uses.

Ammonium acetate-acetic acid buffer solutions, pH 4.0-5.5, was prepared directly from 0.2 M ammonium acetate and 0.2 M acetic acid by adding different volumes of stock solutions to achieve desired pH values.

2.2 MICROBIOLOGICAL METHODS

2.2.1 MEDIA

Unless specified, all media were sterilised by autoclaving for 15 minutes at 121°C, except for media used in the 2-L fermentor and media for SSF. For 2-L fermentations, broth media were transferred into the fermentor first, together with the other accessories, and sterilised for one hour at 121°C. The hull, sawdust and hull-sawdust mixtures as substrates for SSF were sterilised for 30 minutes at 121°C.

Okara or lupin hull (about 10 kg) were ground using a manual grinder (Universal, Warehouse Sales P/L) so all the resulting powder could pass through a one mm size mesh Screen (Endecott, Selby Scientific Laboratory Equipment) then the powder was stored at room temperature in capped glass bottles before use. When lupin hull is referred to in the Materials and Methods, Results and Discussion sections, this term describes the finely ground hull (sieved through one mm mesh) unless otherwise stated. Unground hull, coarse hull ground without mesh sieving and its laminar particles (usually about five mm in diameter) were also used in this study.

2.2.1.1 Potato dextrose agar (PDA)

PDA contained 39 g of PDA powder (Oxoid) dissolved in one L of distilled water. PDA was used to prepare the inoculum in agar, to test fungal growth conditions and maintain the strains.

2.2.1.2 Malt extract agar (MEA)

MEA contained 50 g of MEA powder (Oxoid) dissolved in one L distilled water. MEA was used for the determination of fungal growth condition.

2.2.1.3 Basal medium of Shewale and Sadana (Shewale and Sadana, 1978)

SSBM contained 2.0 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.3 g urea, 0.3 g $MgSO_4.7H_2O$, 0.3 g $CaCl_2$, 5 mg $FeSO_4.7H_2O$, 1.56 mg $MnSO_4.7H_2O$, 0.34 mg $ZnSO_4.7H_2O$, 2.0 mg $CoCl_2$ and 0.33 g Tween 80 in one L of distilled water. Urea was dissolved in distilled water, sterilised separately by passing through the Minisart 0.2 μm syringe filter (SM17597, Sartorius) and adding to the cooled sterilised broth before use.

SSBM was used in SFs for determining enzyme production when the basal medium was supplemented with the different carbon sources. The 500-mL Erlenmeyer flasks contained 200 mL SSBM and 1% (w/v) of the following: arabic acid, carboxylmethylcellulose (CMC), cellobiose, galactose, GA, lupin hull, β-methylglucopyranoside (MG), MCC, pectin, PGA, sawdust or xylan.

Cellobiose, galactose, GA and MG were prepared as 10% (w/v) stock solutions and added aseptically to SBBS after autoclaving separately. Other carbon sources were added to SSBM prior to autoclaving.

2.2.1.4 Glucose yeast extract broth (GYE)

A modified GYE (Tewari *et al.*, 1987) contained 0.5% (w/v) glucose, 0.5% (w/v) yeast extract (Oxoid) and 0.5% (w/v) peptone (Oxoid). Glucose was added from a 10% (w/v) stock solution after sterilisation.

2.2.2 INOCULUM PREPARATION

Three types of inocula were used for different experiments.

2.2.2.1 Potato dextrose agar inoculum

PDA inocula were prepared on PDA plates, where the fungi stock stored at 5°C were transferred onto PDA plates with a sterilised needle and then allowed to grow at 26°C in the dark for approximately one week. The growing edges of the colonies were cut with a sterilised 4-mm core-borer and used as inocula (agar plugs) (Hutchison, 1990). When

inoculated to agar plates, the mycelium of the inoculum piece was placed face down so that this contacted with the agar surface.

PDA inoculum was used to prepare the wheat grain inoculum, broth inoculum, SF and in tests for fungal growth conditions (see following sections).

2.2.2.2 Wheat grain (WG) inoculum

WG inocula were prepared on wheat grain (AWS 1, purchased from Essendon Produce) and used in the preparation of spawn for commercial-scale mushroom cultivation (Stamets and Chilton, 1983; Zeng *et al.*, 1987). Wheat grain kernels (typically 200 g) was immersed in water for about 30 hours until buds appeared and then air dried on paper tissues at room temperature until excess water on the grain surface was removed (usually less than two hours) and mixed with $CaCO_3$ (typically two g). The prepared grain was then transferred into a 1-L Mason jar and capped with a cotton plug. After sterilisation at 121°C for 30 minutes, this was inoculated with 15-20 plugs of 4-mm-diameter PDA inocula, mixed well by shaking and incubated at 26°C until the grain was colonised totally (about 20 days) before use. The Mason jar was wrapped with aluminium foil to prevent light reaching the culture during the incubation period.

2.2.2.3 Glucose-yeast extract broth inoculum

GYE inocula (Tewari *et al.*, 1987) were prepared in 250 mL flasks which contained 50 mL sterilised GYE broth and a few (10-15) glass beads (about eight mm in diameter). This was inoculated with 10 PDA inoculum plugs and incubated in an orbital shaker (Orbital Shaker Incubator, Model 013422, Paton Scientific P/L) at 26°C and 150 rpm for one week before use. The incubator was made light-proof by covering the viewing hole with aluminium foil.

2.2.3 FUNGAL GROWTH ON PLATES

P. ostreatus, *L. edodes* and *F. velutipes* were grown on PDA and MEA plates. Each plate was inoculated in its centre with one PDA inoculum plug. Inoculated plates were placed into plastic bags (about 150 X 400 mm) in groups of up to 10 plates and the plastic bag was closed then incubated at temperatures between 25 and 28°C in a dark incubator or the plastic bags were wrapped in aluminium foil before incubation.

Optimal temperatures for fungus growth were determined by comparing growth at different temperatures ranging from 15 to 40°C, at 5°C increments, for up to three weeks or until the whole plate was colonised, or when the fungi ceased to grow. The diameter of the colonised areas was measured at different times and the results used to determine suitable conditions for routine growth. Average diameter was determined from one measurement (longest distance) for each plate; measurements were made for at least 15 plates for each condition, from 20 plates set up initially for each determination. Measurement of growth was not made for plates where the fungal culture did not grow at all, where growth was extremely slow, or where contamination by bacteria or other fungi had occurred.

pH optima for fungus growth were determined by comparing the growth rates on plates with different starting pH values. The pH values of PDA and MEA media were adjusted after sterilisation and cooling down to about 50°C using sterile 0.1 M HCl or 0.1 M NaOH. The amount of HCl or NaOH added to each was pre-determined from tests with PDA and MEA which gave starting pHs in a range of pH 3.0-8.0, at increments of 0.5 pH units. The plates were inoculated in the centre with a PDA inoculum plug, and then these were allowed to grow at the optimal growth temperature for two weeks. The sizes of the colonised areas

were used to determine the optimal pH for growth. The same measurement techniques were used as described above except at least 12 plates from an initial 20 inoculated were used for each determination.

2.2.4 EFFECTS OF SAWDUST AND HULL ON MUSHROOM SPECIES GROWTH

Potential effects of sawdust and hull on fungal growth were investigated on PDA plates. After inoculation with a PDA inoculum plug, a small amount of sterilised sawdust or hull was placed on the plate about two mm away from the inoculum, using sterilised tweezers. The mycelial reaction towards sawdust or lupin hull, indicating inhibition or enhancement of growth as the colony reached or covered over the sawdust or hull, was recorded over two to three weeks. This procedure was used to evaluate the presence of inhibitors in the substrates (*e.g.* resins and tannins in sawdust) which may have interfered with the interpretation of results for colonisation or fruiting in SSF experiments.

The same method of growth measurement as described in Section 2.2.3 of the Materials and Methods was used. At least nine plates were measured from an initial 10 inoculated.

2.2.5 GROWTH IN SUBMERGED FERMENTATION

2.2.5.1 Small-volume shake-flask submerged fermentation

The Erlenmeyer flasks of 500-mL capacity containing 200 mL SSBM broth supplemented with 1% (w/v) of carbon source and about fifteen 8-mm glass beads were inoculated with five mL GYE inoculum. Flasks were incubated on an orbital shaker at 26°C and 200 rpm.

The small-volume shake-flask SF was performed to determine which enzymes were produced by *P. ostreatus* when grown on the different carbon sources, to study the enzymes produced in the time course of growth on the lupin hull as carbon source for *P. ostreatus* fermentation and to collect the culture filtrate as a source of enzyme for further biochemical study.

For the study of the enzyme induction, only single flasks were used for each carbon source and samples (10 mL) were withdrawn weekly for enzyme assay.

For the study of enzymes produced in the course of hull degradation, 15 flasks were used. Samples (10 mL) were withdrawn at intervals of four days randomly from each of the 15 flasks sequentially for enzyme assay.

For the SF set up specifically for the production of PGase and PALase, 1% (w/v) of lupin hull was used as the carbon source and the culture was harvested after approximately 20 days of growth.

2.2.5.2 Large-volume shake-flask submerged fermentation

Erlenmeyer flasks of five L capacity containing two L SSBM broth supplemented with 1% (w/v) of carbon source and about sixty 8-mm glass beads were inoculated with 50 mL GYE inoculum and then incubated on an orbital shaker at 26°C and 200 rpm. Attempts were carried out to use the large-volume shake-flask SF to determine enzyme production and fungal growth in the one culture vessel. Samples (10 mL) were withdrawn at intervals of four days for enzyme assay.

2.2.5.3 Growth in a 2-L fermentor

Growth of *P. ostreatus* was also conducted in a two L capacity laboratory fermentor (Biostat B, B. Braun Biotech International, GMBH). SSBM supplemented with 1% (w/v) carbon source was firstly sterilised at 121°C for 15 minutes and then inoculated with 200 mL of GYE inoculum to give a total volume of two L. The fermentation conditions were as follows: pH uncontrolled, oxygen 100%, 26°C, stirring rate 120 rpm, antifoam-controlled, uncontrolled light. Samples (about 20 mL) were withdrawn every two days for enzyme assay.

2.2.6 GROWTH IN SOLID-STATE

2.2.6.1 Growth in MacCartney bottles

The MacCartney bottles containing six mL of SSBM and five grams of hull or sawdust, or 2.5 g hull and 2.5 g sawdust, were sterilised then inoculated with two plugs of PDA inoculum. The cultures were maintained at 25°C in darkness for up to 28 days for *P. ostreatus* and 40 days for *L. edodes* and *F. velutipes*, at which time the whole substrate was colonised or no further growth observed. Ten MacCartney bottles were used for each species on each individual substrate.

2.2.6.2 Solid-state fermentation in commercial scale

SSF was also carried out in one L polypropylene (PP) bags (about 180 X 300 mm, made from autoclavable disposal bags, Disposable Products Pty Ltd), a method modified from previous work (Pettipher, 1987). Each bag contained 150 g ground lupin hull, 150 g hardwood sawdust and 400 mL distilled water, where the same batch of materials was used

for all experiments. The mixture was adjusted to pH 4.5 with 0.1 M HCl. After autoclaving at 121°C for 30 minutes, each bag was inoculated with about 10 g (wet weight) of WG inoculum. Bags were incubated in 26°C till the primordia formed and then were cut for mushroom development, which occurred usually in the fourth week of incubation.

2.2.6.3 Conditions for mushroom formation

Fungal growth in MacCartney bottles were conducted to test the ability of the three test species, *P. ostreatus*, *L. edodes* and *F. velutipes*, to grow and form fruiting bodies under laboratory conditions. To stimulate mushroom formation, the cultures were chilled (cold shocked) in a refrigeration unit at 4°C for two days for *P. ostreatus*, and seven days for *L. edodes* and *F. velutipes* after the whole substrate was colonised and before primordia appeared. The lids of the MacCartney bottles were removed, then the bottles were placed vertically in a plastic tray which contained 3-cm-depth water so that the culture was isolated from water by the bottle. The tray was sealed within a plastic cabinet (600mm L X 350mm W X 400mm H) and an air pump was supplied to enrich humidity and air exchange within the cabinet. The whole setup was located in a fume exhaust cabinet with two 100 W fluorescent light operating continuously. The cabinet was opened twenty minutes every two hours during the day for ventilation. The formation of fruiting bodies was recorded.

For the *F. velutipes* grown in the MacCartney bottles, another simpler method was also used. After cold shock in the cool room, the bottles were transferred into a plastic tray containing a little water, then covered with a beaker which isolated the air inside the bottle from the outside so that *F. velutipes* could grow in a closed, high-humidity environment.

The PP bags of *P. ostreatus* and *L. edodes* cultures were also incubated in the plastic cabinet for mushroom fruiting body formation after cold shock in the cool room, as above, and afterwards bags were cut where primordia formed.

P. ostreatus was also grown in a Temperature-Humidity incubator (Humiditherm 8801, Australian Instrument Service Pty Ltd) with a light option which could be used during mushroom fructification. Inoculated PP bags were incubated in the incubator firstly at 26°C, 60% relative humidity (RH) and in darkness for 28 days, when most bags were totally colonised with fungus. This was followed by transfer to 4°C, 60% RH and light for two days, then finally at 18°C, 85% RH and light (two 100 W fluorescent lights, 10 hours per day).

The PP bags of *P. ostreatus* for enzymatic studies were maintained constantly at 25°C without cold shock treatment. The bags were cut where the primordia formed and further incubated in the plastic cabinet.

2.3 ANALYTICAL METHODS

2.3.1 ANALYSIS OF CHEMICAL COMPONENTS OF LUPIN WASTES

Before analysis, ground lupin hull and okara were dried overnight in an oven set at 90°C and stored in closed one L glass bottles at 5°C. The analysis of macromolecular components was based on the methods described previously by Datta (1981) and Oluyemi and Cornelius (1990), where the hull and okara of lupin were fractionated with use of modifications of these extraction techniques as described below.

One gram of dried material was washed with cold distilled water and refluxed at 100°C for two hours in a 500 mL flask with 200 mL distilled water. The supernatant was discarded after centrifugation at 1,000 X g for 10 minutes. The residue was dried at 90°C overnight: the weight loss accounts for the water solubles fraction.

The extracted residues were then continuously refluxed with 5% (w/v) ammonium oxalate for three hours at 90°C to extract the protopectin. Hemicellulose was removed by refluxing the dried residue in 0.5 M H_2SO_4 for two hours at 100°C. The dried residue was further treated with 72% (w/v) H_2SO_4 at room temperature for four hours (the treatment was carried out in a desiccator to prevent dilution of 72% H_2SO_4 by atmosphere moisture) then diluted into 0.5 M H_2SO_4 and refluxed at 100°C for two hours. The weight loss represented cellulose and the residue remaining is lignin and ash.

Five samples of lupin hull and okara were analysed. Results were calculated as the average weight percentage for each component on a dry matter basis, with a standard deviation quoted on the basis of the replicates.

2.3.2 ESTIMATION OF PROTEIN CONCENTRATIONS

2.3.2.1 Dye-binding assay

Coomassie blue G-250 protein assay reagent kit (Pierce, USA) was used for protein estimation as described in the commercial manual. Samples (one mL) containing one to twenty-five μg protein were mixed with one mL Coomassie Protein Assay Reagent and then

the absorbance was read at 595 nm. For samples with higher concentrations of protein (100 to 1,500 μg/mL), 0.1 mL was mixed with five mL of the reagent and absorbance at 595 nm was read. A standard curve was prepared by using the BSA stock (two mg/mL) supplied in the kit at 2.5 to 25 μg/mL for the lower range of protein and 75 to 1,500 μg/mL for the higher range of protein samples.

2.3.2.2 Lowry assay

The method was modified from the original described by Lowry *et al.* (1951). The sample volume was 0.5 mL, contained 0 to 100 μg of protein, to which was added 0.5 mL of solution A (0.1 mL of 5%[w/v] $CuSO_4$, 0.9 mL Na_2CO_3 in 0.5 M NaOH). After 10 minutes at 37°C, 1.5 mL of solution B (one mL Folin-Ciocalteu's reagent plus 10 mL deionised water) was added and the solutions immediately mixed by vortex. Absorbance at 680 nm was recorded after incubation at 52°C for 20 minutes. Standards containing 0 to 100 μg of protein were prepared from a two mg/mL BSA solution, in the appropriate buffer or water. Reagents A and B were prepared immediately before use from the stock solutions.

2.3.3 REDUCING SUGARS ESTIMATION

RS were measured by the dinitrosalicylic acid (DNS) method (Miller, 1959). The reagent contained 75 g potassium-sodium tartrate, 4 g sodium hydroxide and 0.25 g DNS in 250 mL distilled water. Samples (0.5 mL) were added to one mL of DNS reagent and the absorbance at 560 nm measured after heating in a 100°C water bath for five minutes. Standards contained zero to five μmoles of appropriate monosaccharides.

2.3.4 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of proteins was preformed using the Phast system (Pharmacia). Homogenous (7.5%, 12.5%) and gradient (8%-10%) precast gels were used. Appropriately prepared protein samples (approximately 0.5 to 10 μg/mL of protein after dialysis against distilled water) were denatured in the presence of 10 mM Tris-HCl pH 8.0 buffer, 1 mM ethylenediaminetetraacetic acid (EDTA), 2.5% SDS, 5% β-mercaptoethanol and 0.01% bromophenol (final concentrations) by heating the mixture in a Eppendorf tube at 100°C. Tubes were centrifuged for five minutes in an Eppendorf centrifuge then samples (one μL) were applied to gels and electrophoresis performed according to the programmed separation method two. The following solutions were prepared according to the Phast manual (all solutions are %, v/v, except where specified):

Wash	a) 50% ethanol and 10% acetic acid
	b) 10% ethanol and 5% acetic acid
	c) deionised water
Sensitiser	8.3% glutardialdehyde (w/v)
Staining	0.25% silver nitrate (w/v)
Developer	0.04% formaldehyde in 2.5% sodium carbonate
Stop	5% acetic acid
Preserving reagent	10% acetic acid and 5% glycerol

Programmed development method two (for silver staining) was used to detect proteins.

2.4 BIOCHEMICAL METHODS

2.4.1 PREPARATION OF CRUDE ENZYME EXTRACT FROM CULTURE SAMPLES AND STORAGE OF ENZYME EXTRACTS

Extracellular culture fluids from the SF, or the extracts from the SSF, were firstly passed through glass wool then centrifuged at 8,000 rpm, 25 minutes, 4°C (Beckman J2-HS). The supernatant fluid was used as the crude enzyme extract (Chahal, 1985).

For SSF, substrate from different growing regions and different growing stages were taken for preparation of enzyme extracts by two methods. The first approach involved terminating the growth of the fungus in PP bags at weekly intervals for up to six weeks of incubation (when the entire substrate was normally colonised), for estimating the total activity of enzymes in the colonised regions of the lupin hull. The sample was withdrawn from the colonised substrate only and thoroughly mixed. The second method involved sampling different parts of the colonised substrate, using vertical or horizontal transects. For the vertical transects, disc-shaped samples were taken from three areas: the growing tip, an area half-way down the colonised region and another immediately under the inoculum, representing the different growth stages of the colony. For the horizontal transects, samples across the colony were taken from a region half-way between the growing tip and the inoculum or half-way down the bag (once the entire substrate was colonised). Here, the disc-shaped samples were divided into regions representing the centre of growth and the growing edge. During fruiting body formation, the sample was divided into these regions plus an additional one which was mid-way between the growing edge and the centre, enabling estimation of activities during mushroom development.

For SSF, one part (wet weight) of substrate which was colonised by *P. ostreatus* was suspended in four parts of distilled water (w/v) with 0.5% Tween 80 and the mixture were shaken by Gallenkamp 256 flask shaker (Great Britain) at a speed setting of 10 for one hour at room temperature before extracts were filtered through glass wool for further study.

When enzyme extracts were prepared from the mushroom fruiting body, the whole fruiting body or parts of this were crushed using a mortar and pestle before adding water and 0.5% Tween 80 to extract enzymes. One part (wet weight) to four parts water was also used here.

The culture fluid from the SF, or enzyme extracts from SSF prepared as above, were then stored at 5°C for up to one week, or at -4°C for up to one month. The enzyme extracts fractions in the process of purification could also be stored under the same conditions. These storage conditions were determined experimentally by comparing the activity changes after storage in buffers at different pHs and temperatures over extended periods.

2.4.2 DIALYSIS

Dialysis tubing was prepared according to Sambrook *et al.* (1989). Appropriately cut dialysis tubing (10 to 20 cm in length) of various diameters was firstly boiled for 10 minutes in three L of 2% (w/v) $NaHCO_3$ and one mM EDTA (di-sodium salts), pH 8.0, then rinsed thoroughly in distilled water and boiled for 10 minutes in one mM EDTA again, finally submerged in distilled water at 4°C for storage. Tubing was handled with gloves and washed inside and outside with distilled water before use. All samples were dialysed at least twice against 100 volumes of dialysis fluids (distilled water or appropriate buffer) at 4°C.

2.4.3 CONCENTRATING ENZYME SAMPLES

The culture filtrate was concentrated with a nitrocellulose membrane with a MW cut-off of 5,000 Da (SM16667, Sartorius) prior to gel filtration. The Ultrasart cell (Ultrasart cell 50, Sartorius) was connected to a cylinder of oxygen-free nitrogen (CIG, Australia) in order to apply a pressure of up to four bars. A total volume of 300 mL of culture filtrate was concentrated to approximately 15 mL.

The partially purified enzyme from the gel filtration column, ion-exchange column or IEF Rotofor cell was concentrated using a Collodian finger (Sartorius) with a MW cut-off of 5,000 Da. The sample was filled inside the finger and vacuum was applied from outside the finger by a water vacuum unit.

Sartorius centrifuge tubes (SM13229) containing a membrane with a MW cut-off of 5,000 Da were also used for concentrating extracts. Two mL samples were transferred into the sample tube and then placed into the centrifuge tube according to the supplier's instructions. The concentrated enzyme was collected after 20 minutes centrifugation at a speed of 4,000 rpm in a bench-top centrifuge (Econospin H1323, FSE Du Pont Australia Ltd).

All the concentration procedures were performed at 4°C.

2.4.4 PARTIAL PURIFICATION OF ENZYMES

2.4.4.1 Overview

The culture broths were centrifuged at 8,000 rpm (Beckman J2-HS) for 25 minutes at 4°C after filtering through glass wool. The supernatant fluid was then concentrated using the

Ultrasart cell. The concentrated culture filtrate was dialysed overnight against the buffer which would be used in the next step of purification, including Sephacryl S-200 gel filtration or DEAE-Sepharose fast flow ion-exchange chromatography. The fractions from the Sepharose S-200 MW sieve column which contained enzyme activity were then collected and dialysed as described above against distilled water and finally prepared for processing by isoelectric focusing (IEF) using a Rotofor cell for further purification. Fractions from the Rotofor cell which showed activity were rerun in the Rotofor cell using a different pH gradient range. All fractions were analysed for protein, enzyme activity and were run on PAGE to check for homogeneity.

2.4.4.2 Gel filtration

Sephacryl S-200 chromatography was used to partially purify and separate enzyme activities and to estimate the MW of the enzymes. Two Sephacryl S-200 columns were used in this study where both were packed and run under the same conditions but differed only in their total bed volumes. The first column (V_t = 446 mL, V_0 = 165 mL) was used in attempts to purify the enzymes and in general studies of activity and the second column (V_t = 456 mL, V_0 = 176 mL) was used only for determination of enzyme MW.

Sephacryl S-200 was supplied swollen from the manufacture (Pharmacia) and equilibrated in 0.01 M phosphate buffer, pH 7.0. The column (XK 26/100, Pharmacia) was cleaned with 20% ethanol, dried and one end was fitted with an adaptor. The swollen gel was degassed and cooled to 4°C before pouring into the column via a column extension funnel. The gel medium was allowed to settle at 4°C with the column outlet closed and then packed at a downward flow rate of 2.5 mL/minute for the first two hours, then at 4.5 mL/minute for a

further one hour using phosphate buffer (0.01 M, pH 7.0) containing 0.15 M KCl. The column was fitted with a second flow adaptor. The column was further eluted in an upward flow by the same buffer overnight with a constant flow rate of 0.3 mL/minute, which was maintained using a Pharmacia P-3 peristaltic pump.

The 20-fold concentrated culture filtrates (five mL) were dialysed at 4°C against the 0.01 M phosphate buffer pH 7.0, containing 0.15 M KCl and then applied to the bottom of the column via a thin capillary tubing inlet. The fractions (five mL) were collected in a 7000 Ultraroc fraction collector (LKB Bromma, Sweden).

The exclusion volume was determined with blue dextran and the inclusion volume with glucose. Glucose was estimated using the DNS method. MW calibration was made using sweet potato β-amylase (197,000), yeast alcohol dehydrogenase (150,000), BSA V (66,000), ovalbumin (44,000), bovine β-lactoglobulin (36,560), bovine erythrocytes carbonic anhydrogenase (29,000), soybean trypsin inhibitor (22,000), horse heart cytochrome C (12,384) and bovine lung aprotinin (6,500). Each application contained blue dextran, glucose and three protein standards whose eluted volume could be distinguished by reading the absorbance at 280 nm. Each standard was run at least three times. The amounts of standard protein applied were: blue dextran, 10 mg; glucose, 10 mg; sweet potato β-amylase, 500 units; yeast alcohol dehydrogenase, 6 mg; BSA V, 10 mg; ovalbumin, 5 mg; bovine β-lactoglobulin, 10 mg; bovine erythrocytes carbonic anhydrogenase, 10 mg; soybean trypsin inhibitor, 5 mg; horse heart cytochrome C, 5 mg and bovine lung aprotinin, 4 TIU. The column was washed for at least eight hours after each application, or before each use.

2.4.4.3 Ion-exchange chromatography

DEAE-Sepharose Fast Flow gel (Pharmacia) was washed and equilibrated in 0.01 M citrate buffer (pH 5.0) to make the gel suspension approximately 75% (w/v) and then this was transferred into a clean XK16/40 column (Pharmacia) after degassing. The column was packed downward overnight at the flow rate of eight mL/minute in the same buffer. The inlet was fitted with a column adaptor and the gel washed downward with a further 300 mL of the same buffer. The bed volume was about 80 mL.

The culture filtrate (600 mL) was firstly concentrated with the Ultrasart cell to 80 mL, then dialysed against the 0.01 M citrate buffer, pH 5.0, overnight before applying 40 mL (out of about 80 mL) to the column. The column was then washed with 100 mL of the same buffer at the flow rate of one mL/minute. The column was eluted with a linear NaCl gradient starting from 0 to 0.5 M in the same buffer in a total volume of one L. The fractions (five mL) were collected after the 100 mL wash was applied and thereafter; enzyme activity (PALase) was assayed in every third tube by the method using product scanning at 235 nm (see Section 2.4.5.8 of the Materials and Methods). The activity of PALase in each tube was expressed as a percentage of the highest activity in the tested tubes. Samples of initial crude enzyme and pooled fractions were also assayed using the RS method, to enable evaluation of the efficiency of purification by ion-exchange chromatography prior to further purification by Sephacryl S-200 chromatography. Protein in fractions was monitored by absorbance at 280 nm.

The column was washed with 300 mL of the same buffer containing 2 M NaCl after each use, then re-equilibrated in the starting buffer using a 500 mL wash. The concentrations of

the salt in the factions from the ion-exchange column were determined in a Model 2100 TPS digital conductivity meter (A.I.S. Australian Instrument Services Pty/Ltd), which was calibrated with potassium chloride according to the manual before use with a sodium chloride standard curve.

2.4.4.4 Isoelectric focusing (IEF): Rotofor cell

The Rotofor preparative IEF cell (Bio-Rad) was prepared as described in the instruction manual. The device was disassembled and washed with deionised water before use, then assembled as described in the manual. The anode (+) electrode assembly was filled with 25 mL acidic electrolyte (0.1 M H_3PO_4) while the cathode (-) electrode assembly was filled with 25 mL basic electrolyte (0.1 M NaOH). Before running the sample, the temperature of the system was cooled by connecting the cooling finger to a source of external recirculating coolant which was maintained at 4°C.

The column chromatography fractions containing the enzyme activity were selectively pooled and dialysed against deionised water then mixed with ampholytes (Bio-Lyte 3/10, Bio-Rad) and deionised water to make a final volume of 55 mL with a 2% (v/v) ampholytes concentration. This mixture was injected into the sample chamber and the air bubbles trapped in the cell removed. The power source was set to supply a constant power of 12 W and the system run for four hours.

Twenty 12 X 75 mm culture tubes were loaded in the test tube rack and placed inside the harvest box. The box was covered by a lid so that each stainless collecting tube was fitted into the tubes, then the box was connected to a vacuum pump and the vacuum applied.

When the focusing was completed, the black toggle switch was moved to the "harvest" position and the power supply was disconnected. The fractions were harvested by gently putting the needle through the sealing tape into the focusing chamber, which caused all 20 fractions to be simultaneously aspirated from the cell and delivered to the collection tubes. The pH of fractions in each tube was measured using a model-671 pH/temperature meter (Jenco Electronics Ltd, Taiwain) and fractions were analysed for enzyme activities. pH of the reaction mixture of the assays was monitored during the enzyme assay.

The pI of the enzyme was determined by the pH of the fraction which contained the highest enzyme activity.

2.4.5 ENZYME ASSAYS

Unless specified otherwise, all assays for polysaccharide-degrading enzyme activities involved measuring the amount of RS production by the DNS method, following degradation of appropriate substrate by enzyme extracts supplied. Each assay system contained enzyme, substrate, buffer and enzyme activators or inhibitors (as desired), using a standard assay of 0.5 mL. After suitable incubation periods at specified temperatures (conditions determined experimentally), reactions were terminated by the addition of the DNS reagent and heating at 100°C (Section 2.3.3). Controls included: omission of substrate, inclusion of enzyme extract (to determine RS supplied in the extracts or formed from material supplied in the extract during the incubation period); enzyme extracts boiled for 20 minutes to inactivate enzyme (also a measure of endogenous RS); substrate but no enzyme extract (to detect changes in the substrate which was not a result of the enzymatic reaction during the enzyme assay). PALase activity was also detected using a second approach, based on the formation

of a double bond in the product, where this absorbed at 235 nm (Rombouts and Pilnik, 1980). All assays used in the course of this project are described in the following sections.

The definition of enzyme activity described in the literature has varied from author to author (see Sharma, 1987 and Dekker, 1983). In order to compare the activities of enzymes (FPase, CMCase, xylanase, PGase and PALase), it is desirable to use a systematic approach to defining enzyme activity which is based on the amount of RS produced in mole terms rather than on a weight basis of RS termed. In this study, one International Unit (IU) of enzyme activity was defined as the amount of enzyme required for creating one μmole RS within one minute under assay conditions.

2.4.5.1 Filter paper cellulase (FPase)

The assay used was modified from the method described by Mandels and Weber (1969): typically, 0.2 mL of enzyme extract was mixed with 0.2 mL of 0.1 M sodium acetate buffer, pH 5.0, 0.1 mL of distilled water and five mg Whatman No. 1 filter paper, then incubated at 50°C for one hour. The reaction was stopped by adding one mL of DNS reagent followed by heating to 100°C for five minutes.

2.4.5.2 CMCase

CMCase activity was estimated by measuring the increase in RS in 0.5 mL reaction mixtures containing 0.1 mL enzyme extract, 0.1 mL of 1% (w/v) CMC, 0.1 mL of 0.1 M sodium acetate buffer, pH 5.0, and 0.2 mL distilled water, after incubating at 50°C for 30 minutes (Platt *et al.*, 1984).

2.4.5.3 Xylanase

Xylan from the birchwood was used as the substrate for xylanase, the assay contained 0.1 mL of 1% (w/v) xylan, 0.1 mL of enzyme extract, 0.1 mL sodium acetate buffer, pH 5.0, and 0.2 mL distilled water. RS were measured after incubation at 50°C for 30 minutes (Hong, 1976). RS produced was linear over the 30 minutes under the assay conditions.

2.4.5.4 Pectin depolymerase

Pectin-degrading activity in crude enzyme preparations of SF using lupin hull as a carbon source was initially measured using a method based on assays for CMCase and xylanase. The assay (0.5 mL) contained 0.1 mL enzyme, 0.1 mL acetate buffer (0.1 M, pH 5.0) and 0.1 mL 1% (w/v) PGA and 0.2 mL distilled water, with RS being measured after incubating at 50°C for 30 minutes. However, during the development of this project, two *P. ostreatus* enzymes (PGase and PALase) were found and the assay conditions were modified to account for differences in pH optimum, temperature optimum and the requirement of metal ions for PGase and PALase activities, as described in the following sections.

2.4.5.5 Optimising assay conditions for pectin depolymerase activities

Optimal pH for assay of enzymes which degraded PGA was determined using various buffers, with pH ranging from 3.0 to 9.0. The reaction mixture, which contained 0.2 mL of standard buffer (exactly the same as described by Dawson *et al.*, 1986), 0.1 mL of 1% (w/v) PGA, 0.1 mL of crude enzyme made up to 0.5 mL with distilled water, were incubated at 50°C for one hour, then RS measured by the DNS method. The buffers used here included citric acid-Na_2HPO_4 (McIlvaine) buffer solutions, pH 3.0-7.5; citric acid-sodium citrate buffer solutions, pH 3.0-6.0; sodium acetate-acetate acid buffer solutions, pH 4.0-5.5;

succinic acid-NaOH buffer solutions, pH 4.0-6.0; ammonium acetate-acetic acid buffer solution, pH 4.0-5.5; tris(hydroxymethyl)aminomethane-maleate buffer solutions, pH 5.5-8.0; Na_2HPO_4-NaH_2PO_4 buffer solutions, pH 6.0-8.0; Clark-Lubs solutions, pH 6.0-8.0 and Clark-Lubs solutions, pH 8.0-10.0.

The effect of buffer strength on the activity of the enzymes was also studied using the optimal temperature for assay as determined below. The stock Clark-Lubs solution, pH 8.6, and citrate-phosphate buffer, pH 5.0, were used at 0 to 0.3 mL in the assay system. The pHs of the reaction mixtures were measured using a model-671 pH/temperature meter with a micro-pH probe (Jenco Electronics Ltd, Taiwain) at room temperature and recorded.

Optimal temperatures for the enzymes which degraded PGA were determined by performing assays at various temperatures, ranging from 30°C to 65°C with increments of 5°C, using the buffer at appropriate concentration at pH which gave optimum activity. Buffers used were those determined previously as giving highest activity.

Studies on the metal ion requirements for enzymes were carried out by incubating 0.1 mL of stock Clark-Lubs buffer solution (pH 8.6), 0.1 mL of 1% (w/v) PGA, 0.1 mL of enzyme partially purified by column chromatography, 0.1 mL of distilled water and 0.1 mL of different concentrations (0.5 or 1.0 mM) of salt solutions. In preliminary experiments, the ability of the fresh, or spent growth media, to restore activity of the partially purified enzymes were tested by including up to 0.2 mL of heat-treated (30 minutes at boiling water bath) culture filtrates or SSBM broth.

Once the pH and temperature optima were established, kinetic studies were performed which included varying the period of incubation, concentration of enzyme and concentration of substrate. In the standard assays determined through this process, the period of assay and amount of enzyme used ensured that activity was measured during the initial rate phase of the reactions.

2.4.5.6 Polygalacturonase

PGase activity was assayed routinely using the experimentally-determined optimum conditions. A total volume of 0.5 mL reaction mixture contained 0.1 mL enzyme (diluted with distilled water if necessary), 0.1 mL stock citrate-phosphate buffer, pH 5.0, 0.1 mL 1% (w/v) PGA and 0.2 mL distilled water. The reaction mixture was incubated at 55°C for 30 minutes and then the RS measured. When the RS produced was higher than 1.5 μmoles in the 0.5 mL reaction mixture, the enzyme was appropriately diluted and activity was measured again.

2.4.5.7 Pectate lyase (measured by reducing sugar production)

PALase was assayed under the experimentally-determined optimum conditions. A total volume of 0.5 mL reaction mixture contained 0.1 mL enzyme (diluted with distilled water if necessary), 0.1 mL PGA, 0.1 mL stock Clark-Lubs solution, pH 8.6 and 0.2 mL distilled water. Calcium chloride was supplemented to a final concentration of 0.15 mM to activate PALase activity if the enzyme was partially purified by Sephacryl S-200, ion-exchange chromatography or IEF (typically, 0.1 mL PGA was added to 0.4 mL mixture of buffer, enzyme, calcium chloride and distilled water). The reaction was stopped by adding the DNS reagent after 30 minutes incubation at 45°C and the RS produced was measured.

