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PRODUCTION AND SOME PROPERTIES OF LIGNINASE USING FOAM-IMMOBILISED PHANEROCHAETE CHRYSOSPORIUM

BY

NEIL KIRKPATRICK, B.Sc.

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ABSTRACT

Ligninase is an extracellular haemprotein produced in the culture filtrates of white rot fungi which is capable of carrying out some of the reactions associated with the degradation of lignin *in vivo*. The enzyme was first isolated from stationary submerged cultures of *Phanerochaete chrysosporium*. In this thesis, modifications to existing culture methods were investigated in order to facilitate the production of successive harvests of ligninase from the same fungal biomass. In early experiments, the fungus was embedded in agar beneath a liquid medium. However, it was found that the fungus was apparently able to solubilise the agar, and this led to the use of polyurethane foam as an alternative - inert - immobilisation support.

The immobilisation of *P.chrysosporium* in cubes of polyurethane foam resulted in the production of increased and more uniform harvests of ligninase, and also facilitated the use of the same fungal biomass for the production of several successive harvests of ligninase. In addition, foam-immobilised cultures of *P.chrysosporium* could be stored at 4°C for periods of at least two months with no apparent loss in the ability of the fungus to produce fresh successive harvests of ligninase upon culture reactivation.

During the concentration and purification of the isozymes of ligninase produced by foam immobilised P.chrysosporium, it was observed that an apparent loss of enzyme activity could be reversed by the inclusion of a range of metal ions in the standard veratryl alcohol oxidase assay. This effect was shown to be due to the co-concentration of a pH-dependent inhibitor of ligninase; the effects of the latter being reversed in the presence of the metal ions. The inhibitor compound was separated from the isozymes of ligninase during anion-exchange chromatography, and a preliminary analysis has shown it shares a number of properties with uronic acid polysaccharides.

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ABBREVIATIONS

Ca^{2+}	cation of calcium
Co^{2+}	cation of cobalt
DEAE	diethylaminoethyl
FPLC	fast performance liquid chromatography
H_2O_2	hydrogen peroxide
HPLC	high pressure liquid chromatography
K_m	concentration of substrate at which the rate of enzyme catalysis is half-maximal (Michaelis constant)
Kd	kilodalton
La^{3+}	cation of lanthanum
Mg^{2+}	cation of magnesium
Mn^{2+}	cation of manganese
Na^{+}	cation of sodium
S.D.	standard deviation
SDS	sodium dodecyl sulphate
Sp.Ac.	specific activity
TCA	trichloroacetic acid
U	unit of enzyme activity
UV	ultraviolet
V_{max}	maximum rate of enzyme catalysis

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INTRODUCTION

1.1 What is lignin, and why is it so important both ecologically and industrially?

Lignin is the second most abundant organic compound on Earth, the most abundant being cellulose. It is found in all higher plants, including the ferns and club mosses, but has not been detected in other plant groups such as the Bryophyta (true mosses) or the liverworts (Erickson and Miksche, 1974a and b; Miksche and Yasuda, 1978). In wood and vascular plant tissue, lignin is present at a concentration of 20-30% (w/w) and is located in the cell wall material where together with the hemicelluloses, it forms a matrix which surrounds the orderly arrangement of cellulose microfibrils (Sarkanen and Ludwig, 1971). In wood tissue, the lignin acts as a glue which forms the middle lamella layer between adjacent cells.

Lignin itself is an amorphous, three-dimensional polymer, composed of phenyl propane units linked in a variety of different ways. It differs from other naturally occurring polymers such as cellulose or protein, in that it does not have a regular repeating bond along a linear backbone. The reason for this heterogenous structure lies in its biosynthesis which occurs by the free radical polymerisation of precursor aromatic alcohols (Sarkanen and Ludwig, 1971). There are three possible precursor molecules that are known to be involved in the

biosynthesis of lignin, and these are p-hydroxycinnamyl (coumaryl) alcohol, 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, and 3,5-dimethoxycinnamyl (sinapyl) alcohol, see Fig. 1; these give rise to p-hydroxyphenyl, guaiacyl, and syringyl units in the lignin polymer, respectively. The relative balance of these units in lignin enables the lignins of different plant groups to be classified into three major groups: guaiacyl lignin (most conifers, lycopods, ferns, and horsetails), guaiacyl-syringyl lignin (most dicotyledenous angiosperms and some gymnosperms), and guaiacyl-syringyl-p-hydroxyl lignin (highly evolved grasses); Higuchi et al. 1977. Typical examples of the structural arrangement of the monomeric units in conifer and beech lignin are shown in Figs. 2a and 2b, respectively (Adler, 1977; Nimz, 1974). Fig. 3 shows the relative proportion of the different linkages found in spruce lignin (Adler, 1977); the most dominant form of linkage being the B-O-4 ether linkage which accounts for approximately 50% of the total linkages in all angiosperms (Kirk and Farrell, 1987).

From an ecological point of view, lignins are an extremely important group of compounds. Ultimately, the driving force for the maintenance of life on Earth is provided by solar radiation. Autotrophic organisms use this energy, and by the process of oxygenogenic photosynthesis, convert simple inorganic compounds into complex living organic material. After an appropriate period, the living material eventually dies, and when the

p-coumaryl alcohol

coniferyl alcohol

sinapyl alcohol

Fig. 1 Precursor molecules of lignin

Fig. 2a Structural features of conifer lignin

(Adapted from Adler 1977)

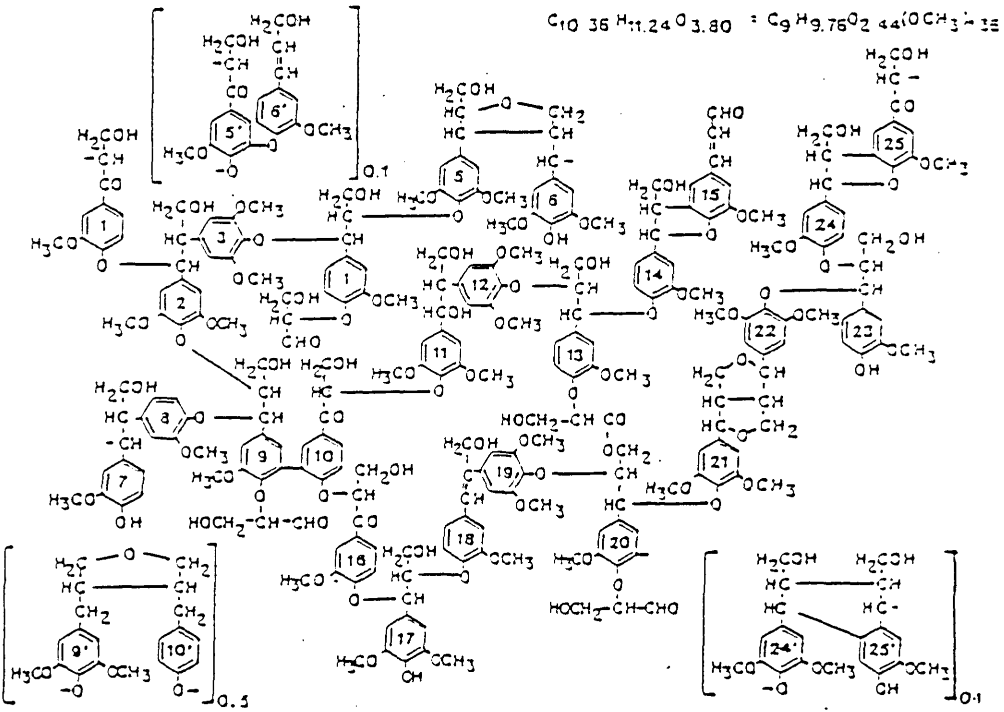


Fig. 2b Structural features of beech lignin

(Adapted from Nimz 1974)

BOND TYPE	STRUCTURE	PROPORTION (%)
Arylglycerol-β-aryl ethers		48
Noncyclic benzyl aryl ethers		6 – 8
Biphenyl		9.5 – 11
1,2- Diarylpropane structures		7
Phenylcoumaran structures		9 – 12
Diphenyl ethers		3.5 – 4

Fig. 3 Relative proportions of the major bonds found in spruce lignin

(Adapted from Adler 1977)

"trapped" carbon and oxygen have been mineralised, they then re-enter the pool of carbon and oxygen as simple inorganic compounds again. In this way, the carbon-oxygen cycle is able to continue. Within this cycle, insoluble aromatic macromolecules synthesized by the higher plants constitute a vast reservoir of carbon and oxygen (Evans, 1977). The latter include lignins and tannins, and these compounds are particularly important since they are often only degraded at a very slow rate; and consequently, they provide the most probable rate-limiting step for the entire carbon-oxygen cycle.

In addition to being important ecologically, lignin is also important industrially. Man has exploited the biosphere's ability to produce vast quantities of lignocellulosic material, and has developed large industries based on the use of this substrate; for example, agriculture, forestry, and the manufacture of paper. Each of these industries generates either directly or indirectly enormous quantities of waste products that contain lignocellulose as a major component. A summary of the waste products that could be used as potential substrates for microbial conversion, and also, a summary of the products that might usefully be produced by microbial conversion, are shown in Tables 1 and 2, respectively.

Table 1 Potential substrates for microbial conversion *

Substrate	Reference
Cattle manure	Bellamy (1972a and b, and 1974)
Urban solid waste	Stutzenberger et al. (1970 and 1971); Updegaff (1971)
Logging and pulpmill wastes	Millett et al. (1970); Mellenberger et al. (1971); Harkin et al. (1974); Pamment et al. (1979)
Agricultural wastes	Chalel et al. (1979); Chandra and Jackson (1971); Dekker and Richards (1973); Peitersen (1975); Ramasay et al. (1979)
Peat	Farnham (1978); Quierzy et al. (1979); Ghosh and Klass (1979)

* Adapted from Crawford (1981)

Table 2 Goals for microbial conversion *

	Reference
Production of chemicals	
Polyphenols	Goldstein (1975)
Organic acids	Scott et al. (1930); Hajny et al. (1951)
Methane	Schmid (1975); Clausen et al. (1977); Hashimoto et al. (1975); Yeck (1979); Commoner (1979); Robbins et al. (1979)
Glucose	Mandels et al. (1971); Brandt et al. (1972); Dhawan and Gupta (1977)
Alcohols or Single cell protein	Bellamy (1974); Dauglulis and Bone (1977); Moo-Young et al. (1979); Stutzenberger (1979)
Delignification of wood chips	Ander and Eriksson (1975 and 1978)
Pretreatment of highly lignified plant residues to increase their digestibility by ruminants	Dekker and Richards (1973); Zadrazil (1977); Ford (1978)
Detoxification of pollutants	Eaton (1985)

*Adapted from Crawford (1981)

1.2 Lignin degradation - How is its degradation studied, and what microorganisms can degrade it?

It can be seen from Figs. 2a and 2b that microorganisms which can degrade lignin must first overcome the difficulties associated with the structure of such a complex polymer. This means that unlike the degradation of other bipolymers, the initial steps in the degradation of lignin must be non-specific and non-hydrolytic, and because of the size and solubility of lignin, this initial attack must also be extracellular. The nature of lignin has also presented difficulties in the study of its degradation. The use of ^{14}C-labelled lignins which are converted to $^{14}CO_2$ provides a relatively simple assay which gives a good estimation of the overall degradation of lignin. However, the results must be interpreted with caution, as there are a number of possible flaws in this approach.