2.4.5.8 Pectate Lyase (measured by absorption of end-products at 235 nm)

The products of reactions using the standard enzyme assay (50°C, pH 5.0 and 40°C, pH 8.6) were scanned to measure absorbances from 190nm to 900nm using one cm-path quartz curvettes in a UV-vis spectrophotometer (Cary 1, Varian). The reaction mixture contained one mL partially purified enzyme from Sephacryl S-200, one mL of 1% (w/v) PGA, two mL of distilled water (supplemented with calcium chloride for PALase with a final concentration of 0.15 mM in the assay) and one mL of stock buffer (Clark-Lubs for PALase and citrate-phosphate for PGase). After 30 minutes incubation at the appropriate temperature, the reaction was stopped by placing tubes in a 100°C water bath for 30 minutes. A sample of the reaction mixture was then transferred into quartz curvettes and scanned. Controls contained all reagents but the enzyme was inactivated by heat-treatment (30 minutes in boiling water bath) before adding this to the assay mixture. The scanning program automatically subtracted absorbance readings in the controls.

Following the detection of the double bond in the unsaturated galacturonates (whether they were mono-, di-, tri- or oligo-galacturonates) which absorb at 235 nm, PALase activity was also assayed by monitoring the continuous increase in absorbance at 235 nm relative to an inactivated enzyme control. The reaction mixture contained 0.8 mL of partially purified enzyme (from MW chromatography fractionation, ion-exchange chromatography or IEF Rotofor cell fractionation), 0.8 mL 1% (w/v) PGA, 0.8 mL stock Clark-Lubs solution, pH 8.6, 0.8 mL of 0.75 mM $CaCl_2$ and 0.8 mL distilled water, and incubated at 40°C for 30 minutes unless specified otherwise. Inactivated enzyme (by boiling water bath) from the same source was used to substitute the active enzyme in the control. The depolymerisation of PGA by PALase was monitored at 235 nm as determined previously.

2.4.5.9 Effects of potential activators or inhibitors on polygalacturonase and pectate lyase activity

Potential enzyme activators or inhibitors were tested using the crude enzyme preparation in a 0.5 mL reaction mixture which contained 0.1 mL enzyme, 0.1 mL 1% (w/v) PGA, 0.1 mL stock buffer (citrate-phosphate, pH 5.0, for PGase and Clark-Lubs buffer, pH 8.6, for PALase), 0.1 mL distilled water and 0.1 mL stock testing solutions. RS were determined after incubation at the appropriate temperature for 30 minutes (determined as an appropriate time for the extract used). Four categories of chemical were used:

a. Salts

The anionic ions were first examined as sodium salts of the acetate, carbonate, chloride, citrate, dihydrogen phosphate, monohydrogen phosphate, hydrogen carbonate, nitrate, sulfate, oxalate and tartrate, at a final concentration of 20 mM in the reaction mixture.

Appropriate salts of the sulfate, chloride and nitrate (anions which did not effect activity) were then tested for their ability to inhibit enzyme activity. Those salts included: Ag^{+}, Li^{++}, Cs^{+}, Al^{+++}, Ba^{++}, Pb^{++}, Hg^{++}, Cr^{+++}, Fe^{++}, Fe^{+++}, K^{+}, Ni^{++}, Cu^{++} and NH_4^{+} at a final concentration of up to 10 mM in the reaction mixture.

b. Chelators

Tri-sodium citrate and EDTA (di-sodium salt) were tested at a final concentration of 10 mM.

c. Metabolism inhibitors

A concentration of 10 mM of the following was included in reaction mixtures: arsenite, arsenate, ferricyanide, cyanide, dithiothreitol, iodoacetamide, mercaptoethanol, metronidazole, phenylmethyl sulfonyl flouride (PMSF) and azide.

d. End-products of reaction

This category included adding five mM concentrations of cellobiose, fructose, galactose, GA, glucose and xylose solutions to reaction mixtures.

CHAPTER 3. RESULTS

3.1 CHEMICAL COMPONENTS OF LUPIN WASTES

The major wastes of lupin processing are the seed hull, since de-hulling is the first step of seed processing. Okara is another fibre residue resulting from the extraction of protein from the de-hulled seeds. The ground hull and okara were analysed for their carbohydrate content as described in Section 2.3.1 of the Material and Methods. Results are shown in Table 3.1.

The hull was composed mainly of cellulose and hemicellulose, with protopectin being present at 8.91%. Note that soluble materials, which presumably would be readily available for microbial conversion, made up 16.91% of the dry weight. Lignin value was further determined because of low content of lignin and ash residue.

Okara carbohydrates were mainly composed of hemicellulose, with a comparatively low cellulose content. Okara was not further utilised as part of this study for mushroom production from lupin wastes.

3.2 GROWTH OF SELECTED MUSHROOMS SPECIES UNDER LABORATORY CONDITIONS

Three strains of mushroom, *P. ostreatus*, *L. edodes* and *F. velutipes*, were tested for their ability to grow and form fruiting bodies on lupin hulls as a substrate under laboratory conditions. Some preliminary experiments were performed to determine growth conditions on laboratory media then to determine if the above species could fruit on different substrates when screened using small-scale cultures.

Table 3.1 Carbohydrates in lupin processing wastes

a. Lupin hull

Fraction	X[a] (%)	S[b]
Water soluble	16.9	4.8
Protopectin	8.9	1.6
Hemicellulose	19.5	0.4
Cellulose	50.1	6.3
Lignin+Ash	4.6	1.3

b. Okara

Fraction	X[a] (%)	S[b]
Water soluble	27.4	5.2
Protopectin	22.3	4.9
Hemicellulose	42.5	1.0
Cellulose	4.9	0.8
Lignin+Ash	3.0	0.9

[a] X: Average percentage of dry weight.

[b] S: Standard deviation, calculated from five replicates.

Experimental details are found in Section 2.3.1.

3.2.1 OPTIMAL CONDITIONS FOR MUSHROOM GROWTH ON LABORATORY MEDIA

The effects of temperature and pH of the medium were investigated by observing the relative rates of growth of the three test species on PDA and MEA media at different temperatures (Table 3.2) and then the pH of the PDA medium was adjusted to values between 3 and 8 (Table 3.3).

Although the optimal temperature and pH for mushroom growth has been well documented (Huang *et al.*, 1987), the different strains had different growth patterns on the laboratory media tested. Table 3.2 showed that the three fungi could hardly survive on both PDA and MEA plates when the temperature of incubation was over 30°C. *P. ostreatus* preferred to grow at a temperature between 25 and 30°C, whereas the other two species (*L. edodes* and *F. velutipes*) preferred between 20 and 25°C.

Mushroom growth on PDA adjusted to different pHs showed that the mushrooms under study could survive in a wide range of pH although the optimum pH was located between 5.0 and 6.0. These results on the effects of pH and temperature on growth are similar as those reported by other researchers (Huang *et al.*, 1987; Tonomura, 1978; Tokimoto and Komatsu, 1978; Zadrazil, 1978).

Subsequent studies on the growth of the three species during SSF were carried out at pH 5.0 and 25°C $\pm$ 2°C. Because ground lupin hull and sawdust were to be tested as substrates (alone or in combinations) for mushroom growth, the fungi were grown on PDA media containing these substrates to test whether growth was stimulated or inhibited. For all of the

Table 3.2 Effect of temperature on mushroom species growth on PDA and MEA plates

a. PDA plates

Species	Period of Incubation (Weeks)	Temperature (°C)				
		20	25	30	35	37
P. ostreatus	1	**	**	**	*	-
	2	***	****	****	*	-
L. edodes	1	**	**	*	*	-
	2	****	****	**	*	-
F. velutipes	1	***	**	**	-	-
	2	****	****	***	-	-

b. MEA plates

Species	Period of Incubation (Weeks)	Temperature (°C)				
		20	25	30	35	37
P. ostreatus	1	*	**	**	*	-
	2	***	****	****	*	-
L. edodes	1	**	***	**	*	-
	2	****	****	**	*	-
F. velutipes	1	**	**	*	-	-
	2	****	****	**	-	-

Growth was scored according to the diameter of the culture after seven and fourteen-days growth on unbuffered media.

- No growth observed;
- * 0 - 1.5 cm;
- ** 1.5 - 2.5 cm;
- *** 2.5 - 4.0 cm;
- **** Plate fully colonised.

Table 3.3 The effect of pH on mushroom species growth on PDA plates

Species	Period of Incubation (Weeks)	pH					
		3.0	4.0	5.0	6.0	7.0	8.0
P. ostreatus	1	**	**	**	**	**	**
	2	***	****	***	****	***	**
L. edodes	1	**	***	**	**	**	*
	2	***	****	****	****	**	**
F. velutipes	1	*	*	**	**	*	*
	2	***	***	****	****	***	*

Growth was scored according to the diameter of the culture after seven and fourteen-days growth at 25°C. See Section 2.2.3 of the Materials and Methods for experimental details.

- No growth observed

* 0 - 1.5 cm

** 1.5 - 2.5 cm

*** 2.5 - 4.0 cm

**** Plate fully colonised

species tested, growth was neither stimulated nor inhibited relative to PDA growth at 25°C, pH uncontrolled (5.3-5.5). This indicated that inhibitors were not present in the substrates, which is common for commercially-obtained sawdust (Stamets and Chilton, 1983).

3.2.2 MUSHROOM GROWTH ON LUPIN HULL

Three species of mushroom were tested for their ability to grow on ground hull, sawdust and a mixture of hull and sawdust in MacCartney bottles for up to six weeks at 25°C.

P. ostreatus could not grow on sawdust alone although there was no evidence of inhibition of mushroom growth when sawdust was incorporated in PDA plates. This may be due to the low nitrogen content of sawdust, which may also explain the inability of *L. edodes* and *F. velutipes* to colonise the entire sawdust substrate within six weeks.

When grown on the ground hull only, the three fungi produced more biomass (thicker white mycelia mass were observed) in the first two weeks than when grown on the mixture of hull and sawdust. Penetration of the mushroom mycelia, however, was slightly slower than when grown on the mixture of lupin hull plus sawdust.

All three species grew well on the mixture of lupin hull and sawdust. Comparing the growth rates on the mixture of hull and sawdust at 25°C, *P. ostreatus* grew slightly faster than the other two species. There was not much differences between the growth rates of *L. edodes* and *F. velutipes*.

Normally, it took four weeks for *P. ostreatus* to colonise the entire substrate in PP bags, whereas *L. edodes* took seven to nine weeks to colonise the same bags.

When unground hull - hemispherical in shape - was used as substrate, the fungi could not grow well due to the uneven distribution of water and less absorption of water by the hull. The ground lupin hull appeared to be powdery and formed layers in the bottles so that the packing conditions in this SSF did not provide enough ventilation for mushroom mycelia growth. The mixture of hull and sawdust could provide the ventilation conditions for mycelium penetration, while holding enough water for mushroom colonisation and probably supplying sufficient nitrogen for fungal growth. Although the major component of lupin hull is cellulosic biomass, analyses performed by Ivana Radojevic (CBFT, VUT) showed 0.3% of the mass is protein.

Although the optimal conditions for mushroom production could also partly depend on the preparation of the substrate, a mixture of lupin hull and sawdust at a ratio of 1:1, with a water content of 65%, and pH adjusted to 4.5 with HCl, was used for further SSF experiments. This choice was consistent with previous commercial mushroom cultivation conditions in which a water content of 65% was shown to be optimal for mushroom growth (Huang *et al.*, 1987).

3.2.3 FRUITING BODY FORMATION ON LUPIN HULL

3.2.3.1 Fruiting body formation in MacCartney bottles

Each of ten MacCartney bottles containing one of three substrates were initially inoculated with each fungus and incubated under the condition described above. When the substrate was

completely colonised with pure cultures (Table 3.4), the bottles were transferred to 4°C for "cold shock" for one week, lids of bottles were then removed, and the bottles left in a moisture-enriched environment for fruiting body formation (referred to Section 2.2.6.3 of the Materials and Methods).

For *P. ostreatus*, nine bottles of hull substrate and eight containing sawdust plus lupin hull were cold shocked: all bottles showed fruiting body formation within two weeks of cold shock. There were no significant differences in the time when the fruiting bodies formed, although a few bottles containing hull plus sawdust produced primordia one or two days ahead of the others. There were significant differences in the yields of mushroom, where mycelia grown on hull only produced larger mushrooms than did the mixture. A second flush of mushrooms was also observed in both the mixture and hull-only substrates.

L. edodes was unable to form primordia within four weeks after cold shock in MacCartney bottles under the laboratory conditions used, and the surface of some cultures (three out of eight from the hull-only, and four out of nine from the mixed substrates) turned brown in colour after cold stock.

F. velutipes primordia formation was achieved by setting the MacCartney bottles in water, then each bottle was enclosed in an inverted glass vessel to increase local humidity. The glass was removed for 30 minutes each day for air exchange. The primordia formed at various times for the different cultures (five out of five for both hull-only and the mixture), varied from one week to three months following cold shock treatment. Two sets of bottles (two hull-only and two of the mixture) where the culture was exposed to air directly and two

Table 3.4 Growth of three mushroom species on hull and sawdust mixtures in MacCartney bottles[a]

Species	Period of Incubation (Weeks)	Sawdust	Hull	Hull + Sawdust (1:1)
P. ostreatus	1	-	*	*
	2	-	**	***
	3	-	**	****
	4	-	****	N
L. edodes	1	-	*	*
	2	*	*	**
	3	*	**	***
	4	**	**	***
	5	**	***	****
	6	***	****	N
F. velutipes	1	*	*	*
	2	*	*	**
	3	*	**	***
	4	**	***	****
	5	**	****	N
	6	**	N	N

[a] See Section 2.2.6.1 of the Materials and Methods for experimental details.

Growth was scored on the following:

- \- No growth
- * Start growth
- ** Grow over 1/3 media
- *** Grow over 2/3 media
- **** Grow over whole media
- N No further observed changes

sets of closed bottle (two hull-only and two of the mixture) were unable to form primordia, indicating that local humidity was important for forming primordia.

3.2.3.2 Fruiting body formation on commercial scale

In the first trial for fructification of *P. ostreatus* in PP bags, the contamination rate with other fungi was zero (judged by visual observation and odour) during vegetative growth. The culture bags were cut horizontally in the middle, then the upper parts of the PP bag was removed to expose the culture to air. This resulted in contamination by other fungi during the late stages of mushroom development (around the sixth or seventh week). For mushroom growth in the plastic cabinet for fructification, the average contamination rate was 20% up to the first flush of mushrooms being harvested. The contaminated culture could still form and develop a normal fruiting body. In later experiments, PP bags were cut only where the primordia formed. This reduced the contamination rate to zero both in the plastic cabinet and temperature-humidity incubator during the first flush.

Thirty PP bags of the mixture of lupin hull and sawdust (1:1 ratio, as described in Section 2.2.6.2 of the Materials and Methods) were set up and inoculated with *P. ostreatus*, then incubated in darkness at 25°C. For the thirty bags tested, eight formed primordia before the substrate was fully colonised. These bags were cut where the primordium formed and four bags were incubated in a plastic cabinet and the other four bags in the temperature-humidity incubator (see Section 2.2.6.3 of the Materials and Methods), without cold stock treatment. The other 22 bags were cold shocked (4°C) for two days after the substrate was fully colonised with *P. ostreatus*, and incubation continued in a plastic cabinet (six bags) or

temperature-humidity incubator (sixteen bags). The bags were cut to allowed fruiting body formation only when and where primordium formed, with a total of ten bags incubated in the plastic cabinet and twenty bags in the temperature-humidity incubator. These species formed fruiting bodies within six weeks (30 out of the 30 bags tested) (see Fig. 3.1 for photographs of mushrooms). Mushroom harvested from the plastic cabinet were weighted: a total of 435 g of oyster mushrooms were harvested from the first flush of five bags. Mushrooms were well shaped and of good visual quality (Fig. 3.1a). For cultures incubated in the temperature-humidity incubator, mushrooms showed abnormally pale colour and they had a longer stipe (Fig. 3.1b). When compared to mushrooms produced in the plastic cabinet, mushroom formed in the temperature-humidity incubator were poorer quality (see Fig. 3.1c).

For *L. edodes*, ten PP bags of the same substrate mixture as described above were inoculated and incubated in darkness at 25°C. When the substrate was fully colonised (usually after seven weeks of growth), the bags were cold stocked for one week at 4°C. The bag was then cut in the middle, the upper parts of the bag removed and the culture bag set vertically in the plastic cabinet for initiation of primordia and formation of fruiting bodies for up to four weeks. *L. edodes* was able to form primordia between 10 to 20 days growth at room temperature after cold shock treatment at 4°C (four out of ten bags), but these were not able to develop into normal mushroom fruiting bodies at room temperature when tested for up to 30 days incubation, at which time the substrate was contaminated by a green fungus. Due to the variability in the period for *F. velutipes* to form primordia and the lack of incubation facilities to maintain high moisture levels needed for mushroom development during large-scale culture, *F. velutipes* was not further tested for mushroom development at large scale.

Figure 3.1 *P. ostreatus* harvested from the mixture of lupin hull and sawdust

The substrate contained 150 g ground lupin hull, 150 g hardwood sawdust and 400 mL distilled water, with pH adjusted to 4.5 with 0.1 M HCl. After inoculation with WG inoculum (10 g, wet weight), cultures were incubated at 26°C, in darkness, for four weeks, then cold shocked at 4°C for two days after the whole substrate was colonised with fungus. The cultures were finally incubated at 25°C. PP bags were cut to allow mushroom development when primordia appeared.

Incubation conditions in the plastic cabinets and the Temperature-Humidity incubator are described in Section 2.2.6.3 of the Materials and Methods.

a. Mushrooms grown in a plastic cabinet

b. Mushrooms grown in the Temperature-Humidity incubator

c. Comparison of mushrooms grown in a plastic cabinet (right) and the Temperature-Humidity incubator (left)

3.3 POLYSACCHARIDE DEGRADING ENZYMES OF *P. OSTREATUS*

3.3.1 ENZYMES PRODUCED BY *P. OSTREATUS* DURING DEGRADATION OF LUPIN HULL IN SUBMERGED FERMENTATION

Although chemical analysis revealed that lupin hull contained some water-extractable sugars, the main components of lupin hull were polymeric polysaccharides which could not be utilised directly by *P. ostreatus* without degradation into a form which could pass through the fungus cell wall. Because this fungus could grow on lupin hull, it was apparent that degradatory enzymes were secreted to enable growth. Results presented in this section relate to investigation on the enzymes involved in the degradation of this substrate in SF.

In initial experiments, it was considered that the major enzymes present would be hydrolases, so that enzymes were assayed using methods described previously for four enzymes (FPase, CMCase, xylanase and PGase), where all assays were carried out at pH 5.0 and 50°C (Mandels and Weber, 1969; Platt *et al.*, 1984; Hong, 1976; Sharma, 1987). When *P. ostreatus* was grown on lupin hull as a sole carbon source using small-volume shake-flask SF, all four of these enzymes were detected and PGase showed the highest activity compared with cellulase and xylanase activities. Results which show typical relative activities over the first two weeks of growth are shown in Fig. 3.2. In the first two weeks of growth, mycelia formed visible spherical pellets (diameter about 0.8 cm) with some particles of lupin hull attached and suspended in the media. Fungal biomass was not otherwise monitored. Samples from triplicate flasks were assayed at the end of first and second weeks of growth. Similar patterns of enzyme production were seen: low CMCase and FPase activities and xylanase activity over the first two weeks of culture, with PGase being the dominant activity.

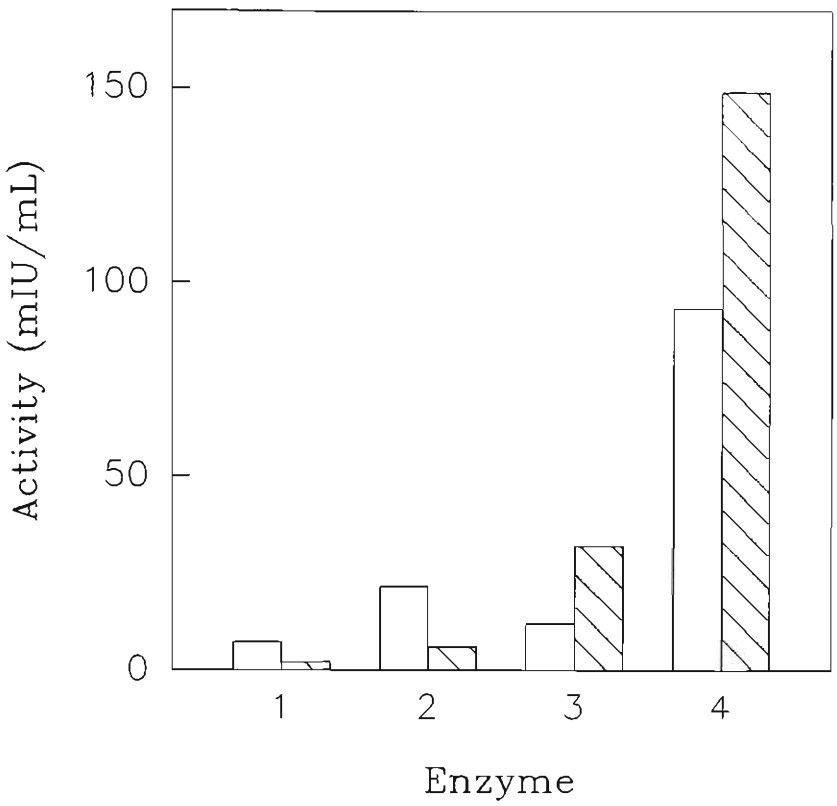


Figure 3.2 Preliminary studies of the polysaccharide-degrading enzymes produced by *P. ostreatus* grown on lupin hull in submerged fermentation

The standard small-volume shake-flask method was used in this study (see Section 2.2.5.1 of the Materials and Methods). A total volume of 200 mL SSBM in a 500 mL flask supplemented with two grams of ground lupin hull and glass beads was inoculated with five mL GYE inoculum, then incubated on an orbital shaker with 25°C and 200 rpm. Three flasks were sampled at the end of week one (▭) and the same three sampled at the end of week two (▨). Results of enzyme assays for these triplicate samples were averaged. RS production was measured with the DNS method to determine enzyme activity. All enzyme assays except FPase were carried out with an assay mixture of 0.1 mL acetic acid-sodium acetate (0.1 M, pH 5.0), 0.1 mL of 1% (w/v) substrate (CMC for CMCase, xylan for xylanase, PGA for pectinase), 0.1 mL crude enzyme and 0.2 mL distilled water, incubated at 50°C for 30 minutes. FPase was assayed using five mg Whatman No.1 filter paper with the same buffer and crude enzyme in 0.5 mL for 60 minutes.

1. FPase; 2. CMCase;
3. Xylanase; 4. Pectinase.

Further investigation of the extracellular enzymes in culture fluids was made in small-volume shake-flask SF after characterisation of the biochemical properties of PGase and PALase, which revealed the optimal assay conditions for these enzymes (see Section 3.4.1). A typical profile of extracellular enzyme production by *P. ostreatus* during growth on lupin hull is shown in Fig. 3.3. This confirmed that both FPase and CMCase activities were relatively low and FPase was synthesised only in the first two weeks of growth. The peak of CMCase activity occurred several days after that of FPase. Xylanase, PGase and PALase increased in activity after the peak of cellulase and their activities were much higher than that of cellulase. The highest volumetric activity detected in small-volume shake-flask SF was PGase, then PALase, xylanase, CMCase and with FPase normally the lowest activity detected.

When SF was conducted in the 2-L Braun Bio-stat laboratory fermentor, the profile of enzymes produced was similar to that seen for the small-volume SF in the first two weeks, however, the levels of PGase and PALase were lower and the particles of lupin hull were still suspended undegraded on the surface of the culture fluid (lifted up by air agitation) and adhered to the wall of fermentor above the culture fluids. Also, the pH sensor, air sparging duct and sampling pipe were easily blocked by fungus pellets. Because of these technical problems, the fermentor system was not the best option for monitoring the enzyme changes in this SF and this approach was abandoned in favour of the small-volume shake-flask approach.

Many attempts were also made to determine the enzyme production profile in large-volume shake-flask culture (two L of medium in a five L flask). This configuration was desirable

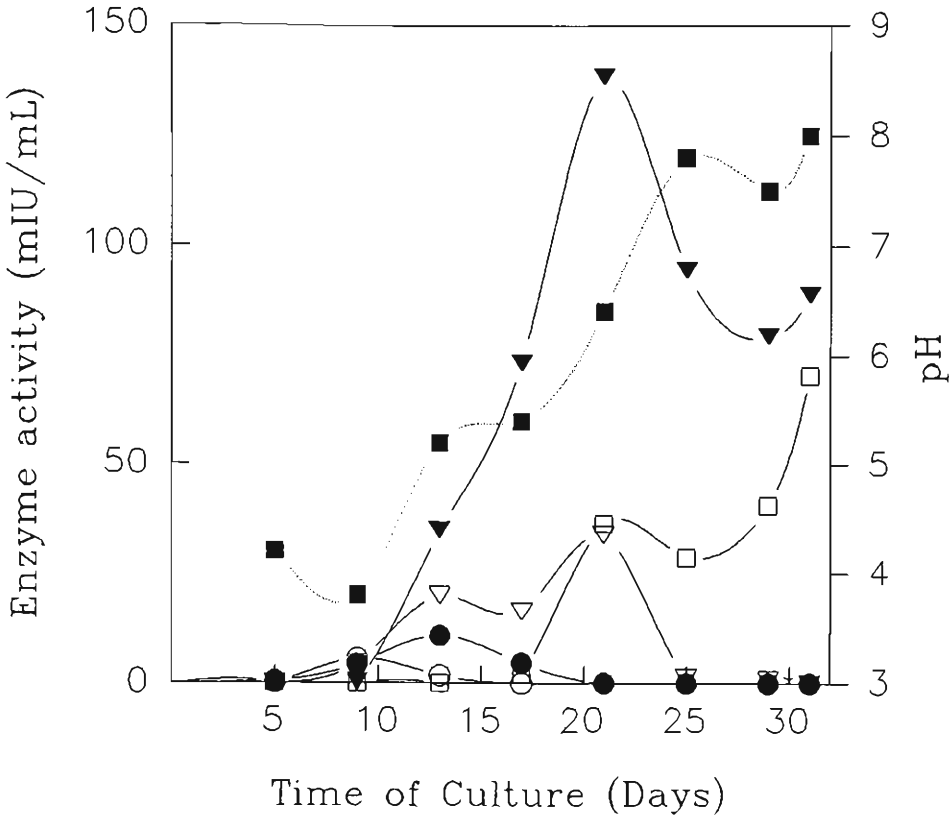


Figure 3.3 Enzymes produced by *P. ostreatus* during degradation of lupin hull in submerged fermentation, using optimal enzyme assay conditions

Samples were withdrawn from individual standard small-volume shake-flasks (see Section 2.2.5.1. of the Materials and Methods). Standard assays applied for all enzymes, using RS production to detect all activities and optimum conditions for PGase and PALase (see Section 2.4.5).

(■) pH;
(○) FPase;
(●) CMCase;
(▽) Xylanase;
(▼) PGase;
(□) PALase.

for study because it would have enabled sequential sampling from the one vessel rather than setting up parallel flasks for harvesting at different times of incubation (with the possibility of variability being introduced for the latter in inoculation, media composition or culture conditions). However, frequent withdrawal of samples often lead to contamination of cultures over the one-month incubation period, so that complete production profiles could not be constructed. Similar patterns of enzyme production (low FPase and CMCase, high xylanase, PGase and PALase) were seen for the large-volume shake-flask SF as seen for the small-volume culture over the first two weeks of incubation.

3.3.2 ENZYMES INDUCED BY VARIOUS CARBON SOURCES

Attempts were made to find out whether different carbon sources for fungus growth caused changes in the enzyme profiles seen during SF. Enzymes detected during four weeks of growth on 12 different carbon sources, including polymeric and monomeric substrates, are shown in Fig. 3.4.

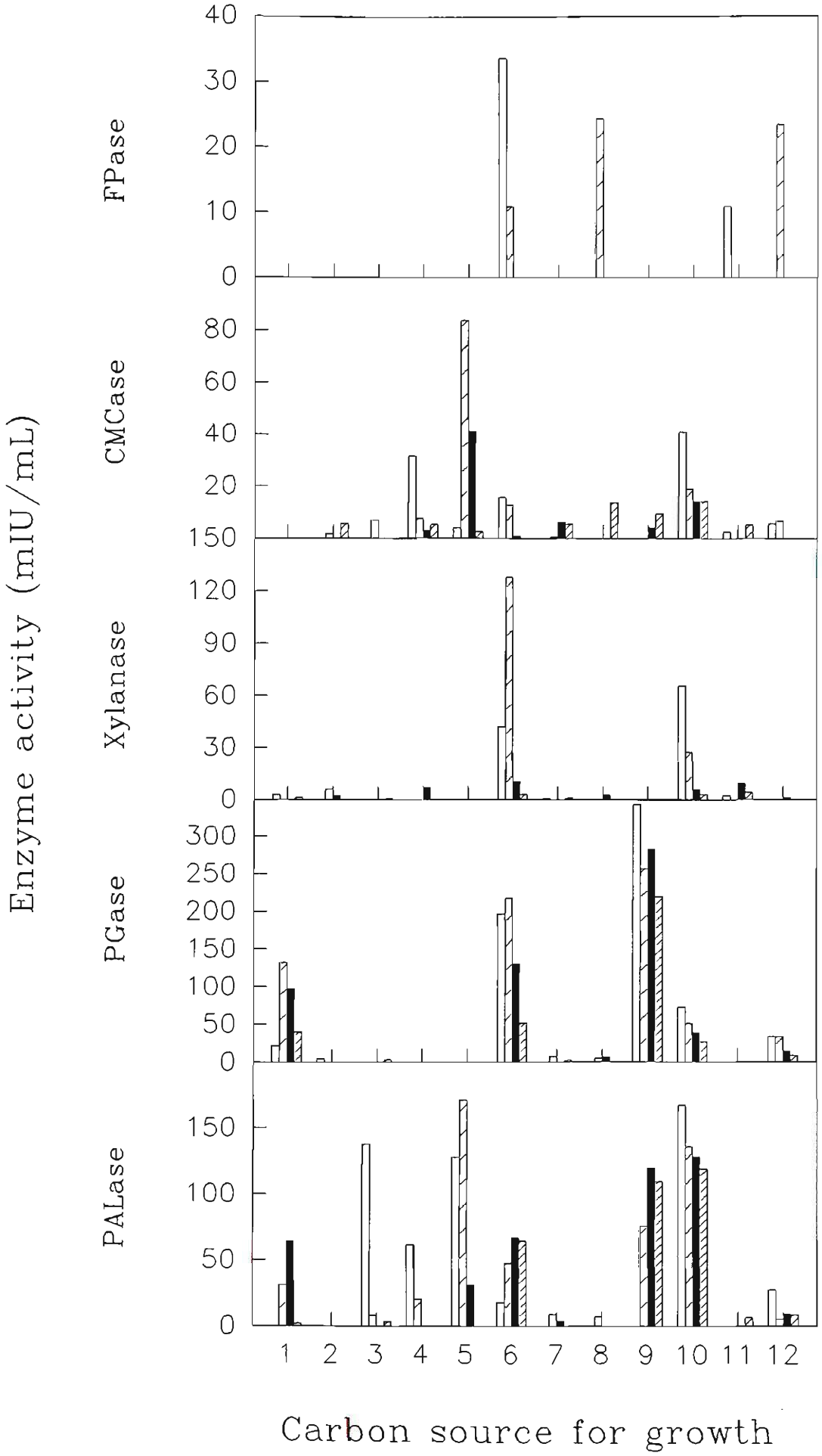
P. ostreatus was able to produce FPase, CMCase, xylanase, PALase and PGase in different amounts when grown on different substrates. When compared to the other enzymes, FPase activity was relatively lower and it always appeared within the first two weeks of growth. Lupin hull, MCC, sawdust and xylan were better substrates for stimulating FPase production. When PGase was produced in SF, activity was higher than PALase except with PGA as carbon source, although PALase was produced on substrates which failed to stimulate production of high levels of PGase (such as cellobiose, galactose, GA). Pectin, lupin hull, arabic acid and sawdust were good substrates for stimulating PGase. Amongst the substrates which could induce CMCase activity, including CMC, cellobiose, galactose, GA, hull of

Figure 3.4 Polysaccharide-degrading enzymes from *P. ostreatus* grown on a range of carbon sources

A total of 12 small-volume shake-flasks (see Section 2.2.5.1 of the Materials and Methods) contained 200 mL SSBM and glass beads supplemented with two grams of carbon sources in each flask. Sample was withdrawn and analysed from each flask at the end of week one (), week two (), week three () and week four ().

Carbon sources were:

1. Arabic acid;
2. CMC;
3. Cellobiose;
4. Galactose;
5. GA;
6. Lupin hull;
7. MG;
8. MCC;
9. Pectin;
10. PGA;
11. Sawdust;
12. Xylan.


Enzyme activity (mIU/mL)
FPase
CMCase
Xylanase
PGase
PALase
40
30
20
10
0
80
60
40
20
150
120
90
60
30
0
300
250
200
150
100
50
0
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12
Carbon source for growth

lupin, MG, MCC, pectin, PGA, sawdust and xylan, the GA surprisingly gave the highest yield of the CMCase.

Compared with the other substrates, lupin hull was a good substrate for producing xylanase activity as was PGA. These polymeric solid substrates were better than xylan for stimulating production of xylanase. When *P. ostreatus* was grown on arabic acid, lupin hull, pectin, PGA or xylan, it produced a reasonable level of PGase. Although PALase could be induced by most growth substrates, GA, lupin hull, pectin and PGA could induce relatively higher levels of PALase.

Of the 12 substrates tested, lowest enzyme activity was found in media using CMC, MG, xylan or sawdust as carbon source. This may be a reflection of the experimental approach used, with sampling points at weekly intervals which may not have detected fluctuations in enzyme activities, or these substrates could not support fungal growth as well as the other substrates. It was noted earlier that *P. ostreatus* failed to grow on sawdust in small SSF (Table 3.4): better aeration and substrate availability in SF allowed growth but this was relatively poor.

3.3.3 ENZYMES PRODUCED IN SOLID-STATE FERMENTATION

3.3.3.1 Changes in extracellular enzyme activity during the life cycle of *P. ostreatus*

Typical enzyme activity changes in the colonised substrate prepared by wheat grain inoculum are shown on the Fig. 3.5, where the enzymes produced were extracted from the whole colonised substrate at the times indicated.

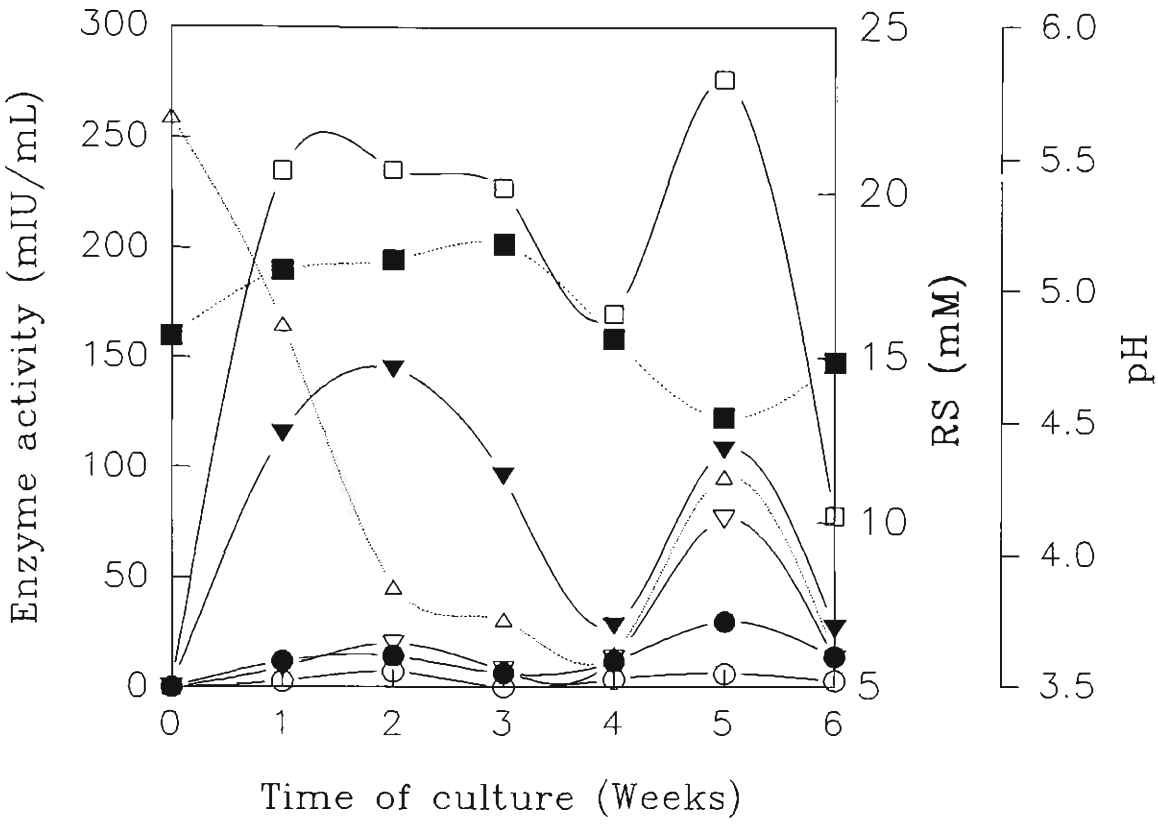


Figure 3.5 Extracellular enzymes of *P. ostreatus* during solid-state fermentation using wheat grain inoculum

PP bags containing SSF substrate (150 g lupin hull, 150 g sawdust, 400 mL distilled water and pH adjusted to 4.5 with 0.1 M HCl) were inoculated with 10 g WG and incubated at 26°C till primordia appeared between 25 and 28 days growth without cold shocked. Bags were then cut to allow mushroom development and incubated in a plastic cabinet (see Section 2.2.6.3 of the Materials and Methods). For each week, one bag was randomly selected and sampled. After removing the WG inoculum, uncolonised substrate and mushroom fruiting bodies (if any), substrate colonised with *P. ostreatus* was used to prepare crude enzyme extracts as described in Section 2.4.1 of the Materials and Methods. pH, RS and enzyme activity present were measured in the extracts.

(■) pH; (△) RS; (○) FPase;
(●) CMCase; (▽) Xylanase; (▼) PGase;
(□) PALase.

Normally, fungus growth was visible within 48 hours after inoculation. It took about three to five weeks to colonise the whole substrate, at which time biomass was visible on the surface of bags and formed a white "skin" which adhered to the PP bag. In the meantime, some small, grape-like primordia appeared in the thickening "skin", especially in the "aged" parts of the culture. After primordia initiated, enzymatic studies were only performed on cultures where primordia appeared about the same stage of growth (around 25 days, without cold shock). In the fourth week, the primordium enlarged and developed. The bags were cut to allow the further differentiation of the primordium and were incubated in a plastic cabinet (see Section 2.2.6.3 of the Materials and Methods) at the 28th day. The mushroom fruiting bodies were fully developed at the end of fifth week and decayed at the end of the sixth week.

The five enzymes assayed were all present during the degradation of lupin hull in SSF. Normally, the enzymes increased in activity in the first two weeks of growth and formed the first peak of activity at the end of the second week then declined to the lowest activities between the third and fourth weeks of growth, when the substrate was fully colonised and primordia appeared. Small differences were seen between the profiles of enzyme activity thereafter: FPase, CMCase and xylanase activities appeared to increase by the fourth week at which time PGase and PALase activities were at their lowest and had not started to increase to their second peak of activity.

The five enzyme activities increased again during the development of fruiting bodies and formed a second peak at the fifth week when the fruiting bodies were fully developed, then dropped by the sixth week, when the fruiting bodies started to decay. FPase exhibited low

activity, which was similar to the pattern seen in SF. PALase exhibited the highest activity of the five enzymes assayed. When compared with the first peaks seen during vegetative growth, CMCase and xylanase showed higher activities in the second peak when fruiting, whereas the PGase and PALase demonstrated similar levels of activities in the two peaks. The RS present in the substrate dropped during vegetative growth, then increased when the enzymes increased their activities during fruiting and reaching peak levels when the enzymes did also. The pH of the substrate extract fell during fruiting.

In SSF of lupin hull by *P. ostreatus*, generally, PALase exhibited the highest activity, followed by PGase, xylanase and CMCase activities, with FPase showing the lowest level of activity (Fig. 3.5).

3.3.3.2 Wheat grain inoculum compared with glucose yeast extract broth inoculum

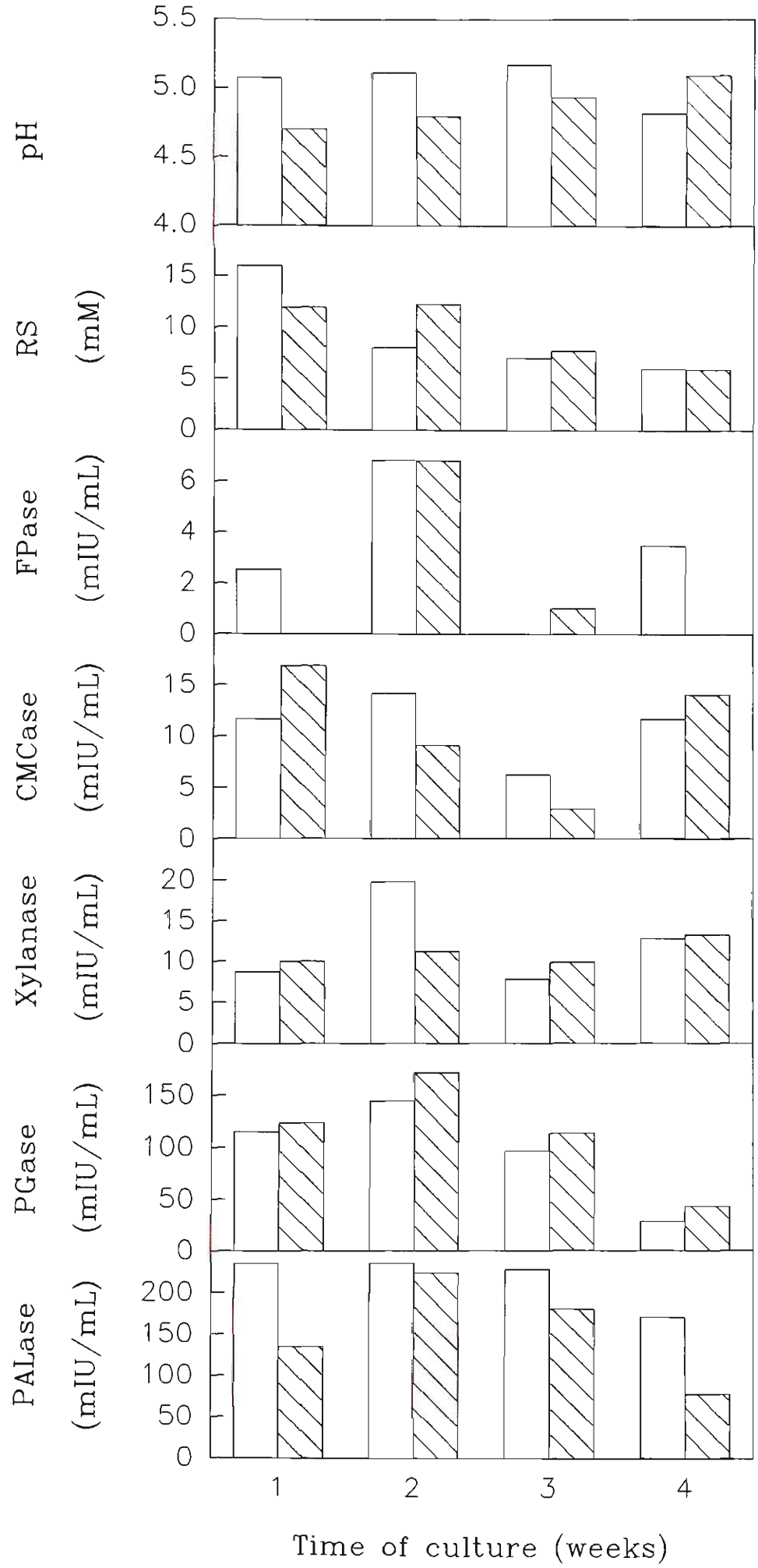
Fig. 3.6 compares the extracellular enzyme activity of *P. ostreatus* in aqueous extracts of the substrate following inoculation with cultures grown in GYE broth or WG. The pattern of enzyme activity, pH values, RS levels were essentially similar. The WG inoculum was used routinely in all subsequent experimentation for SSF.

3.3.3.3 Enzyme activity in different growth regions during solid-state fermentation

To gain same insight into the role of each enzyme detected in substrate utilisation in SSF, two types of experiments were performed to sample and extract the substrate at different

Figure 3.6 The effect of inoculum type on enzymes produced by *P. ostreatus* during solid-state fermentation on lupin hull

Substrate (prepared according to Section 2.2.6.2 of the Materials and Methods) was inoculated with 10 g WG inoculum () or 10 mL GYE inoculum (), then incubated at 26°C. For each week, one PP bag was randomly chosen for crude enzyme preparation (see Section 2.4.1 of the Materials and Methods) after removing WG inoculum and uncolonised substrate. pH, RS and enzyme activity present were measured in the extracts.


pH
5.5
5.0
4.5
4.0
RS (mM)
15
10
5
0
FPase (mIU/mL)
6
4
2
0
CMCase (mIU/mL)
15
10
5
0
Xylanase (mIU/mL)
20
15
10
5
0
PGase (mIU/mL)
150
100
50
0
PALase (mIU/mL)
200
150
100
50
0
1
2
3
4
Time of culture (weeks)

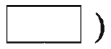
growth regions of the fungal colony growing on the lupin-sawdust mixture. The first type of experiment involved extracting enzymes from samples taken at regions between the inoculum and the growing tip (vertical, disc-shape sample removed from bags during vegetative growth over four weeks - Fig. 3.7). The second type of experiment removed sampling from the centre of the growth or from the circumference of growth as the inoculum spread across the substrate following inoculation (horizontal transects - Fig. 3.8).

In vertical transects, pH was lower at the growing tip compared to other zones sampled at the same time, RS were normally at higher levels in the newly colonised substrate except in samples from the first week of culture. FPase and CMCase demonstrated higher activities in the aged colony in the first two weeks. Total FPase or CMCase activity seemed to be lowest at the third week, which is similar to the results presented in Fig. 3.5. In the second week, all of the enzymes exhibited highest activity in the aged growth and lower activity in the growing tip. With the exception of PGase, all of the enzymes showed a relative increase in activity in the growing tip at week four, which may correspond to the decrease of free RS in the substrate at the same time.

Fig. 3.8 shows results obtained for horizontal transects, where activities may be influenced by changes in temperature, light and humidity. It is hard to generalise about the results obtained here, as they represent an average both across and through the culture. There was little change in pH across the culture (centre to edge); the pH generally fell at weeks five and six, corresponding to development of mushroom fruiting bodies. Free RS declined during vegetative growth and showed a small rise as formation of mushroom fruiting bodies

Figure 3.7 Enzyme activities detected in extracts from different growth regions between the growing tip and inoculum - vertical transects

SSF substrate preparation, inoculum and culture conditions are described in Section 2.2.6.2 and extracellular extract preparation in Section 2.4.1 of the Materials and Methods. One bag was sampled each week.

() Growing tip;

() Colony between growing tip and WG inoculum;

() Colony near inoculum.

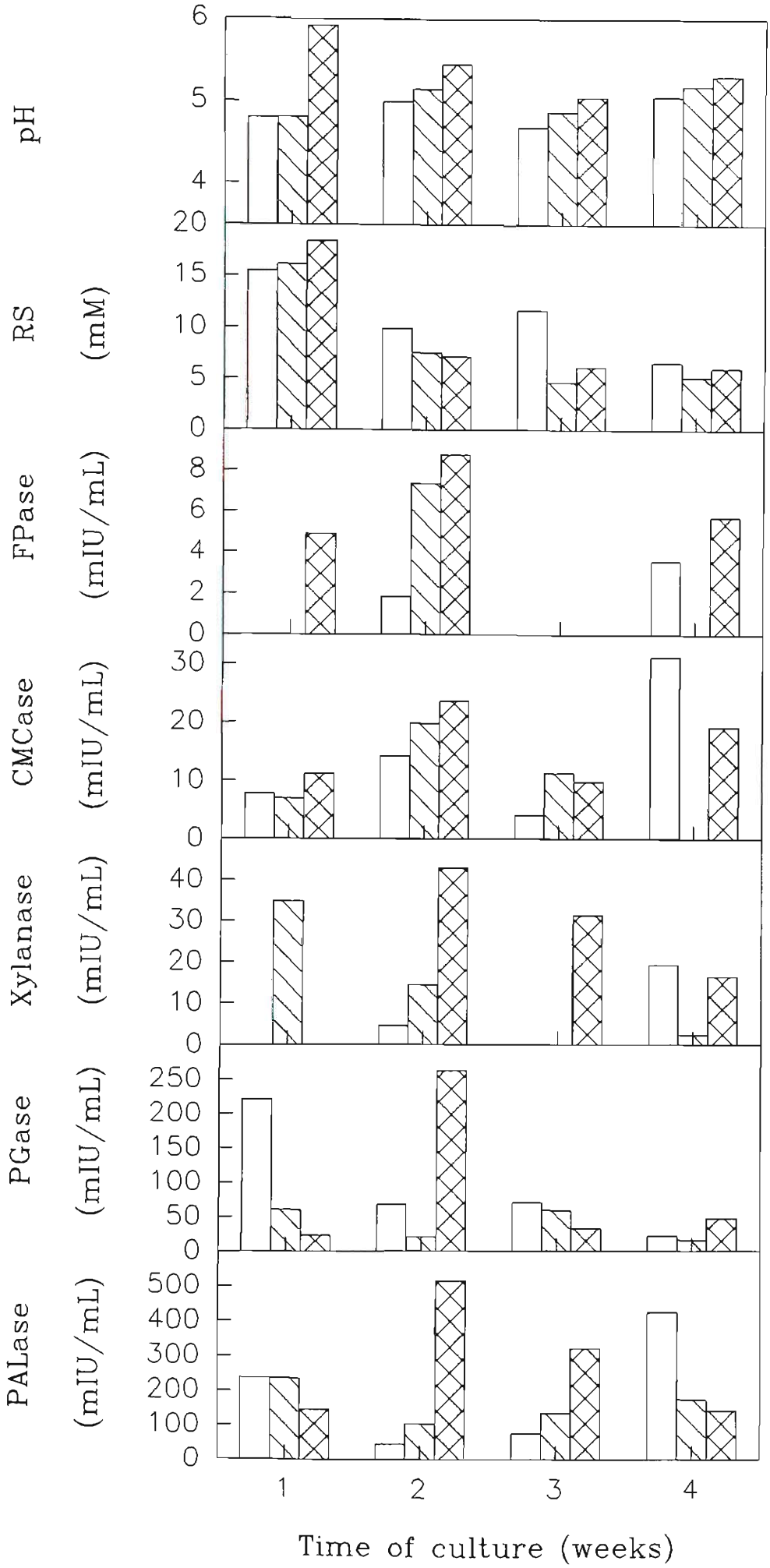

pH
RS (mM)
FPase (mIU/mL)
CMCase (mIU/mL)
Xylanase (mIU/mL)
PGase (mIU/mL)
PALase (mIU/mL)
Time of culture (weeks)

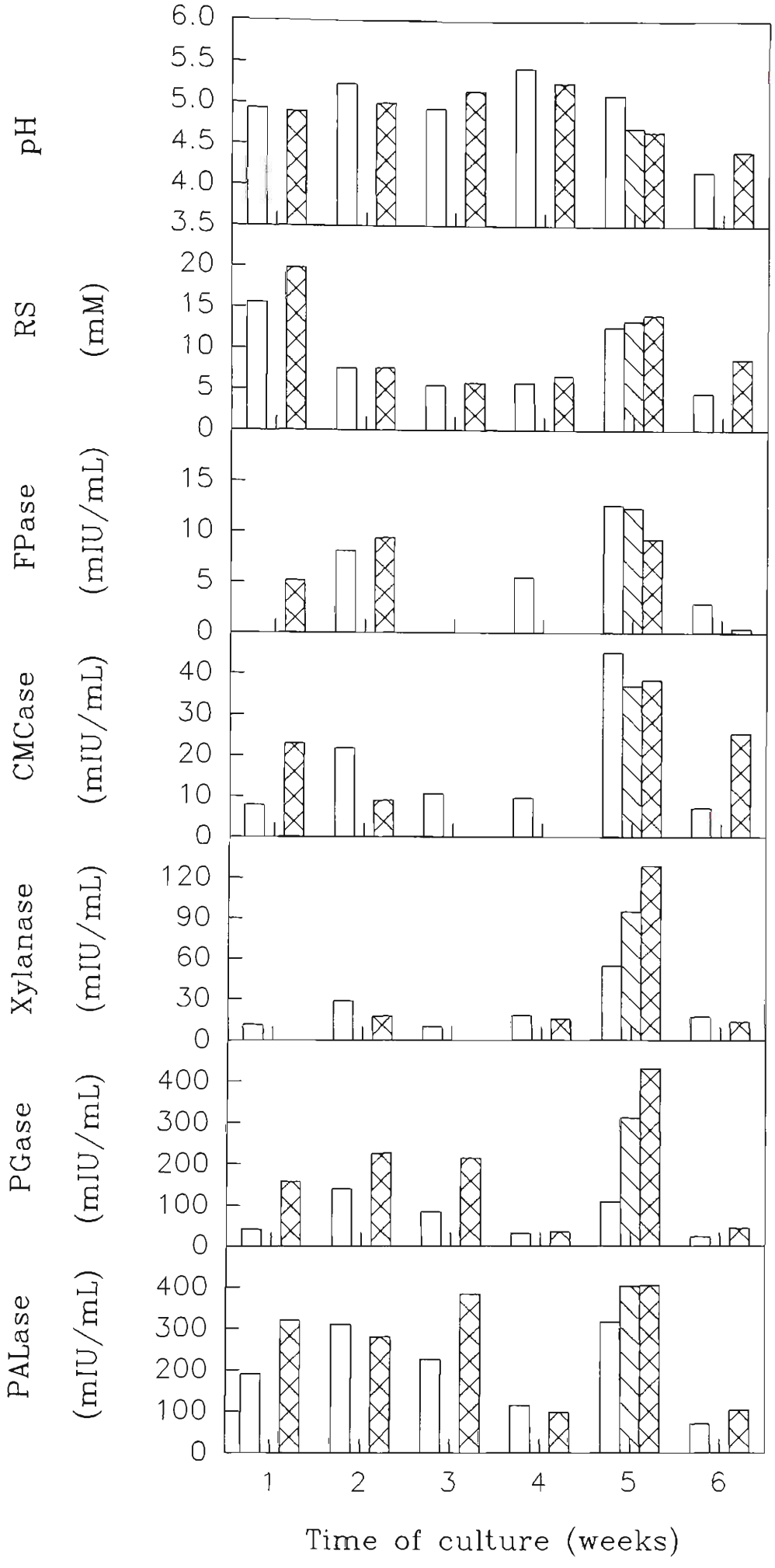
Figure 3.8 Enzyme activities detected in extracts from different growth regions between the centre and outer layer - horizontal transects

SSF substrate preparation, inoculum and culture conditions are described in Section 2.2.6.2 and 2.2.6.3 and extracellular extract preparation in Section 2.4.1 of the Materials and Methods. One bag was sampled each week.

() Outer layer;

() Between outer layer and centre (only tested in week five);

() Centre.


pH
RS (mM)
FPase (mIU/mL)
CMCase (mIU/mL)
Xylanase (mIU/mL)
PGase (mIU/mL)
PALase (mIU/mL)
6.0
5.5
5.0
4.5
4.0
3.5
20
15
10
5
0
15
10
5
0
40
30
20
10
0
120
90
60
30
0
400
300
200
100
0
400
300
200
100
0
1
2
3
4
5
6
Time of culture (weeks)

progressed in week five. RS levels were always higher in the centre than in the outer layer throughout the six weeks of growth. PGase activity was higher in the centre and lower in the outer layer. During the fruiting stage, FPase and CMCase were higher in the outer layer and lower in the centre, whereas xylanase, PGase and PALase were always higher in the centre.

3.3.3.4 Enzyme activity in mushroom fruiting bodies

Table 3.5a shows the level of enzymes formed in fruiting bodies of different size during development and Table 3.5b shows the enzyme activities distributed in different parts of the mushrooms. Studies on the biochemical changes of the fruiting body during development of the mushrooms showed the following:

a. The pH increased during the development of the mushroom fruiting bodies from the primordium stage to the medium-sized stage, then decreased when mushrooms started to decay.

b. The free RS of the fruiting body declined slightly with the development of the fruiting body, from a starting point of 12.65 mM in crude extracts of primordia at week four, then decreasing to 10.60 mM at the fully developed stage; RS levels in the primordium were much higher than in the substrate (5.83 mM) in week four, however, the levels in the mushroom fruiting bodies were similar to that in the substrate (11.20 mM) during fruiting in week five.

Table 3.5 Enzyme activity in *P. ostreatus* mushrooms

[a] Harvested at week four of culture, white skin-like mycelium in the outer layer of the culture.

[b] Harvested at week five from different PP bags, sizes of mushrooms were defined on the width of basidiocarp:

Small Mushrooms: 1-3 cm;

Medium Mushrooms: 4-6 cm;

Large Mushrooms: > 6 cm.

[c] Units of measurement are mM for RS and mIU/mL for enzyme activity in extracts.

[d] Mushrooms (basidiocarp width > 8 cm) were harvested in week five and stipes were isolated, then the gill was separated from the basidiocarp and which left the remaining pigmented surface of the basidiocarp.

Preparation of extracts is described in Section 2.4.1 of the Materials and Methods.

a. Enzyme activity in *P. ostreatus* mushroom of different size

Parameter	Biomass[a]	Primordia[b]	Small Mushrooms[b]	Medium Mushrooms[b]	Large Mushrooms[b]
pH	5.37	5.10	5.30	6.34	5.65
RS[c]	5.16	12.65	12.03	10.62	10.60
FPase[c]	0	12.59	12.20	0	3.80
CMCase[c]	19.49	45.14	25.29	7.21	1.89
Xylanase[c]	31.75	54.68	58.56	32.94	83.20
PGase[c]	35.72	108.36	50.24	0	1.34
PALase[c]	46.06	314.09	183.05	0	0

b. Enzyme levels in different parts of the mushroom fruiting body

Parameter	Stipe[d]	Gill[d]	Surface[d]
pH	5.55	5.40	5.29
RS[c]	10.48	8.18	11.85
FPase[c]	6.85	2.90	0
CMCase[c]	0	10.17	4.72
Xylanase[c]	33.43	67.10	36.85
PGase[c]	0	10.00	0
PALase[c]	0	0	4.71

c. CMCase levels were much higher during the development stage of the mushroom. Activity declined with the development of the mushroom fruiting body, whereas xylanase showed highest activity in the fully developed mushroom.

d. PGase and PALase showed highest activity in primordia, then the activities decreased and finally disappeared when the mushrooms were developed into medium size. Their activities were also found to be low in different parts of the mushroom tissues.

e. Xylanase activity remained at high levels during mushroom development and was demonstrated to be a major enzyme in different parts of the mushroom tissue.

3.4 PROPERTIES OF POLYGALACTURONASE AND PECTATE LYASE FROM *P. OSTREATUS*

Before attempting purification and characterisation of pectinase activities, some basic information on the assay and stability of enzyme activity was determined, as described in the following section. Once the optimal conditions for assay of the two enzymes (PGase and PALase) were determined, these were applied in analysis of enzyme production in the culture (see earlier sections) and for partial purification of the enzymes plus characterisation of their physical and biochemical properties. In some experiments, crude enzyme was used while in others enzyme partially purified by Sephacryl S-200 chromatography was used (for details see Section 2.4.4.2).

3.4.1 DETERMINING ASSAY CONDITIONS

3.4.1.1 Optimum pH for assay

The pH optimum for assay of the enzyme(s) which degraded PGA was initially estimated using 14-day-old culture extract of *P. ostreatus* from small-volume shake-flask SF (Fig. 3.9a). RS production was monitored after incubation at 50°C for one hour. Using buffers (citrate-phosphate, sodium acetate-acetic acid, succinic acid-NaOH and ammonia acetate-acetic acid) over the range of 3.0 to 7.5, a sharp peak of activity was seen at pH 5.0 and citrate-phosphate buffer gave the highest activity. A slight increase in activity was observed when the pH was higher than 7.0 using citrate-phosphate buffer and this was confirmed by using Tris buffer, Na_2HPO_4-NaH_2PO_4 buffers and Clark-Lubs solutions (KH_2PO_4-NaOH, pH 6.0-8.0). A second peak of activity was found at pH 8.6 when Clark and Lubs solutions, pH 8.0-10.0, were used. The enzyme with the optimum of pH 5.0 was confirmed as PGase and the enzyme with a optimum of pH 8.6 was confirmed as PALase in later experimentation. The pH optimum of PGase was checked using pooled peak activity from Sephacryl S-200 chromatography (see Fig. 3.15), which validated the pH optimum as 5 for this enzyme (Fig. 3.9b).

Although using several buffer solutions indicated the same optimum pH for PGase, citrate-phosphate gave the highest activity. When the ionic strength of the buffer in the assay was increased by adding more buffer to the standard assay, it was shown that the greatest activity was obtained when 0.2-0.3 mL of citrate-phosphate buffer (prepared from 0.1 M citric acid and 0.2 M di-sodium hydrogen phosphate) was used (Fig. 3.10). When higher

Figure 3.9 Effect of pH on PGA degrading activity of *P. ostreatus*

a The source of crude enzyme extract was a two-week culture (lupin hull-SSBM in small-volume shake-flask SF), filtered and centrifuged (see Section 2.4.1 of the Materials and Methods). A reaction mixture of 0.5 mL contained 0.1 mL extract, 0.1 mL of 1% PGA (w/v), 0.2 mL standard buffer solution (see Section 2.4.5 of the Materials and Methods), which was incubated at 50°C for 60 minutes. Enzyme activity was estimated from increased RS detected by the DNS method. pH in the reaction mixture was measured at 25°C after mixing.

b Partially purified enzyme from Sephacryl S-200 chromatography (see peak around fraction 60, Fig. 3.15) was first dialysed against distilled water then assayed. The reaction mixtures (0.5 mL) included 0.1 mL enzyme, 0.1 mL 1% (w/v) PGA, 0.2 mL standard citrate-phosphate buffer and 0.1 mL distilled water. The enzyme activity was estimated from increased RS at 55°C for 30 minutes, detected by the DNS method. pH in the reaction mixture was measured at 25°C after mixing.

(▽) Citrate-phosphate buffer;

(△) Acetic acid-sodium acetate buffer;

(▲) Acetic acid-ammonium acetate buffer;

(■) Succinic acid-NaOH buffer;

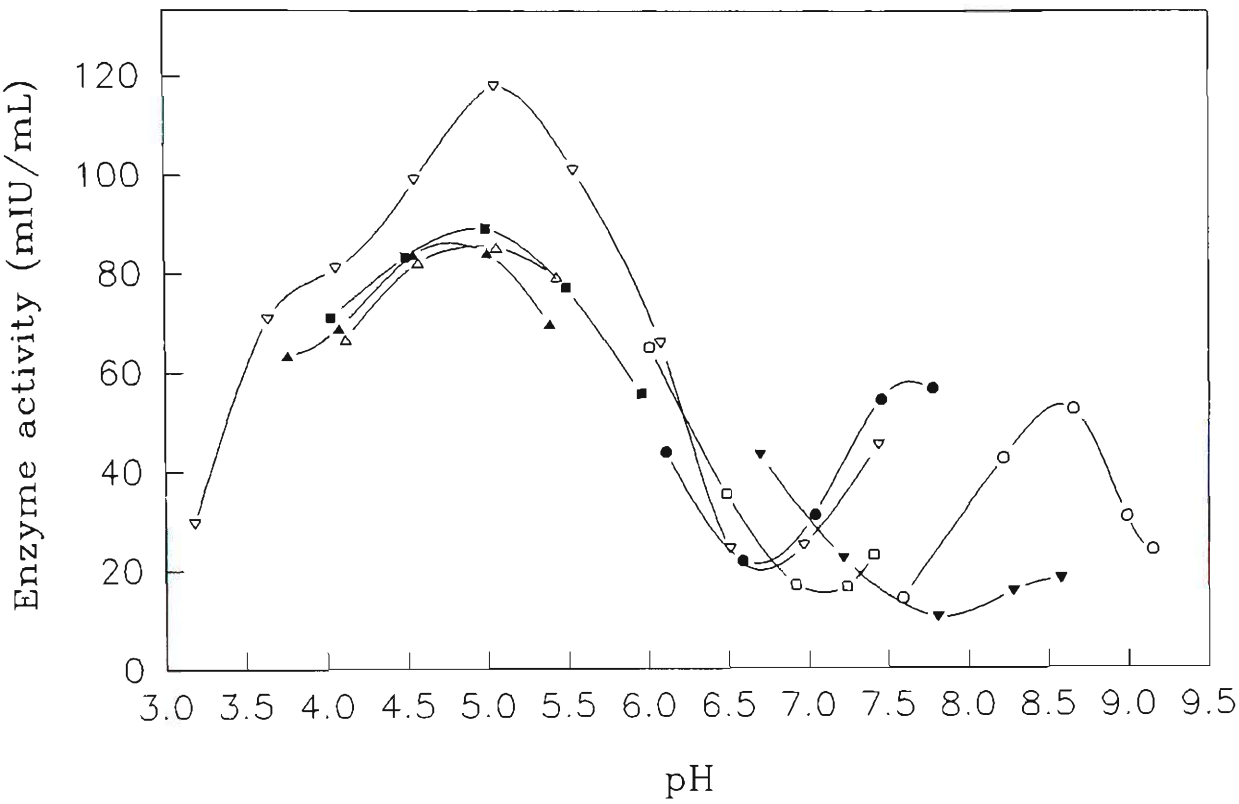
(□) Clark-Lubs (K phosphate) buffer solution;

(●) Na_2HPO_4-NaH_2PO_4 buffer;

(▼) Tris-maleate buffer;

(○) Clark-Lubs (boric acid) buffer solution.

a. pH optimum of PGA degrading activity in a crude enzyme preparation



b. pH optimum of partially purified enzyme from Sephacryl S-200 chromatography

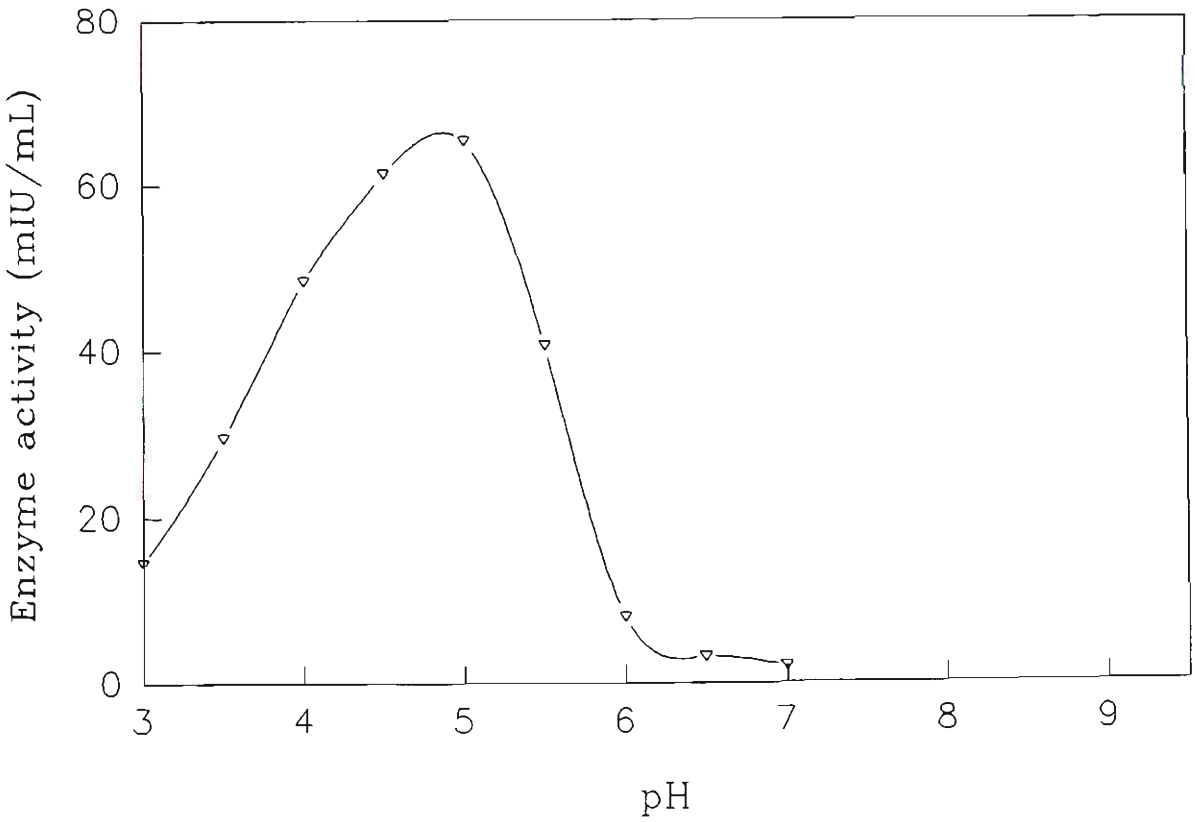
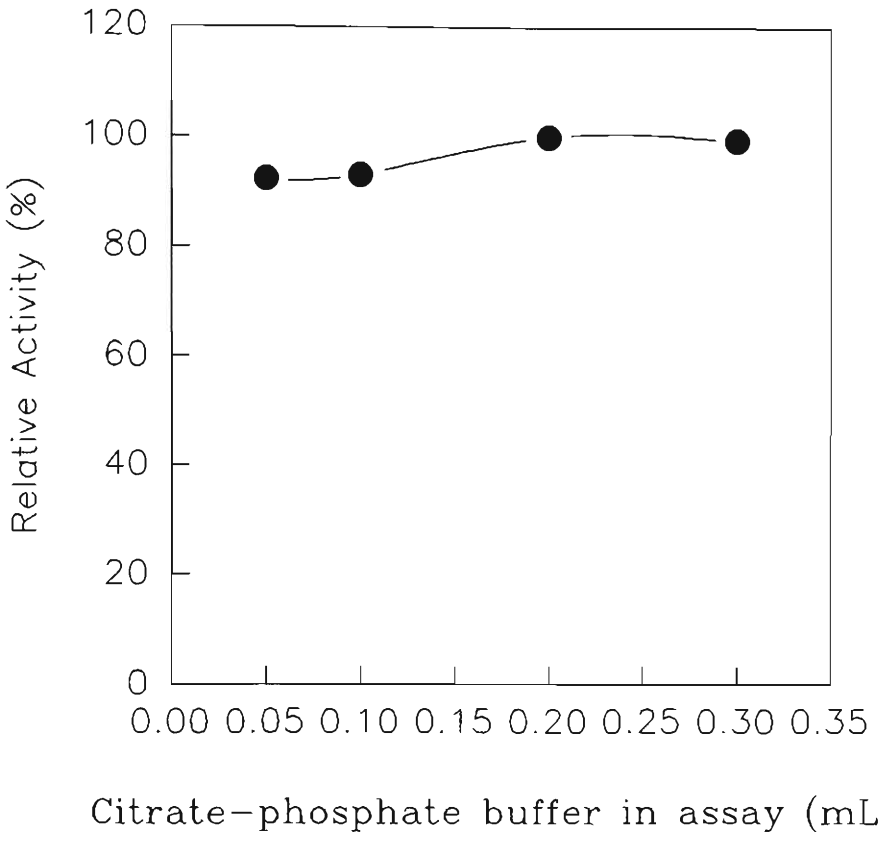


Figure 3.10 Effect of ionic strength of buffer on activity of PGase and PALase

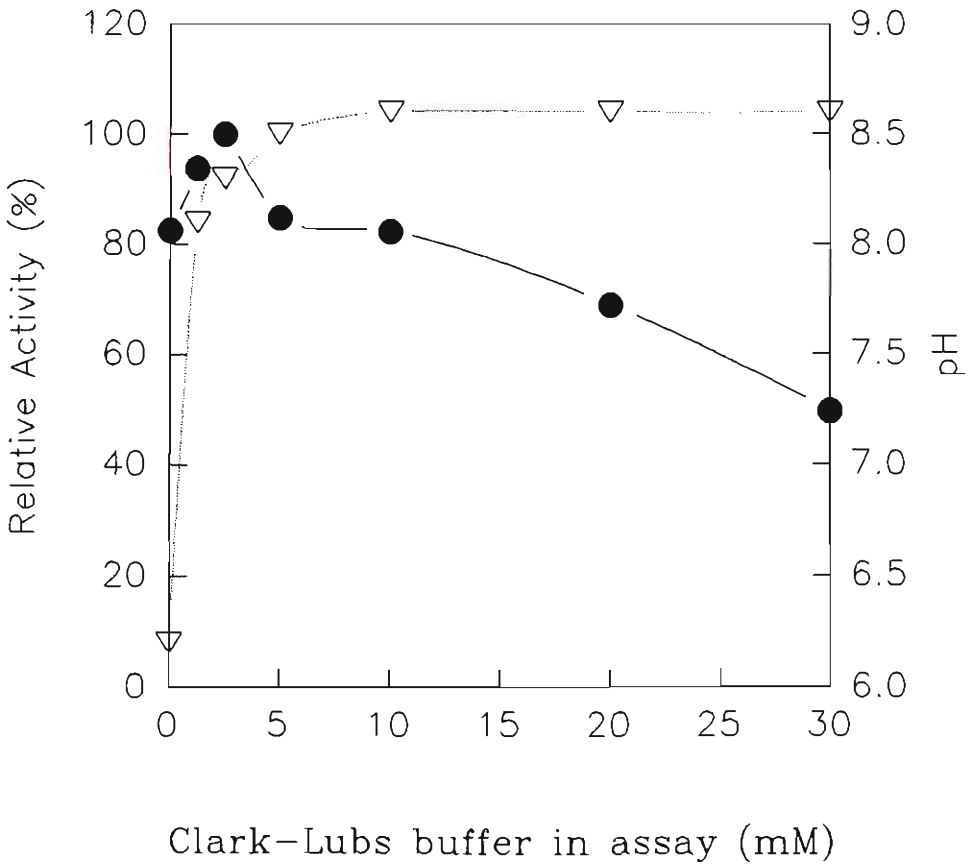
A reaction mixture of 0.5 mL contained 0.1 mL crude enzyme, 0.1 mL 1% (w/v) PGA, standard citrate-phosphate buffer, pH 5.0 for PGase or Clark-Lubs solution, pH 8.6 for PALase and distilled water. The activity was measured after incubating at optimum temperature (55°C for PGase and 45°C for PALase) for 30 minutes and relative activity was based on the highest activity obtained.