The preparation of ^{14}C-labelled lignins can either be *in vivo* (Crawford and Crawford, 1976; Haider and Trojanowski, 1975) or *in vitro* (Haider and Trojanowski, 1975; Kirk et al., 1975). During the preparation of *in vivo* labelled lignins, it is essential to ensure that labelled precursor material does not become incorporated into non-lignin components such as protein or aromatic acids that are not part of the structure of lignin itself (Benner et al., 1984; Crawford, 1981). In addition, when using labelled lignins prepared *in vitro*, it is essential that the lignin synthesized is indeed polymeric (Crawford, 1981). It should also be pointed out that

during the degradation of lignin, a proportion of the lignin is degraded to water-soluble compounds and thus when using labelled lignins, some of the label will always remain in the aqueous phase (Leisola et al., 1983a; Reid et al., 1982); and also, that some of the bonds in lignin can be relatively unstable during prolonged incubation in hot, acidic, or alkaline conditions, and this can lead to the release of small fragments, which are then able to be degraded more easily, thus leading to conclusions which are not necessarily applicable to an in vivo situation (Kirk and Farrell, 1987). An alternative approach to the use of labelled lignin, is to study the degradation of lignin model compounds. These are low molecular weight compounds that contain chemical structures that are known to occur in lignin. The information that is gained about the degradation of these may then provide useful information about the degradation of lignin itself (Muranaka et al., 1976). Once again though, the results obtained should be interpreted with caution, and it should be remembered that whereas the lignin model compounds are usually low molecular weight and water soluble, and can thus be degraded intracellularly, lignin itself is macromolecular and water insoluble, and its degradation must therefore fulfil the criteria outlined above, i.e. it must be extracellular, non-specific and non-hydrolytic (Haars and Huttermann, 1980). Of all the model compounds investigated, one of the simplest to use with isolated preparations of enzymes is veratryl alcohol.

The use of veratryl alcohol was first reported by Tien and Kirk (1984). They noted that during the oxidation of the B-O-4 dimer model compound, 1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1, 3-diol, approximately 15% of the compound was oxidised to the C-alpha ketone, and that this reaction was analogous to the oxidation of veratryl alcohol to veratraldehyde. The main advantages of using veratryl alcohol as a model compound for the degradation of lignin, are that in addition to it being commercially available, its oxidation to veratraldehyde can also be monitored spectrophotometrically. Thus, unlike more complex model compounds that require sophisticated techniques to interprete what products have been formed upon their degradation, it is possible to monitor the oxidation of veratryl alcohol as it occurs.

Having discussed the methods by which lignin degradation can be studied, and also the criteria which must be fulfilled for the results obtained to be applicable to an in vivo situation, it is now appropriate to discuss those microorganisms which by a combination of macroscopic and microscopic observation, and isolation from "rotted-lignin" environments, in conjunction with the techniques discussed above, have been shown to degrade lignin. It should be stated that at present, using this approach, there is no convincing evidence which suggests that lignin degradation can occur to any significant level in anaerobic environments such as in

lake sediments, sea-beds or anaerobic soils (Zeikus et al., 1982; Colberg and Young, 1985; Holt and Jones, 1983; Odier and Monties, 1983). Thus, it would appear that the microbial degradation of lignin is confined to aerobic conditions only.

The degradation of lignin by microorganisms can be conveniently divided into bacterial and fungal systems. Of the bacterial systems that have been studied, it can generally be concluded that aerobic bacteria are not able to bring about rapid or extensive lignin degradation. The actinomycetes are the most extensively studied group of bacteria, with the maximum conversion of ^{14}C-labelled lignin to $^{14}CO_2$ being c.20%. In one report, *Streptomyces viridosporus* and *S. setonii* were able to reduce the lignin content of spruce, maple and *Agropyron* by 32-44%, as determined by performing a chemical analysis of the insoluble residues (Antai and Crawford, 1981), and in another report, oxidative alterations that resembled those brought about by white rot fungi (see below) were observed when *S. viridosporus* had been incubated in the presence of spruce phloem (Crawford et al., 1982). In other studies, the production of "acid-precipitable polymeric lignin", or APPL, has been reported (Crawford et al., 1983; McCarthy et al., 1986; Petty and Crawford, 1985). These preparations of lignin contain different relative concentrations of carbohydrate, and thus, may be formed as a result of polysaccharide degradation rather than lignin degradation (McCarthy et al. 1986).

In studies using other groups of bacteria, it has not been possible to provide conclusive evidence of significant lignin degradation. However, in one set of experiments, Deschamps et al., (1980) did show that a range of bacteria were apparently able to bring about rapid rates of Kraft lignin degradation, although since they did not use ^{14}C-labelling, it cannot be discounted that they may simply have been observing adsorption of the lignin to the cells.

The degradation of lignin by fungi is generally much more rapid and extensive than that observed for bacteria. It is possible to sub-divide wood fungi into three categories depending on the macroscopic and microscopic appearance of the wood after fungal colonisation: white rot, soft rot and brown rot. The white rot fungi are the most prolific lignin degrading fungi, and consequently, because this group has been more thoroughly investigated than the other groups, it will be dealt separately in Section 1.3 of this Introduction.

The soft rot fungi have not been studied extensively, and as a result, little is known about their enzymology. However, it has been shown that, at least for some species of Ascomycetes and Fungi Imperfecti, wood polysaccharides are preferentially degraded rather than the lignin (Kirk, 1984; Kirk and Cowling, 1984).

The brown rot fungi have also not been studied very extensively, although it is known that they secrete

enzymes that breakdown wood polysaccharides, and that they are able to demethylate aromatic methoxyl groups, and to a lesser extent, bring about aromatic ring hydroxylation and ring cleavage (Kirk, 1984; Kirk and Adler, 1970). Consequently, in studies using ^{14}C-labelled lignins, more $^{14}CO_2$ was produced when the label was present in the aromatic methoxyl groups rather than in the side chains or the aromatic ring itself (Haider and Trojanowski, 1975; Kirk et al., 1975). Because of these characteristics, brown rot fungi do not lead to a significant mineralisation of the lignin polymer.

1.3 Lignin degradation by the white rot fungi

The white rot fungi are the most efficient lignin degrading fungi, and consequently, are the most thoroughly studied of all the lignin degrading microorganisms. Typically, white rot fungi invade the lumen of wood cells where they secrete enzymes which lead to the degradation of lignin as well as of other wood cell components. Studies using electron microscopy have shown that the degradation of lignin can actually occur at a distance away from the fungal hyphae (Blanchette, 1984; Blanchette and Reid, 1986; Otjen and Blanchette, 1986). During the degradation of lignin, it has also been shown that a number of oxidative changes occur at the structural level, including the opening of aromatic ring structures (Chen and Chang, 1985), and together, these result in the depolymerisation of lignin (Chen et al., 1982; Chen et al., 1983; Reid, 1985; Reid et al., 1982; Tai et al.,

1983; Terazawa et al., 1983) and the release of fragments which have a molecular weight of 1000 Kd or less (Faix et al., 1985; Leisola et al., 1983a).

Most white rot fungi are basidiomycetes (Crawford, 1981), and of these, Phanerochaete chrysosporium (also referred to as Sporotrichum pulverulentum) has been studied the most extensively. In the following sections, various aspects of lignin degradation by this fungus will be discussed in more detail.

1.3.1 Culture conditions and microbial physiology

The optimisation of the culture conditions for lignin degradation by P.chrysosporium has revealed that lignin is not a growth substrate and that its degradation is a secondary metabolic process that requires the presence of a co-substrate, and that is optimal under a high oxygen tension and in the presence of inducer molecules.

Lignin alone is not able to serve as the sole source of carbon and energy, and its degradation will only occur in the presence of a co-metabolizable substrate such as cellulose, hemicellulose, or glucose. This observation has been made using a variety of lignin preparations, and for a number of different fungi in addition to P. chrysosporium (Kirk et al., 1976; Kirk and Fenn, 1982; Drew and Kadam, 1979). It has also been demonstrated that the degradation of lignin by P.chrysosporium will only occur under conditions of

nitrogen (Keyser et al., 1978; Kirk et al., 1978; Jeffries et al., 1981), carbon or sulphur (Jeffries et al., 1981) limitation and is thus a secondary metabolic process. This observation has also been made for a number of other white rot fungi (Hatakka et al., 1983; Leatham and Kirk, 1983; Odier and Roch, 1983). In wood itself, it is likely that lignin degradation is naturally triggered by the depletion of nitrogen (Cowling and Merrill, 1966), and consequently, most of the in vitro studies of lignin degradation have been carried out under conditions of nitrogen limitation. Jeffries et al. (1981) observed that carbon limitation led to autolysis and only transient lignin degradation; however, in recent studies, carbon limitation has become the preferred method of triggering secondary metabolism because it leads to the production of increased titres of ligninase (See Section 1.3.2.1) and decreased levels of extracellular polysaccharide (Faison and Kirk, 1985; Leisola et al., 1985a). Sulphur limitation has not been widely used; probably because it is not so easy to demonstrate that it has been depleted.

The relative concentration of molecular oxygen has also been shown to have a profound effect upon the degradation of lignin. In the presence of an increased concentration of oxygen, there is an increase in the rate of lignin degradation which has been explained by the increased production of ligninase (See Section 1.3.2.1), and also of those systems which produce H_2O_2 (see Section

1.3.2.4) (Faison and Kirk, 1983; Faison and Kirk, 1985); and, in addition, there is also an increase in the relative efficiency of the existing ligninolytic system which has been explained by the increased availability of oxygen for degradative reactions and also for H_2O_2 production (Kirk et al. 1986a).

Veratryl alcohol is a natural secondary metabolite produced by P.chrysosporium (Lundquist and Kirk, 1978) which has also been shown to be produced by a number of other white rot fungi including Coriolus versicolor (Kawai et al., 1987) and Phlebia radiata and Pycnoporus cinnabarinus (Hatakka 1986). It has been demonstrated that the presence of this compound, or of lignin itself, results in an apparent induction of the complete ligninolytic system including ligninase (Ulmer et al., 1984; Faison and Kirk, 1985) and this has led to the proposal that veratryl alcohol is a natural inducer of the ligninolytic system (Leisola et al., 1984). It has also been proposed that radical cations of veratryl alcohol can act as redox mediators which would be able to initiate a chain reaction of auto-oxidation in lignin by the removal of single electrons (Palmer et al., 1987).

1.3.2 Biochemistry

In recent years, there has been significant progress in the biochemistry of lignin degradation, and consequently, in this section, details of some of these aspects will be dealt with separately.

1.3.2.1 Ligninase (Lignin Peroxidase)

In 1983, the discovery of an extracellular H_2O_2-requiring enzyme in the culture filtrates of P. chrysosporium that was able to carry out reactions previously only described for whole cultures, was independently reported by two research groups (Glenn et al., 1983; Tien and Kirk, 1983). This discovery was extremely significant since not only was this enzyme able to catalyse the oxidation of lignin model compounds, but more importantly, it was able to partially depolymerise ^{14}C-labelled methoxylated lignins. The enzyme was subsequently referred to as "ligninase" and was shown to have a molecular weight of c.42Kd and to be a glycosylated haemprotein containing one mole of protohaem IX per mole of enzyme (Tien and Kirk, 1983; Tien and Kirk, 1984; Kuwahara et al., 1984). The incorporation of $^{18}O_2$ into C-alpha - C-beta cleavage products of lignin dimer model compounds led to the initial conclusion that this enzyme was a unique H_2O_2-requiring oxygenase (Gold et al., 1984; Tien and Kirk, 1984). However, it has now generally accepted that the consumption of oxygen can be explained by the binding of oxygen to intermediate free radicals (Yamazaki and Yokota, 1973) or by the involvement in an oxygen shuttle in the cycling between compound III and the ferrous form of the enzyme (Smith et al., 1982), and that all the experimental evidence obtained so far is consistent with the view that ligninase is in fact a peroxidase (Harvey et al., 1985a and b; Hammel et al., 1985).