(●) Activity of enzyme; (▽) pH of reaction mixture.

a. PGase



b. PALase



concentrations of Clark-Lubs pH 8.6 solution were used, less PALase activity was detected. However, the pH of the assay system could not be maintained when concentrations were lower than five mM (concentration refers to boric acid). Also, when the concentration of boric acid was lower than 2.5 mM, the activity of PALase decreased. The standard assay for PGase and PALase therefore used the equivalent of 0.25 mL of citrate-phosphate buffer, pH 5.0 (prepared from 0.1 M citric acid and 0.2 M di-sodium hydrogen phosphate) and 0.2 mL of 0.05 mM Clark-Lubs buffer solution, pH 8.6 (prepared according to Dawson *et al.*, 1986) for all subsequent analyses and purification procedures.

3.4.1.2 Optimum temperature for assay

By estimating RS produced at different incubation temperature up to six hours with the pH optima found as above, the optimum temperature for the PGase was found to be 50-55 °C and the PALase was 40-45°C (Fig. 3.11). The optimum temperature for the PALase was also estimated to be 40°C using the assay which detected degradation products at 235 nm (Fig. 3.12).

Temperature affected both the initial rates of reaction and the extent of reaction, as indicated by determining the average velocity of reaction over six hours incubation (Fig. 3.11). For both PGase and PALase, a higher initial rate was seen at temperatures above the defined optima but the extent of reaction (total product converted) decreased. This is clearly demonstrated in Fig. 3.12 for PALase, which shows that at temperatures above 45°C, the reaction stops rapidly.

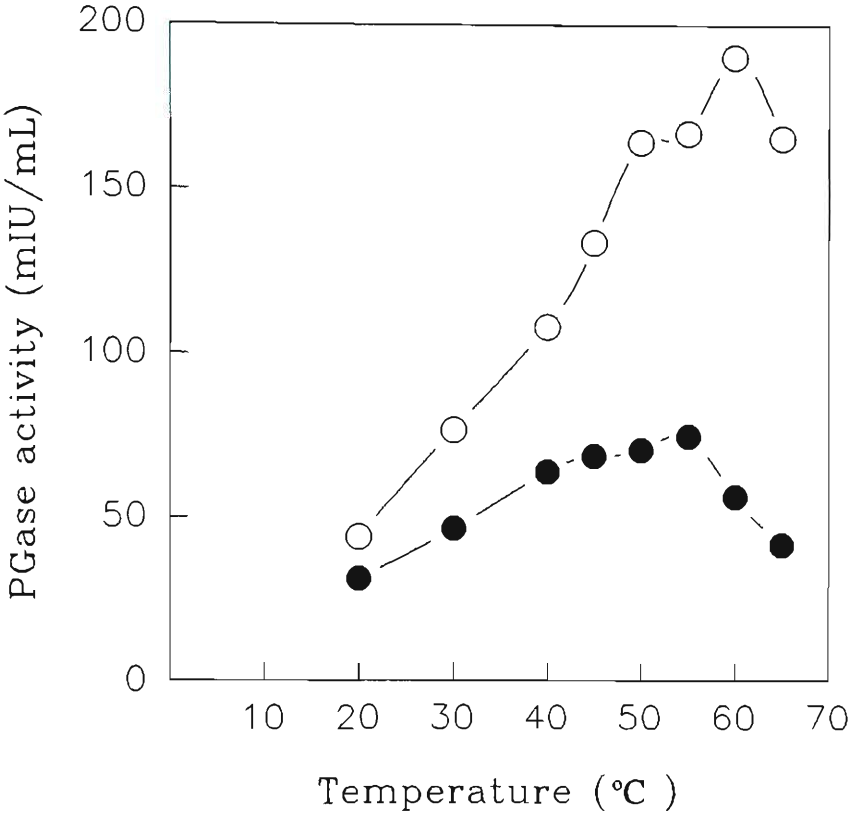
Figure 3.11 Effects of temperature on PGase and PALase activities of *P. ostreatus*

Culture (14-day-old) extract (0.1 mL) was incubated with 0.1 mL 1% (w/v) PGA, 0.2 mL standard buffer (citrate-phosphate, pH 5.0 for PGase and Clark-Lubs, pH 8.6 for PALase) and 0.1 mL distilled water for up to six hours. Enzyme activity was estimated by increased RS.

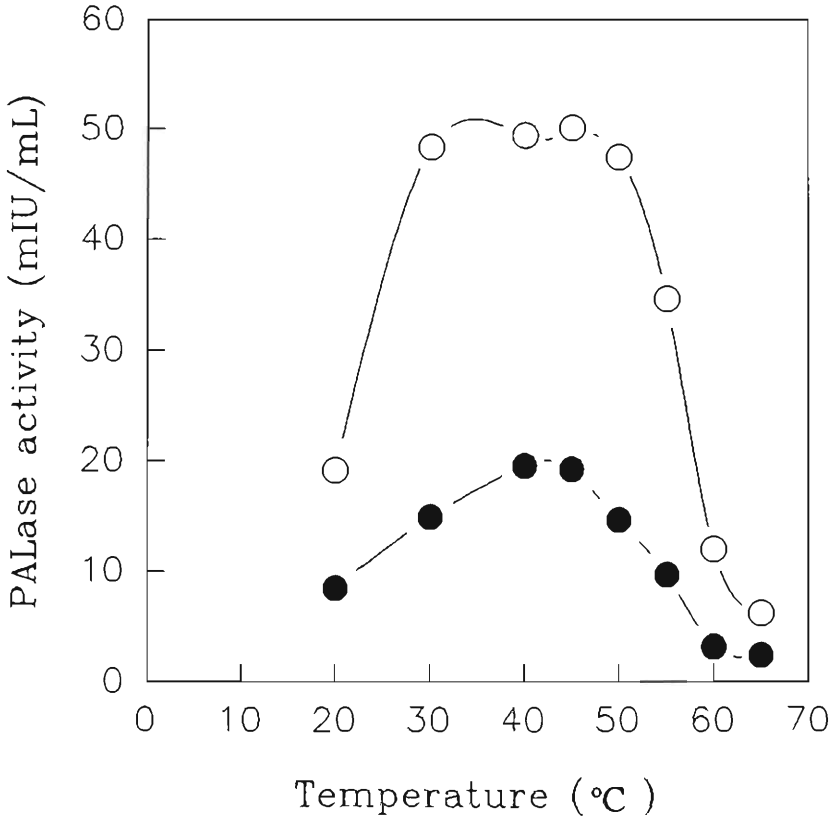
(○) Initial velocity of reaction in the first 30 minutes;

(●) Average velocity over six hours.

a. PGase



b. PALase



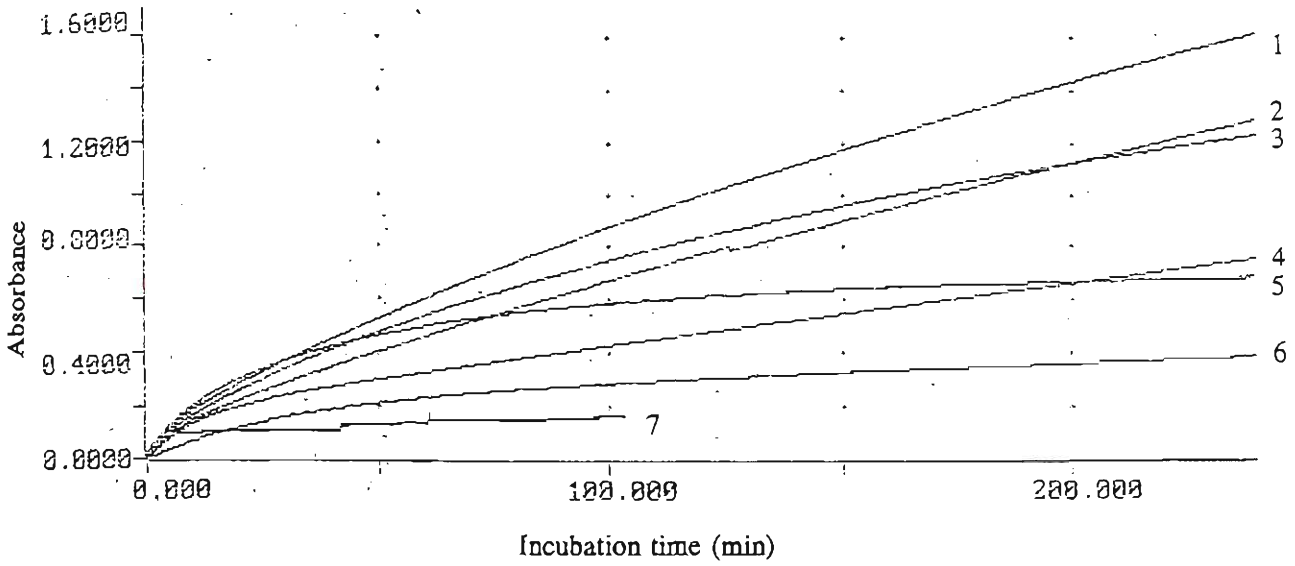


Figure 3.12 Effect of temperature on PALase activity of *P. ostreatus* as assayed by scanning product formation at 235 nm

Pre-warned culture extract, stock Clark-Lubs buffer pH 8.6, 1% (w/v) PGA and distilled water were mixed at a ratio of 1:1:1:2 and scanned at 235 nm. Controls contained the same amount of enzyme which had been boiled to eliminate activity. Scans for the latter were subtracted automatically from test assays.

1. 40°C;
2. 38°C;
3. 42°C;
4. 30°C;
5. 45°C;
6. 20°C;
7. 50°C.

3.4.1.3 Relationship between initial rate of reaction and amount of enzyme extract used

The relationship between the rate of reaction and the amount of enzyme used was determined by varying the enzyme concentration in assays monitored over four hours. The time course of PGA degradation showed that enzymatic degradation of PGA by PGase was a straight line over the first 30 minutes of reaction when RS was less than 1.5 μmoles in 0.5 mL of reaction mixture (Fig. 3.13a). A linear relationship was seen between initial rate of PGase and volume of crude extract used up to 0.2 mL of extract, using a shorter incubation period (Fig. 3.14a): assays were therefore run thereafter for 30 minutes and tests reassayed with less enzyme if RS produced exceeded 1.5μmoles. RS produced by PALase, however, was not directly proportional to the time (Fig. 3.13b), which was also confirmed in assays where products were measured by scanning absorbance of reactions at 235 nm (Fig. 3.12). Initial rates were therefore only estimates of initial rate averaged over a 30 minutes period. Again, the relationship between initial rate and amount of enzyme failed at high enzyme levels used (Fig. 3.14b) so that assays were limited thereafter to 30 minutes and volumes of crude extract did not exceed 0.1 mL in assays.

3.4.1.4 Metal ion requirement for pectate lyase

In early attempts to purify PGase and PALase, little activity of PALase was recovered after Sephacryl S-200 chromatography when only buffer and PGA were used in assays, compared to about 90% recovery of the PGase applied. One explanation was that the crude extract contained components important for activity and that these were removed during gel

Figure 3.13 Kinetics of reactions with different incubation times for assays of PGase and PALase

A two-week-old culture filtrate was used as crude enzyme for testing suitable incubation times for assay. A total volume of 0.5 mL of reaction mixture contained 0.1 mL of 1% (w/v) PGA, 0.2 mL standard buffer (citrate-phosphate, pH 5.0 for PGase and Clark-Lubs, pH 8.6 for PALase), crude enzyme and distilled water. The reactants were incubated at 55°C for PGase and 45°C for PALase for up to six hours. Increases in RS level were monitored.

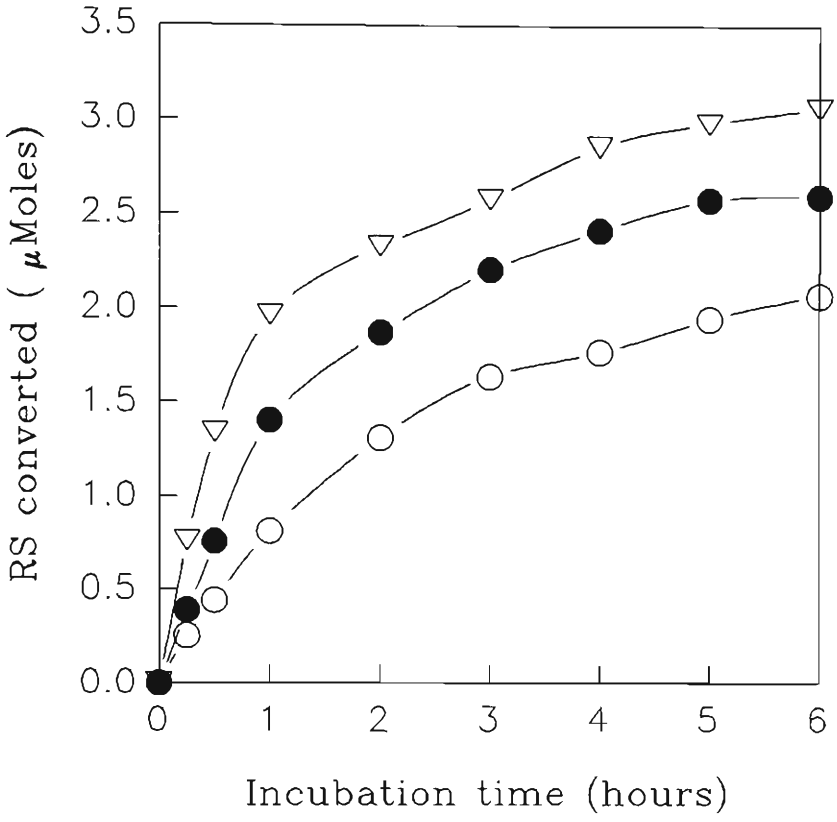
Volume of crude enzyme used:

(▽) 0.2 mL;

(●) 0.1 mL;

(○) 0.05 mL.

a. PGase



b. PALase

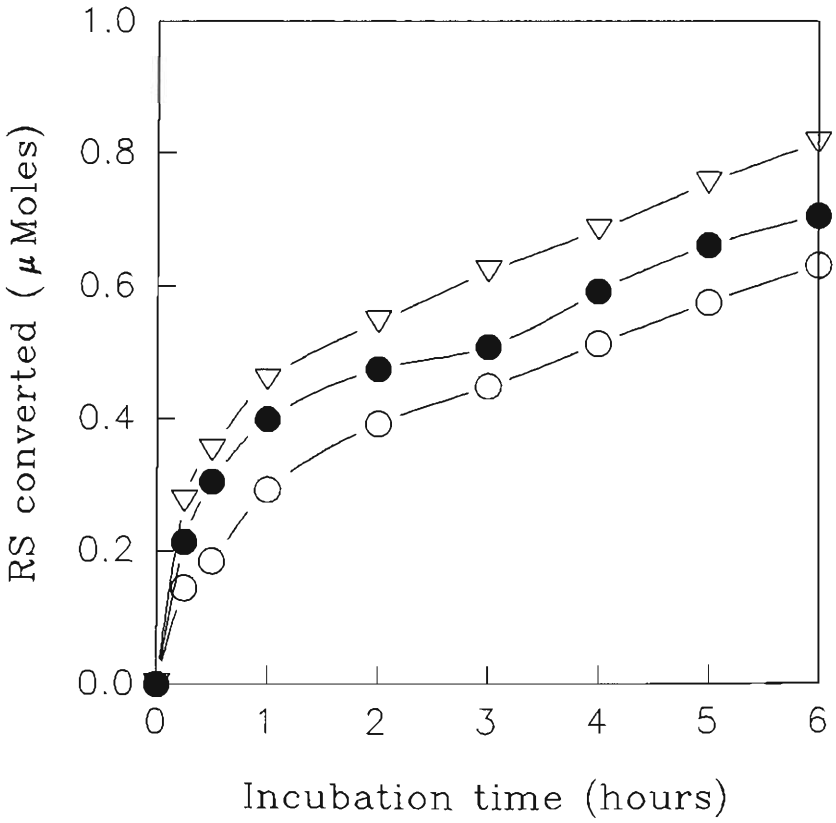


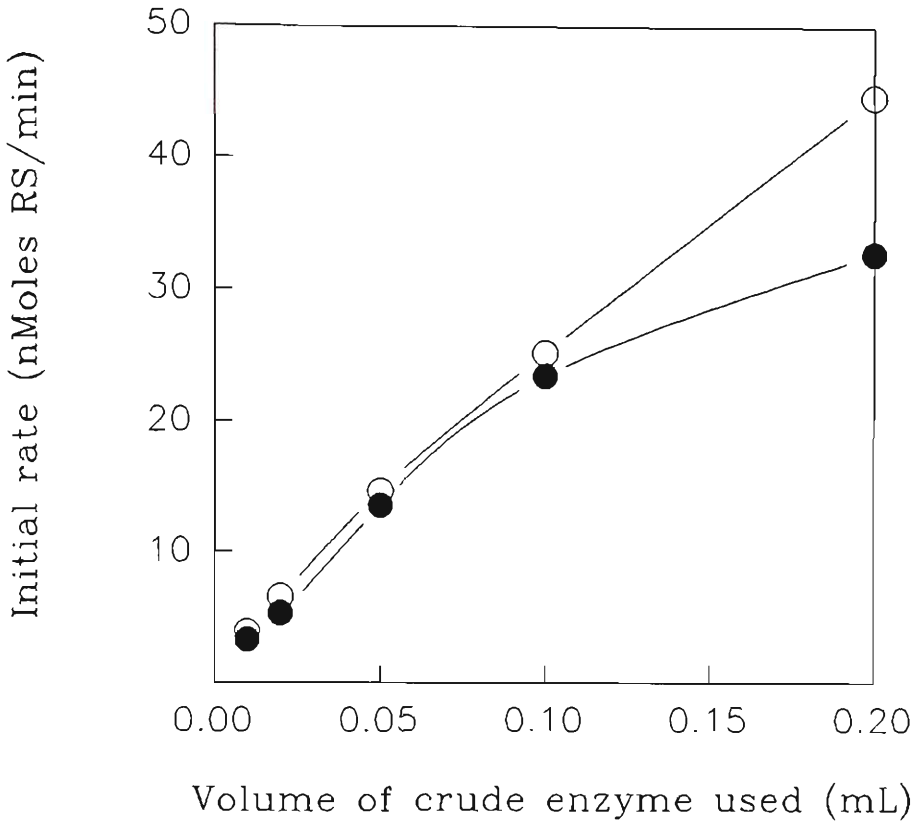
Figure 3.14 Kinetics of reactions with different amounts of enzyme in assays of PGase and PALase

A reaction mixture with a total volume of 0.5 mL contained 0.1 mL of 1% (w/v) PGA, 0.2 mL standard buffer (citrate-phosphate, pH 5.0 for PGase and Clark-Lubs, pH 8.6 for PALase), crude enzyme preparation and distilled water, incubated at 55°C for PGase and 45°C for PALase for up to six hours.

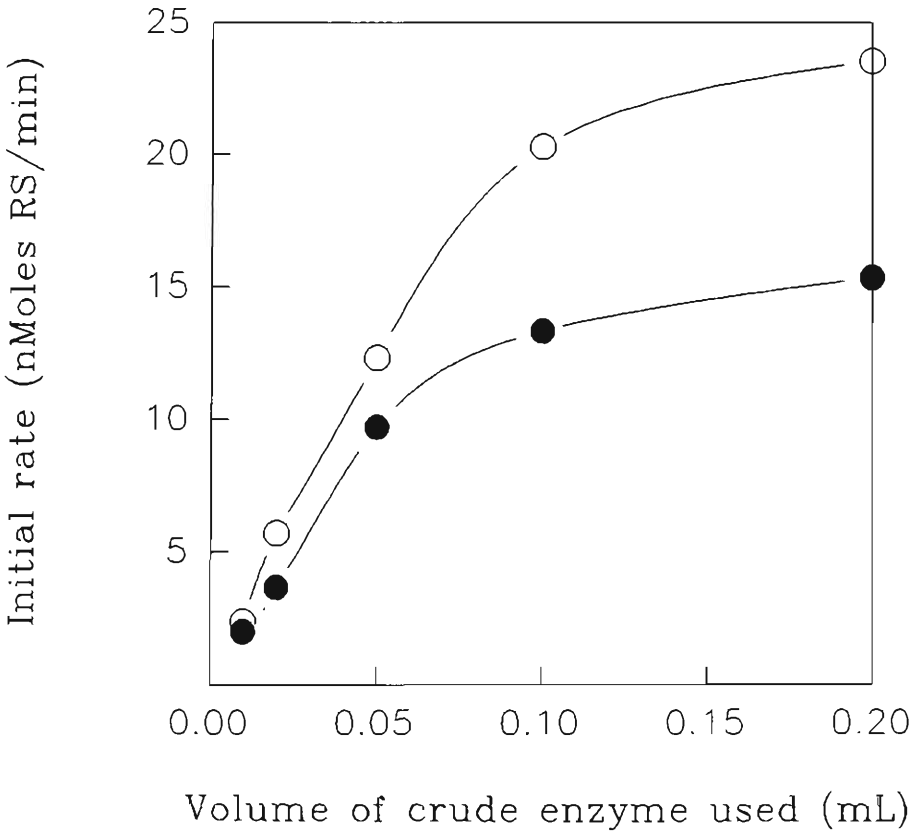
(○) Average Rate over 30 minutes;

(●) Average Rate over 60 minutes;

a. PGase



b. PALase



filtration. The re-activation of PALase was first carried out by supplementing assay mixtures with boiled culture filtrates (Fig. 3.15). Once the PALase activity was found in column fractions, further studies on the re-activation of activity by the boiled culture filtrate, SSBM, mineral salts and their concentration were conducted (Fig. 3.16).

Although the boiled culture filtrate should contain the same salts as the SSBM, the boiled culture filtrate restored PALase activity better than SSBM, noting that activity was impaired if volumes greater than 0.1 mL of either was used (Fig. 3.16a).

By supplementing reaction mixtures with the individual salts of the SSBM at 0.5 mM or 1.0 mM, it was shown that the calcium chloride gave the highest recovery of activity over the K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4$, $MnSO_4$, $CoCl_2$, $ZnSO_4$, $CuSO_4$, $FeSO_4$ and $FeCl_3$. The highest activity was seen when 0.5 mM $CaCl_2$ was used in the final assay mixture and 1.0 mM appeared to be less effective (Fig. 3.16b).

Further studies involved testing $CaCl_2$ supplementation at different concentrations up to 1.0 mM: the best concentration for re-activation of PALase was between 0.1 mM and 0.2 mM and higher concentrations inhibited activity (Fig. 3.16c). Calcium chloride was added at a final concentration of 0.15 mM to assays of PALase activity routinely, which was particularly important when the enzyme used was partially purified from column chromatography or IEF Rotofor cell fractions.

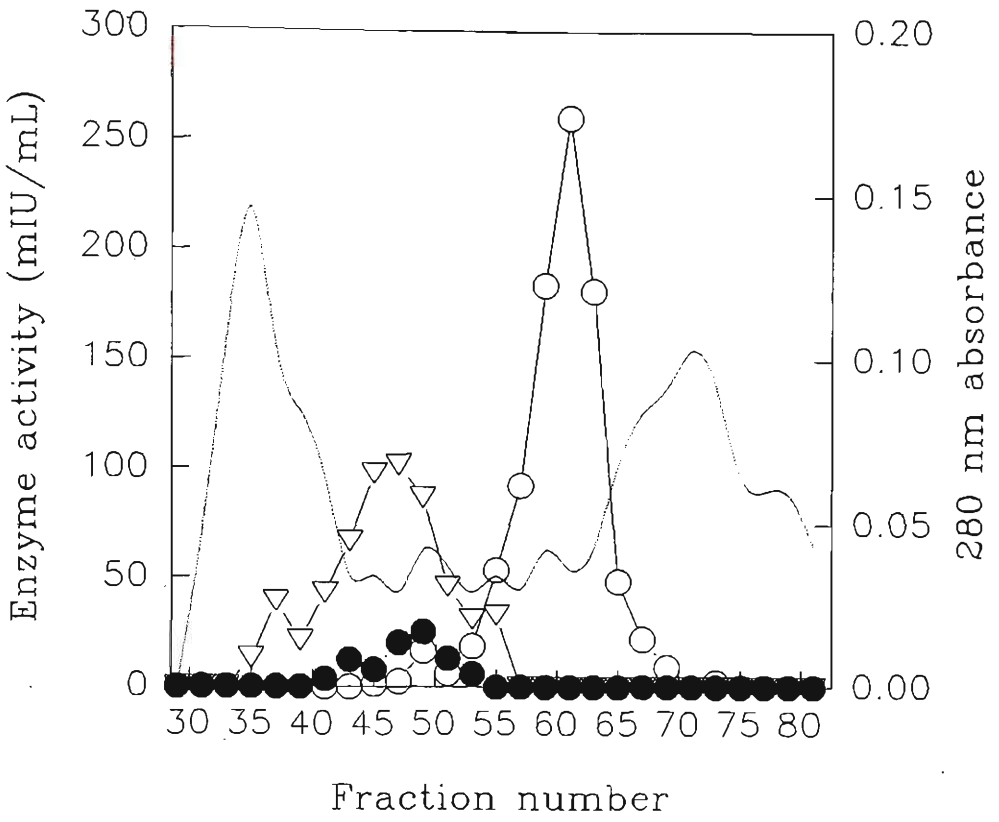


Figure 3.15 Re-activation of PALase activity after chromatography

A 20-fold concentrated culture extract was applied to Sephacryl S-200 column 1 (for experimental details see Section 2.4.4.2 of the Materials and Methods). Fractions were examined for PALase activity and absorbance at 280 nm was continuously monitored in the outflow (.....). PALase activity was initially assayed in a system containing 0.1 mL of column fraction, 0.1 mL stock Clark-Lubs buffer pH 8.6, 0.1 mL 1% (w/v) PGA and 0.2 mL distilled water, 45°C, (●) and the standard PGase assay was used (○). Boiled culture filtrate (0.1 mL) was added to the PALase assay (distilled water reduced to 0.1 mL to make total volume 0.5 mL), PALase activity (▽) was found.

Figure 3.16 Re-activation of PALase activity

A reaction mixture with a total volume of 0.5 mL contained 0.1 mL of partially purified enzyme (pooled from Sephacryl S-200 column 1 fractions 45-47, Fig. 3.15), 0.1 mL stock Clark-Lubs buffer pH 8.6, 0.1 mL of 1% (w/v) PGA and various supplementations, made up to the total volume with distilled water and then incubated at 45°C for 30 minutes. Activity of enzyme was estimated from RS production using the DNS method. Relative activity was compared to the highest activity obtained in the same category of tests.

a. (●) Volume of boiled culture filtrate added to assay;

(○) Volume of mineral salts mixture supplied in SSBM added to assay.

b. Individual ion at 0.5 mM (▨) and 1.0 mM (□) in final concentration.

1.	K_2HPO_4;	2.	$(NH_4)_2SO_4$;	3.	$MgSO_4$;	4.	$CaCl_2$;
5.	$MnSO_4$;	6.	$ZnSO_4$;	7.	$CoCl_2$;	8.	$CuSO_4$;
9.	$FeSO_4$;	10.	$FeCl_3$;	11.	Control (without additional ions).		

c. PALase used:

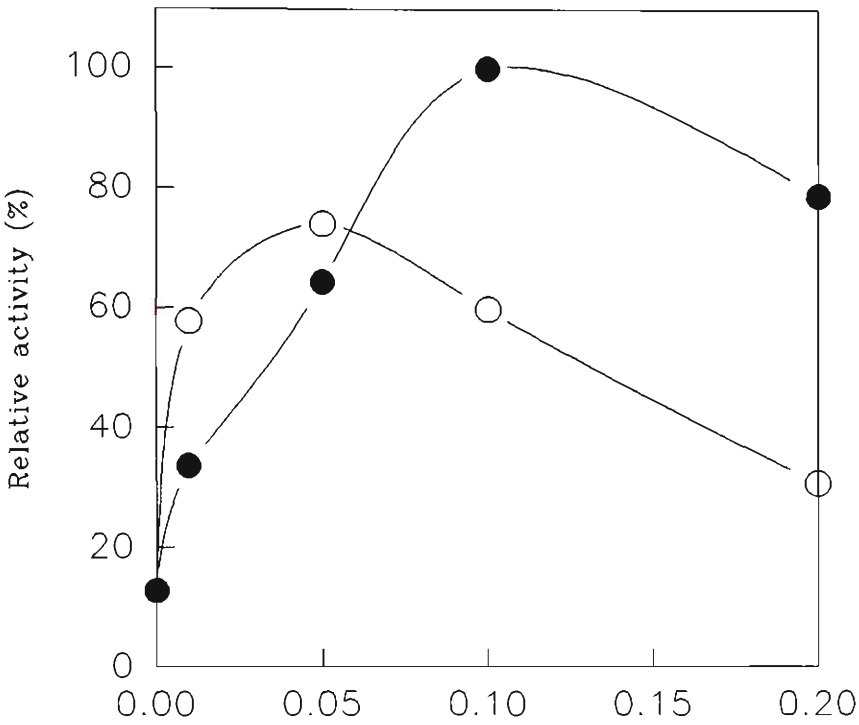
(○) 1:10 diluted enzyme (equivalent to 10 μL pooled fraction);

(●) 1:5 diluted enzyme (equivalent to 20 μL pooled fraction);

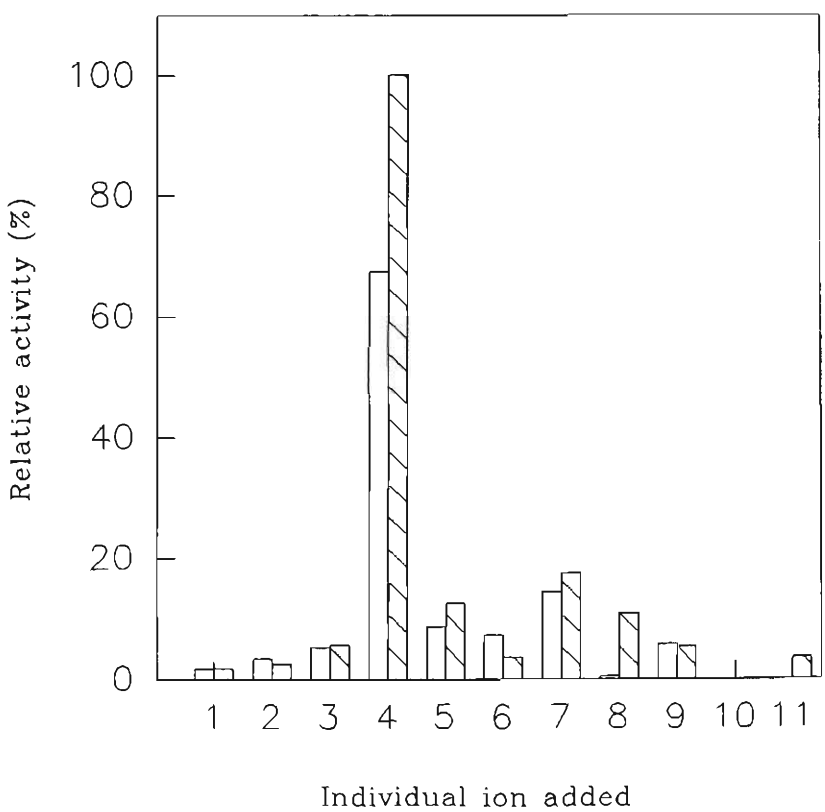
(▽) 1:2 diluted enzyme (equivalent to 50 μL pooled fraction);

(▼) Undiluted enzyme (equivalent to 100 μL pooled fraction).

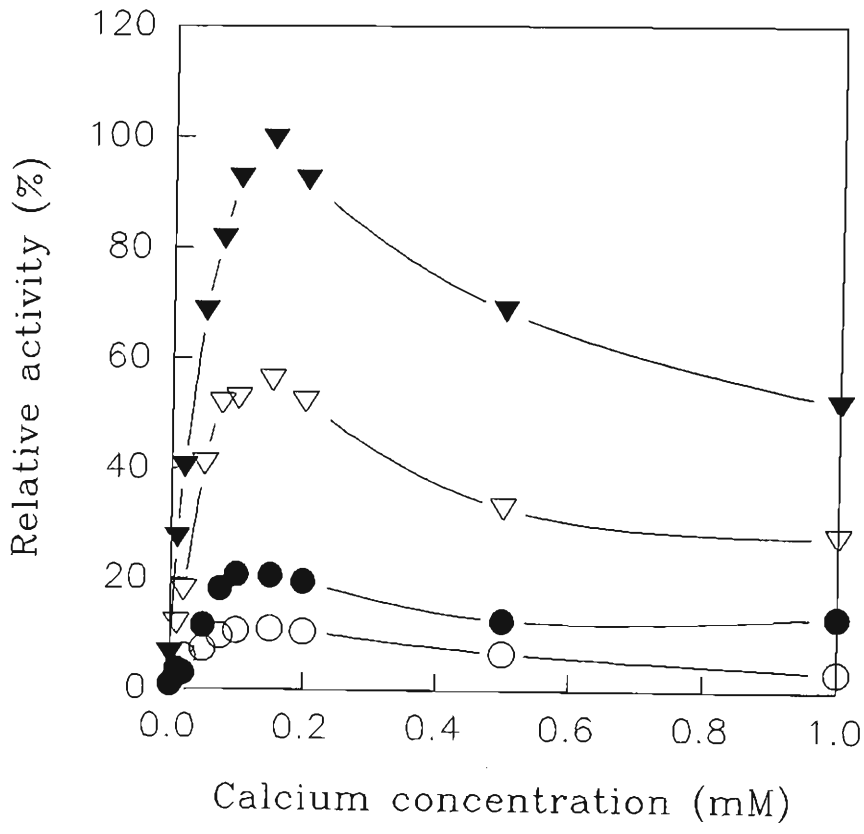
a. Re-activation of PALase activity using different volumes of boiled culture filtrate and mineral salts mixture



b. Re-activation of PALase activity by individual salts



c. Enzyme activity detected using various amounts of PALase and calcium chloride



3.4.1.5 Detection of degradation products by absorbance

Enzymatic products of PGA degraded by the PGase and PALase assayed at their optimal pH and temperature were scanned for the absorbance from 190 nm to 900 nm (Fig. 3.17). The product of PALase exhibit a peak in absorbance at 235 nm, which is characteristic for this class of enzyme. Products of PGase did not have any absorbance peak. Scanning also indicated that no lyase activity was found when assays were performed at pH 5.0, validating the observation that the pectinase activity with a pH optimum of 5.0 was the PGase.

3.4.2 STABILITY OF ENZYMES

3.4.2.1 pH stability of enzymes

Attempts were made to determine the stability of pectinase activity in crude extracts when stored at room temperature. The results were not informative because the level of RS increased during storage, presumably due to continued degradation of soluble substrate in the crude extract, so that background levels were high after three days storage. When partially purified enzyme was available from Sephacryl S-200 (see Section 2.4.4.2), this experiment was repeated. The results (Fig. 3.18) showed that both PGase and PALase in partially purified state from the Sephacryl S-200 column was stable for up to 72 hours storage at room temperature in citrate-phosphate buffer and Clark-Lubs buffer at pHs in the range of 3.0 to 9.0. The PGase retained 90% of its original activity in buffers of pH 3.0 to 9.0. The PALase retained 70% from 5.0 to 9.0, relative to initial activity.

3.4.2.2 Thermostability of enzymes

Using culture extract as the crude enzyme source, it was shown that PGase and PALase demonstrated different stability to heat exposure (Fig. 3.19). These results indicated that

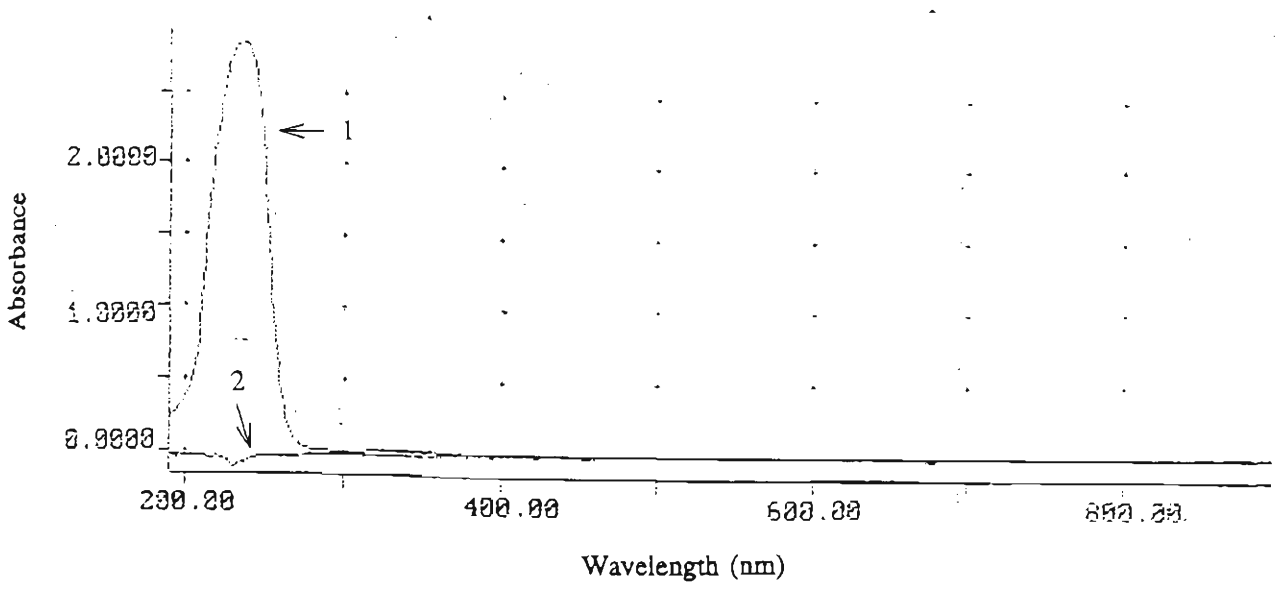


Figure 3.17 Scanning of enzymatic products of PGA degraded by PGase and PALase

Experimental details are given in Section 2.4.5.8 of the Materials and Methods. PGase and PALase activities in a crude extract were assayed under optimal conditions for each enzyme. The control for the scanning products of PGA degraded by PGase and PALase was the reaction mixture containing boiled crude extract in place of active crude enzyme. Scans of the controls were subtracted from scans of tests automatically.

1. Absorbance of products produced by PALase;
2. Absorbance of products produced by PGase.

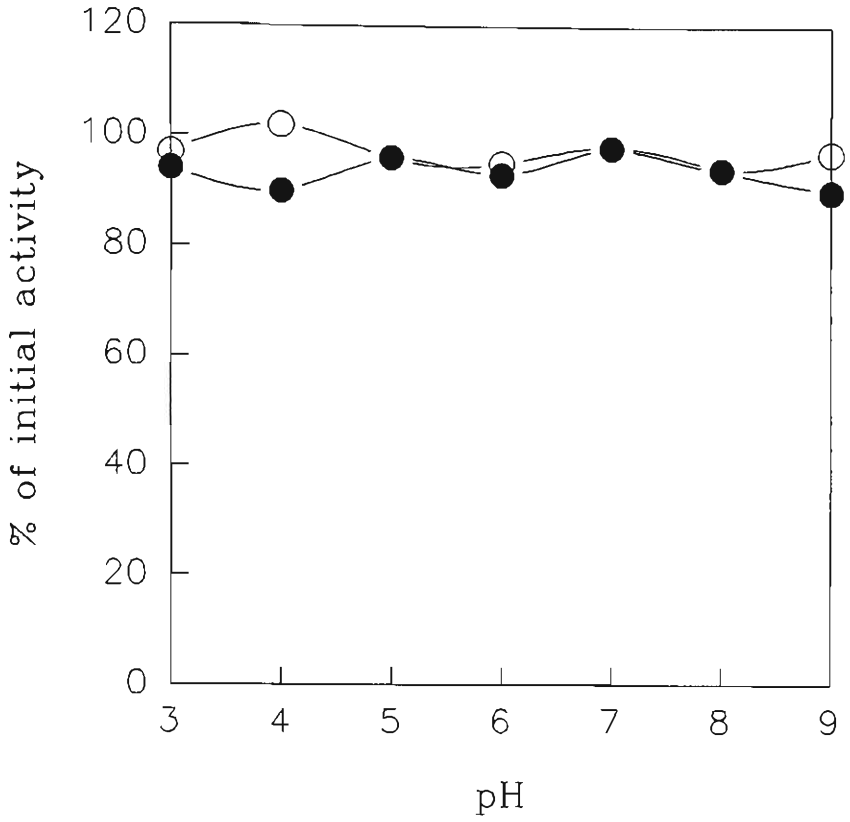


Figure 3.18 Stability of PGase and PALase in citrate-phosphate and Clark-Lubs buffer at different pH values

Partially purified enzymes collected from Sephacryl S-200 column fraction were first dialysed against distilled water, then mixed with standard citrate-phosphate buffer (prepared from 0.1 M citric acid and 0.2 M di-sodium hydrogen phosphate), pH 3.0-7.0 and 0.05 M Clark-Lubs buffer (concentration referred to boric acid), pH 8.0-9.0 at ratio of 3:1 (v/v). The initial activities of enzymes was assayed immediately after mixing and used to compare the retained activities of enzymes after 72 hours.

(○) PGase;

(●) PALase.

Figure 3.19 Thermostability of PGase and PALase

A series of tubes was set up where each contained two mL of culture extract and two mL of stock buffer (citrate-phosphate buffer pH 5.0 for PGase and Clark-Lubs buffer for PALase). Tubes were incubated in water baths with different temperatures and samples withdrawn from the bath after appropriate periods and immediately cooled in an ice-water bath. Assay mixtures (0.5 mL) contained 0.2 mL of incubation mixture, 0.1 mL PGA and 0.2 mL distilled water. Activity detected after heat treatment was expressed as a percentage of the activity assayed before heating (t = 0).

(○) 30°C;

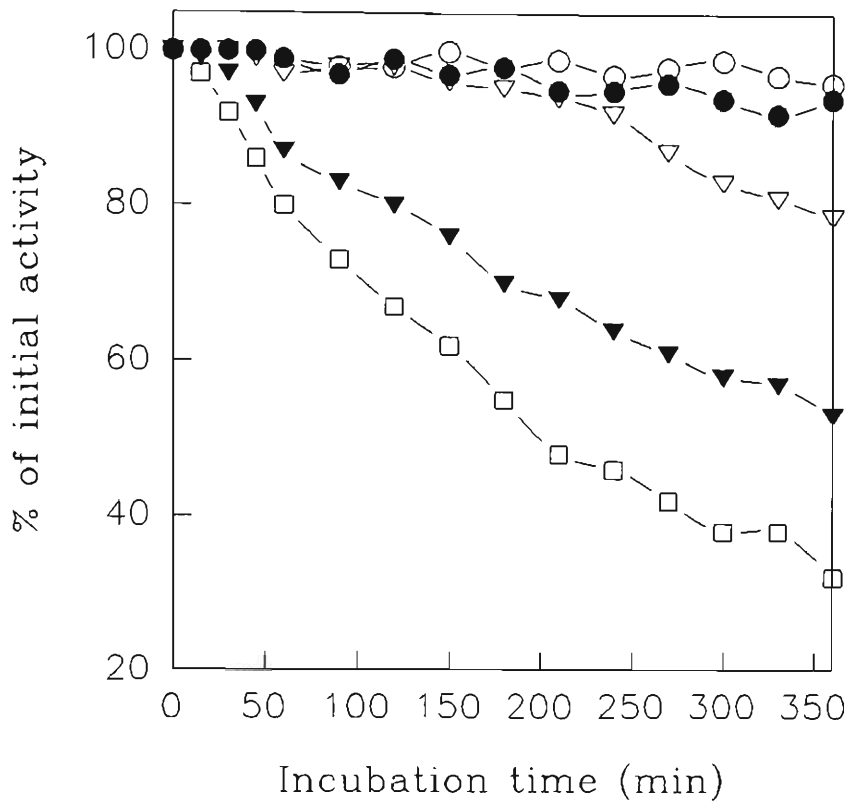
(●) 40°C;

(▽) 50°C;

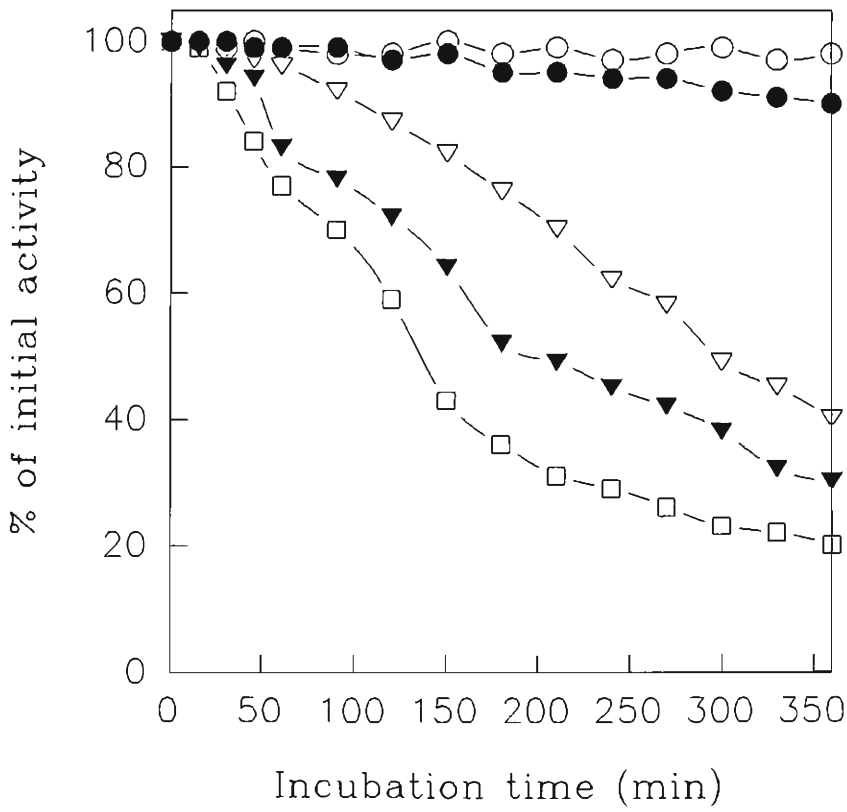
(▼) 60°C;

(□) 70°C.

a. PGase



b. PALase



PGase was stable below 50°C at pH 5.0, whereas PALase was stable under 40°C at pH 8.6. PGase retained 85% of its initial activity at 50°C after six hours and 50% of original activity at 60°C after six hours. PALase retained 90% of activity at 40°C after six hours, but only 40% at 50°C. These observations are consistent with those obtained for determining the temperature optimum for assay. For PGase, the extent of degradation significantly decreased above 60°C, which corresponds to loss of enzyme activity as shown here. Similarly, for PALase, the extent of degradation decreased dramatically above 50°C.

3.4.2.3 Denaturation of enzymes following heating in a boiling water bath

During preliminary experiments, it was apparent that the level of reducing sugar for controls of the pectinase assay (crude extract boiled for five minutes to inactivate enzymes and enable detection of endogenous RS) were high and the kinetics of assays could not be interpreted. One possible explanation was that the activities in the crude extract were surviving boiling. This was tested as described in Fig. 3.20. PALase in crude extracts was inactivated by heat-treatment at 100°C in a boiling water bath for six minutes, whereas PGase retained some activity up to ten minutes. It should be noted that the temperatures in the tubes were not monitored and it is likely that the extracts took some time to reach 100°C.

3.4.3 GEL FILTRATION OF CULTURE EXTRACTS ON SEPHACRYL S-200

3.4.3.1 Estimation of molecular weight by chromatography

A concentrated culture extract of *P. ostreatus* was applied to a Sephacryl S-200 column (column 2, note this column was not the same column as column 1 in Section 3.4.1.5), which was pre-calibrated for MW determination, so that the approximate size of the enzyme could be estimated (Fig. 3.21). Activity of PGase eluted in a peak corresponding to a MW of

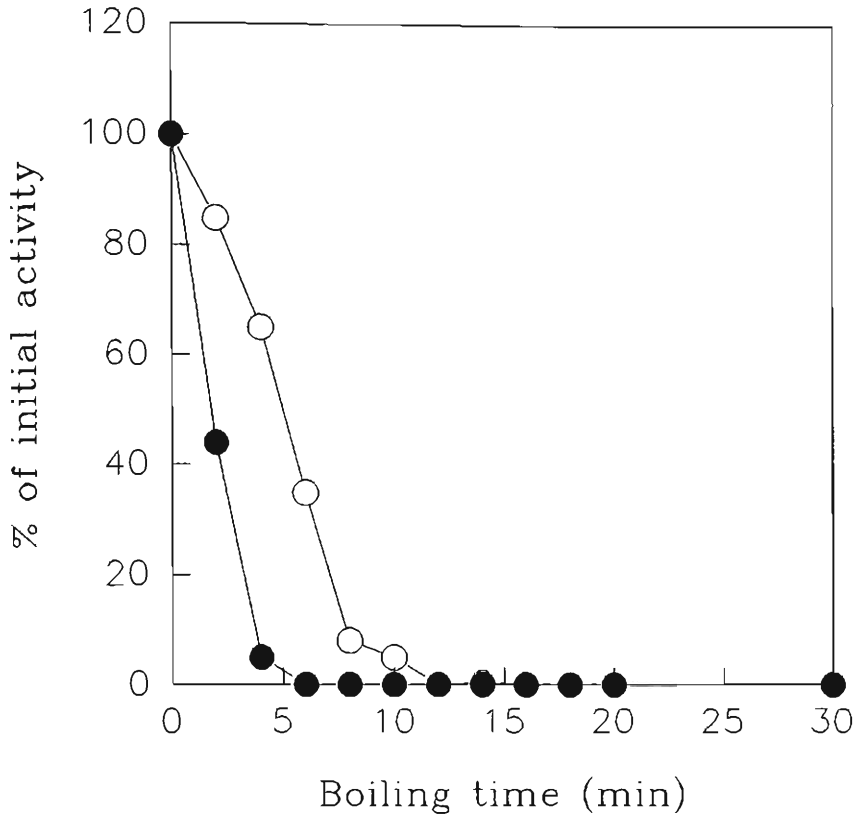


Figure 3.20 Survival of PGase and PALase activity during boiling

A series of tubes was set up where each contained four mL of culture extract. Tubes were placed in a boiling water bath for various periods then immediately transferred to an ice-water bath. The treated culture extract was used as crude enzyme and standard PGase and PALase assays were applied and activity expressed as a percentage of the activity assayed before heating (t = 0).

(○) PGase;

(●) PALase.

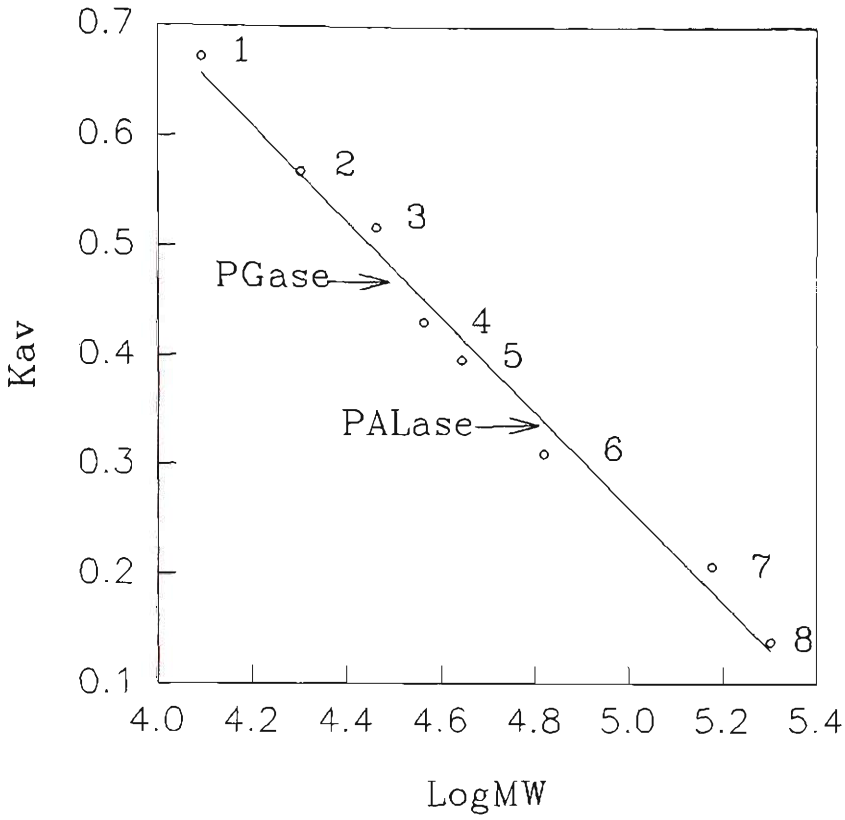


Figure 3.21 Molecular weight of PGase and PALase by Sephacryl S-200 chromatography

For experimental details see Section 2.4.4.2 of the Materials and Methods.

1. Cytochrome C;
2. Trypsin inhibitor;
3. Carbonic anhydrase;
4. β-lactoglobulin;
5. Ovalbumin;
6. BSA-V;
7. Alcohol dehydrogenase;
8. β-amylase.

36,500; activity of PALase, detected in an assay system containing 0.15 mM calcium chloride, elute in a peak corresponding to a MW of 66,000.

3.4.3.2 Partial purification of enzymes by chromatography

Attempts were made to purify pectinase by Sephacryl S-200 (Fig. 3.22). Relative to the total activity applied, 95% of PGase was recovered in the fractions. The specific activity of PGase in the pooled active fractions was 37,435 mIU/mg of protein, which was 34-fold that of the crude enzyme (Table 3.6). For PALase, 110% of activity was recovered after assaying by adding 0.15 mM $CaCl_2$ (see 3.4.12). The specific activity of PALase in the pooled active fractions was 10,320 mIU/mg of protein, which was 16-fold that of the crude enzyme. Further purification by IEF was attempted using the Rotofor cell but estimations of purification factors could not be made due to the low protein levels present (note that the fractions which contained PGase and PALase had low absorbance at 280 nm and the residual ampholytes interfered with protein measurement).

3.4.4 ION-EXCHANGE CHROMATOGRAPHY OF PECTATE LYASE

Dialysed culture filtrate containing PALase was applied to a DEAE-Sepharose Fast Flow ion-exchange column after the pI of PALase was determined in the IEF Rotofor cell (see Section 2.4.4.3 of the Materials and Methods). Three peaks of activity were found with a total of 64% of activity applied being recovered (Fig. 3.23). Peak one probably corresponds to PALase which did not bind to the column and was eluted during washing (tubes 1-16 correspond to the initial 80 mL of the wash). The highest activity appeared in the third peak with 23% of the original activity found here (tubes 75-87). The specific activity in the pooled fractions (tubes 80-82) was 28,995 mIU/mg of protein, which was 66-fold the

Table 3.6 Partial purification of enzymes by Sephacryl S-200 chromatography

A 14-day *P. ostreatus* SSBM culture grown on lupin hull as sole carbon source was harvested and filtered through glass wool and centrifuged at 10,000 x g (8,000 rpm). The supernatant was used as crude enzyme for testing the initial enzyme activities. A total volume of 160 mL of culture filtrate was concentrated 20-fold to 8 mL using an Ultrasart Cell 50 (for details see Section 2.4.3), which was used as the enzyme source for testing PGase and PALase activities of the concentrated culture filtrate after 20-fold dilution with distilled water. A 5 mL sample of these culture concentrates was firstly dialysed at 4°C against the 0.01 M Na_2HPO_4-NaH_2PO_4 buffer pH 7.0, containing 0.15 M KCl, then this was applied to Sephacryl S-200, eluting with the same buffer. Three tubes, fraction 45 to 47 for PALase and 60 to 62 for PGase around their activity peaks, were pooled and analysed.

a. PGase

Enzyme Source	Volume (mL)	Pooled (mL)	Activity (mIU/mL)	Protein (μg/mL)	Total Units (mIU)	Specific Activity (mIU/mg Protein)	Yield (%)	Purification factor
Culture Filtrate	100	-	275.8	253.2	27,580.0	1,089.6	100	0
Concentrated Filtrate	5	-	5,324.9	3,001.2	26,624.5	1,774.2	96.5	1.6
S-200	-	15	190.6	5.1	2,859.0	37,435.4	10.4	34.4

b. PALase

Enzyme Source	Volume (mL)	Pooled (mL)	Activity (mIU/mL)	Protein (μg/mL)	Total Units (mIU)	Specific Activity (mIU/mg Protein)	Yield (%)	Purification factor
Culture Filtrate	100	-	159.5	253.2	15,950.0	630.0	100	0
Concentrated Filtrate	5	-	3,203.4	3,001.2	16,017.0	1,067.4	100.4	1.7
S-200	-	15	241.3	23.4	3,619.5	10,320.0	22.7	16.4

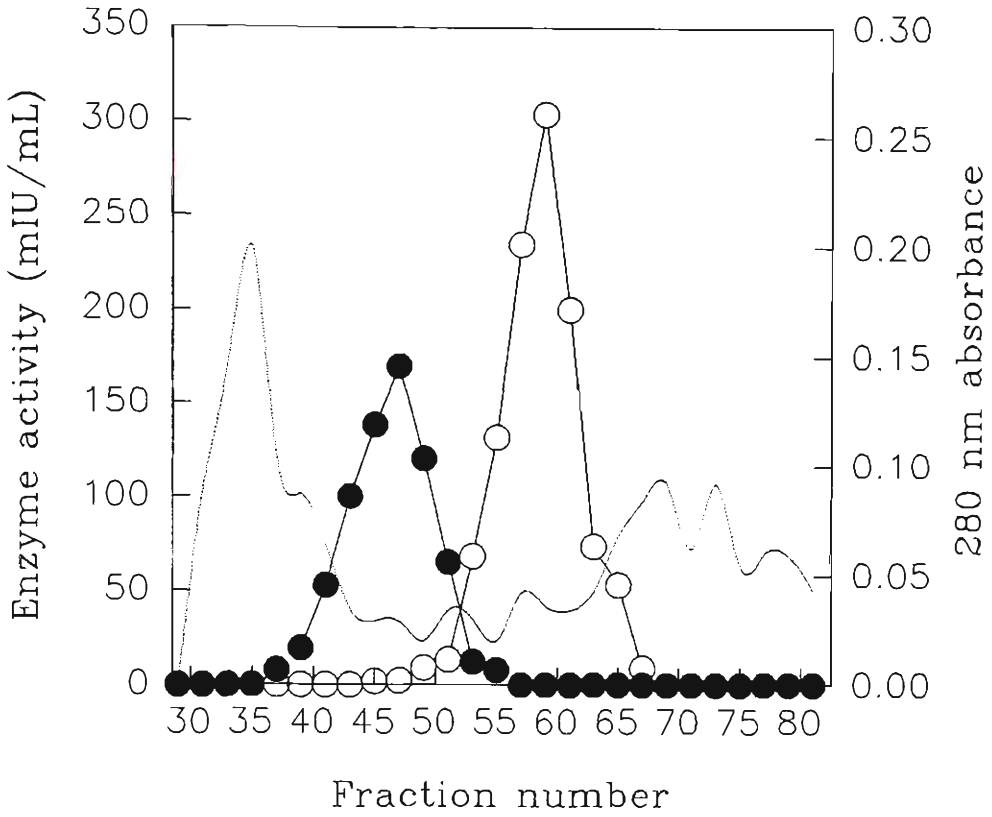


Figure 3.22 Column chromatography of culture fluid for PGase and PALase on Sephacryl S-200

A 20-fold concentrated culture extract (five mL) was applied to Sephacryl S-200 (for experimental details, see Section 2.4.4.2 of the Materials and Methods). Fractions were examined for PGase and PALase activities using the assay detecting RS production and PALase assays contained 0.15 mM $CaCl_2$.

(——) 280 nm absorbance;

(○) PGase;

(●) PALase.

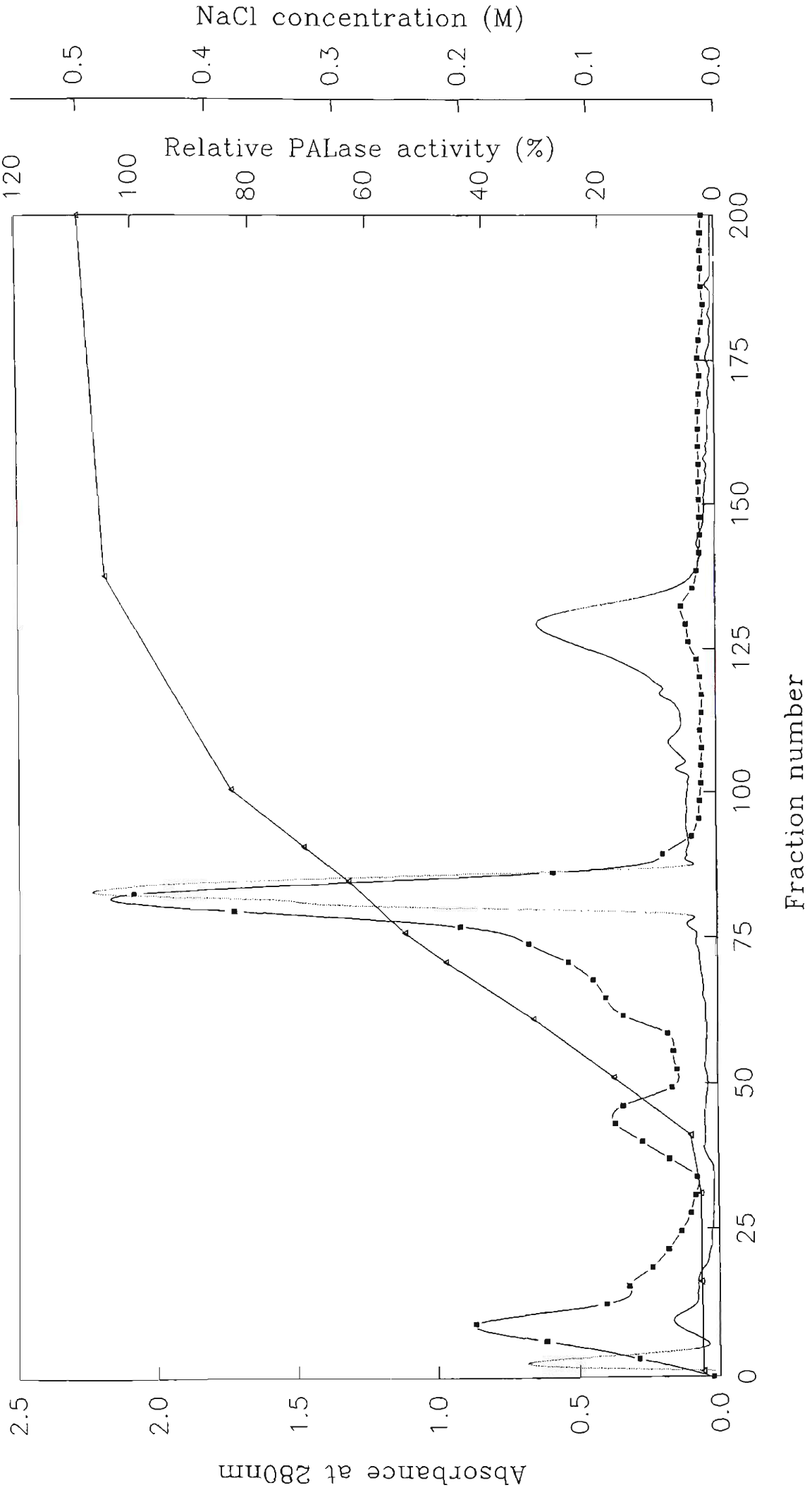
Figure 3.23 Ion-exchange chromatography of culture fluids for purification of PALase

For experimental details, see Section 2.4.4.3 of the Materials and Methods.

(·····) 280 nm absorbance;

(○) NaCl concentration;

(●) Relative PALase activity, assayed by detecting degradation products from absorbance at 235 nm. Activity was expressed as a percentage of the maximum activity detected (in fraction 81).


NaCl concentration (M)
0.5
0.4
0.3
0.2
0.1
0.0
Relative PALase activity (%)
120
100
80
60
40
20
0
Absorbance at 280nm
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
175
200
Fraction number

original. This pooled fraction was applied to Sephacryl S-200 column 1, however the recovered PALase activity and protein concentration in this pooled fraction was relatively low (Table 3.7). The yield of PALase after ion-exchange chromatography was low, which may have been associated with loss of activity during the procedures. This could have arisen due to inherent instability of the protein or may have been associated with lack of reactivation of activity due to irreversible loss of a prosthetic group which is required for activity (*e.g.* a metal ion, despite exogenous supplementation with calcium chloride). It is also most likely that only a proportion of the PALase in crude extracts binds to the DEAE column. Results reported in Section 3.4.5 indicate that PALase activity had two pI values: under the buffer conditions used, only one of these activities (pI 4.5) would have bound to the DEAE column. This issue remains unresolved and it was not investigated further as part of this thesis.

PGase activity was not assayed as this did not bind to this ion-exchange gel (see pI results in Section 3.4.5).

3.4.5 ISOELECTRIC FOCUSING (ROTOFOR CELL) OF ENZYMES

Preliminary experiments using the IEF Rotofor cell used dialysed crude culture filtrates and results showed broad peaks of enzyme activity and lots of precipitated protein in the IEF Rotofor cell, which suggested that lower protein levels should be loaded or partially purified enzyme should be used. An IEF Rotofor cell run was performed using partially purified enzyme from the Sephacryl S-200 (column 1, tubes 40-65), containing both PGase and PALase (Fig. 3.15). Results indicated that the pI of PGase of *P. ostreatus* was pH 7.5 (Fig. 3.24), with 92% of the activity retained. Two peaks of PALase activity were found (pH 4.5

Table 3.7 Partial purification of PALase by ion-exchange chromatography and Sephacryl S-200 chromatography

Enzyme Source	Volume (mL)	Pooled (mL)	Activity (mIU/mL)	Protein (μg/mL)	Total Units (mIU)	Specific Activity (mIU/mg Protein)	Yield (%)	Purification factor
Culture Filtrate	500	-	80.8	183.6	40,400	439.9	100	0
Ion-exchange	15	-	232.0	8.0	3,480	28,995.0	8.6	65.9
S-200	-	5	17.5	Undetectable	87.5	ND	0.2	ND

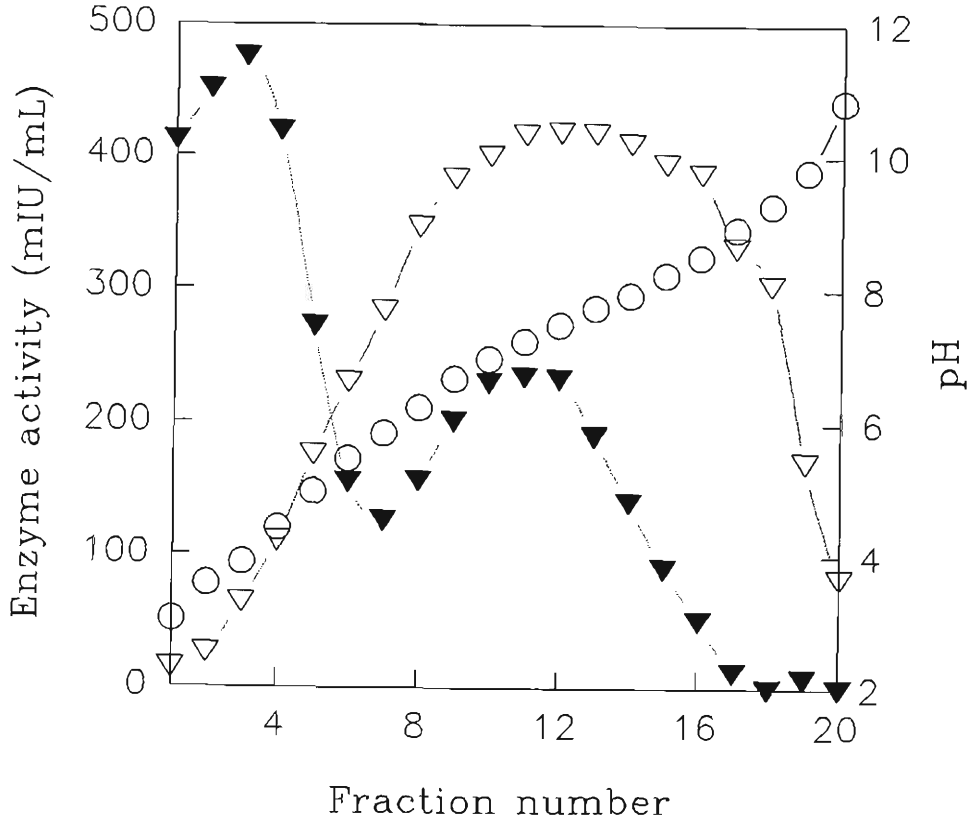
ND Not determined

A two-week-old *P. ostreatus* SSBM culture grown on lupin hull as sole carbon source was harvested and filtered through glass wool and centrifuged at 10,000 x g (8,000 rpm). The supernatant was used as the source of crude enzyme for detecting initial enzyme activity. A total volume of 600 mL of culture filtrate was then concentrated to 50 mL using the Ultrasart Cell 50 (for details see Section 2.4.3). After dialysis against 0.01 M citric acid-sodium citrate buffer, pH 5.0, overnight, 40 mL was applied to DEAE-Sepharose Fast Flow gel. The column was then washed with 100 mL of the same buffer at the flow rate of one mL/minute and eluted with a linear NaCl gradient starting from 0 to 0.5 M in the same buffer. Fractions with highest activity (80-82) were collected and analysed, then applied to Sephacryl S-200 after appropriately concentrating using a Collodian finger (for details see Section 2.4.3 of the Material and Methods). Only little PALase activity was found in fractions and protein concentrations were undetectable. PALase assays from ion-exchange chromatography and Sephacryl S-200 chromatography were supplemented with 0.15 mM $CaCl_2$.