A series of spectroscopic studies have revealed that the haem group in ligninase is similar to the hexacoordinate haem in hydrated myoglobin, and that it is distinct from the pentacoordinate haem in horseradish peroxidase (Andersson et al., 1985). It has also been shown that the iron in the haem is high spin Fe III (Andersson et al., 1985; Kuila et al., 1985; Paszczynski et al., 1986), and that the fifth ligand in the pentacoordinate is histidine (Andersson et al., 1985; Kuila et al., 1985). The formation of compounds I and II has also been demonstrated spectroscopically for ligninase (Renganathan and Gold, 1986), and it was shown that the conversion of compound I (the 2-electron oxidized form) to compound II (the 1-electron oxidized form) required the equivalent of 1-electron; for example, from a 1-electron substrate such as phenol, or from a 0.5 equivalent of a 2-electron substrate such as veratryl alcohol. Thus, it has been concluded that ligninase shares a number of properties with classical peroxidases such as horseradish peroxidase. In fact, in recent studies, it has been demonstrated that the H_2O_2-oxidized states of ligninase are even more oxidizing than the analogous states of horseradish peroxidase (Hammel et al., 1987).

During the oxidation of veratryl alcohol to veratraldehyde by ligninase, it has been demonstrated that in the presence of excess veratryl alcohol, there is a stoichiometry of one mole of veratraldehyde produced to one mole of H_2O_2 consumed (Tien et al., 1986). The

results of this study indicated that H_2O_2 first reacted with the enzyme, and the oxidized enzyme then reacted with the veratryl alcohol; i.e. the oxidation of veratryl alcohol occurred via a "ping-pong" mechanism. The inability to detect substrate free radicals led to the conclusion that veratryl alcohol was oxidized by two rapid one electron oxidations without the release of free radical cations.

In Section 1.3.1, it was mentioned that the radical cations of veratryl alcohol could play a key role in the degradation of lignin *in vivo*. Experimentally, it has been demonstrated that veratryl alcohol was able to facilitate the increased oxidation of anisyl substrates, and also benzo(a)pyrene by ligninase (Harvey et al., 1986; Haemmerli et al., 1986b, respectively). Harvey et al. (1986) have proposed that this was due to the formation of radical cations of veratryl alcohol by ligninase which are able to act as diffusible 1-electron oxidants, and then react with other substrates remote from the enzyme active site. However, Kirk and Farrell (1987) argue that the inability of Tien et al. (1986) to detect substrate free radicals means that it is more likely that veratryl alcohol is either simply protecting the enzyme from inactivation by the anisyl substrates themselves, or that it is acting as an electron relay whilst at the enzyme active site, or even that it might be bringing about an alteration of the enzyme configuration.

In fact it was proposed by Schoemaker et al. (1985) that the oxidation of all susceptible compounds by ligninase was able to be explained by the formation of cation radicals, and this was backed up with the evidence that the chemical 1-electron oxidation by iron phenanthroline or ceric ammonium nitrate of veratryl alcohol or lignin model dimer compounds gave the same products as oxidation by ligninase (Harvey et al., 1985a). Similarly, Kersten et al. (1985) and Hammel et al. (1985 and 1986a), have shown that ligninase will oxidize susceptible aromatic nuclei by one electron, thus producing a cation radical which can then undergo a variety of non-enzymatic reactions.

The factor which determines whether or not a given substrate is susceptible to oxidation by ligninase, is its oxidation potential (Hammel et al., 1986b), and this in turn is influenced by the ease with which it can give up electrons; the tighter a compound holds on to its electrons, the less susceptible it is to oxidation by ligninase. C-alpha carbonyl groups are strongly electron withdrawing and hence tend to inactivate aromatic nuclei to oxidation by ligninase (Kirk and Farrell, 1987), whereas the presence and relative abundance of alkoxy groups, tend to make compounds increasingly more susceptible. Thus, syringyl lignin is more easily oxidized than guaiacyl lignin, and guaiacyl lignin is more easily oxidized than p-hydroxyphenyl lignin (see Section 1.1).

After the formation of a cation radical, a number of different reactions can follow depending on the relative position and abundance of substituent groups (Kirk et al., 1986b). These include nucleophilic attack by water or internal hydroxyl groups, or the loss of the acidic proton at the C-alpha position, or C-alpha - C-beta cleavage. These initial reactions are then followed by a variety of sequential reactions which accounts for diversity of products that can be formed during the degradation of lignin (Higuchi, 1987). These include the addition of molecular oxygen to carbon-centered radicals, one electron oxidation or reduction, and in the absence of molecular oxygen which is able to scavenge radicals, the coupling of radicals (Hammel et al., 1986a; see Kirk and Farrell, 1987).

The production and characterisation of ligninase forms the basis of this thesis and is thus discussed in detail in The Discussion of this thesis (see Section 4). However, for the sake of completeness, it should be stated that when this thesis was commenced in 1984, the production of ligninase was in nitrogen limited non-induced stationary cultures which produced relatively low activities of c.5U.l^{-1} based on the oxidation of veratryl alcohol (Tien and Kirk, 1984). Since its initial discovery, strategies for the scale up of ligninase production have been approached from a variety of different directions. For example, the addition of veratryl alcohol to cultures has facilitated the

production of yields of ligninase of up to $120U.l^{-1}$ in nitrogen limited cultures incubated in larger flasks and with culture agitation (Leisola et al., 1985a). In the same publication, it was also reported that carbon limitation led to a significant increase in the production of ligninase with yields of up to $670U.l^{-1}$ being produced. Similarly, Jager et al. (1985) reported that in the presence of detergents it was possible to scale up the production of ligninase in agitated cultures. In other reports, the use of mutant strains of P. chrysosporium has been reported. Buswell et al. (1984) isolated one strain designated INA-12, which was able to produce ligninase activities of $400U.l^{-1}$ when grown on glycerol, and Kirk et al. (1986c) used a strain designated SC-26 which was able to produce ligninase in a rotating disc fermentor. Other research groups have adopted a similar approach to that presented in this thesis. For example, Paszczynski et al. (1985) has immobilised P. chrysosporium on the roughened interior walls of a 201 carboy, and recently, Linko et al. (1986) have reported the immobilisation of P. chrysosporium in agar or agarose gels. In both of these immobilisation techniques, the same fungal biomass has been used for the production of successive harvests of ligninase.

In a number of reports on the production of ligninase, it has also been shown there are a number of proteins which exhibit ligninase activity (Leisola et al., 1985b; Leisola et al., 1987; Jager et al., 1985;

Renganathan et al., 1985; Kirk et al., 1986c; Paszczynski et al., 1986). The variation in the number of isozymes separated and a comparison of their properties are discussed further in The Discussion to this thesis; see Section 4.

1.3.2.2 Manganese Peroxidase

In 1984, Kuwahara et al. reported the isolation of an extracellular peroxidase from the ligninolytic culture fluids of P. chrysosporium. This enzyme had a molecular weight of 46Kd and catalysed the oxidation of phenol red, o-dianisidine and polymeric dyes, in the presence of H_2O_2, Mn^{II} and lactate. The presence of lactate was reported to lead to a 3-20 times stimulation in the oxidation of susceptible substrates (Glenn and Gold, 1985). Huynh and Crawford (1985) were also able to isolate a similar enzyme; however, although the enzyme they isolated had a molecular weight of 45-47Kd, and also required H_2O_2 and Mn^{II}, it did not require lactate and did not catalyse the oxidation of phenol red. Neither of these enzymes catalysed the oxidation of veratryl alcohol.

Despite these differences, subsequent purification and characterisation (Glenn and Gold, 1985; Paszczynski et al., 1985) have revealed that the two enzymes are either identical or are two isozymes; both enzymes containing one mole of high-spin ferric iron protohaem IX per one mole of enzyme, and functioning by the oxidation of Mn^{II} to Mn^{III} which then in turn is able to oxidize

the organic substrate. Paszczynski et al. (1986) have recently shown that the Mn peroxidase they isolated is a glycoprotein containing 17% carbohydrate and a high proportion of acidic amino acids.

The isolation of similar enzymes has also been reported by Leisola et al. (1987a) who were able to separate six Mn peroxidases on the basis of differences in their isoelectric points.

It is interesting to note that manganese triacetate (Mn^{III} ions) will oxidize lignin on its own without the presence of the enzyme. This is due to the greater oxidation potential of the Mn^{III} ions compared to that of the enzyme.

1.3.2.3 Phenol Oxidases

This group of enzymes includes laccase and was shown to play an important role in lignin degradation by Ander and Eriksson (1976) who demonstrated that a phenol oxidase-less mutant of _Sporotrichum pulverulentum_ (= _P. chrysosporium_; Burdsall and Eslyn, 1974; Raeder and Broda, 1984) was unable to degrade lignin, whereas the wild type strain and a revertant strain of the phenol oxidase-less mutant were both able to degrade the lignin. Laccase is produced by most white rot fungi and is a blue copper oxidase that catalyses the 1-electron oxidation of phenols to phenoxy radicals, accompanied by the transfer of four electrons to oxygen (Reinhammer 1984). The 1-electron oxidation of lignin related phenols leads to

C-alpha oxidation, limited demethoxylation and aryl - C-alpha cleavage (Kirk and Shimada, 1985). It has also been shown that some degradative reactions can occur with various phenolic lignin model compounds, especially with syringyl compounds (Kirk and Shimada, 1985; Higuchi, 1987). The most significant activity of laccase oxidation, however, is the coupling/polymerisation of lignin related phenols and isolated lignins (Lobarzewski et al., 1982; Kamaya and Higuchi, 1983).

Ander and Eriksson (1976 and 1978) have suggested that the role of phenol oxidases *in vivo* was to either detoxify low molecular weight phenols that are released during lignin degradation; to trigger lignin degradation by some unknown reaction; to regulate the production of lignin- and polysaccharide-degrading enzymes; or to act in concert with cellobiose:quinone oxidoreductase (Westermark and Eriksson, 1974) to regulate the degradation of lignin.

It is interesting to note that unlike the majority of white rot fungi, *P. chrysosporium* does not produce laccase; however, isolated ligninase will also lead to the polymerisation of phenols and isolated lignins (Haemmerli et al., 1986a). In an attempt to elucidate the mechanism whereby ligninase does not lead to the polymerisation of veratryl alcohol *in vivo*, Leisola et al., (1987b) have suggested a pathway involving oxidations by peroxidases, reductions and reactions with activated oxygen species, in which veratryl alcohol is

oxidized to radical cations by ligninase, of which c.85% are then further oxidized to veratraldehyde, and c.15% are then oxidized to quinones or lactones, and then of these products, the veratraldehyde is reduced back to veratryl alcohol by an aryl alcohol/NADP-oxidoreductase, and the quinones and lactones are then metabolized to carbon dioxide.

In contrast to this model, however, Palmer (Personal communication) has suggested that in vivo, radical cations of veratryl alcohol probably interact directly with the lignin, thus resulting in the simultaneous formation of radical cations of the lignin, and also the return of the radical cations of veratryl alcohol back to their ground state, without veratraldehyde ever being formed.