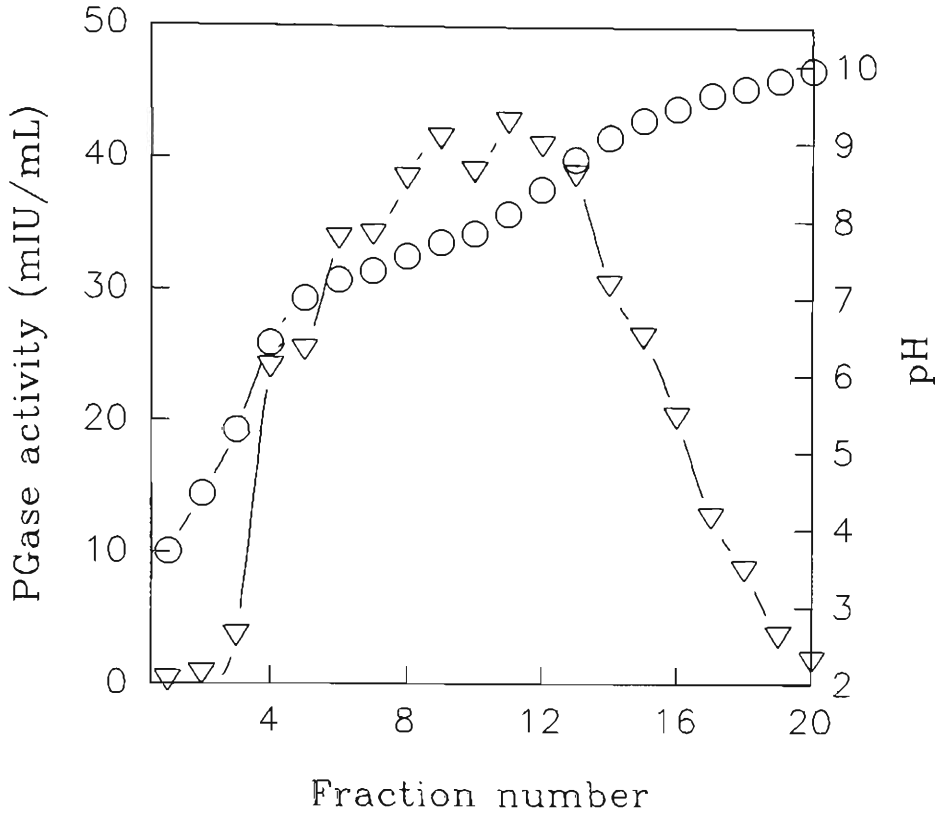
Figure 3.24 pI determination of PGase and PALase from *P. ostreatus* by IEF

Fractions from Sephacryl S-200 chromatography (Fig. 3.15) with PGase and PALase activities were pooled and dialysed against distilled water. This was applied into the IEF Rotofor cell after mixing with ampholytes. The fractions from the IEF Rotofor cell were analysed for pH (○), volume and activities of PGase (▽) and PALase (▼) (RS method). PALase was assayed in the presence of 0.15 mM $CaCl_2$.

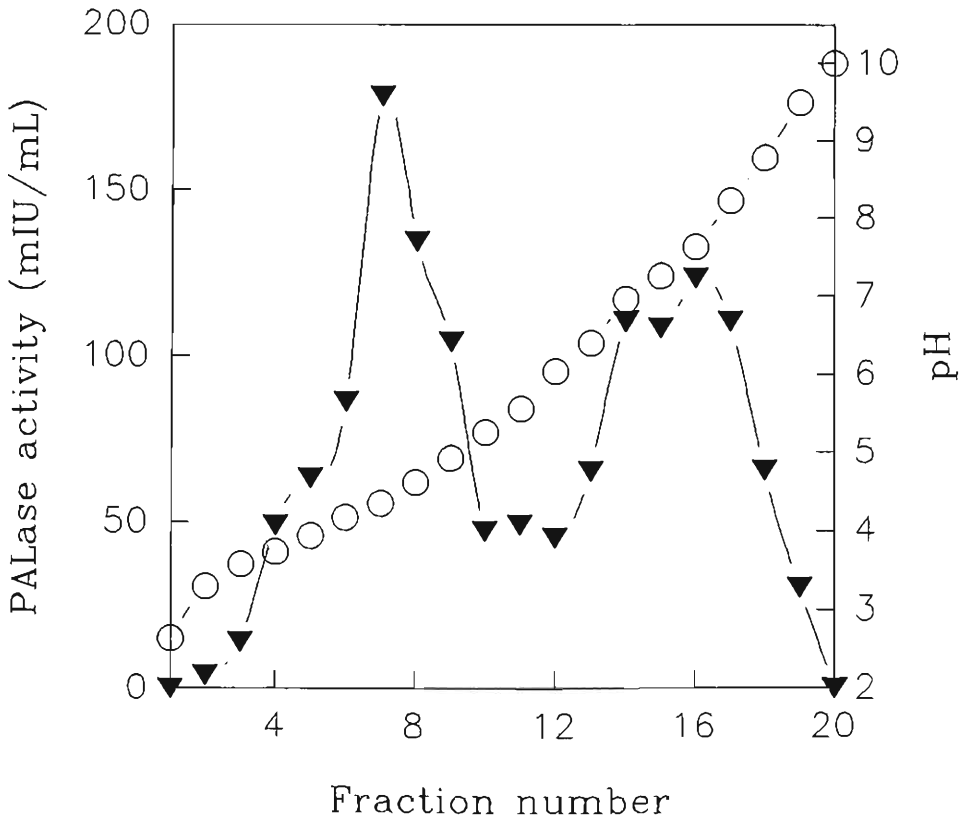
a. IEF of fractions 40-65 from Sephacryl S-200 Chromatography



b. IEF of fractions 61 from Sephacryl S-200 Chromatography (peak of PGase eluted)



c. IEF of fractions 44-47 from Sephacryl S-200 Chromatography (peak of PALase eluted)



and pH 7.0), where the peak at pH 4.5 represented 58% of the activity applied and the peak at pH 7.0 represented 41% of the original activity.

The IEF Rotofor cell preparation from the Sephacryl S-200 fraction 61 which contained the highest level of PGase activity is also shown in Fig. 3.24. The pI of PGase was confirmed as 7.5. An IEF Rotofor cell prepared from the single PALase peak fractions (column 1, tube 46) of Sephacryl S-200 confirmed that the pI of PALase has two peaks at 4.0-4.5 and between 7.0 and 7.5. The activity ratio of the two peaks was 1:1.

Due to difficulties in removing ampholytes and the small volume of enzymes obtained, the protein concentration could not be estimated by assay so that no information on specific activity was obtained.

3.4.6 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF POLYGALACTURONASE AND PECTATE LYASE

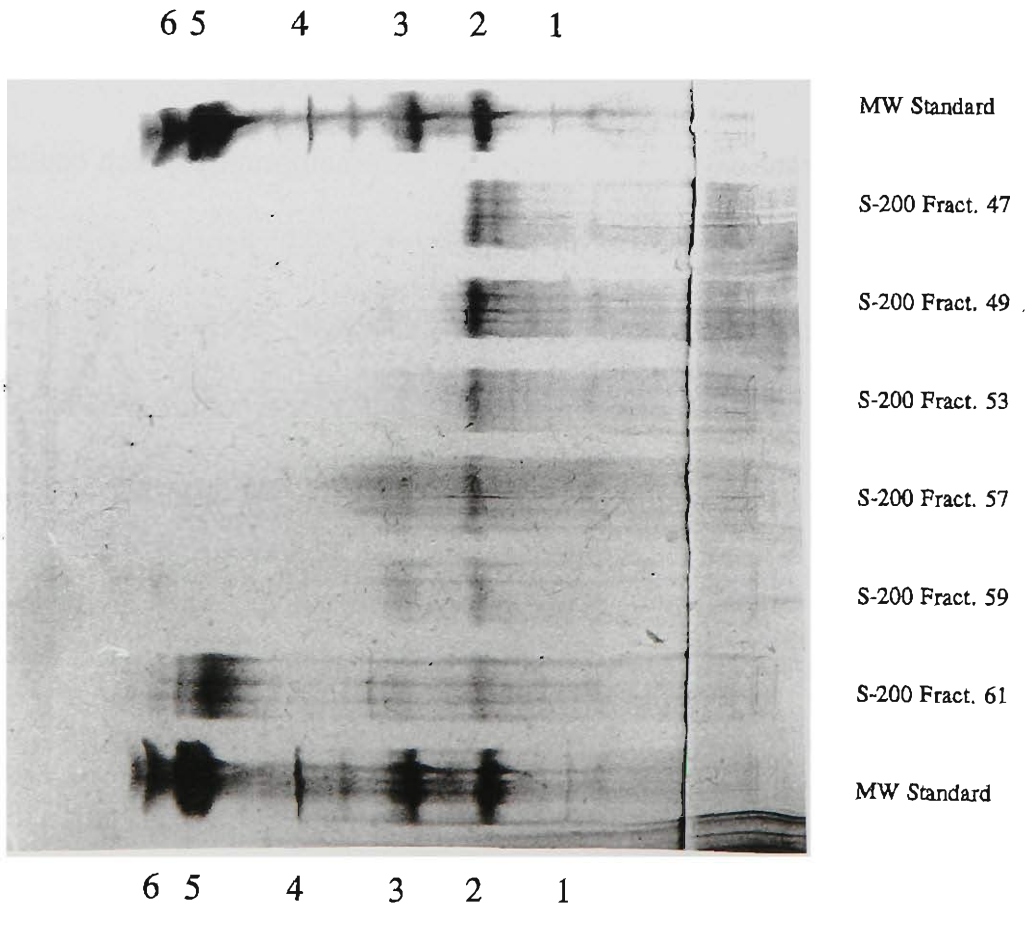
PAGE of culture fluids, the fraction from ion-exchange chromatography which contained the highest activity of PALase, the fraction from Sephacryl S-200 chromatography with the highest PALase activity and the fraction of Sephacryl S-200 with the highest PGase activity is shown in the Fig. 3.25. PAGE analysis across the PALase to the PGase peaks on Sephacryl S-200 (Fig. 3.15) showed a clear band corresponding to the peak of PALase in tube 47 which declined but persisted to tube 61 (Fig. 3.25a). This band corresponded to the MW marker of BSA, which has a MW of 66,000 Da. A second feint and dispersed band commenced in fraction 57 and remained in fraction 61: as the MW was consistent with that seen for PGase activity from Sephacryl S-200 gel filtration, it is likely that this band

Figure 3.25 Polyacrylamide gel electrophoresis of PGase and PALase at different stages of purification by column chromatography

About 10 ng of protein was applied to PAGE gels and stained with silver according to the supplier's instructions (Phast manual, Pharmacia). Concentrated culture filtrate was applied to Sephacryl S-200 chromatography firstly, then the fractions with PGase and PALase activity (fractions 40-65, Fig. 3.15) were pooled and applied to the IEF Rotofor cell. The fractions from the Rotofor cell were dialysed and applied to PAGE after concentrating. Similarly, fractions from DEAE chromatography were concentrated then applied. MW markers are:

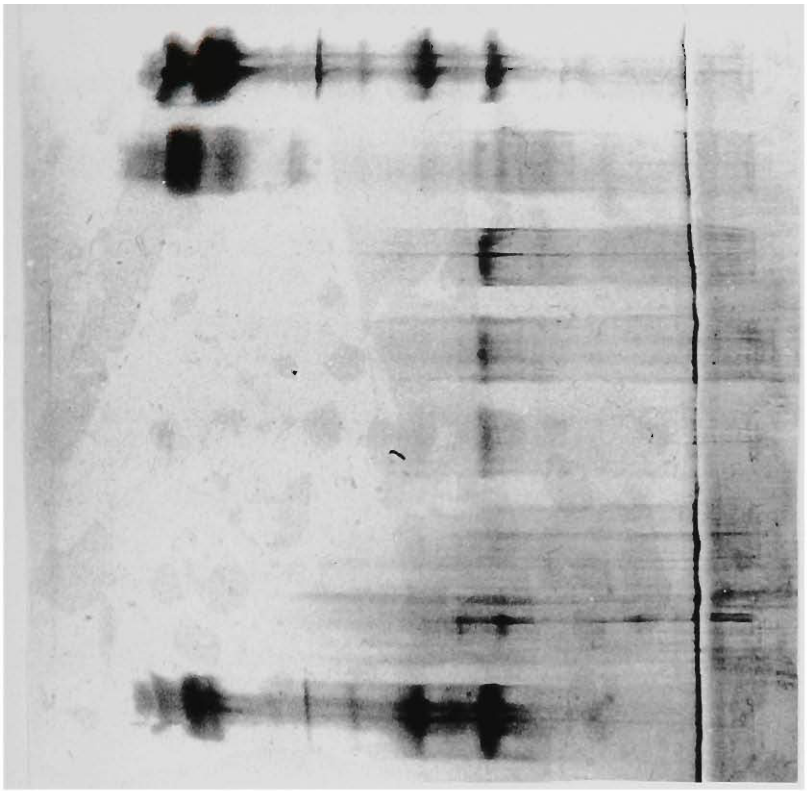
1. Rabbit muscle phosphorylase B - 97.4 kDa;
2. BSA - 66.2 kDa;
3. Ovalbumin - 45 kDa;
4. Carbonic anhydrase - 31 kDa;
5. Soybean trypsin inhibitor - 21.5 kDa;
6. Hen egg white lysozyme - 14.4 kDa.

a. PAGE of Sephacryl S-200 (Fig. 3.15) sample across the peaks of PALase and PGase



b. PAGE of crude extract, Sephacryl S-200 fractions (Fig. 3.15) and peaks of PALase and PGase from IEF (Fig. 3.24a)

6 5 4 3 2



MW Standard

Culture filtrate

S-200 Fract. 47

S-200 Fract. 49

S-200 Fract. 57

Rotoforcell Fract. 3

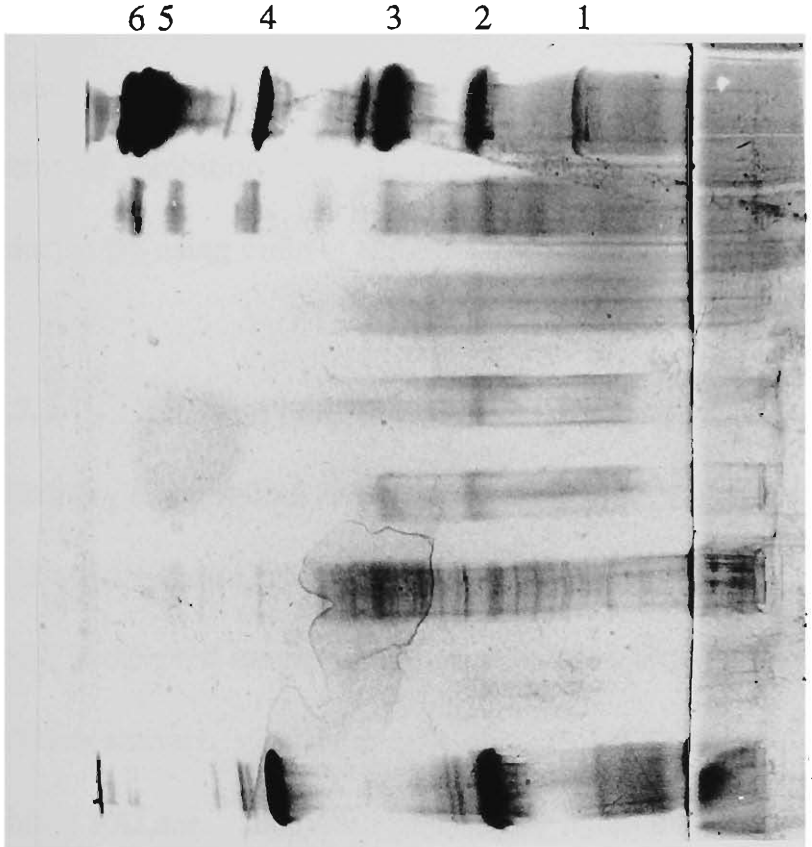
Rotoforcell Fract. 16

MW Standard

6 5 4 3 2

c. PAGE of DEAE-Sepharose fractions (Fig. 3.23) and IEF fractions (Fig. 3.24b)

6 5 4 3 2 1



MW Standard

Culture filtrate

DEAE Sepharose Fract. 81

DEAE 81, applying S-200 Fract.49

DEAE 81, applying S-200 Fract. 49

Rotoforcell Fract. 14

Rotoforcell Fract. 9

MW Standard

4 2

corresponds to PGase protein. The results here, however, are difficult to interpret without further purification of the proteins to show which band corresponds to the activities, which would enable confirming the relationship between activity and bands seen on PAGE. The bands seen for the peak of PALase activity on DEAE-Sepharose chromatography (Fig. 3.25c) had the same MW as the major protein detected after Sephacryl S-200 chromatography. PAGE analysis of IEF Rotofor cell fractions following Sephacryl S-200 chromatography (Fig. 3.25) showed smears rather than bands, probably due to residual ampholytes. PAGE results suggested that the MW of PGase was between 36-40 kDa (as the PAGE bands had a similar migration as ovalbumin) and the MW of PALase was around 66 kDa, which corresponds to the result obtained from Section 3.4.3.1 for gel filtration.

3.4.7 ACTIVATORS OR INHIBITORS OF ENZYMES

Several compounds were screened for their potential effects of activating or inhibiting PALase and PGase to evaluate whether activity could be further improved or whether patterns of inhibition could further differentiate the observed activities. This study was conducted by using culture extract as the source of crude enzyme.

3.4.7.1 Effects of cations and anions on activities of pectinases

Preliminary experiments were carried out to choose the anion components of the salts which would not affect the activity of the enzymes (Fig. 3.26a). By using sodium salts at level of 20 mM in the final assay system, it was shown that a number of salts affected either PALase or PGase activity. For example, sodium carbonate inhibited PGase and sodium oxalate inhibited PALase. Inhibition of PALase by sodium citrate was expected, considering that this enzyme required Ca^{++} for good activity which is sequestered in presence of citrate and

Figure 3.26 Inhibition of PGase and PALase by salts

Inhibition of enzymes (in a crude preparation) was conducted by adding potential inhibitors into the assay system, then the activity of the enzymes was compared to the original activity without inhibitor.

(☐) PGase; (▨) PALase.

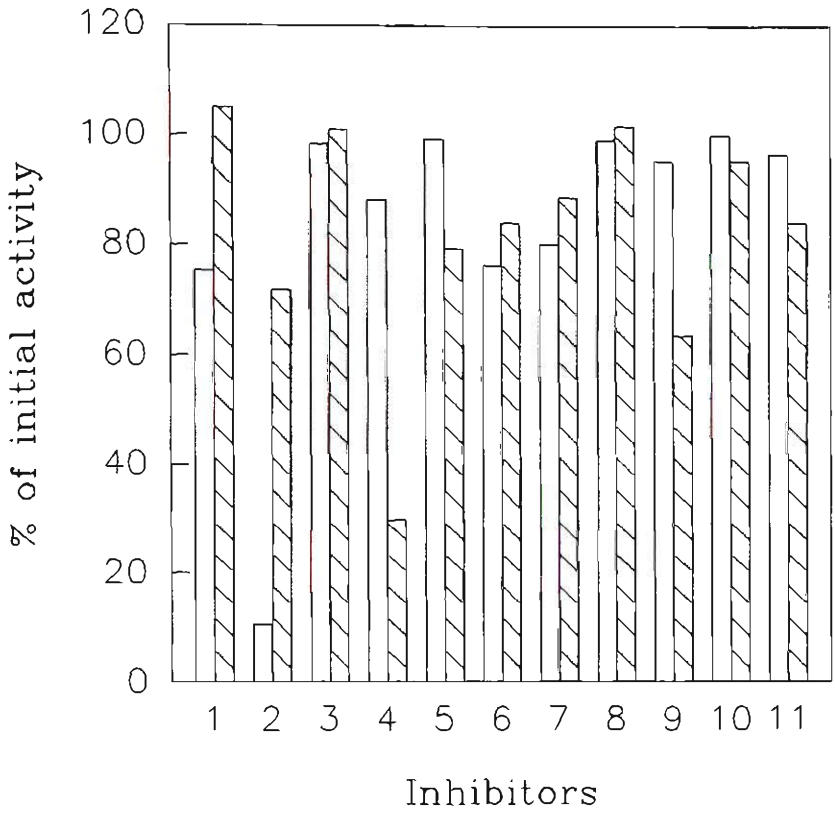
a. A final concentration of 20 mM of each salt was present in the reaction mixture.

1.	Sodium acetate;	2.	Sodium carbonate;
3.	Sodium chloride;	4.	tri-Sodium citrate;
5.	Sodium dihydrogen phosphate;	6.	Sodium hydrogen carbonate;
7.	di-Sodium hydrogen phosphate;	8.	Sodium nitrate;
9.	Sodium oxalate;	10.	Sodium sulphate;
11.	Sodium tartrate.		

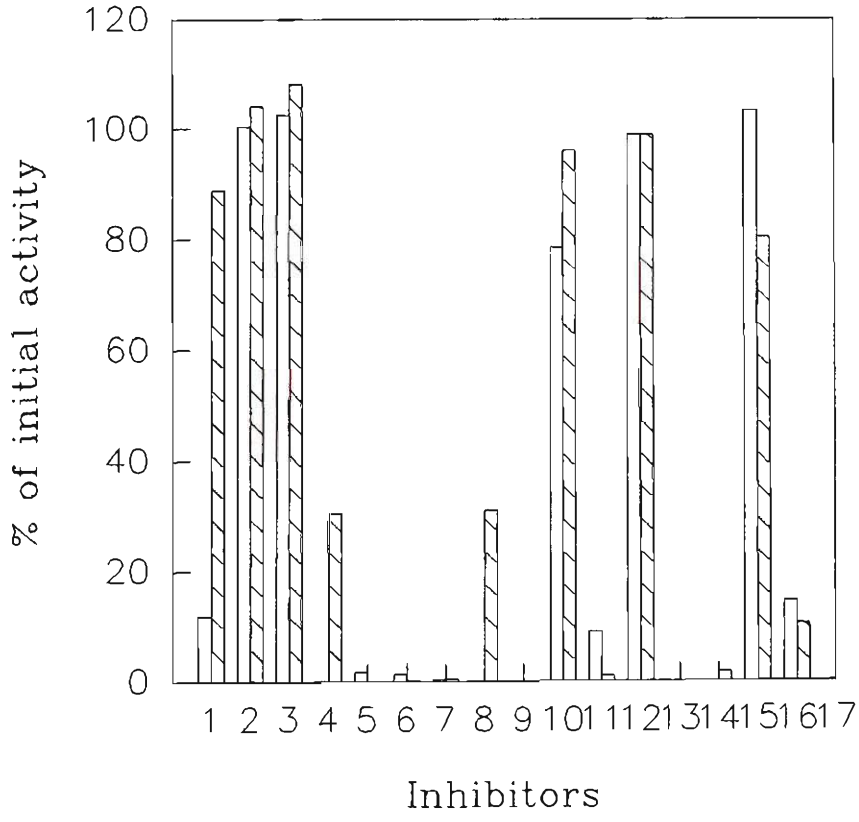
b. A final concentration of 10 mM of each salt was present in the reaction mixture.

1.	$AgNO_3$;	2.	$LiSO_4$;	3.	$CsCl_2$;	4.	$AlCl_3$;
5.	$BaCl_2$;	6.	$Pb(NO_3)_2$;	7.	$HgCl_2$;	8.	$Cr(NO_3)_3$;
9.	$FeCl_3$;	10.	K_2HPO_4;	11.	$Ni(NO_3)_2$;	12.	$(NH_4)_2SO_4$;
13.	$Fe(NO_3)_2$;	14.	$CuSO_4$;	15.	KH_2PO_4;	16.	$FeSO_4$.

a. Inhibition by sodium forms of various anions



b. Inhibition by cations



oxalate ions. Activity detected in the presence of chloride,nitrate and sulfate salts was 95% of both control activities. Whereas the other acids, such as acetate, hydrogen phosphate, dihydrogen phosphate, bi-carbonate and tartrate inhibited either PGase or PALase or both.

Further studies on potential inhibition of enzymes by metal ions were carried out by using the chloride, sulfate and nitrate salts at 10 mM in final assay mixtures. The results revealed that most of the heavy metal ions, such as Pb^{++}, Hg^{++}, Fe^{+++}, Fe^{++} and Cu^{++}, totally inhibited both PGase and PALase activity, where the activity exhibited was below 20% of control levels. Ag^{+}, Al^{+++}, Cr^{+++} inhibited PGase more than PALase. Li^{++}, Cs^{++}, K^{+}, Na^{+} and NH_4^{+} did not inhibite either enzyme (Fig. 3.26b).

3.4.7.2 Effects of metabolic inhibitors on pectinases

In most cases, metabolic inhibitors did not affect the activity of PGase and PALase when used at 10 mM in the assay system (Fig. 3.27). The activities of both enzymes retained 90% of the original in the presence of dithiothreitol, iodoacetamide, 2-mercaptoethanol, metronidazole, PMSF, cyanide and azide. Activities of both enzymes were totally inhibited by ferricyanide, possibly due to Fe^{+++} inhibition of the enzymes. The partial inhibition by arsenite and arsenate on PGase (30-40%) was due to a shift in pH of the reaction mixture. Activation of PALase (20%) by metronidazole antibiotic is interesting but not otherwise investigated.

3.4.7.3 Effects of chelators on pectinases

PGase could be differentiated from PALase activity on the basis of reaction to chelators. In the presence of 10 mM citrate, PGase retained 78% of its activity, whereas PALase only

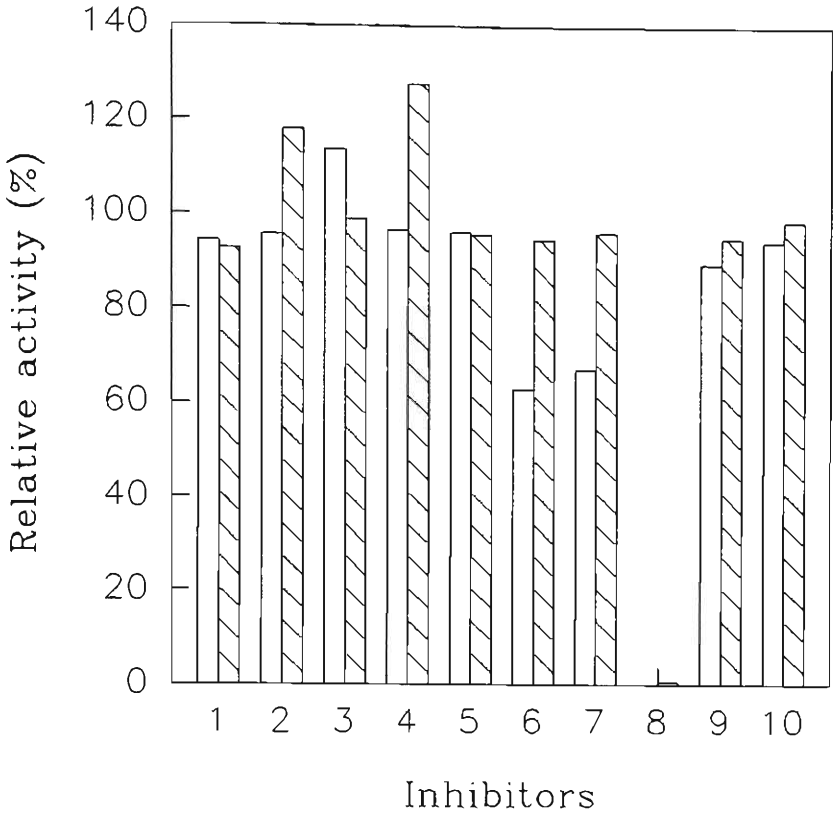


Figure 3.27 Inhibition of PGase and PALase by metabolic inhibitors

Inhibition of enzymes (in a crude extract) was conducted by adding potential inhibitors into the assay system, then the activity of the enzymes was compared to the original activity without inhibitor. A final concentration of each compound was 10 mM in reaction mixtures.

(▭) PGase; (▨) PALase.

1.	Dithiothreitol;	2.	Iodoacetamide;	3.	Mercaptoethanol;
4.	Metronidazole;	5.	PMSF;	6.	Arsenite;
7.	Arsenate;	8.	Ferricyanide;	9.	Cyanide;
10.	Azide.				

retained 20% of its original activity. The same concentration of EDTA did not inhibit PGase, but did inhibit activity of PALase by up to 80% (Fig. 3.28). These observations are consistent with the requirement of PALase for Ca^{++} ions.

3.4.7.4 Effects of carbohydrates as potential end-product inhibitors of pectinases

The effects of several RS at five mM on PGase and PALase was examined (Fig. 3.29). Fructose, galactose, glucose and xylose did not inhibit either enzyme activity, as the activities observed for PGase and PALase were between 90%-102% of the controls. GA partially inhibited both enzymes, as activities of 67% of controls were seen. Cellobiose inhibited PALase (50%) more than PGase (20%).

3.4.8 EXTENT OF SUBSTRATE DEGRADATION BY POLYGALACTURONASE AND PECTATE LYASE

The extent of reaction was explored for PGase and PALase by analysing the end-products after extended degradation periods, when maximum conversion of substrate into product would have occurred. The end-products of PGase and PALase were withdrawn after 24 hours reaction at 50°C, pH 5.0 and 40°C, pH 8.6 when the level of RS was no longer increasing. The RS at the end of reaction was 8.29 mM and 3.80 mM for the PGase and PALase, respectively, using culture extract as the source of crude enzymes. Theoretically, total digestion of the PGA supplied in the assay would produce approximately 8.6 mM of GA, based on the weight of substrate supplied and the MW of a monomeric product. The saccharification conversion rate of PGase from *P. ostreatus* against PGA was estimated as 96.40%. In this case, the production would be mostly monosaccharides (GA). The degree of digestion of PGA by PALase was estimated as 44.18%. The average length of PALase

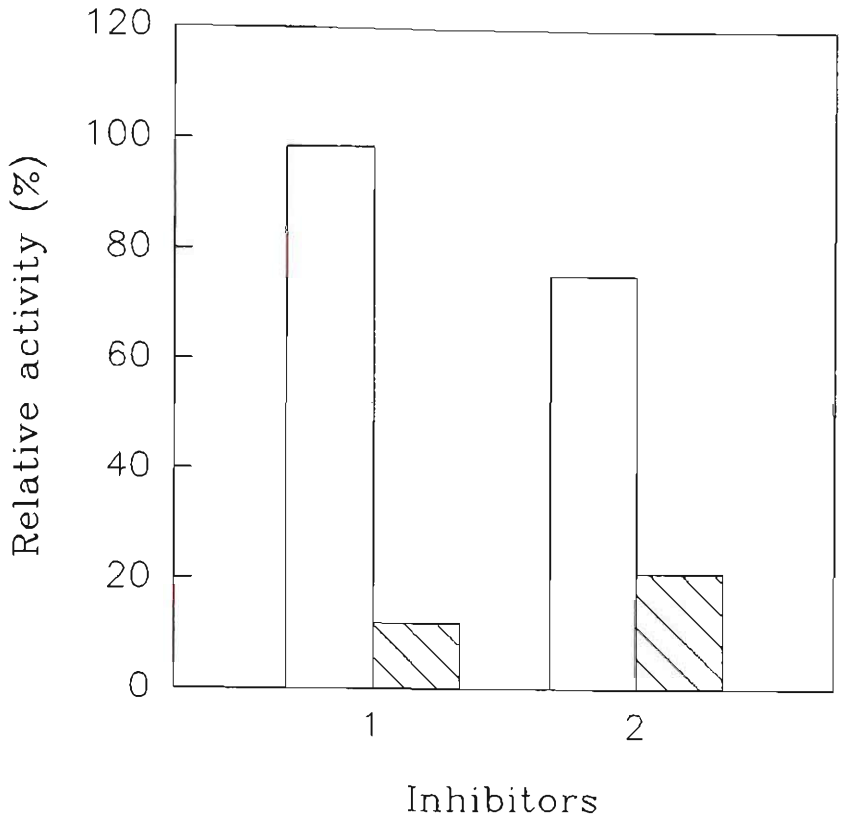


Figure 3.28 Inhibition of PGase and PALase by chelators

Inhibition of enzymes (in crude preparation) was conducted by adding sodium-EDTA (1) or sodium citrate (2) into the assay system, then the activity of the enzymes was compared to the original activity without the potential inhibitors. A final concentration of 10 mM was present in the reaction mixtures.

(▭) PGase; (▨) PALase.

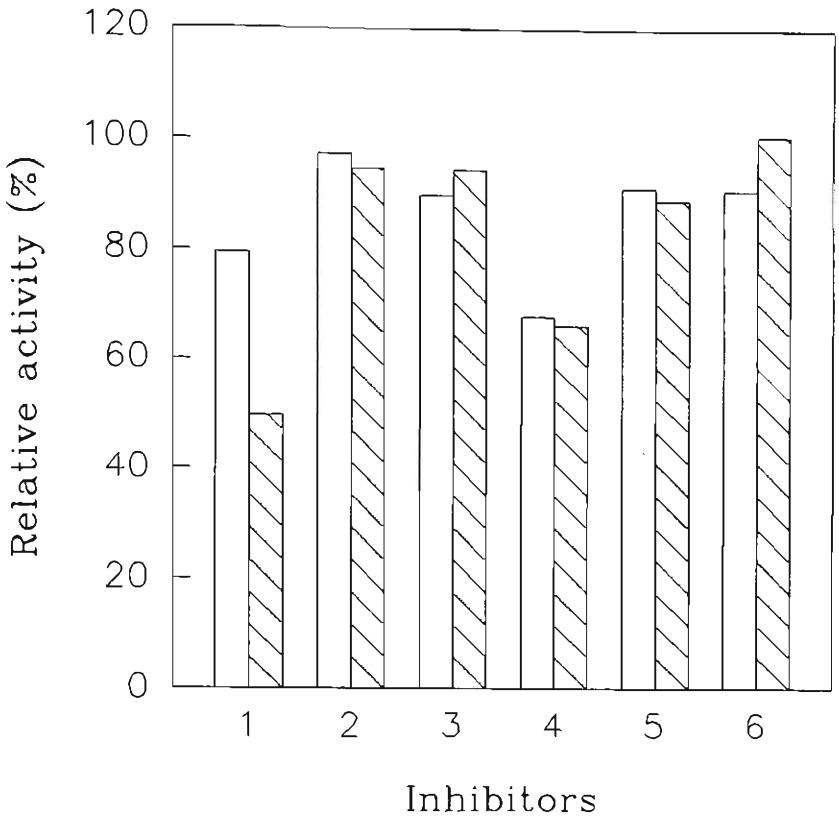


Figure 3.29 Inhibition of PGase and PALase by carbohydrates

Inhibition of enzymes (in crude preparation) was conducted by adding potential inhibitors into the assay system, then the activity of the enzymes was compared to the original activity without potential inhibitors. A final concentration of five mM was presented in reaction mixtures.

(▭) PGase; (▨) PALase.

1. Cellobiose; 2. Fructose; 3. Galactose;
4. GA; 5. Glucose; 6. Xylose.

product would most likely be a disaccharide. These results should be confirmed by HPLC analysis of the degradation products.

3.4.9 K_m OF POLYGALACTURONASE

Fig. 3.30 shows a double reciprocal plot of the initial velocities obtained when the crude enzyme extract was incubated with varying concentration of PGA. The K_m estimated for the PGase against PGA in crude culture extract was about 0.27 mg/mL.

The K_m of the PALase was hard to determined due to the non-linear relationship between the reduced sugars produced and incubation time, as shown in Section 3.4.1.2 and 3.4.1.3.

3.4.10 PECTIN DEGRADATION BY PECTATE LYASE

Partially purified PALase from the DEAE ion-exchange chromatography was tested for activity against pectin by scanning the absorbance increases at 40°C and 235 nm. The absorbance increased dramatically at the beginning of the reaction, then maintained at a certain level and finally increased again (Fig. 3.31). These results indicated that PALase could degrade native pectin; the kinetics of the conversion remain open to interpretation, considering that partially purified enzyme was used.

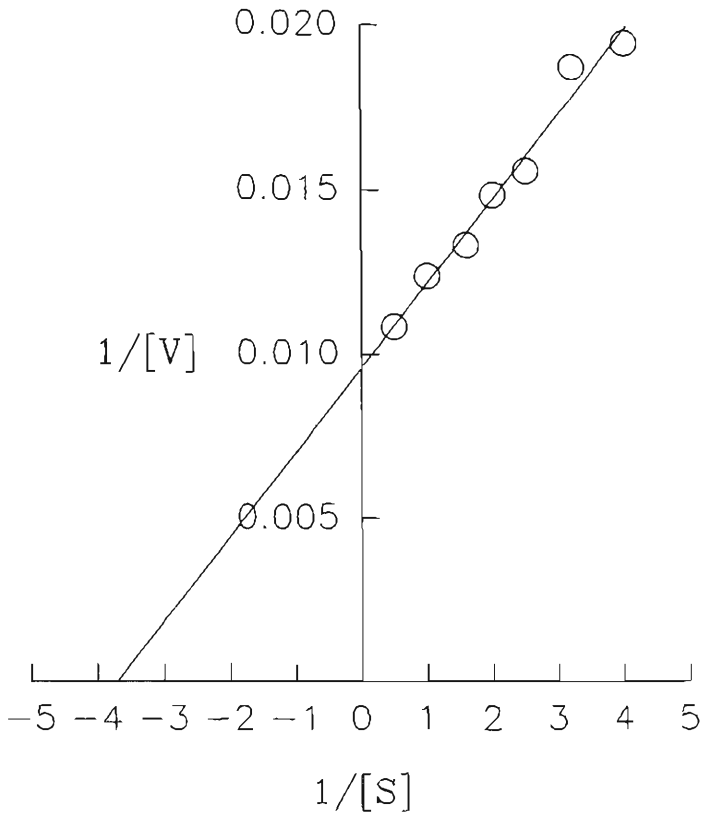


Figure 3.30 Determination of K_m with double-reciprocal plots for PGase from *P. ostreatus* in a crude culture extract

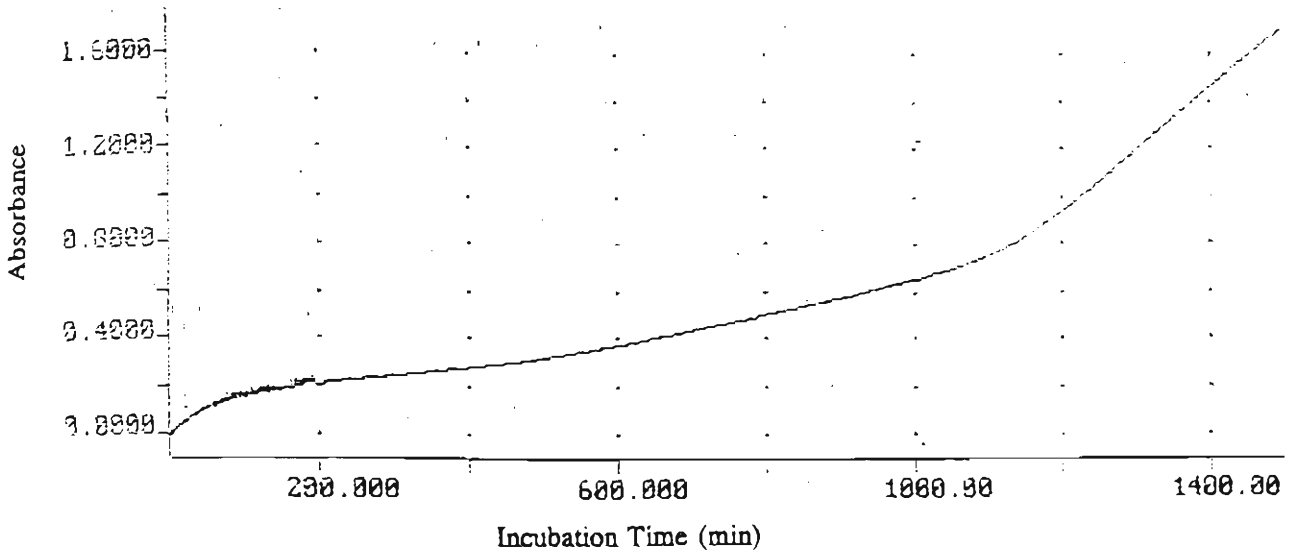


Figure 3.31 Monitoring pectin degradation by PALase at 235 nm

Partially purified PALase from the ion-exchange column (fraction No. 80), 1% (w/v) citrus pectin, stock Clark-Lubs buffer pH 8.6, and distilled water were mixed at a ratio of 1:1:1:2 after equilibrating all components at 40°C, the absorbance at 235 nm was monitored at 40°C. Denatured (by boiling for 30 minutes) PALase from the same source was used to substitute active PALase in the same system as a control. The control was automatically subtracted from the test results.

CHAPTER 4. DISCUSSION

4.1 MUSHROOM GROWTH

4.1.1 GROWTH ON PLATES

Environmental factors, such as temperature, pH and water content in the substrate are important to commercial mushroom cultivation and most of these factors have been studied thoroughly for several commercial mushroom species (Zadrazil, 1978; Stamets and Chilton, 1983). However, many of these studies reported differences in optimal growth conditions for the same species, possibly due to the characteristics of the various strains tested. For instance, the study of Wu (1987) reported that the most suitable temperature for *P. ostreatus* growth was between 20 and 30°C and the optimum was 24±2°C, whereas Zadrazil (1978) reported the optimum temperature for *P. ostreatus* was 30°C. These relatively small differences in growth conditions could have significant effects on the growth and ability of a particular strain to form fruiting bodies. For this reason, it was necessary to investigate the effects of these basic factors on the particular mushroom species and strains studied in this thesis. It was found that the *P. ostreatus* strain used could hardly survive on both PDA and MEA plates when the growth temperature was over 30°C and that this strain preferred to grow at temperatures between 25 and 30°C, a range which falls between those reported above.

For *L. edodes*, the optimum temperature was recorded between 24 and 27°C by Wu *et al.* (1987) and 25°C by Tokimoto and Komatsu (1978), and the optimum temperature for *F. velutipes* was between 22 and 26°C (Tonomura, 1978; Guo, 1987). In this study, the optimum temperature for both *L. edodes* and *F. velutipes* growth were determined as 20 to 25°C, which was similar to results in these previous reports.

The effect of pH on growth of the mushroom species tested showed that these species could survive in a wide range of pH, although the optimal pH was located between 3.0 and 7.0. These results are quite similar to those in other reports (Huang *et al.*, 1987; Tonomura, 1978; Tokimoto and Komatsu, 1978): *P. ostreatus*, *L. edodes* and *F. velutipes* were reported to grow over pH ranges of 3.0 to 7.2, 3.0 to 7.0 and 3.0 to 8.4, respectively (Huang *et al.*, 1987). Normally, lignicolous mushrooms (mushrooms which grow on wood, such as *Pleurotus*, *Lentinus* and *Flammulina*) prefer to grow at lower pHs than terricolous mushrooms (terrestrial mushrooms, such as *Agaricus*), which relates to the nature of the extracellular enzymes produced and their adaptation to their environment and substrate. The main component of wood is cellulose: fungal cellulases and other polysaccharidases typically have their highest activities between pH 4 and 5. Where less lignocellulose and more protein is present in the substrate available to terricolous mushroom growth, proteinase activity was found to be highest between pH 7 and 8 (Huang *et al.*, 1987).

Although pH of the substrate can affect growth for some mushroom species, it is a less crucial factor for growth of other species. It has been reported that for *L. edodes*, the initial pH of the media did not seriously affect mycelial growth over the range of pH 3 to 6 (Tokimoto and Komatsu, 1978). During growth of *L. edodes*, several organic acids are produced, including acetic, succinic and oxalic acids (see Tokimoto and Komatsu, 1978). These acids lower the pH and probably form a buffering system which maintains favourable conditions for the hydrolysing enzymes to provide substrates for further growth of the fungus.

For further studies on the three species used in this thesis, growth in SSF was carried out at pH 4.5, 25$\pm$2°C.

4.1.2 MUSHROOMS GROWTH IN SOLID-STATE CONDITIONS

4.1.2.1 Growth

This study indicated that lupin hull could be a good substrate and/or nitrogen supplement for mushroom growth. When sawdust, lupin hull or mixtures of hull and sawdust were inoculated with mushrooms, it was shown that the fungi preferred the mixture of hull and sawdust.

The physical properties of the substrate were important for mushroom growth. This study showed that the lupin hull alone served as a poor substrate for mushroom growth. When lupin hull was used alone, the substrate had poor aeration and uneven water distribution. Incorporation of sawdust in the substrate may improve areation and provide sufficient water for mycelial growth, as suggested by Tonomura (1978). In other experiments using *P. ostreatus* cultivated on cocoa shell waste (an agricultural waste similar to lupin hull), sawdust was also incorporated in the substrate preparation (Pettipher, 1987). In Pettipher's experiments, the substrate contained 40% cocoa shell waste, 40% sawdust, 19% oatmeal and 1% high viscosity CMC. Distilled water was added to the dry mixture to give a moisture content of 60% (w/w) and the pH was adjusted to 3.5 with 1 M HCl. Each bag contained 333.33 g (average) of dry substrate mix and was surface-inoculated with 5-10% (w/w) maize-colonised *P. ostreatus* and incubated at 25°C. The average time for mycelial growth to fully colonise the substrate was seven weeks (range five to nine weeks) in his study. In studies reported in this thesis, it was found that full colonisation of the substrate (300 g dry weight of sawdust and lupin hull) took four to five weeks. This indicated that the growth rate of the *P. ostreatus* strain used on lupin hull and sawdust was much faster than that seen on the mixture of cocoa shell waste, sawdust, oatmeal and CMC.

However, *P. ostreatus* could not initiate growth on sawdust alone in this study. It has been proven that some chemicals (resins, turpentine and tannins) in wood may inhibit mushroom growth and conifer wood can contain high concentrations of these (Stamets and Chilton, 1983). Hardwood sawdust is generally more suitable for mushroom growth because it contains less of these inhibitors (Stamets and Chilton, 1983). Results in this thesis showed that there was no evidence of inhibition of mushroom growth when hardwood sawdust was incorporated into PDA plates. Lack of growth on sawdust alone may be due to the low nitrogen content of sawdust, which may also explain the inability of *L. edodes* and *F. velutipes* to colonise the entire sawdust substrate over six weeks in MacCartney bottles.

Although the three species tested are all able to grow on wood in nature, the substrates and techniques adopted for artificial cultivation of these three species are different. *L. edodes* is grown commercially on wood logs, however, cultivation methods using lignocellulose wastes (such as sawdust, bagasse, cotton seedshell) supplemented with a nitrogen source (rice bran, wheat bran) have been developed for many years (Wu *et al.*, 1987). *F. velutipes* was traditionally grown on sawdust plus rice bran or wheat bran (Guo, 1987). Although sawdust, cotton seed shell, bagasse, maizecob and other agriculture wastes are also used depending on the availability of lignocellulose wastes locally, *P. ostreatus* is commonly grown on cereal straw. Chemical analysis revealed that rice straw and wheat straw contained (respectively) 27.3% and 50.1% cellulose; 19.7% and 29.0% hemicellulose, 17% and 13.7% lignin (see Rajarathnam and Bano, 1989). Analysis performed on lupin hulls indicated that this substrate contained 50.08% cellulose, 28.39% non-cellulose polysaccharides (19.48% hemicellulose and 8.91% protopectin) and 4.62% lignin, which was comparable to wheat straw except lupin

hull had a lower lignin content. Nitrogen in the hull-sawdust substrate initially was estimated to be 0.025% (w/w) (0.05% of nitrogen content in lupin hull according to Ivana Radojevic, CBFT, VUT). Wu (1987) had shown that for *P. ostreatus*, the optimal nitrogen content in the substrate for vegetative growth was 0.016 to 0.064% and the optimum nitrogen content for fructification was 0.016 to 0.032%. The lupin hull-sawdust mixture provided nitrogen levels within these optimum ranges.

4.1.2.2 Fructification

The formation of primordium is a complex process which depends upon the mushroom (species, strains), the condition of the inoculum and environmental factors (such as temperature, light, moisture, pH, ventilation, composition of substrate) (Eger, 1978; Zadrazil, 1978; Wu, 1987). Results reported in this thesis described a limited screening program that aimed at identifying which of the three mushroom species selected for study could, indeed, grow and form mushroom on lupin hull as a substrate. Initial tests involved growing the species under different conditions in MacCartney bottles, using different combinations of ground hull and sawdust, cold stock treatment and observing mushroom formation with and without elevated humidity.

P. ostreatus and *F. velutipes* were able to fruit in the MacCartney bottles under moisture-enriched environments at room temperature, following cold shock treatment at 4°C. *L. edodes* was unable to form primordia under the same conditions.

In these experiments, all of the *P. ostreatus* cultures were able to form primordia at room temperature in MacCartney bottles within five weeks of growth on a mixture of lupin hull

plus sawdust or hull alone, without cold shock treatment; no growth was observed on the sawdust substrate under the same growth conditions. This demonstrated that the lupin hull was a good carbon and nitrogen source for *P. ostreatus* vegetative growth and for fruiting body formation.

An important process for *F. velutipes* fructification in commercial media prepared from sawdust or other wastes is "scratching" or "cleaning" of the aged mycelia and inoculated spawn (Tonomura, 1978; Guo, 1987). In this process, the aged spawn mycelium which is exposed to air is scratched and removed, the surface was then made smooth. This process is normally performed before cold shock treatment, which occurs when 90% of the media is colonised. This practice is believed to enhance mushroom production and to speed up fructification; it is also thought to reduce the chance of contamination. It was difficult to "scratch" and "clean" the aged spawn in the small-scale experimentation used in the screening program so that this process was not used in these experiments. After cold shock treatment, fruiting bodies of *F. velutipes* appeared variously within one week and three months in a moisture-enrich environment. This species was unable to produce primordia without the moisture-enrich conditions, indicating that *F. velutipes* requires ventilation and wet conditions to develop its fruiting bodies.

The reason why *L. edodes* could not form primordia in the MacCartney bottles is unknown. It is suspected that *L. edodes* might require more ventilation and/or wetter condition and/or longer periods for fructification than *P. ostreatus* and *F. velutipes*, which the MacCartney bottles and the experimental conditions used could not provide.

Due to the variability in the period for *F. velutipes* to form primordia and the lack of incubation facilities to maintain the high moisture levels during large-scale culture, *F. velutipes* was not tested for growth in large-scale trials using plastic bags. *P. ostreatus* and *L. edodes* were subsequently tested at large-scale for mushroom formation.

In large-scale cultivation on lupin hull plus sawdust in PP bags, 100% of *P. ostreatus* cultures formed fruiting bodies compared to about 40% of the *L. edodes* cultures. These results also indicated that the mixture of lupin hull and sawdust could be a substrate for both *P. ostreatus* and *L. edodes* fruiting body formation. It also showed that *P. ostreatus* was the best of the three fungi for cultivation and fruiting body formation under these laboratory conditions. Failure of *L. edodes* to develop further primordia into fruiting bodies may have been due to the high temperatures used during fructification, although other environmental factors (such as moisture, light and ventilation conditions) may also be involved in the development of mushroom fruiting bodies (Wu, 1987).

The effect of temperature on the formation of fruiting bodies has been thoroughly studied (Guo, 1987; Huang *et al.*, 1987; Tokimoto and Komatsu, 1978; Tonomura, 1978; Wu, 1987; Wu *et al.*, 1987; Zadrazil, 1978). Primordia differentiation of *P. ostreatus*, *L. edodes* and *F. velutipes* occurs over a wide range of temperatures between 13-30°C, 8-21°C and 5-20°C respectively, with the optima reported over the ranges of 13-24°C, 10-12°C and 12-15°C. The optima for development of fruiting bodies is reported as slightly different from that of primordia differentiation, this being 15-19°C, 8-16°C and 3-20°C, respectively. Optimum temperatures for mushroom growth and fructification are summarised in Table 4.1.

Table 4.1 Temperatures for different mushroom growth stages (Guo, 1987; Huang *et al.*, 1987; Wu, 1987; Wu *et al.*, 1987)

Species	Temperature (°C)					
	Mycelium growth		Primordium differentiation		Fruiting body development	
	Range	Optimum	Range	Optimum	Range	Optimum
P. ostreatus	3-33	22-26	5-30	13-24	13-28	15-19
L. edodes	5-32	24-27	8-21	10-12	5-24	8-16
F. velutipes	3-34	23-24	5-20	12-15	3-20	10-16

In the experiments described in this thesis, the temperature used for fruiting body development of *L. edodes* was room temperature (22-25°C), which is at the upper limit for both primordia differentiation and fruiting body development. In commercial cultivation of this species in sawdust-bag systems, mushrooms are produced in most of the culture bags even when these are lightly contaminated, indicating that under optimal culture conditions mushrooms form readily. For the formation of *L. edodes* primordia on sawdust substrates, temperature and moisture is important: low temperature and high humidity are essential conditions for differentiation. The highest temperatures reported for primordium differentiation has varied from strain to strain, but this is normally between 18 and 20°C (Wu, 1987). High humidity and good ventilation conditions are also required when the fungus is grown at high temperatures, otherwise, the primordium would simply die under dry conditions, or development would be inhibited by excessive CO_2 produced from expiration during this phase of rapid metabolism (Wu, 1987). However, *F. velutipes* is reported to require even lower temperatures for formation of fruiting bodies, yet fruiting bodies were obtained at the same temperature for experiments in MacCartney bottle. Strain variation might account for the differences observed.

Light is also an important factor in formation of mushroom fruiting bodies. In general, light is necessary for fruiting, but it inhibits mycelial vegetative growth (Eger, 1978; Guo, 1987; Huang *et al.*, 1987; Tokimoto and Komatsu, 1978; Tonomura, 1978; Wu, 1987; Wu *et al.*, 1987). Both quality and quantity of light are important. It is well established that wood-rotting fungi require light of wavelengths shorter than 520 nm for fruiting and pileus expansion (Eger, 1978). Light sources with a high proportion of blue light are most suitable for *P. ostreatus*. The light intensity and the amount of light (light intensity X light duration)

are critical for fruiting initiation. There is a minimum requirement and an optimum beyond which primordia formation is rapidly inhibited. In commercial cultivation, fungal cultures are incubated in darkness during vegetative growth and exposed to shaded natural light or artificial light during fructification. Low levels of light (30 Lux) are sufficient for initiation of *P. ostreatus* primordium formation, and ambient natural light, or 10 lux, in the 370 to 420 nm range, is sufficient for initiation and development of *L. edodes* fruiting bodies (Wu *et al.*, 1987; Stamets and Chilton, 1983). In the experiments reported in this thesis, about 30% of *P. ostreatus* cultures produced primordia in a dark incubator before they were moved to the cool room for cold shock treatment. All of these primordia appeared on the substrate at a position near the inoculum, where the mycelium was relatively aged. The formation of *P. ostreatus* primordium in dark condition could be due to the light exposure accumulated during routine checking of growth (every other day for about 10-20 minutes) which may have initiated the process of primordium formation.

Ventilation could also affect mushroom growth and fructification. Both oxygen and carbon dioxide are required in fungal growth. *P. ostreatus* mycelial growth is stimulated by high CO_2 concentrations in the air (Zadrazil, 1978). A CO_2 concentration in the air of up to 28% (v/v) stimulates vegetative growth of *P. ostreatus*. The high CO_2 tolerance of *P. ostreatus* might help protect mycelial growth against other microorganisms, which either cannot grow or die off at higher concentrations (Zadrazil, 1978). A higher CO_2 level has also been reported to stimulate *A. bisporus* vegetative growth (San Antonio and Thomas, 1972; Wiegant *et al.*, 1992). Oxygen is also essential for mycelial growth. In SF of *Pleurotus* mycelium biomass, the growth rate immediately decreases to zero if the supply of oxygen is cut off (Zadrazil, 1978). In SSF, air circulation is necessary for *Pleurotus* mushroom

production during fructification. At this stage of the growth cycle, the presence of 1-2% (v/v) of CO_2 may cause abnormal fruit body development (Zadrazil, 1978). In the experiments described in this thesis, abnormal mushrooms of *P. ostreatus* were obtained following cultivation in the temperature-humidity incubator, which had light control and internal air circulation. Compared with normal mushrooms, abnormal ones showed longer stipe, were dry on their surface and pale in colour, observations which were similar to those described in the literature for this species when grown under insufficient ventilation conditions (Zadrazil, 1978). However, *L. edodes* is less sensitive to CO_2. It is reported that increasing the CO_2 level in the environment seriously affected the development of *P. ostreatus* and *A. bisporus*, but that this had little effect on *L. edodes*, *F. velutipes* and *A. auricula* (Huang *et al.*, 1987).

Many factors influence the successful production of fruiting bodies, including the quality of the substrate supplied. One parameter to evaluate the suitability of the substrate is the biological efficiency (BE), which is expressed as the average production yield from the raw materials. Although the average yield of *P. ostreatus* on wheat straw or rice straw can reach one kg fresh weight of mushrooms per kg of dry weight of straw substrate, only the first two flushes are harvested for the best commercial values (Chen, 1988; Stamets and Chilton, 1983). The average yield was reported to be 50% fresh weight mushroom relative to the dry weight of substrate when *P. ostreatus* was grown on a substrate of 40% cocoa shell waste, 40% sawdust, 19% oatmeal and 1% CMC (Pettipher, 1987). When the oyster mushroom *P. sajor-caju* was cultivated on paddy straw supplemented with cottonseeds, the percentage conversion of substrate into fruiting bodies (on a dry weight basis) was 12.82 g/100 g substrate (Bisaria *et al.*, 1987b). In the experiments described in this thesis, only the quality

of lupin hull as the substrate was studied and the small amount of mushrooms harvested in these experiments was not sufficient to make statistically supported statements about yields relative to other substrates previously used for commercial mushroom production. Further studies on the BE of lupin hull for mushroom production would be required, under more precisely controlled conditions and in larger quantities, to enable better evaluation of using lupin wastes for commercial production of oyster mushrooms. These studies did demonstrate that lupin hull could be used as a substrate for oyster mushroom development, where, under the experimental conditions used, this species grew better than the other species trialled. Optimisation studies were not pursued, when it was discovered that the major enzymes produced on this substrate were pectinases. Because of the novelty of this observation, this area of study became the major part of the research of this thesis.

4.2 POLYSACCHARIDE-DEGRADING ENZYMES OF *P. OSTREATUS*

Based on the results from the chemical analysis, culture filtrates of *P. ostreatus* grown on lupin hull as sole carbon source were assayed for cellulase (FPase and CMCase), hemicellulase (xylanase) and pectinase (PGase), the enzymes expected to be secreted to degrade the corresponding polysaccharide components of the substrate.

Preliminary studies showed that very low activity of cellulase was present in the first two weeks of growth, whereas pectinase and xylanase activity dominated the culture at about the second to third week of growth and xylanase activity disappeared after the fourth week. It is noted that pectinase of *P. ostreatus* has not been characterised before and the tests for PGase activity were conducted at pH 5.0, 50°C for 30 minutes. Further characterisation of

P. ostreatus revealed that a second pectic enzyme - PALase - was also involved in the degradation of lupin hull.

Studies on the polysaccharidases produced by *P. ostreatus* in the course of degradation of lupin hull were carried out subsequently in SF and SSF after characterisation of PGase and PALase activities.

4.2.1 SUBMERGED FERMENTATION

In SF, three kinds of vessels were used: small-volume shake flask (200 mL broth in 500 mL flask), large-volume shake flask (two L broth in five L flask) and a two L capacity Braun fermentor. The small-volume shake flasks provided the best results for studying enzyme production, because contamination problems were encountered with the large-volume shake flask cultures due to frequent sampling, and difficulties with a buoyant particulate substrate in the stirred fermentor vessel. Similar conditions were described previously by Chaga *et al.* (1972). In submerged cultivation of *A. niger* for the production of pectic enzymes, the optimum temperature was 29°C and optimum amount of cultivation medium was 60 mL in a 250 mL glass vessel mounted in an excentric shaker at 220 rpm.

Typical results for the production of polysaccharide-degrading enzymes in 500 mL flask culture of *P. ostreatus* growing on lupin were shown in Fig. 3.3. The peak of cellulase activity occurred before the others, followed by xylanase, then PGase and finally PALase (using optimum assay conditions for PGase and PALase). In the course of degradation, the PGase showed highest activity, whereas PALase showed the second highest activity and this occurred at the time in the growth when an increase in pH also occurred. This observation

is interesting because the PALase has a high pH optimum for activity which would have been favoured by the culture conditions observed. Both enzymes exhibited their activities in culture fluids for up to one month incubation. Xylanase activity was detected for a short period during the second and third week of culture, whereas FPase only appeared in the first week of culture. CMCase activity was detected at low levels up to the third week of culture.

Previous studies by other researchers on polysaccharidases produced by other fungal species have shown that the pattern of enzyme activity presented in the course of submerged culture growth can vary with different carbon sources. Tewari *et al.* (1987) used corn-cob, groundnut shell, sugarcane bagasse, wheat straw and cellulose for studying cellulase production by *T. reesei* and found that in SF CMCase had its highest activity around six to ten days of growth, followed by a decrease in activity. FPase production appeared to have the same pattern, except when using corn-cob as carbon source, where FPase activity gradually increased till the last sample was examined at the 14th day of culture. Similar patterns of cellulase production occurred in the early growth stages of *P. ostreatus* cultured on lupin hull. In another fungus, *Alternaria macrospora*, the maximum activity of endo-PGase and PALase were found in about 12 to 14 days of initiating growth, whereas cellulase activity was maximum in six to ten days in the same growth medium (Padmanaban and Narayanasamy, 1977). Earlier appearance of the maximum cellulase activity followed by the peaks in PALase and PGase was also observed for *P. ostreatus*, as reported in this thesis, which possibly resulted from the induction of cellulase by cellulose as the major component of hull, at the beginning of colonisation. This cellulose degradation may expose other polysaccharides, such as hemicellulose and pectin, for microbial attack. Further degradation of hemicellulose and pectin provided more accessible products from the lupin hull for

microbial conversion. Presumably, the role of the cellulase, xylanase and pectin-depolymerase in the culture of *P. ostreatus* is to degrade the corresponding substances in the media, cellulose, xylan and pectin. The higher observed levels of PGase and PALase in the media of *P. ostreatus* grown on lupin hull may indicate a preference for degradation of the pectic substances present in lupin hull. It is not known if the PALase and PGase action provides the fungus with the energy and carbon sources needed to grow and survive, or if these activities merely make other polysaccharides, such as cellulose, available for enzyme attack. By using a crude enzyme solution from *Aspergillus sojae* culture to degrade soybean cell wall (SCW) (a substrate similar in composition and source to the lupin hull used in the study here), it was found that the SCW was more easily degraded and that the degree of degradation was much higher if a strain of *A. sojae* with higher pectinase activity was used (Kikuchi *et al.*, 1971). This indicated that pectinase plays an important role in disruption of SCW. Synergistic action of cellulase, hemicellulase and pectinase has also been observed in the degradation of plant cell walls when commercial enzymes were used to degrade the cell wall polymers of carrot in food processing (Schoenfeld and Behnke, 1991). Treating membrane tissue of grapefruit segments with pectinase and cellulase was reported to decrease the content of xylose and glucose significantly more than the sum of the separate enzyme activities. The synergistic effect obtained by the combination of pectinase and cellulase showed that the pectin present sterically "masked" the hemicellulose and cellulose. Breaking down the barrier posed by pectin with pectinase, or chemical treatment, allowed a significant increase in the hydrolysis of the hemicellulose and cellulose. Preparation of alcohol-insoluble solids from fresh tissue modified the structure of the pectin in the cell wall and prevented its steric hindrance to breakdown of hemicellulose and cellulose. Extraction of pectic substances from the tissue by NaOH greatly increased cellulose hydrolysis by cellulase (Ben-Shalom,

1986). Enzymatic hydrolysis of carrot tissue also highlight the need for use of pectinase enzymes to free cell wall cellulose from pectin cementing materials and allow cellulase to be effective (Anastasakis *et al.*, 1987). The pectic enzymes detected in *P. ostreatus* during growth on lupin hull may perform a similar function in degradation of lupin hull.

4.2.2 INDUCTION OF POLYGALACTURONASE AND PECTATE LYASE IN *P. OSTREATUS*

FPase, CMCase, xylanase, PGase and PALase were all detected in SSF and SF of lupin hull by *P. ostreatus*, and the pectin-depolymerases were the major enzyme activity detected in this study. Since it could be argued that the major degradative enzymes were present due to the particular strain variety used, or due to the nature of the substrate being utilised, experiments were performed to evaluate which component may induce the individual enzymes. This study was particularly interesting as the PGase and PALase of *P. ostreatus* had not been characterised before. There was no attempt made to evaluate strain variation in this study, as such a study was beyond the scope of this thesis.

In the study described in this thesis, PGase of *P. ostreatus* was induced by pectin and PGA, however, it could not be induced by either GA or galactose. Production of PALase by *P. ostreatus* occurred during growth on most of the carbon sources tested, including non-galactose-derived substrates, *i.e.* MG and xylan. However, derivatives of galactose, such as pectin, PGA, GA and galactose, were the most effective inducers, noting that growth on the polymeric substrates, such as pectin and lupin hull, resulted in relatively high persistence of PGase activity in culture fluids.

Induction of cellulase and xylanase have been studied previously for various fungi (Riou *et al.*, 1991; Yazdi *et al.*, 1990), including *Pleurotus* species. Cellulose powder was found to be most effective in the production of Avicelase and β-glucosidase, while CMC (sodium salt) was good for the production of CMCase; glucose (1%) completely repressed the synthesis of enzymes in *P. sajor-caju* (Rajarathnam and Bano, 1989). PGase and PALase activities were found in the culture fluid when a hybrid strain of *P. ostreatus* and *P. florida* was grown on flax shive in SF at 20°C for 25 days. The activities of PGase and PALase were equivalent to 475 and 175 μg GA/hour/mL culture fluid at 30°C (Sharma, 1987). No further information on induction of pectinase by *Pleurotus* has been reported.

Literature studies have shown that various factors may affect the production of pectic enzymes produced by various microorganisms. Most important factors are type and volume of inoculum, pH, temperature, nitrogen and carbon sources (Call *et al.*, 1985; Mehta *et al.*, 1991; Riou *et al.*, 1992; Galiotou *et al.*, 1993; Schwan and Rose, 1994). Using spore (*A. niger*) concentrations in the range 10^2 - 10^8 spores/L of substrate as inoculum in SF, pectinase activity (measured by the Apple Juice Depectinising Assay) and PGase showed the highest values with the smallest inoculum, whereas PLase showed higher activity when a higher spore concentration was used (Friedrich *et al.*, 1990). When grown on pectins with various degrees of esterification, *Aspergillus sp.* CH-Y-1043 produced maximum endo- and exo-pectinases at pH 2.5 - 3.5 (Aguilar *et al.*, 1991). In screening 72 strains of *Candida* for extracellular enzymes, peptone was found to be the best nitrogen source for the production of pectic enzymes and highest enzyme activities were achieved with succinic acid (aerobic) and inulin (anaerobic) as carbon sources; enzyme production under aerobic conditions was

considerably increased at pH 3.0; and addition of inducers (pectins or pectin fibre) led to only very slightly increased enzyme production (Call *et al.*, 1985).

The only parameter studied as part of this thesis was the effect of carbon source on induction of polysaccharidases of the strain of the *P. ostreatus* under investigation, although it was appreciated that the pH of culture and level and type of nitrogen source may have influenced the profile of enzymes produced.

Induction of pectinase by carbon source may vary depending on the species under study. Generally, GA, citrus pectin, apple pectin, beet pulp are considered common substrates for inducing of pectin-hydrolysing enzymes (Murad and Foda, 1992). However, none of these substrates of pectin-hydrolysing enzymes could affect the level of PGase formation by the yeast *Kluyveromyces lactis* (Murad and Foda, 1992). When another yeast, *Kluyveromyces marxianus*, was grown on media using different sugars as carbon sources, it was found that glucose- and sucrose-containing media produced similar PGase and ethanol production, whereas growth in galactose- and fructose-containing medium retarded secretion of PGase and ethanol but had no effect on growth. Growth and PGase secretion, however, were considerably retarded in xylose-containing media (Schwan and Rose, 1994). Early studies by Phaff (1947) revealed that PGase and PEase are strongly induced in *P. chrysogenum* by pectin, pectin acid, D-galacturonate, mucate and L-galacturonate. Swinburne and Corden (1969) and Spalding and Abdul-Baki (1973) demonstrated that *P. expansum* produced PGase and PLase when cultured on apple tissue. Formation of both enzymes was repressed by a variety of sugars and also by galacturonate and glutamate. Dialysis of apple medium resulted in a greatly increased level of enzyme production (Spalding and Abdul-Baki, 1973). Similar

repression of the synthesis of inducible PGase by *A. niger* was reported by Maldonado *et al.* (1989). The synthesis of PGase was repressed by glucose, even in the presence of the inducer. The production of PGase started once glucose was used up, or when the mycelium was washed free of glucose and incubated in a glucose-free medium containing the inducer. This proved the reversibility of the repression mechanism. *P. digitatum* produces PLase constitutively at a relatively constant rate, and is independent of culture conditions (Lobanok *et al.*, 1977). *G. lactis* produced extracellular PGase in media containing pectate, pectin, or GA as inducers and synthesis of the enzyme was strongly repressed by glucose (Pardo *et al.*, 1991). A study by Riou *et al.* (1991) on the production of cell wall-degrading enzymes by the phytopathogenic fungus *S. sclerotiorum* showed that PGase activities were produced constitutively on all of the substrates tested, including glucose, β-MG, xylose, sorbose, sophorose, cellobiose, Avicel, CMC, HMC, citrus pectin, apple pectin, Na^+-PGA, galactan, arabinogalactan, laminarin and xylan, where citrus pectin, apple pectin, PGA, galactan and arabinogalactan showed higher induction than the other carbon sources. Two different PALase forms (differentiated by MW and K_m) were found with the different carbon sources used to cultivate *T. curvata* (Stutzenberger, 1991).

Results reported in this thesis for *P. ostreatus* show patterns similar to those reported for other fungal species, where the carbon source influenced the level, and persistence of both PGase and PALase activities. Induction of both PGase and PALase of *P. ostreatus* by PGA, pectin and pectin-containing substrate (lupin hull) was similar to results reported for several other fungi and bacteria (Tierny *et al.*, 1994; Pardo *et al.*, 1991; Stutzenberger, 1991). However, the effects of GA on induction of PGase by *P. ostreatus* was different to results reported for other fungal species, such as *S. sclerotiorum* and *G. lactis*, where GA induced

PGase of both *S. sclerotiorum* and *G. lactis* (Pardo *et al.*, 1991; Riou *et al.*, 1991). Preferred substrates for induction of PGase by *P. ostreatus* were polymeric carbon sources, such as arabic acid, PGA, pectin, whereas PALase was produced more generally on a variety of carbon sources.

4.2.3 SOLID STATE FERMENTATION OF LUPIN HULL

4.2.3.1 Total enzyme activity changes

Studies on the enzyme activity changes during the sporophore of mushroom development have been mainly concerned with cellulase and xylanase activities due to the dominance of these components in the substrates used (Rajarathnam and Bano, 1989). Most reports showed a gradual increase in enzyme activities in the substrate during the vegetative stage (Turner *et al.*, 1975; Rajarathnam *et al.*, 1979; Leatham, 1985; Wang and Wang, 1991) and one study reported the lack of detection of cellulase activity during vegetative colonisation by *P. ostreatus* (Iwahara *et al.*, 1981). All studies reported that there was a dramatic increase of cellulase and xylanase activities during fructification and decreases in activities after harvest. There are two differences in the results reported in this thesis when compared with these previous studies: firstly, a slight decrease in enzyme activities was detected between late vegetative growth stage and before the development of mushroom fruiting body stage and, secondly, changes in PGase and PALase activities were detected.

Results for enzyme production in SSF of the lupin hull and sawdust mixture were shown in Fig. 3.5. In the first two weeks, substantial decreases in RS levels in the substrate were attributed to the intake of free mono-, di- and oligo-saccharides by fungal cells due to rapid growth, which is similar to conclusions reached in other reports (Valmaseda *et al.*, 1991).

By studying the degradation of straw, two phases of fungal growth in SSF were suggested. In SSF of wheat straw by *P. ostreatus*, the "colonisation phase" was characterised by a strong increase in respiratory activity and a decrease in extractable free sugars, extractives and *in vitro*-digestibility. Its duration was estimated to be 10 days for *P. ostreatus*. The "degradation phase" represented fungal attack on the lignin and polysaccharides and an increase in straw digestibility and protein content. In studies reported in this thesis, a dramatic decrease of RS in the substrate (soluble in distilled water) was accompanied by an increase in extracellular polysaccharide-degrading enzymes at the beginning of fungal growth. In the work of Rajarathnam *et al.* (1987), estimation of the sugars (soluble in 70% alcohol) formed during growth of *P. flabellatus* on wheat straw in SSF showed that glucose, xylose, arabinose and cellobiose and total sugars increased continuously until the end of the mycelial growth phase, which indicated a progressive saccharification of substrate during fungal growth. The total sugar (soluble in 70% alcohol) increased from the initial 0.82% (dry weight) to 2.95% at the twelfth day of growth when the substrate was fully colonised by *P. flabellatus*. In the results reported in this thesis, RS in the water extracts decreased from the initial 22.30 mM (equivalent to 2.00% of glucose based on w/w of dry substrate) to 5.99 mM (equivalent to 0.54% of glucose) at the fifth week, when substrate was fully colonised by *P. ostreatus*. These contrasting results may be a reflection of the differences in substrates used, initial RS level in the substrate, techniques used (especially the solvent used for extraction) and species under test. In the results reported in this thesis, increasing enzyme activity implied that there is a progressive saccharification of substrate during fungal growth, where the degradation products are then transferred into the fungal cell.

In the third week of *P. ostreatus* growth on lupin hull, a time just before the formation of primordia, RS decreased slowly and enzyme activities also declined. To my knowledge, this phenomena has only been reported for exocellulase of *L. edodes* (detected by cellulose azure degradation), however, CMCase activity did not decrease before fructification of *L. edodes* (Leatham, 1985). A decline in the activities of CMCase, β-glucosidase and xylanase was also reported during SSF of wheat straw by *P. ostreatus* during vegetative growth, however, no further observations were made in the fructification stage (Valmaseda *et al.*, 1991). Such declines probably reflected the internal metabolic adjustment of the fungus to achieve a ready-fruiting state, or could be attributed to the accumulation of inhibitors (such as CO_2, *etc.*), or other physical parameters (*e.g.* light, *etc.*). This question was not investigated further here.