1.3.2.4 Production of H_2O_2

The origin of H_2O_2 production in ligninolytic cultures of P. chrysosporium has received particular attention in recent years. For example, Forney et al. (1982a and b) demonstrated that in the presence of added glucose, ligninolytic cultures produced H_2O_2, and that the likely origin for the production of this H_2O_2 was peroxisome-like structures located just beneath the hyphal cell walls. Glucose oxidase activity in ligninolytic mycelia (Reddy et al., 1983) was subsequently demonstrated to be glucose-1-oxidase (Kelley and Reddy, 1986a), and it was shown that mutants lacking this enzyme

were unable to degrade ^{14}-C labelled lignin to CO_2, whereas the wild type strain and a revertant strain were both ligninolytic. In addition, it was also observed that when P. chrysosporium was grown on a range of sugars, glucose oxidase activity was only found in those cultures that were ligninolytic (Kelley and Reddy, 1986b; Reddy and Kelley 1986). From these results, Kelley and Reddy concluded that glucose oxidase was the primary source for H_2O_2 production in ligninolytic cultures of P. chrysosporium.

However, other potential sources for the production of H_2O_2 have also been demonstrated. For example, Eriksson et al. (1986) have purified an intracellular glucose-2-oxidase, and Greene and Gould (1984) concluded that the production of H_2O_2 that they observed by washed starved mycelia was due to fatty acyl CoA oxidase. In addition, the Mn peroxidase described in Section 1.3.2.2, has also been shown to oxidize a range of substrates coupled to the reduction of O_2 to H_2O_2 (Paszczynski et al., 1985); and more recently, Kersten and Kirk (1987) have demonstrated the presence of an extracellular glyoxal oxidase which is able to oxidize a range of alpha-hydroxy carbonyl- and dicarbonyl compounds also coupled to the reduction of O_2 to H_2O_2. Hence from the results of a number of research groups, it seems likely that H_2O_2 can be produced from a variety of different sources.

1.3.2.5 Other enzymes

The study of partially degraded lignin by ^{13}C-nmr spectroscopy has revealed that during the degradation of lignin, there is a decrease in the number of methoxyl-, phenolic- and aliphatic hydroxyl groups, accompanied by the cleavage of aromatic nuclei with the resultant formation of aliphatic carboxyl-containing residues, and also the formation of new C-alpha carbonyl and carboxyl groups, and of alkoxyacetic acid, phenoxyacetic acid and phenoxyethanol structures (See Chen and Chang, 1985). In addition, it has also been reported that there is a significant increase in the content of aliphatic hydrocarbon structures (Haider and Trojanowski, 1975; Chua et al., 1982; Chen and Chang, 1985).

Although it is now possible to attribute many of these observations to ligninase, it also indicates that there are a number of other enzymes which play a role in the degradation of lignin which have yet to be isolated. For example, since the formation of aromatic carboxyl groups has not been observed in any of the studies with ligninase and lignin model compounds, it may mean that there is an undiscovered aromatic aldehyde oxidase; and similarly, there may also be undiscovered enzymes involved in the production of the alkoxyacetic acid, phenoxyacetic acid and phenoxyethanol structures. Kirk and Farrell (1987) suggest that the aliphatic hydrocarbons are probably derived from covalently bound lipids, and are thus not formed directly from the lignin

itself. It is not known at present whether ligninase is able to account for the decreased methoxyl content of lignin (Ander and Eriksson, 1975; Chen and Chang, 1985), nor is it known how brown rot fungi are able to demethylate methoxyl groups in lignin (Kirk and Adler, 1970).

In addition, there may also be a number of enzymes involved in the regulatory mechanism by which ligninase and phenol-oxidizing enzymes do not lead to the polymerization of lignin or phenolic products of lignin in vivo (Reid et al., 1982; Chua et al., 1983; Faix et al., 1985; see also Haemmerli et al., 1986a and Section 1.3.2.3).

1.3.2.6 Activated oxygen species

Before the discovery of ligninase, it was widely believed that the oxidative degradation of lignin was largely due to the direct action of activated species of oxygen. The latter includes hydrogen peroxide; superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$); and of these, only singlet oxygen and hydroxyl radicals are sufficiently reactive per se to be considered as potential oxidants of lignin. Subsequent investigations, however, were able to clearly discount either of these species from a direct involvement in lignin degradation (Kirk et al., 1983; Kirk et al., 1985).

1.3.3 Genetics and Molecular biology

A clear understanding of the genetics of lignin degrading microorganisms will not only provide useful information about the mechanisms by which primary and secondary metabolism are controlled, but will also facilitate strain improvement and eventually, the cloning of recombinant ligninolytic enzymes.

As in other areas of lignin degradation, *P. chrysosporium* has been the most extensively studied ligninolytic microorganism, its ability to produce abundant quantities of conidia being particularly advantageous for the production of mutant strains. When Gold and Cheng (1979) worked out the physiological conditions required for fruiting body formation, they found that fruiting was controlled by glucose and nitrogen metabolite repression, and that adenosine-3', 5'-cyclic monophosphate was able to reverse the effects of glucose repression.

In an attempt to improve selective lignin degradation, Eriksson et al. (1983) isolated mutants that were unable to produce cellulase, and then used these to degrade the lignin in wood (Johnsrud and Eriksson 1985) and synthetic lignins (McCarthy et al., 1984). However, a comparison of several strains, revealed that there was no correlation between lignin degradation and ligninase production (Kirk et al., 1986a), thus emphasizing that there are still fundamental problems that require to be resolved.

From this observation, it is also evident that it is necessary to consider all the activites involved in the degradation of lignin before using recombinant DNA technology to facilitate the scale up of the production of ligninolytic enzymes. As a first step, however, a number of laboratories have been extensively concerned with the cloning of ligninase, and it has recently been reported that one of the isozymes of ligninase has been successfully cloned (Maione et al., 1987). The recombinant ligninase exhibits the same activity with two lignin model compounds as the equivalent native isozyme, and with Kraft lignin and milled wood lignin, the recombinant ligninase exhibits substrate-dependent peroxide uptake, as well as probable C-alpha oxidation and demethoxylation as indicated by ionization difference spectroscopy.

1.4 **Aims of this thesis**

As mentioned in Section 1.3.2.1, when this thesis was commenced in 1984, the production of ligninase was only possible in relatively low quantities. This meant that it was necessary to dedicate a lot of time to producing ligninase, and also, that it was still only possible to produce it in relatively low quantities for the characterisation of its properties. Hence, the main aim of this thesis was to develop an improved method of ligninase production that would not only lead to the production of greater yields of ligninase, but that would also be less tedious and time consuming than the existing method of ligninase production. In addition, using the

"new" method of ligninase production, it was also planned to purify the isozymes of ligninase produced and then to compare them by a partial characterization of their properties.

2. MATERIALS AND METHODS

2.1 Growth and maintenance of *Phanerochaete chrysosporium* on solid media

2.1.1 Reactivation of a lyophilised stock culture of *P. chrysosporium*

A stock culture of *P. chrysosporium* strain ATCC 24725 (also referred to as *P. chrysosporium* strain BKM-F-1767) was obtained from The American Type Culture Collection, Rockville, Maryland, U.S.A. This was supplied as a lyophilised powder contained within a double vial system. The latter consist of an inner vial which holds the lyophilised fungus and a hermetically sealed outer vial which maintains sterility in the presence of a dessicant. In order to reactivate lyophilised fungi supplied in this manner, the outer vial, which is made of soft glass, is opened by first gently heating around the neck to facilitate breaking the glass. Approximately 3ml of sterile distilled water are then aseptically added to the lyophilised material and the resultant suspension is then allowed to soak for at least 30 minutes before being transferred to an appropriate agar surface.

After the resuspension of *P. chrysosporium*, a single loopful of the fungal suspension was aseptically transferred onto slopes of 3.9% Potato Dextrose agar (Difco) which were then incubated at 24°C for one week.

2.1.2 General maintenance and serial sub-culturing

After reactivation of the lyophilised stock culture of P. chrysosporium, the fungus was routinely maintained at 30°C in 300ml medical flat bottles containing 50ml of 2.0% Malt agar (Oxoid) and 2.0% Bacto agar (Oxoid). The fungus was then serially sub-cultured every 10-14 days by the aseptic transfer of a loopful of fungal growth.

2.1.3 Preparation of spore suspensions for inoculation of liquid cultures

Liquid cultures of P. chrysosporium were inoculated with spores washed from 14 day old sub-cultures of the fungus (see Section 2.1.2). The spore suspension was obtained by the addition of approximately 10ml of sterile distilled water to each medical flat bottle. The entire fungal growth was then scraped off the surface using a sterile rod, and the resultant suspension filtered through three layers of sterile muslin to remove fragments of mycelia.

To maintain a controlled inoculum size, the density of the spore suspension was determined using a haemocytometer and the spores diluted accordingly prior to inoculation. A standard inoculum size was used throughout such that the concentration of spores immediatelyafter inoculation was 2.5×10^5 spores per ml.

2.2 Ligninase production using stationary submerged cultures of *P. chrysosporium*

2.2.1 Introduction

In the early reports on the production and properties of ligninase, *P. chrysosporium* was incubated in low volume non-induced stationary submerged cultures (Tien and Kirk, 1983 and Glenn et al., 1983). In this section, methods for the induction of ligninase activity are described using either 5-hydroxy methyl furfural, a compound formed during the autoclaving of glucose, or organosolv lignin. In addition, as a first step towards using the same fungal biomass for more than one harvest of ligninase, alternative methods for the production of ligninase are described in which cultures of *P. chrysosporium* are immobilised in either agar or polyurethane foam.

2.2.2 Measurement of ligninase activity

The level of ligninase activity in the extracellular culture fluid of stationary cultures of *P. chrysosporium* was measured as described by Tien and Kirk (1984). In this assay, the conversion of veratryl alcohol to veratraldehyde is monitored spectrophotometrically at 310nm. Each assay contained 0.7ml of the extracellular culture fluid, 20mM potassium phosphate buffer pH 2.75, 0.8mM veratryl alcohol (Aldrich) and 0.21mM hydrogen peroxide in a total volume of 1.0ml, and was performed at room temperature in a Perkin Elmer PE-555 spectrophotometer. The initial rates of reaction were measured

for all assays of veratryl alcohol oxidase activity, using an extinction coefficient for veratryl alcohol of 9.3mmol^{-1}cm-1. One unit was defined as that amount of enzyme required to produce 1 μmole of veratraldehyde from veratryl alcohol per minute.

It was found that for stationary cultures, it was necessary to decant the entire culture supernatant of flasks to obtain an accurate determination of ligninase activity. Three replicates were set up for each measurement of activity.

2.2.3 Immobilisation of P. chrysosporium in agar

In Section 2.2.1, it was described that in the early reports on the production and properties of ligninase, P. chrysosporium was incubated in low volume non-induced stationary submerged cultures. Typically, 10ml cultures in 125ml Erlenmeyer flasks were incubated at 39°C without agitation and were purged with 100% oxygen at a flow rate of 0.5l per minute for 5 minutes every 3-4 days. Inoculum preparation and medium composition are described in section 2.1.3 and Appendix I, respectively.

The immobilisation of P. chrysosporium in agar is a simple adaptation of the standard stationary liquid culture technique in which 2.0% purified agar (Oxoid) is added to the liquid medium prior to autoclaving at 121°C for 15 minutes. When the medium cools to approximately 50-55°C, it is inoculated with the standard spore inoculum (See Section 2.1.3) and 10ml aliquots are then dispensed

into sterile 125ml Erlenmeyer flasks. After 24 hours incubation at 39°C, 10ml of sterile distilled water are aseptically added to the flasks and they are then treated the same as for the standard stationary liquid cultures.

2.2.4 Comparison of ligninase activity obtained when using an autoclaved or filter-sterilised stock solution of glucose

The medium composition used for the production of ligninase activity in stationary cultures is outlined in Appendix I. Routinely, five times concentrated stock solutions of the medium components were autoclaved at 121°C for 15 minutes and stored at 4°C until required. To prepare medium for a ligninase production experiment, aliquots of the stock solutions were mixed in the appropriate proportions and 10ml aliquots were then dispensed into 125ml Erlenmeyer flasks and autoclaved at 121°C for 15 minutes.

When comparing the results of a number of experiments, it was observed that apparently more ligninase activity was produced when using autoclaved glucose rather than filter-sterilised glucose. This observation was further investigated by setting up two batches of medium. One batch of medium was prepared as above; autoclaved stock solutions of all the medium components were mixed together and re-autoclaved after being dispensed into the Erlenmeyer flasks. The other batch of medium was prepared in a similar manner except that filter-sterilised glucose was added to the rest of

the medium components after they had been appropriately mixed and autoclaved, and then allowed to cool.

This experiment was carried out using the standard liquid culture technique and also using the immobilisation in agar method outlined in Section 2.2.3.

The conversion of a proportion of glucose molecules to 5-hydroxy methyl furfural as a result of heating, especially in acidic conditions, was first demonstrated by Haworth and Jones (1944). Hence, in an attempt to mimic the effects of autoclaving, it was then decided to examine whether the addition of a range of concentrations of 5-hydroxy methyl furfural to filter-sterilised glucose agar-immobilised cultures of P. chrysosporium, would lead to the induction of increasing titres of ligninase activity. The final concentration range investigated was 2.5-10.0μM.

2.2.5 Induction of ligninase activity using organosolv lignin

The ability of organosolv lignin to induce ligninase activity was investigated using stationary liquid cultures and agar-immobilised cultures of P. chrysosporium. For both methods of culture, the lignin was added before autoclaving at a final concentration of 0.2% w/v.

To determinine the effect of removing particulate material from the lignin preparation, in one stationary liquid culture experiment, a ten times concentrated stock

solution of the lignin was made up in distilled water and then filtered through Whatman Number 1 filter paper before being appropriately diluted in the medium.

2.2.6 Determination of the glucose concentration in the extracellular medium using the glucose oxidase/ peroxidase method

The basis of this assay is a two step enzymatic conversion in which the concentration of glucose is determined by the production of hydrogen peroxide in the first reaction, which then leads to the stoichiometric production of a chromophore (a quinoneimene dye) in the second reaction (see below).

Glucose oxidase:

$$2 \text{ Glucose} + 2\ O_2 + H_2O \longrightarrow 2 \text{ Gluconic Acid} + 2\ H_2O_2$$

Peroxidase:

$$2\ H_2O_2 + \text{4-Aminophenazone} + \text{Phenol} \longrightarrow \text{Quinoneimene Dye} + 4\ H_2O$$

Method

Per assay:

3.9 ml 4-Aminophenazone (33mg/100ml 0.1M Potassium Buffer pH7.0)

3.9 ml Phenol (100mg/100ml 0.1M Potassium Buffer pH7.0)

1.15ml 0.1M Potassium Buffer pH7.0

20.0µl Peroxidase (Grade II, lyophilised = 100U/mg)

20.0µl Glucose oxidase (Grade I, lyophilised = 200U/mg)

10.0µl Test Sample

Total Volume = 9.0ml

After mixing thoroughly, assay mixtures were incubated at 37°C for 30 minutes. Samples were read against a reagent blank at 505nm in a Perkin Elmer PE-555 spectrophotometer.

Using a standard of D-glucose (BDH), a calibration curve was obtained over the range 0.33-2.00% (w/v) for each set of assays, with samples being first routinely boiled for 2 minutes to inactivate any interfering enzyme activity.

2.2.7 Determination of the carbohydrate concentration in the extracellular medium using the phenol/sulphuric acid method

The phenol/sulphuric acid method of carbohydrate determination was developed by Dubois et al. (1956) and is a useful assay for "total carbohydrate" content. It is a simple colourimetric assay in which heating with concentrated sulphuric acid leads to the hydrolysis of polysaccharides to form monosaccharides. The latter are then dehydrated to form furfural derivatives (from pentoses) or hydroxy methyl furfural derivatives (from hexoses), which then react with the phenol reagent to form a stable coloured compound which can be measured spectrophotometrically.

The advantage of this assay for "total carbohydrate" content over alternative methods is that the phenol reagent gives approximately the same degree of reaction with all sugars.

Method

Using glass test tubes, 30µl of sample containing the equivalent of up to 70µg of glucose was mixed with 420µl of a 5% phenol solution in distilled water. 3.0ml of concentrated sulphuric acid was then squirted in directly on to the surface of the latter before being rapidly mixed using a whirlimixer.

The tubes were then allowed to stand for 10 minutes at room temperature before being read at 488nm (hexoses) or 480nm (pentoses) against an appropriate blank containing water instead of sugar.

For each determination of carbohydrate content using this method, a calibration curve was set up using glucose over the range 0-70µg per 30µl aliquot.

It was found that the reproducibility of the phenol/sulphuric acid assay was dependent upon the fast and thorough mixing of the reagents at each step of the assay.

2.2.8 Immobilisation of *P. chrysosporium* in a range of polyurethane foams

In Section 2.2.3, an adaptation of the standard stationary liquid culture conditions was described in which *P. chrysosporium* was immobilised in a medium containing purified agar. It will be shown in the Results section of this thesis, however, that agar was not an ideal immobilisation support.

As an alternative support matrix, a range of polyurethane foams were investigated. Five polyurethane foams were obtained as a gift from Professor D.O.Hall (Kings College, University of London) and discs of these were cut so that they would occupy the same volume as 10ml medium in a 125ml Erlenmeyer flask. The effect of including these in otherwise standard stationary liquid cultures of P. chrysosporium was then investigated; ligninase activity being measured as described in Section 2.2.2. Before using the discs of foam, however, they were first washed three times in distilled water and then autoclaved in distilled water to ensure that any contaminating compounds that might interfere with the production of ligninase activity would be leached.

The polyurethane foams investigated were all originally obtained from Caligen Foam Ltd., Accrington, United Kingdom and had the designated codes 3300A, 4200A, H4200, 74165 AO and PR 22/60.

2.3 Ligninase production using agitated cultures of P.chrysosporium

2.3.1 Introduction

In Section 2.2.1, it was reported that ligninase was first isolated from low volume non-induced stationary submerged cultures of P. chrysosporium. A significant step towards the scale up of the production of ligninase was made when it was observed that increased levels of ligninase activity were produced in cultures induced with

lignins or low molecular weight aromatic compounds (Faison and Kirk, 1985; Leisola et al., 1985a; see also Sections 2.2.4 and 2.2.5). In addition, using cultures of P. chrysosporium induced with veratryl alcohol, it was also shown that it was possible to scale up the production of ligninase further by using agitated cultures in which both flask size and culture volume had been increased (Leisola et al., 1985a).

In this section, it is described how the immobilisation of P. chrysosporium in polyurethane foam first mentioned in section 2.2.8, was adapted to increased volumes of medium in agitated culture conditions. Using foam-immobilised cultures of P.chrysosporium, culture conditions are described which facilitate the production of successive harvests of ligninase from the same fungal biomass, and also, for the storage and reactivation of mature pellets of P.chrysosporium. These procedures mean that the tedious and skilled procedure of generating fresh fungal biomass for each fresh harvest of ligninase is effectively avoided.

2.3.2 Measurement of ligninase activity

Ligninase activity was measured essentially as described in Section 2.2.2; except that since the level of ligninase activity was generally significantly higher after the induction of ligninase activity by veratryl alcohol, it was only necessary to use 0.1ml of the extracellular culture fluid in the standard veratryl alcohol oxidase assay.

Also, since the production of ligninase had been scaled up both in terms of culture volume and flask size (see Section 2.3.3), it was no longer necessary to decant the entire contents of individual flasks for each assay of ligninase activity. A standard procedure was adopted of aseptically removing 1.0ml aliquots of the extra-cellular culture fluid from at least three flasks for each measurement of ligninase activity and, consequently, flasks were able to be used for several assays over the course of an experiment without a significant reduction in the volume of the culture.

2.3.3 Immobilisation of P. chrysosporium in polyurethane foam

In Section 2.2.8, it was described how polyurethane foam was used as an immobilisation support for stationary cultures of P. chrysosporium. In this section, it is described how polyurethane foam was used to immobilise P. chrysosporium using agitated cultures.

The control method of ligninase production, in which ligninase activity was produced in agitated batch cultures of P.chrysosporium in the absence of any immobilisation support, was essentially as described in Haemmerli et al. (1986b). 1l Erlenmeyer flasks containing 600ml of medium were inoculated with a spore suspension as described in Section 2.1.3. The cultures were then incubated at 39°C and 150 r.p.m./2.5cm radius under an air atmosphere for 48 hours. After this period, the fungal biomass within each flask was concentrated

three times by aseptically decanting 400ml of the extracellular medium and filter-sterilised veratryl alcohol was then added to give a final concentration of added veratryl alcohol in the flasks of 1.0mM. After purging the cultures with 100% oxygen for 10 minutes at a flow rate of 1l/minute, they were then re-incubated at 39°C but with a reduced agitation rate of 60 r.p.m./2.5cm radius. Medium composition was as described in Appendix I.

The immobilisation of P. chrysosporium in polyurethane foam in agitated cultures was achieved by cutting the foam into 1cm cubes and then adding forty five of these to 1l Erlenmeyer flasks. The flasks were then treated in precisely the same manner as for the control flasks which contained no cubes of foam. Before using the cubes of foam, they were first washed and autoclaved in distilled water as described in Section 2.2.8. The polyurethane foam used for this work was that designated Code 3300A.

2.3.4 Optimisation of conditions for the semi-continuous production of ligninase using foam-immobilised P. chrysosporium

To determine the optimum conditions for the continued successive production of harvests of ligninase using foam-immobilised cultures of P. chrysosporium, an experiment was carried out in which the extracellular medium from four sets of flasks was aseptically decanted when the cultures reached peak ligninase activity in the

initial batch culture, and was then replaced with 200ml of either undiluted, two-fold diluted, or five-fold diluted sterile batch culture medium, or, lastly, buffer alone. For each set of flasks, the buffer concentration was maintained as described in Appendix I.

At the same time as replacing the extracellular medium, the cultures were also induced with veratryl alcohol (final concentration of 1.0mM) and purged with 100% oxygen as described in Section 2.3.3., before being re-incubated at 39°C with an agitation rate of 60 r.p.m./ 2.5cm radius. The level of ligninase activity in the cultures was then monitored over the following 8 day period.

The results of this experiment showed which dilution of the batch culture medium was likely to be suitable as a standard replacement medium and the effect of using this dilution of the medium to produce continued successive harvests of ligninase every 24 to 48 hours, whilst maintaining the cultures at 39°C and 60 r.p.m./ 2.5 cm radius, and also inducing with 1.0mM veratryl alcohol and purging with oxygen at each replacement of the medium, was then investigated.

Having found the most suitable dilution of the batch culture medium to facilitate the continued production of successsive harvests of ligninase (see Section 3.2.3), it was then decided to determine whether it was necessary to maintain an agitation rate of 60 r.p.m./2.5 cm

radius for the continued production of successive harvests of ligninase. This was carried out using cultures that had been stored and then reactivated as described in Section 2.3.5 (see below); except with one set of cultures incubated at an agitation rate of 60 r.p.m./2.5 cm, and the other at 150 r.p.m./2.5cm radius, all other culture conditions remaining unaltered. Three replicates were set up for each agitation rate.