RS and the main extracellular enzyme activities (PGase and PALase) reached their lowest level at the end of fourth week of *P. ostreatus* growth on lupin hull, accompanied by full colonisation of the substrate in the plastic bag and formation of primordia, which fully developed into fruiting bodies at the fifth week, when enzyme activities reached another peak. As a result of extracellular enzyme activity, the level of RS in the substrate also increased from the fourth week and formed a peak at the fifth week of growth. An increase in cellulase and hemicellulase activities during fructification has been observed by several authors (Turner *et al.*, 1975; Rajarathnam *et al.*, 1979; Leatham, 1985; Wang and Wang, 1991). By using antibody techniques, it was found that the increase in enzyme activities in the substrate was due to the synthesis of new enzymes by the fungus (Wood, 1993). This suggested that the change of extracellular enzyme activity in the substrate was regulated by synthesis and secretion of the enzyme protein. During fructification of the basidiomycetes,

carbohydrates were transferred from the vegetative mycelium to build up the mushroom fruiting body. An increase in extracellular polysaccharide-degrading enzyme activity by *P. ostreatus* from the fourth to fifth weeks probably indicated increased biosynthesis of enzyme. As a result of the increase in enzyme activity, more polysaccharides in the substrate were degraded into an accessible form which made more sugars available for the construction of the mushroom fruiting body. Mushrooms were fully developed at the fifth week and extracellular enzyme activity plus the level of RS reached a peak in the substrate at the same time. When mushrooms decayed at the sixth week, there was no further need for translocation of sugars so the level of synthesis of enzymes declined again, and the enzyme activity dropped. In SSF of wheat straw by *P. flabellatus*, a continuous increase of monosaccharides (glucose, xylose and arabinose) during the mycelial growth phase was followed by a decrease during fructification; glucose showed larger decreases than the pentose sugars. However, there was an increase in content of cellobiose and higher oligosaccharides after harvesting the fruiting bodies (Rajarathnam *et al.*, 1987). These results strongly supported the hypothesis that the assimilation or/and translocation of these monosaccharides occurred during fructification, and unused higher oligosaccharides remained in the substrate. It is still unknown how the fruiting process is switched on and how enzyme synthesis is regulated although some of factors, such as light, temperature, CO_2 or O_2 level, may be involved (see Section 4.1.2.2).

There is no doubt that both PGase and PALase play a similar physiological role as the other polysaccharidases, that of providing energy and building blocks for fungal growth. Previous reports (Turner *et al.*, 1975; Rajarathnam *et al.*, 1979; Leatham, 1985; Wang and Wang, 1991) which described changes in cellulase and hemicellulase activities during the life cycle

of mushrooms were similar to the changes noted in this thesis for xylanase activity. Activities of FPase were lower in SSF for *P. ostreatus* grown on lupin hull. No significant differences in activity of FPase during the life cycle of *P. ostreatus* were observed here, however, CMCase showed similar patterns of production to xylanase activity.

Compared with the other enzymes, PGase and PALase showed relatively higher levels of activity at the beginning of the colonisation. Both showed similar peak levels during vegetative growth and the sporophore stage, which is slightly different to patterns seen for the other enzymes (where the first peak was normally lower than the second one). Two possibilities may explain these observations:

a. Different carbon sources are needed by *P. ostreatus* during vegetative growth and fructification. In SSF of wheat straw, glucose was the highest monosaccharide found in the extracts (70% alcohol) of substrate during the life cycle of *P. flabellatus*, and glucose showed the largest decrease of the monosaccharides during fructification (Rajarathnam *et al.*, 1987). These results implied that glucose was important during the construction of mushroom fruiting bodies. Further evidence supporting this is that the fruiting body of mushrooms contains certain levels of cellulose, *e.g.* fresh mushrooms of *Hydnum repandum* contain 7.6% cellulose (Ertan and Guelyavuz, 1993). An increase in FPase and CMCase activities during fructification provides more glucose for the synthesis of this cellulose during the construction of the fruiting body. Degradation products of both PGase and PALase reported in this thesis may provide the main energy and carbon sources for building up new cells in vegetative growth of *P. ostreatus*. In sporophore, a different balance of saccharides may be

required for construction of the fruiting body due to the different compositions between vegetative mycelium and the fruiting body. However, there is insufficient information on the comparative compositions of vegetative mycelium and the fruiting body to support this hypothesis directly. As shown in Fig. 3.5, only activity of PGase was lower in the fructification peak relative to the peak seen in vegetative growth. FPase, CMCase and xylanase activities were much higher during fructification. Simultaneous increases in cellulase and hemicellulase activities in the fruiting stage have also been reported in *Agaricus*, *Lentinus* and *Pleurotus spp.* (Turner *et al.*, 1975; Rajarathnam *et al.*, 1979; Leatham, 1985; Wang and Wang, 1991).

b. Compositional changes occur in the substrate during vegetative growth. The pectin component originally present of about 4% of the dry weight of the substrate (mixture of lupin hull:sawdust at a ratio of 1:1). High activities of PGase and PALase in the colonisation phase caused a gradual decrease of accessible pectin components. During fructification, there would be less accessible pectin to cause induction of PGase, noting that substrate studies in SF showed that polymeric substrates induced PGase activity. On the other hand, more accessible xylan and cellulose were exposed and this was available to induce the corresponding enzymes. Studies on the induction of enzymes by various carbon sources (see Section 3.3.2) indicated that PALase of *P. ostreatus* was also induced by galactose and GA. During sporophore in SSF, the substrate had less accessible pectin components and more pectin degradation products. A lesser pectin component may induce less PGase and PALase, whereas more pectin degradation products may induce more PALase but not PGase.

4.2.3.2 Enzyme activity changes in different growing regions

Studies by Jablonsky (1981) on the biochemical changes in the different regions during SSF of *P. ostreatus* and *P. florida* on shredded corn cobs showed a conspicuous humidity gradient in the substrate at the time before primordia initiation and fruiting body formation, when the water content as well as the pH were highest in the surface layers and lowest in the central part of the experimental plots. No further explanation was made of these results.

Enzyme activities in different regions of growth of *P. ostreatus* were studied as part of this thesis. In vertical transects, attempts were made to detect any differences in the enzymes secreted by mycelium of different ages. RS level was highest in the growing tip from the second week onwards. This indicated a continuous assimilation of free RS by the fungus. In the first week, relatively small quantities of sample were available and low FPase, CMCase, and xylanase activities were detected, which made it difficult to compare activities accurately in different areas. Lower RS in the growing tip at this stage was due to the available glucose from the WG inoculum. Relatively higher activities of PGase and PALase were detected and these showed a gradient which decreased from the growing tips to the aged inoculum, which may have resulted from inhibition of activity caused by higher levels of water-soluble starch in the inoculum (prepared from wheat grain), plus induction by the accessible pectin components in the newly-colonised substrate. At this stage, colonisation was the main activity, where carbon sources were provided by the inoculum and free saccharides from the substrate, as described by Valmaseda *et al.* (1991) as the "colonisation phase" when *P. ostreatus* initiated growth in SSF of wheat straw. During this stage, induction of enzymes would also occur, where expression of the enzymes might be affected by the presence of free sugars (see Section 4.2.2). In the second week, a gradient of enzyme

activity was also observed but the tip showed the lowest enzyme activities and the aged colony showed the highest activity. There are several possible explanations for these results. Firstly, this occurred as a result of saccharides in the substrate inducing or repressing enzyme synthesis: lower RS levels in the aged colony induced higher enzyme production, whereas higher RS in growing tip suppressed enzyme synthesis. Secondly, differences existed in the qualities and quantities of biomass between the growing tip and the aged colony, which might result in different patterns of enzyme secretion.

In horizontal transects, RS level was always higher in the centre of the colonised substrate and lower in the outer layer, probably because of local enzymatic degradation of the substrate. It is well recognised that the temperature in the centre of the substrate is normally higher than in outer layers in the growth stage, due to the intensive metabolic activity and the diffusion of heat in the outer layers to the environment (Zadrazil, 1978). Results presented in this thesis (see Section 3.4.1.2) showed that both PGase and PALase activities increased at higher temperatures (temperature optima were both above 40°C). Higher temperatures in the centre could result in higher rates of degradation of polysaccharides and hence higher RS levels. Higher activities of FPase, CMCase, PGase and PALase were found in the centre of the substrate in the first week. A reverse result was found in the second week, when CMCase, xylanase and PALase showed highest activity in the outer layer: light penetration, water distribution and temperature might be associated with these observations. A surprising result was obtained in the fifth week or fructification stage: the three major enzymes showed increasing activities from the outer layer to the centre (where no conclusion could be made regarding the activities of FPase and CMCase because of the relatively lower activities found). These observations may be associated with changes in environment factors,

such as light availability, lower temperature and air exchange (noting that the bag was cut for fructification) and water evaporation. However, a more likely explanation for these observations is that changes were associated with physiological changes occurring during mushroom development. On the one hand, higher temperatures and more extracellular enzyme activity in the centre may result in more free saccharides being produced in the centre than in the outer layers. After assimilation by fungal cells, a gradient of free sugar levels within the cells could also be formed, with a lower level of sugars in the mycelium cell distributed in the outer layer of substrate. On the other hand, higher temperatures in the centre may also result in more evaporation of water than in the outer layers. The evaporated water would then condense into water droplets due to the lower temperature in the outer layers of substrate, as was seen frequently on the walls of the PP bags or bottles during mushroom growth in the laboratory, or as observed on bark of tree logs in nature. Jablonsky (1981) reported similar physical effects for growth in SSF of *P. ostreatus* and *P. florida* on shredded corn cobs, where a water gradient formed which resulted in less water in the centre and higher water levels on the surface. Because water molecules can pass the cell wall membrane easily, uneven moisture distribution in the substrate could also cause uneven water content between the mycelium cells in different parts of the substrate, resulting in different rates and/or pathways of metabolism in the different regions. Furthermore, it was observed that a pH gradient also occurred between outer and inner growth regions, which was also similar to the results reported by Jablonsky (1981), who noted higher pH at the surface. Lower water content and higher free sugars in the mycelium cells in the centre of substrate in contrast to higher water content and lower free sugars in cells in the surface, which could form a dynamic gradient for translocation of free sugars from the centre to the surface where the primordium occurred.

4.2.3.3 Enzyme activity in the mushroom fruiting body

In the results presented in this thesis, RS levels and most of the enzyme activities generally decreased with the development of mushroom fruiting bodies, except for xylanase activity which showed irregular changes. These results do not correspond to observations in other reports for *Pleurotus tuber-regium*, *Tricholoma lobayensis*, *Termitomyces robustus* and *Lentinus subnudus*, which might be due to species variation and/or the nature of the substrate supplied. By studying enzyme activities for endocellulase, amylase, lipase, peroxidase, proteinase, catalase, polyphenol oxidase and glucose-6-phosphatase in various fruiting stages of *P. tuber-regium* and *T. lobayensis*, Kadiri and Fasidi (1990) found that both mushrooms showed increases in all enzyme activities from the very young to the mature fruiting stages, except for glucose-6-phosphatase. Increases in endocellulase activity as the sporophore matured were also observed for *T. robustus* and *L. subnudus* (Fasidi and Kadiri, 1991). Endocellulase activity in the mushroom fruiting body was claimed to be associated with the nutritional value of mushrooms as a food for the enzyme was associated with the sugar level and the metabolism of carbohydrates in the fruiting bodies (Kadiri and Fasidi, 1990; Fasidi and Kadiri, 1991).

Results in this thesis also showed that CMCase activity was higher in the gill of *P. ostreatus* basidiocarp, which does not correspond to the observation from *P. tuber-regium*, *T. lobayensis*, *T. robustus* and *L. subnudus*, where endocellulase activity was found to be higher in the stipes than in the pilei (Kaddiri and Fasidi, 1990; Fasidi and Kadiri, 1991).

Surprisingly, PGase and PALase, the two dominant extracellular enzymes in the substrate, were rarely detected in the fruiting body after the mushrooms had developed into medium

size (width > 4 cm). Pectic compounds are mainly present in the cell wall of higher plants. Extracellular PGase and PALase of *P. ostreatus* plays a role in degradation of the substrate and both enzymes were no longer needed for fruiting body development since the substrate (PGA) for their activities was probably absent in the fruiting body of *P. ostreatus*. The presence of FPase, CMCase and xylanase in nearly every fruiting stage and in almost all parts of the *P. ostreatus* fruiting body suggested that they have roles not only in degradation of the substrate but in the development of mushroom fruiting body structure as well.

4.2.4 COMPARISON OF SOLID-STATE FERMENTATION AND SUBMERGED FERMENTATION OF LUPIN HULL BY *P. OSTREATUS*

In SSF, PALase activity was higher than that of PGase, whereas the reverse observation was obtained in SF. Similar observations have been made for commercial fermentations of other pectinases: for example, SSF culture generally produces higher yields of polymethygalacturonate lyase than SF culture (Ward, 1985). This may indicate subtleties in regulation relating to water availability. The substrate of both PGase and PALase are the same - PGA. PGase is a hydrolase which require water to hydrolyse the C-C bond, whereas PALase is a lyase which does not required water for its action. The fungus produced more PGase to degrade the PGA in SF, which is consistent with abundant water being available for hydrolysis. In SSF, the water content is less and less PGase was produced. The regulation of this process is unknown and very little research has been done on pectin metabolism in fungi (Rombouts and Pilnik, 1980).

4.3 BIOCHEMICAL PROPERTIES OF POLYGALACTURONASE AND PECTATE LYASE OF *P. OSTREATUS*

Pectin-depolymer enzymes are known to be produced by numerous fruit species (Giovannoni *et al.*, 1992; Abu-Sarra and Abu-Goukh, 1992; Downs *et al.*, 1992), plant-pathogenic and saprophytic fungi (Riou *et al.*, 1991), bacteria (George *et al.*, 1991) and yeasts (Murad and Foda, 1992). Initial experiments reported in this thesis revealed that the pectin depolymerisation activity of *P. ostreatus* was the main enzyme produced during degradation of the polysaccharide components of lupin hull, which suggested that pectin depolymerase activity is important for the biodegradation of lupin hull by this mushroom species (a white-rot fungus). However, there have been relatively few studies on pectin-depolymerisation enzymes of this, and other mushroom species. Only two studies have reported the detection of mushroom pectinases: one report indicated that pectin-depolymerisation activity was found in the culture filtrates of a hybrid strain of *P. ostreatus* and *P. florida* when grown on flax shives (Sharma, 1987). The other study demonstrated the presence of PGase activity in a sawdust substrate during cultivation of *L. edodes* (Leatham, 1985). The properties of the enzymes responsible for pectin-depolymerisation by these mushroom species were not described.

Preliminary experiments were performed using an assay system which detected the formation of RS from the PGA substrate following incubations with culture filtrates of *P. ostreatus* at 50°C (the temperature normally used for assay of other cellulases and xylanase). Two peaks of activity were found when using a range of different buffers for assay, corresponding to activities with optima at pH 5.0 and pH 8.6. These results implied that two enzymes might be produced during degradation of lupin hull by *P. ostreatus*. Further studies confirmed the

production of two different enzyme types: one had an acidic pH optimum and demonstrated hydrolase activity (polygalacturonase [PGase]), whereas the second had an alkaline pH optimum and showed lyase activity (pectate lyase [PALase]). Both PGase and PALase degraded PGA and created reducing ends.

An acidic pH optimum for the PGase of *P. ostreatus* is similar to other reports for PGase enzymes found in fruit, fungi, bacteria, yeasts and commercial preparations of this enzyme (see Rombouts and Pilnik, 1980), as seen from the following published information. The optimum pH of a purified endo-PGase produced commercially by *A. niger* was reported as 4.5 (Labavitch and Rae, 1977). For eight commercial pectic enzyme preparations, highest activity of PGase was found in pH ranges of 4.4 to 5.6 (Schoenfeld and Behnke, 1991). For bacterial PGases, optimum pH for activity was 5.5 for both *Lactobacillus plantarum* and *Lactobacillus acidophilus* enzymes (Sakellaris *et al.*, 1989; Kim *et al.*, 1991). Studies on the pectic enzymes endo-PGase I and II purified from crude enzyme preparations of *A. niger* showed that endo-PGase I degraded protopectin best at pH 5 whereas endo-PGase II was active over a pH range of 5 to 6 (Hara *et al.*, 1986). pH optima for two exo-PGases of *A. niger* were reported as 3.8 and 4.8 (Hara *et al.*, 1984). For other fungal PGases, optimum pHs for enzyme activity were found to be 4.0, 5.0, 5.0 and 5.0-5.5 for PGase from *R. oryzae*, *R. stolonifer*, *G. lactis* and *T. viride*, respectively (Chung *et al.*, 1992; Pardo *et al.*, 1991; Manachini *et al.*, 1987; Petrich and Boucaud, 1990). An optimum pH at 5.5 and 4.5 was also found for PGase I and PGase II produced by the yeast *A. pullulans* (Manachini *et al.*, 1988). The pH optimum of 5.0 for PGase from *P. ostreatus* reported in this thesis was obtained using various buffers, where citrate-phosphate buffer was found to give the highest activity for the assay employed in this study.

The results presented in this thesis also showed that the optimum pH for *P. ostreatus* PALase was pH 8.6, using the Clark-Lubs buffer system. This buffer was the only choice available as no other system could buffer at pH 9.0 without significant effects by temperature. High optimum pH for PALase activity is similar to previous reports for other PALases. For example, an inducible extracellular exo-PALase from *Erwinia dissolvens* was found to have an optimum pH of 8.5 (Castelein and Pilnik, 1976); optimum activity of PALase produced by *B. subtilis* occurred at pH 8-9 (Chesson and Codner, 1978); maximal activity of purified endo-PALase from *P. fluorescens* was found at pH 9.4 (Rombouts *et al.*, 1978). Endo- and exo-PALase of other species reviewed by Rombouts and Pilnik (1980) also indicated that optimum pH was mostly between 8.0 and 9.5. To date, only one PLase (not PALase) has been reported to have an acid pH optimum, which was an enzyme with an optimum of pH 5.0 produced by *A. pullulans* (Manachini *et al.*, 1988).

The experiments for detecting optimum temperature for enzyme activity were carried out using buffers at pH 5.0 and pH 8.6 after the optimal pH for PGase and PALase action were determined. PGase of *P. ostreatus* was found to have an optimum temperature for assay at 55°C, whereas PALase preferred to degrade PGA at 45°C when assayed using RS production and 40°C when detecting the production of the end-product with an absorption maximum at 235 nm. Optimal temperatures for other PGase and PALase activities vary from species to species in previous reports (see Table 1.2). For PGase produced by the bacteria *L. acidophilus* and *L. plantarum*, optimal temperatures for enzyme activity were 30°C and 35°C (Sakellaris *et al.*, 1989; Kim *et al.*, 1991). Both PGase I and II of yeast *A. pullulans* have an optimum temperature at 50°C (Manachini *et al.*, 1988). For fungal PGase, optimum temperature for enzyme activity secreted by *R. oryzae* and *T. viride* was 40°C and 50°C

(Chung *et al.*, 1992; Petrich and Boucaud, 1990). For PALase activity, optimum temperature for the enzyme from *B. subtilis* was found at 60°C to 65°C (Chesson and Codner, 1978); and an inducible extracellular exo-PGase from *E. dissolvens* was reported having an optimum temperature at 43°C (Castelein and Pilnik, 1976). A higher optimum temperature for PGase activity relative to PLase has also been reported for enzymes secreted by the yeast *A. pullulans*. Optimum temperatures for assay of purified *A. pullulans* PGase and PLase were 50°C and 40°C, for enzymes made during growth on a medium containing apple pectin as carbon source (Manachini *et al.*, 1988). Thermostability studies presented in this thesis also indicated that the PGase was more stable than PALase.

Results obtained from column chromatography of *P. ostreatus* culture filtrates gave direct evidence that there were two different enzymes present in the culture filtrates. The elution volume of PGase corresponded to a MW of 36,500 Da and activity could be assayed directly using PGA without addition of metal ions. In contrast, the peak of PALase activity corresponding to a MW of 66,000 Da was only found when assays were supplemented with additional mineral salts. SDS PAGE gels also suggested that the MW of *P. ostreatus* PGase was 36,500 Da, which is similar to that found for enzymes made by other species (see review by Rombouts and Pilnik, 1980). For the bacterium *L. plantarum*, two PGases were found to have the same MW of 32,000 Da as determined by Sephadex G-100 gel filtration (Sakellaris *et al.*, 1989). The PGase produced by the fungus *R. oryzae* had a MW of 47,000 Da as estimated by SDS-PAGE (Chung *et al.*, 1992); the exo-PGase of *G. lactis* showed a MW close to 53,000 Da by gel filtration (Pardo *et al.*, 1991); two exo-PGases produced by *A. niger* during growth on mandarin peel were 66,000 and 63,000 Da (Hara *et al.*, 1984). For PALase, different MWs have been reported across species and different enzymes are

produced following growth on different substrates. For example, *B. subtilis* produces a PALase with a MW of 33,000 Da (Chesson and Codner, 1978). Two different PALases of *T. curvata* are induced by different carbon source: the MW of PALase produced during growth on a protein-extracted lucerne fibre was 35,000 Da compared to 56,000 Da for the enzyme produced on pure pectin (Stutzenberger, 1991).

The pI of PGase produced by *P. ostreatus* was reported to be 7.5 in this thesis using data from the IEF Rotofor cell, which appears to be a little higher than results for other PGases. In the commercial enzyme preparation Pectinex Ultra SP-L, five major endo-PGase isoenzymes had pIs of 3.2, 3.6, 3.7, 4.4 and 4.5 (Okai and Gierschner, 1991). Two endo-PGases of *E. carotovora* were reported to have pI value at 10.7 and 3.9 (George *et al.*, 1991). The results presented in this thesis showed that PALase secreted by *P. ostreatus* had two pIs at 7.0 and 4.5, both appeared to be much lower than previous reports for the enzymes from other species. Six extracellular endo-PALase with pI of 10.2, 9.6, 9.5, 9.4, 9.2 and 8.9 were found in *E. carotovora* (George *et al.*, 1991). The lowest pI of PALase was reported in *P. fluorescens*, which produced two PALases: the major one had a pI of 10.0 and the minor one had a pI of 6.7 (Liao, 1989). The pI of PALase in previous reports mostly appeared to be between 8.9 of *E. carotovora* (George *et al.*, 1991) and 10.3 of *P. fluorescens* (Rombouts *et al.*, 1978; see Rombouts and Pilnik, 1980). The characteristics of lower pI for the *P. ostreatus* PALase may be of industrial use for recovery of the enzymes, since the enzyme protein would have lower solubility and it would be easier to precipitate from solutions with a pH the same as its pI values. The data for pI values of *P. ostreatus* pectinases needs to be clarified by further experimentation. Detection of two pI values for PALase activity was determined following Sephacryl S-200 gel chromatography, using peaks

of activity for each of the enzymes and the pooled activity across the two pectinase peaks. Similar data was generated using these two approaches. However, it is still not clear whether the different pI values determined for PALase activity represent different forms of the same enzyme (same MW, different pI) or two distinct enzymes. Data from SDS-PAGE of the Sephacryl S-200 fractions did not clarify this situation due to the low levels of protein present. Larger-scale purification would need to be carried out to provide sufficient initial activity and protein to clarify this situation.

The metal ion requirement for PGase and PALase activities was also investigated in this thesis. In early attempts to purify the enzymes by Sephacryl S-200 chromatography, little activity of PALase was recovered compared to PGase, where most of the activity was recovered using the standard assay which detected RS production. This suggested that the PALase had specific requirements for activity which were present in the crude filtrate but may have separated from the enzyme during chromatography. By adding denatured culture filtrates to the assay mixture, a peak of PALase activity was detected in fractions. By using metal ions which were in the culture filtrate (SSBM) to restore PALase activity at pH 8.6, Ca^{++} was found to be best for restoration of enzyme activity at 150 μM in the assay mixture. A linear relationship was observed between PALase activity restored and calcium ion concentration used in the assay mixture when this was lower than 0.10 mM. A slight inhibition of PALase by calcium was observed when concentrations were higher than 0.20 mM. A requirement for calcium ions by PALase activity has been reported for other similar enzymes (see Rombouts and Pilnik, 1980). For example, PALase activity from *E. carotovora* requires Ca^{++} at 0.2 mM (Yoshida *et al.*, 1991). The inhibition of PGase by

calcium ions reported in this thesis is also similar to that in other reports on PGase from other species (Conway *et al.*, 1988; Vazquez *et al.*, 1993).

The results presented here showed that most heavy metal ions, such as Pb^{++}, Hg^{++}, Fe^{+++}, Fe^{++}, Ni^{++} and Cu^{++} totally inhibited both PGase and PALase of *P. ostreatus* at 0.05 M. Inhibition of PGase and PALase by heavy metal ions has varied between microorganisms (Manachini *et al.*, 1987; Kotzekidou, 1990; Chung *et al.*, 1992). At a concentration of 1 mM, Cu^{++}, Pb^{++}, Zn^{++} and Mn^{++} completely inhibited the activity of PGase secreted by *R. oryzae* (Chung *et al.*, 1992), which is similar to the results presented in this thesis. K^+ and Na^+ did not inhibit either enzyme when concentrations were lower than 0.2 M. Both K^+ and Na^+ ions were reported to stimulate *R. arrhizus* endo-PGase activity in the range of 0.05 to 0.10 M (Liu and Luh, 1978). For *A. niger* PGase, K^+, Na^+ and NH_4^+ ions at concentrations from 0.0001 M to 0.1 M increased activity whereas Ag^+ and Hg^{++} completely inhibited PGase activity (Popov, 1975). Inhibition of enzyme activity by heavy metal ions is due to the natural structure of the enzyme. Generally, the active site of enzyme contains important acidic or basic groups of amino acid, such as amino acid α-COOH, aspartic acid, glutamic acid, amino acid α-NH_2, arginine, tyrosine and lysine (Fersht, 1985). Metal ions might interact with these groups and reduce the catalytically active form of enzyme. Inhibition of PALase by chelators reported in this thesis is consistent with the observed requirement for Ca^{++} by this enzyme. There was not much difference between the two enzymes when challenged by metabolic inhibitors, whereas end-product inhibitors showed some differential responses by these enzymes: PGase showed more resistance to cellobiose than PALase.

Spectrophotometric analysis of the end-product of the two enzymes was performed by scanning between 190 and 900 nm. Results showed that the end-products of PGA degradation by culture filtrates under acidic conditions did not have optical absorbance, whereas the end-products of PGA degraded by culture filtrates in assays at pH 8.6 had a peak absorbance at 235 nm. End-product absorbance at 235 nm is a result of the formation of the double bond at C4 - C5 at the new non-reducing ends of the PGA molecule following reactions with PALase (Pilnik and Voragen, 1991). In contrast, PGA degraded by PGase only results in hydrolysis with the creation of reducing ends rather than producing a double bond. Absorbance at 235 nm was used to assay PALase using a recording UV spectrophotometer, the molar extinction coefficient being 4,600 /cm/mM (Rombouts and Pilnik, 1980). However, in this study, RS measurement was also used to assay PALase activity because of the convenience of this assay and the ability to differentiate this activity from PGase on the basis of metal ion requirement plus optimum temperature and pH of assay. Whichever assay was used, the kinetics of the reaction velocity of PALase appeared to include an initial burst phase, as described by Tipton (1992). This could be due to the changes in the degree of polymerisation during degradation of PGA and it is possible that the *P. ostreatus* PALase prefers to degrade the longer chains of PGA, so that the rate of reaction declines rapidly as the reaction proceeds.

The mode of action of PLases and PALases has been studied previously for other enzymes (Pilnik *et al.*, 1974). Activity measurements on pectins which varied in their degree of amidation showed that PLase is very specific for methoxyl groups and that the enzyme did not discriminate between -COOH and -$CONH_2$ groups, which was in contrast with PALase

which are very specific for free carboxyl groups. Analysis of breakdown products obtained by the degradation of pectins with various degrees of methylation showed that the breakdown products of PLase action had a methylated unsaturated galacturonide unit at the non-reducing end, whereas PALase produced oligomers with a non-methylated unsaturated galacturonide unit at the non-reducing end. Comparison of the β-elimination reaction of the pectin (75% methoxylated) and PGA by *P. ostreatus* PALase partially purified by ion-exchange chromatography showed that the degradation of PGA was faster and more continuous than the degradation of pectin when monitoring the absorbance at 235 nm (refer to Fig. 3.31). Absorbance showed degradation of pectin occurred at the beginning of the reaction, then this levelled off and then finally increased again. This indicates that this partially purified lyase is a PALase rather than PLase and it prefers PGA as substrate. The initial degradation rate of pectin might be attributed to the presence of unmethylated carboxyl group in the pectin at an expected ratio of 25%. The reaction stopped once this unmethylated carboxyl group had been utilised. The final increase (after seven hours reaction) of absorbance could be due to several reasons, such as pectin demethylated during prolonged heating at 40°C.

The K_m value of PGase from *P. ostreatus* was found to be 0.27 mg/mL, which appears to be lower than those reported for similar enzymes from other microorganism. Most fungal PGases have a K_m value from 0.54 mg/mL to 1.7 mg/mL (see Rombouts and Pilnik, 1980 and Table 1.2) although a higher K_m for the PGase produced from *L. acidophilus* was reported, this being 4.0 mg/mL (Kim *et al.*, 1991), and a lower K_m was reported for the exo-PGase of *G. lactis* as 0.09 mg/mL (Pardo *et al.*, 1991).

4.4 IMPLICATION AND APPLICATION PROSPECTS OF THIS STUDY

This study demonstrated that lupin hull could be used as a substrate for specialty mushroom cultivation and pectin-depolymerase were found as the main enzymes present during degradation of this waste.

Furthermore, some properties of the pectinases made during cultivation on lupin hulls have been determined, indicating that at least two novel enzymes are produced based on their biochemical properties following partial purification. Although pectin was a relative minor component of the lupin hull, relatively high levels of PALase and PGase were produced, opening up some interesting prospects for the future application of these enzymes.

Firstly, it would be interesting to evaluate the use of the enzymes or the fungus for degrading other pectic wastes, such as orange and apple processing wastes (including rinds, peels and pith). One possibility would be to evaluate the ability of *P. ostreatus* to grow on these wastes and produce mushrooms, with and without supplementation with other cellulosic biomass such as lupin hull. This could be done in the absence of any further knowledge of the enzymes concerned, given the composition of the wastes and the knowledge of the enzymatic capacity of the strain used in this study. The enzymes may have utility alone for pretreatment of these wastes in other waste minimisation processes.

Secondly, the enzymes of *P. ostreatus* described in this study may have broad application in the food industry and other industry sectors. The main application of pectinases is in the food and juice industry, where enzymes from *A. niger* are in commercial use currently.

Degradation of pectin is a feature common in several fungi but pectinase production by mushroom species has not been studied thoroughly previously. To evaluate the utility of the *P. ostreatus* pectinase in industry, it would be necessary to complete the study of the nature of the enzymes present in terms of the number of different enzyme types present and their versatility in terms of substrate range (*i.e.* ability to degrade other pectic substrates encountered in the food industry). Further purification and characterisation of the enzymes on a larger scale than attempted in this thesis would be required. The use of mushroom species in this way is attractive, as a waste material would be used for producing both enzymes for industrial use and mushrooms as a commercial by-product. Furthermore, the mushrooms may have further use in medicine, considering the under-explored area of bioactive compounds from mushroom species (noting that anti-cancer drugs are being produced commercially from *L. edodes* in Japan [Fukushima, 1989]).

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APPENDIX COMMERCIAL SUPPLIERS OF CHEMICALS, MEDIUM CONSTITUENTS AND ACCESSORIES

A. Alphabetical List of Chemicals, Medium Constituents and Accessories.

Reagent	Source
Acetamide	AJAX
Agar	Oxoid
Ammonium oxalate	AJAX
Acetone	AJAX
Ampholytes	Bio-Rad
β-Amylase (sweet potato)	Sigma
Aprotinin (Bovine lung)	Sigma
Arabic acid	Sigma
Blue dextran	Sigma
Boric acid	BDH
Bromophenol	AJAX
BSA	
(fraction V for MW marker)	Sigma
(for Protein assay)	Pierce
Carbonic anhydrogenase (bovine erythrocytes)	Boehringer
Cellobiose	Sigma
CMC	Sigma
Coomassie blue G-250 protein assay reagent kit	Pierce
Cytochrome C (Horse heart)	Boehringer
DEAE-Sepharose Fast Flow gel	Pharmacia
Dinitrosalicylic acid	Sigma
Dithiothreitol	Sigma
EDTA (di-sodium salt)	Sigma
Folin-Ciocalteu's reagent	AJAX
Formaldehyde	AJAX
Fructose	AJAX
GA	Sigma
Galactose	AJAX

Glucose	AJAX
Glutardialdehyde	Sigma
Glycerol	AJAX
Iodoacetamide	Sigma
β-Lactoglobulin (Bovine)	Sigma
Maleic acid	Sigma
MCC	Sigma
β-Mercaptoethanol	Sigma
Metronidazole	Sigma
MG	Sigma
Ovalbumin	Sigma
Pectin	Sigma
Peptone	Oxoid
PGA	Sigma
PDA	Oxoid
PMSF	Sigma
Potassium cyanide	Aldrich
Potassium ferricyanide	Aldrich
Potassium sodium tartate	BDH
SDS	Mallinck
SDS polyacrylamide gel	
Homogenious (7.5%, 12.5%)	Pharmacia
Gradient (8%-10%)	Pharmacia
Sepharose S-200	Pharmacia
Soybean trypsin inhibitor	Sigma
Sodium arsenate	Sigma
Sodium arsenite	Sigma
Sodium azide	Sigma
Succinic acid	Sigma
Tris(hydroxymethyl)aminomethane	Sigma
Tween 80	AJAX
Xylan	Sigma
Xylose	AJAX
Yeast alcohol dehydrogenase	Sigma
Yeast Extract	Oxoid

B. Location of Supplier

Name	Address
AJAX Chemicals Ltd.	18 Hamlet St, Cheltenhem, Vic 3192.
Aldrich	Sigma - Aldrich Pty Ltd, Unit 2, 10 Anella Ave, Castle Hill, NSW 2154.
A. I. S. Australian Instrument Services Pty Ltd.	Factory 2, 21 Stud Rd, Bayswater, Vic 3153.
B. Braun Melsunger AG, Australia	10 Arcae Rd, Box Hill Nth, Vic 3129.
Beckman instruments P/L.	24 College St, Gladesville, NSW 2111.
Bio-Rad Laboratories P/L.	Unit 11, 112-118 Talavera Rd, Nth Ryde, NSW 2113.
Boehringer Mannheim Biochemica Australia P/L.	26-28 Ellingworth Parade, Box Hill, Vic 3128.
Disposable Products P/L.	16 Park Way, Technology Park, S.A. 5095.
Essendon Produce P/L.	16 Russell St, Essendon, Vic 3040.
FSE Du Pont Australia Ltd.	Medical Products Dept. Healthcare Block C, Centre Court Industrial Estate, 25 - 27 Paul St, Nth Northside, NSW 2113.
Hofert A. J.	91 Type St, Burnley, Vic 3121.
Linbrook	21/170 Forster Rd, Mount Waverley, Vic 3149.
Mallinckrodt Australia P/L.	1/126-134 Fairbank Rd, Clayton, Vic 3168.
Merck*	207 Colchester Rd, Kilsyth, Vic 3137.
Millipore	PO Box 721, Richmond, Vic 3121.
Oxoid	PO Box 220, West Heidelberg, Vic 3081.
Paton Scientific P/L.	17 Enterprise Avenue, Victor Harbor, S.A. 5211.
Pharmacia Australia P/L.	4 Byfield St, Nth Ryde, NSW 2113.
Pierce	PO box 117, Rockford, IL USA 61105.
Selby Scientific Laboratory Equipment	368 Ferntree Gully Rd, Notting Hill, Vic 3168.
Sartorius Australia P/L.	PO Box 84, Chadstone Center, Chadstone, Vic 3148.
Sigma - Aldrich Pty Ltd.	Unit 2, 10 Anella Ave, Castle Hill, NSW 2154.
Varian Australia P/L.	679 Springvale Rd, Mulgrave, Vic 3170.
Warehouse Sales P/L.	262 Hampshire Rd, Sunshine, Vic 3020.

* BDH distributor