Similarly, to determine whether veratryl alcohol was necessary for the continued production of successive harvests of ligninase, veratryl alcohol was omitted from the replacement medium from one set of foam-immobilised cultures, and the resultant effect upon the production of fresh successive harvests was then examined; all other culture conditions were as described in Section 3.2.3.

In addition, the effect of first washing the cubes of foam-immobilised P. chrysosporium in sterile distilled water, and then omitting the veratryl alcohol from fresh replacement medium, was also investigated. The cubes of foam-immobilised P.chrysosporium were washed by agitating the cultures for two successive periods of 5 minutes at 200 r.p.m./2.5 cm radius.

Finally, using the culture conditions optimised by the above set of experiments, it was then decided to determine whether increasing the biomass of the fungus within individual flasks would lead to even higher titres of ligninase. This was investigated using two different

methods. The first method investigated was to increase the surface area of the foam whilst maintaining the same total volume of the foam for each individual flask (see Section 3.2.2). This was done by taking forty five 1cm cubes of foam, and then cutting each of these into eight smaller cubes before setting up the foam-immobilised cultures (see Section 2.3.3).

The second method investigated was to aseptically transfer forty five 1cm cubes of foam-immobilised *P. chrysosporium* from one set of flasks to a second set of flasks which already contained forty five 1cm cubes of foam-immobilised *P.chrysosporium*. In all other respects, the culture conditions were as described in Section 3.2.3.

2.3.5 Storage and reactivation of foam-immobilised cultures of P. chrysosporium

To determine whether it was possible to store foam-immobilised cultures of *P. chrysosporium*, the extracellular medium from a number of foam-immobilised cultures was aseptically decanted, and the flasks were then stored at 4°C for a period of two months.

After this period, fivefold diluted sterile batch culture medium containing 1.0mM veratryl alcohol was aseptically added to the flasks, which were then re-incubated at 39°C and 60 r.p.m./2.5cm radius (see also Section 2.3.4). Ligninase activity was measured as described in Section 2.2.2.

2.3.6 Electron Microscopy of foam-immobilised *P. chrysosporium*

To determine the pattern of growth of *P. chrysosporium* in the cubes of polyurethane foam, a number of cubes containing pellets of the fungus were examined by scanning electron microscopy using samples which had been prepared by critical point drying (Anderson 1951).

2.4 Purification of the isozymes of ligninase

2.4.1 Concentration of the extracellular proteins of *P. chrysosporium*

The standard method used to concentrate the extracellular proteins of *P. chrysosporium* was by a combination of ultrafiltration and lyophilisation with resuspension in a smaller volume of solution. Towards the conclusion of this work, however, a third method of concentration was introduced as an additional step and this was acetone precipitation.

Pooled extracellular medium from a number of cultures set up in the same way, was first concentrated by passing it through an Amicon hollow-fibre concentrator at 4°C. Using a cartridge with a 10 Kd exclusion size, pooled extracellular medium was concentrated approximately ten times by this method before being dialysed overnight at 4°C against distilled water.

The second stage of concentration was by lyophilisation with resuspension in a smaller volume of solution. The concentrated material obtained after

ultrafiltration was lyophilised overnight using a Edwards Modulyo freeze-drier and the residue resulting from this procedure was then resuspended in a minimum volume of distilled water before being stored at -20°C.

The third method of concentration introduced towards the conclusion of this work was acetone precipitation; an additional feature of this method of concentration being the removal of unwanted carbohydrate still present in the preparation. This was carried out as described by Kuwahara et al. (1984).

Acetone was first added to 25% (v/v) to precipitate out carbohydrate, and then to 66% (v/v) to precipitate out protein. The carbohydrate was removed by winding the strands formed, if any, around a glass rod, and after the addition of the required amount of acetone, the resultant suspension was then centrifuged at 15000g for 20 minutes to spin down the precipitated protein. The latter was then resuspended in a minimum volume of distilled water and was dialysed overnight against distilled water at 4°C to remove all traces of acetone. After dialysis, protein solutions were stored at -20°C until required for further experimentation.

2.4.2 Anion-exchange chromatography of the isozymes of ligninase using tris-acryl DEAE sepharose

The initial separation of the isozymes of ligninase was by the adsorption of concentrated ligninase to a tris-acryl DEAE sepharose column and then eluting with an

increasing concentration of sodium chloride in 20mM tris-succinate buffer pH 5.0. The preparation of ligninase used for this separation was obtained from concentrating the first five harvests from semi-continuous cultures of foam-immobilised P. chrysosporium (see Section 3.2.3) by ultrafitration, lyophilisation/resuspension in a smaller volume of solution and then acetone precipitation (see Section 2.4.1). The five harvests were treated individually for the initial concentration and were then pooled for the acetone precipitation step.

Using a 10mm x 80mm column with a bed volume of 6.3ml, 10ml of concentrated ligninase (c.300 Units) was loaded onto the column; the remainder of this enzyme preparation was stored at -20°C for additional experimentation. The column was then washed with 0.01M sodium chloride in 0.01M tris-succinate pH 5.0 until no further material with an absorbance at 280nm or any veratryl alcohol oxidase activity (see Section 2.2.2) could be eluted. A step of 25ml of 0.05M sodium chloride in 0.01M tris-succinate pH 5.0 was then applied, followed by a linear gradient from 0.05M to 0.3M sodium chloride in 0.01M tris-succinate pH 5.0 over a two hour period. The separation was then completed by three successive 25ml steps of 0.3M, 0.5M and, finally, 1.0M sodium chloride, all in 0.01M tris-succinate pH 5.0.

The flow rate was 62.0 ml per hour and 2.0 ml fractions collected throughout. The eluate off the column was continuously monitored at 280nm using a LKB UV

flow cell, and every third fraction to be collected was then assayed for veratryl alcohol oxidase activity under a variety of different conditions as described in Section 2.6.2.

2.4.3 Anion-exchange chromatography of the isozymes of ligninase using Mono-Q FPLC

In Section 2.4.2, it was described how the isozymes of ligninase were initially separated using tris-acryl DEAE anion-exchange chromatography. In Section 3.3.1, it will be revealed how this resulted in only a partial purification of the different isozymes present. Consequently, in order to purify the isozymes of ligninase further, a second method of chromatography was performed on each of the individual "partially pure" isozymes already separated. The method chosen was Mono-Q FPLC anion-exchange chromatography and this was performed as described in Kirk et al. (1986c).

First, the appropriate fractions containing the partially pure isozymes of ligninase resulting from the initial purification as outlined in Section 2.4.2, were pooled and then dialysed against distilled water. Each was then lyophilised overnight before being resuspended in 1.25ml of 10.0mM sodium acetate buffer pH 6.0. All samples were filtered through 0.45μm filters (Millipore) before being loaded onto the column.

The FPLC apparatus used consisted of a Liquid Chromatography Controller (Pharmacia - LCC 500), 2 Pumps

(Pharmacia - P-500), Single Path Monitor (Pharmacia - UV-1; Control Unit and Optical Unit), Fraction Collector (Pharmacia - Frac-100), and column (Pharmacia - HR 5/5 Mono Q; Bed size 5 x 50mm). The mobile phase consisted of a linear gradient from 10.0mM to 0.5M sodium acetate pH 6.0, and was applied over a 40 minute period at a flow rate of 2.0 ml per minute with the eluate being constantly monitored at 280nm. Fractions were collected only when the absorbace at 280nm exceeded a given minimum threshold with the maximum volume collected per tube being 2.0ml.

2.5 Characterisation of the properties of concentrated ligninase before anion-exchange chromatography

2.5.1 SDS-polyacylamide gel electrophoresis of the extracellular proteins from successive harvests of foam-immobilised P. chrysosporium

To determine whether the same range of extra-cellular proteins were produced at each successive harvest using the foam-immobilised P. chrysosporium, the extracellular medium from five successive harvests was concentrated and then compared by SDS-polyacrylamide gel electrophoresis. Culture conditions were as described in Section 3.2.3 and the concentration of the extracellular proteins was as described in Section 2.4.1.

Samples were prepared by boiling 2 volumes of protein with 1 volume of an SDS/2-mercaptoethanol mixture for 3 minutes such that 50ul of the final mixture contained c.4.75 x 10^{-2} units of ligninase. The proteins were then separated using a LKB discontinuous electrolyte

system (Laemmli, 1970) employing a 3.7% acrylamide stacking gel and a 10.0% acrylamide resolving gel.

Each gel was run for approximately 1 hour at a constant current of 25.0 mA using a running buffer of 0.025M tris/0.192M glycine pH 6.8 containing c.0.1% bromophenol blue to visualise the running front of the gel. When the latter had reached the interface with the resolving gel, the gel was then run at a constant current of 30.0 mA for a further 3 hours or so until the running front reached about 0.7cm from the bottom of the gel (N.B. Dimensions of gels were: Stacking gel - c.3.5cm long; Resolving gel - c.11.0cm long; Width - 20.0cm; Thickness - 0.25cm).

A range of markers of known molecular weight were run simultaneously (see Appendix II) so that the molecular weight of the unknown proteins was able to be determined; the proteins being visualised after overnight staining in 0.03% Coomassie Blue (Brilliant Blue R).

2.5.2 Spectral properties

The UV/visible absorption spectra of concentrated preparations of ligninase were routinely performed between 250nm and 650nm using a Perkin Elmer PE-555 spectrophotometer. Samples were diluted, if necessary, such that when they were placed in quartz cuvettes with a 1cm light path, the maximum absorbance did not exceed 1.5 Absorbance Units.

2.5.3 Determination of pH optima

The pH optimum of concentrated preparations of ligninase were determined by measuring the initial rates of veratryl alcohol oxidase activity between pH 2.0 and pH 4.4. Veratryl alcohol oxidase assays were carried out as described in Section 2.2.2 using phosphate buffer over the entire pH range.

2.5.4 Effect of metal ions on ligninase activity

The effect of metal ions upon ligninase activity was investigated using concentrated ligninase preparations produced from agitated cultures of *P. chrysosporium*. Ligninase activity was measured throughout as described in Section 2.2.2.

Initially, the effect of a range of metal ions was investigated using a concentrated ligninase preparation which had been produced by pooling five successive concentrated harvests from a foam-immobilised semi-continuous culture of *P. chrysosporium* (see Section 2.4.2). In this initial investigation, the concentration of the metal ions was 1.0mM. In additional experiments using the same preparation of ligninase, the effect of concentration of one of the metal ions upon ligninase activity was also investigated. From the latter results, a standard concentration of one metal ion was chosen for all future experiments involving the effect of metal ions upon ligninase activity.

As a comparison, the effect of metal ions was then investigated upon concentrated ligninase prepared from agitated cultures of P. chrysosporium which contained no cubes of foam, and also, using concentrated ligninase preparations from each of the first five successive harvests using foam-immobilised semi-continuous cultures of P. chrysosporium.

In addition, to determine whether the effect of metal ions upon ligninase activity was pH-dependent, a pH optimum determination experiment was carried out as described in Section 2.5.3 using the pooled concentrated ligninase preparation referred to above, in the presence and absence of 1.0mM Mg^{2+}.

2.5.5 Determination of the K_m for H_2O_2 in the presence and absence of Mg^{2+}

The K_m for H_2O_2 was determined by first plotting the effect of concentration of H_2O_2 against the rate of veratryl alcohol oxidase activity and then performing a Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934) to facilitate the calculation of the K_m value.

This experiment was carried out using pooled concentrated ligninase produced from the semi-continuous culture of P. chrysosporium (see Section 2.4.2) and, other than the variation in H_2O_2 concentration, ligninase activity was measured as described in Section 2.2.2.

To determine whether metal ions had any effect upon the K_m for H_2O_2, the experiment outlined above was carried out in the presence and absence of 1.0mM Mg^{2+}.

2.5.6 Determination of the K_m for veratryl alcohol in the presence and absence of Mg^{2+}

The K_m for veratryl alcohol was determined by first plotting the effect of a range of different concentrations of veratryl alcohol upon the rate of veratryl alcohol oxidase activity and then performing a Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934) to facilitate the calculation of the K_m value.

This experiment was carried out using pooled concentrated ligninase produced from the semi-continuous culture of P. chrysosporium (see Section 2.4.2); and other than the variation in veratryl alcohol concentration, ligninase activity was measured as described in Section 2.2.2.

To determine whether metal ions had any effect upon the K_m for veratryl alcohol, the experiment outlined above was carried out in the presence and absence of 1.0mM Mg^{2+}.

2.5.7 Determination of the protein concentration using the Bradford method of protein estimation

The concentration of protein in the concentrated ligninase preparations referred to in this section were determined using the method of Bradford (1976). Bovine Serum Albumin (Fraction V) was used as the standard.

2.5.8 TCA precipitation of concentrated ligninase preparations in order to determine the % carbohydrate present relative to protein in the proteins present in the ligninase preparation

It was found that to determine the real % carbohydrate present relative to protein in the proteins present in the ligninase preparation, it was necessary to separate the proteins from contaminating carbohydrate also present in the concentrated ligninase preparations. This was achieved by precipitating the proteins out of solution using a 10.0% TCA solution pre-heated to c.70.0°C. Hot TCA was added to concentrated ligninase preparations until no further material was able to be precipitated out of solution. Precipitated material was then removed by centrifugation and resuspended in a minimum volume of distilled water; the concentration of carbohydrate and protein in the resuspended material was then determined as described in Sections 2.2.7 and 2.5.7, respectively.

2.6 Characterisation of the properties of the isozymes of ligninase after purification by anion-exchange chromatography

2.6.1 Determination of pH optima

The determination of the pH optima of the isozymes of ligninase was carried out as described in Section 2.5.3. To confirm the accuracy and reproducibility of these results, five veratryl alcohol oxidase assays were performed for each pH value over a critical region of the pH curve of one isozyme, and also, the pH optimum of one of the isozymes of ligninase was determined not only

using 20.0mM phosphate buffer, but also using 20.0mM glycine/HCl buffer over the same pH range.

2.6.2 Effect of metal ions on ligninase activity at pH 2.5 and pH 3.0

The effect of metal ions on ligninase activity was examined directly on the isozymes of ligninase as they were separated by tris-acryl DEAE sepharose chromatography (see Section 2.4.2). During the purification procedure, every third fraction collected was assayed essentially as described in Section 2.2.2, except that 20.0mM phosphate buffer was used at pH 2.5 and pH 3.0 and all assays were carried out both in the presence and absence of 1.0mM Mg^{2+}. The final pH of the assay itself was confirmed using pH indicator paper.

2.6.3 Assessment of purity and determination of the molecular weights of the isozymes of ligninase

The assessment of the purity of the isozymes of ligninase after two series of anion-exchange chromatography, and also the determination of the molecular weight of each of the isozymes, were simultaneously resolved by SDS-polyacrylamide gel electrophoresis. This was carried out as described in Section 2.5.1.

2.6.4 Determination of the isoelectric points of the isozymes of ligninase

To determine the isoelectric points of the isozymes of ligninase, flat-bed isoelectric focusing was carried out as described by Winter et al. (1977). The

isozymes were focused on a pH gradient from pH 3.5 to pH 9.5, using LKB 1804-101 PAG plates on an LKB isoelectric focusing apparatus. After focusing for 90 minutes at 10°C with a constant power being applied of 30.0W, the actual pH gradient was measured using a Pye surface pH electrode, and the protein bands were then visualised by staining with Coomassie Blue (Brilliant Blue R).

2.6.5 Amino acid analysis of an isozyme of ligninase

The amino acid analysis of the most abundant isozyme of ligninase, Isozyme I (See Section 3.3), was determined using a Chromaspek amino acid analyser (Rank-Hilger Ltd., Margate, Kent). The sample to be analysed was injected onto a column packed with 8% cross-linked sulphonated polystyrene resin beads. The amino acids were then separated by eluting with a pH gradient formed using lithium buffers. On elution from the column, the amino acids were reacted with a mixture of 2-mercaptoethanol and ortho phthalyldialdehyde in borate buffer at pH 10.0, to form iso-endol amino acid derivatives. The latter are lightly fluorescent compounds which are detected by the instrument's built-in fluorimeter.

The instrument was calibrated by injecting a mixture containing 50.0nM of each of 24 amino acids. Sample amino acid determinations were made by comparing peak areas obtained from the sample and the calibration mixture using an automatic peak integrator. All samples contained Nor-leucine as the internal standard.

2.7 Isolation and a partial characterisation of the properties of an inhibitor of ligninase

2.7.1 Introduction

In The Results section of this thesis, it will be shown that the last fraction to be eluted from the tris-acryl DEAE sepharose anion-exchange column contained an inhibitor of ligninase activity. In this section, the methods used to investigate the properties of this inhibitor are described.

2.7.2 Demonstration of the inhibition of ligninase

The inhibition of ligninase by the last fraction eluted from the tris-acryl DEAE sepharose anion-exchange column was demonstrated by assaying one of the isozymes of ligninase in the presence of a range of concentrations of the test material. Ligninase activity was measured as described in Section 2.2.2.

2.7.3 Effect of pH on the inhibition of ligninase activity in the presence and absence of 1.0mM Mg^{2+}

To determine whether the inhibition of ligninase was pH-dependent, and also to determine whether the presence of Mg^{2+} ions had any effect on the inhibition of ligninase, purified ligninase (see Section 2.4) was assayed as described in Section 2.5.3, except in the presence of the minimum inhibitory concentration of the inhibitor as determined by the method described in Section 2.7.2, and also in the presence and absence of 1.0mM Mg^{2+}.

2.7.4 Determination of the presence of uronic acids

To determine whether it was possible to detect uronic acids in the inhibitor material, a specific assay for the determination of uronic acids was carried out as described by Scott (1979). In this assay, uronic acids are hydrolysed with concentrated sulphuric acid to produce 5-formyl-2-furancarboxylic acid. The latter then reacts with a colourimetric reagent, 3,5-dimethylphenol, to produce a characteristic chromophore with a maximum absorbance at 450nm.

Using this assay, the difference in the absorbance readings between 450nm and 400nm was measured in the presence and absence of borate ions. An increase in the difference in the absorbance in the presence of borate ions, means that the uronic acids detected have unsubstituted threo hydroxyl groups at the C-3 and C-4 positions (Scott 1979).

2.7.5 HPLC analysis of the inhibitor containing material

To gain information on the number of components present in the inhibitor fraction, and also to assess whether it would be possible to rapidly purify different components present in sufficient quantities for a more specific identification, an aliquot of the material was analysed using an isocratic HPLC system.

The HPLC apparatus used consisted of a Varian 2050 variable lambda detector; a Varian 2010 pump; a Varian 4270 integrator and a C18 Micro Pak MCH-10 4 x 300mm column and the system was used with a mobile phase of 25%

acetonitrile in distilled water applied at a constant flow rate of 1.0 ml per minute. The inhibitor material was first filtered through a Millipore 45µm filter before being loaded onto the column and the components separated were then monitored at a wavelength of 230nm.

2.7.6 Effect of heparan sulphate on the inhibition of ligninase activity

It will be shown in The Results section of this thesis, that it was indeed possible to show that uronic acid(s) were present in the inhibitor fraction (see Section 3.5.5). To determine whether a typical example of a polymeric uronic acid was able to similarly inhibit ligninase, the veratryl alcohol oxidase activity of a purified isozyme of ligninase was determined in the presence of a range of concentrations of heparan sulphate. The veratryl alcohol oxidase activity was determined essentially as described in Section 2.2.2, except that all the assays were carried out in the presence of 20mM phosphate buffer pH 2.5, and also, 100µl of a series of tenfold dilutions of a 5.0mg.ml^{-1} heparan sulphate was added, the final volume of the assay mixtures being 1.0ml. The stock solution of heparan sulphate was made up in distilled water, and similarly, distilled water was also used to carry out all dilutions of this stock solution.

In addition, to determine whether any inhibition of ligninase caused by the presence of heparan sulphate was pH-dependent, ligninase activity was also measured in the presence of a single concentration of heparan sulphate, at pH 3.0 (using 20mM phosphate buffer pH 3.0); and finally,

to determine whether any inhibition of ligninase activity caused by the heparan sulphate was affected by the presence of Mg^{2+} ions, ligninase activity was also measured in the presence of a single concentration of heparan sulphate, at both pH 2.5 and pH 3.0, and in the presence of 1.0mM Mg^{2+}.

2.7.7 Effect of low concentrations of the inhibitor on the K_m for veratryl alcohol

In an attempt to determine the nature of the inhibition of ligninase caused by the inhibitor fraction eluted from the tris-acryl DEAE sepharose column, the K_m for veratryl alcohol was determined in the presence of two different concentrations of the inhibitor material at which ligninase activity was partially inhibited, but by different extents, when assayed essentially as described in Section 2.2.2, except in the presence of 20mM phosphate buffer pH 2.5. The K_m for veratryl alcohol was also determined in the absence of inhibitor.

These experiments were carried out using purified ligninase (see Section 2.4) by the method outlined in Section 2.5.6. The two concentrations of inhibitor used were 5µl and 10µl of 100 times diluted material (relative to the concentration of the material first isolated off the tris-acryl DEAE sepharose column; see Section 3.3.1) in a total assay volume of 1.0ml.

3. RESULTS

3.1 Production of ligninase using stationary submerged cultures of *P.chrysosporium*

3.1.1 Production of ligninase in the presence and absence of agar as an immobilisation support

The immobilisation of *P. chrysosporium* in agar was investigated primarily as a first experiment to determine whether it would be possible to use the same fungal biomass to produce more than one harvest of ligninase. However, having immobilised the fungus in the agar, the initial objectives were first to determine whether the agar-immobilised cultures were still able to produce ligninase, and secondly to determine whether the agar was a stable immobilisation support.

To answer the first question, two sets of flasks were set up as described in Section 2.2.3. One set of flasks contained "ordinary" liquid cultures of *P. chrysosporium* (Tien and Kirk, 1983); and the other set contained cultures immobilised in agar. Ligninase activity in the extracellular culture fluids was then measured every 24 hours as described in Section 2.2.2. The results are presented in Fig.4.

For both cultures, no ligninase activity was detected during the first two to three days after inoculation. After this period, ligninase showed an almost symmetrical rise and fall in activity with the

Fig. 4 Production of veratryl alcohol oxidase activity by stationary cultures of *P.chrysosporium* incubated with and without agar as an immobilisation support

Culture conditions were as described in Section 2.2.3, with veratryl alcohol oxidase activity being measured as described in Section 2.2.2. Each point represents the mean of three replicates; S.D.= ±10%.

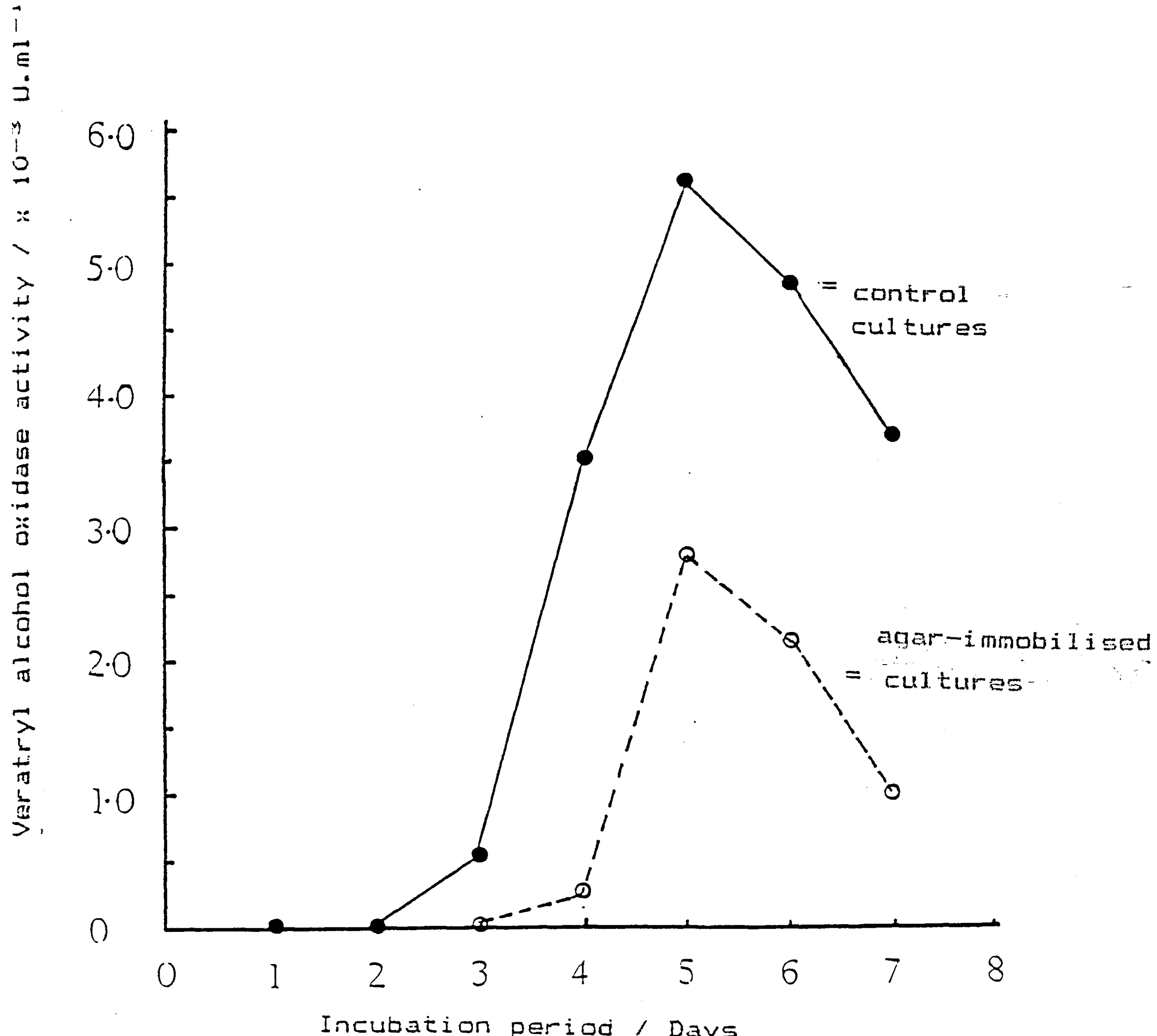


Fig. 4 Production of veratryl alcohol oxidase activity by stationary cultures of *P.chrysosporium* incubated with and without agar as an immobilisation support

maximum activity being detected five days after inoculation. It should be pointed out that the liquid cultures of P. chrysosporium produced approximately twice the level of ligninase activity compared to the agar-immobilised cultures; a difference which may reflect the dilution effect of the distilled water top layer used in the agar-immobilisation technique.

Having established that agar-immobilised cultures of P. chrysosporium were indeed able to produce ligninase activity, albeit at a lower level than the liquid culture control flasks under the culture conditions used, the next question to answer was to determine whether the agar was a stable immobilisation support. Since agar is composed predominantly of chains of galactose sugars (Davis, 1980), it was decided that at least one method of assessing the stability of the agar would be to compare the level of the free carbohydrate in the extracellular medium produced by the two methods of culture. This was measured every 24 hours as described in Section 2.2.7.

As a control, carbohydrate levels were monitored in uninoculated flasks incubated in the same way to ensure that the agar was not leaching sugars which would be detected by the carbohydrate assay. In addition, since the carbohydrate assay is non-specific (Dubois et al., 1956), and thus also responds to the glucose provided in the medium (Appendix I), a specific assay was carried out to monitor the consumption of glucose throughout the course of the experiment (see Section

2.2.6). For comparison, the concentration of extracellular carbohydrate and glucose in standard liquid cultures was also determined.

The results of both of sets of these assays for liquid and agar-immobilised cultures are presented in Fig.5. Calibration curves for the Phenol/Sulphuric Acid method for the determination of carbohydrate concentration, and for the Glucose Oxidase/Peroxidase method were linear over the range of 0-70µg, and 0-1%, respectively.

It can be seen that for the liquid cultures of _P. chrysosporium_, the level of total carbohydrate in the extracellular medium does not differ significantly from the level of glucose during the seven day period monitored. However, for the agar-immobilised cultures, after an incubation period of three days, the level of carbohydrate is approximately twice that of the glucose concentration; and the difference between the two levels then remains constant over the rest of the period monitored. Since the level of carbohydrate detected in the uninoculated flasks did not differ significantly from the glucose concentration present (data not shown), this suggests that during the first three days of incubation, _P. chrysosporium_ was able to partially solubilise the agar immobilisation support. Thus, it was concluded that the use of agar to facilitate the continued production of successive harvests of ligninase from the same fungal biomass of _P. chrysosporium_ was not ideal, and that an

Fig. 5 Comparison of the extracellular glucose and total carbohydrate levels in stationary liquid and agar-immobilised cultures of *P.chrysosporium*

Culture conditions were as described in Section 2.2.3. Extracellular glucose concentration was measured by the glucose oxidase / peroxidase method as described in Section 2.2.6, and the extracellular total carbohydrate concentration was measured by the phenol sulphuric acid method as described in Section 2.2.7. Each point represents the mean of three replicates; S.D.= ±5%

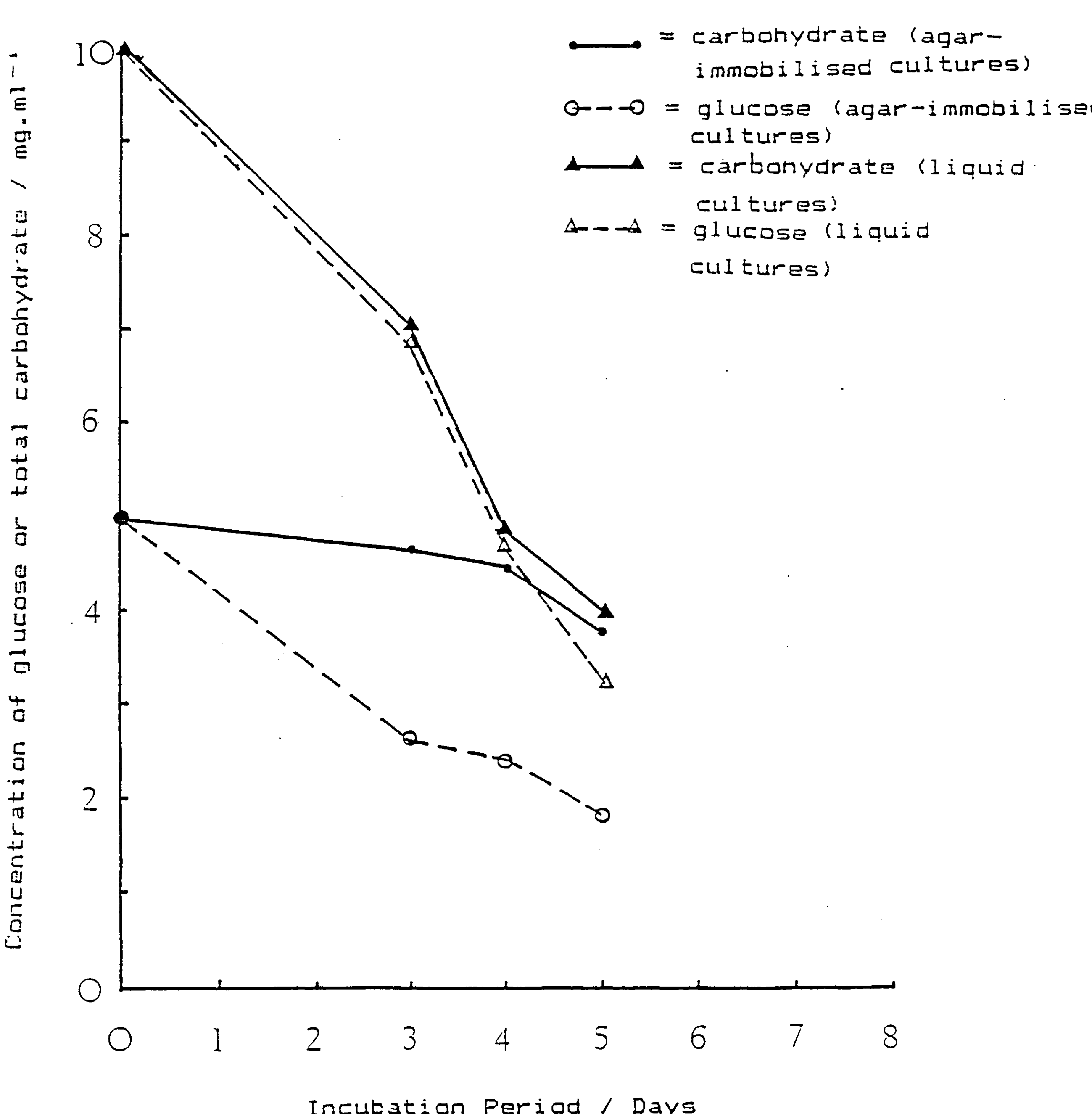


Fig. 5 Comparison of the extracellular glucose and total carbohydrate levels in stationary liquid and agar-immobilised cultures of *P.chrysosporium*

alternative - inert - immobilisation support was required; see Section 3.1.4.

It should be noted that the level of glucose detected at the time of inoculation was twice that in the liquid cultures compared to that in the agar-immobilised cultures, and that this was due to the dilution effect of the distilled water top layer as mentioned above.

3.1.2 Production of ligninase when using autoclaved or filter-sterilised glucose

Throughout the course of this work, the same experiments were often repeated several times to be certain that the results obtained were reproducible and that the conclusions drawn from them were at least consistent. Whilst collating data for the results presented in Section 3.1.1, the observation was made that more ligninase activity was produced when using autoclaved glucose in the medium than when using filter-sterilised glucose (See Appendix I).

The results presented in Figs.6 and 7 illustrate the differences in the levels of ligninase produced when autoclaved or filter-sterilised glucose was used in either liquid, or agar-immobilised cultures, respectively. For each set of data, however, the production of ligninase follows the same pattern with time; a lag period of two to three days followed by a relatively sharp rise and fall in the production of ligninase with the maximum activity being reached five days after inoculation.

Fig. 6 Comparison of veratryl alcohol oxidase activity produced by stationary liquid cultures of *P.chrysosporium* incubated using medium containing an autoclaved or filter-sterilised source of glucose

The effect of using autoclaved or filter-sterilised glucose upon the production of veratryl alcohol oxidase activity was carried out as described in Section 2.2.4. Each measurement of veratryl alcohol oxidase activity (see Section 2.2.2) represents the mean of three replicates; S.D.= ±10%.

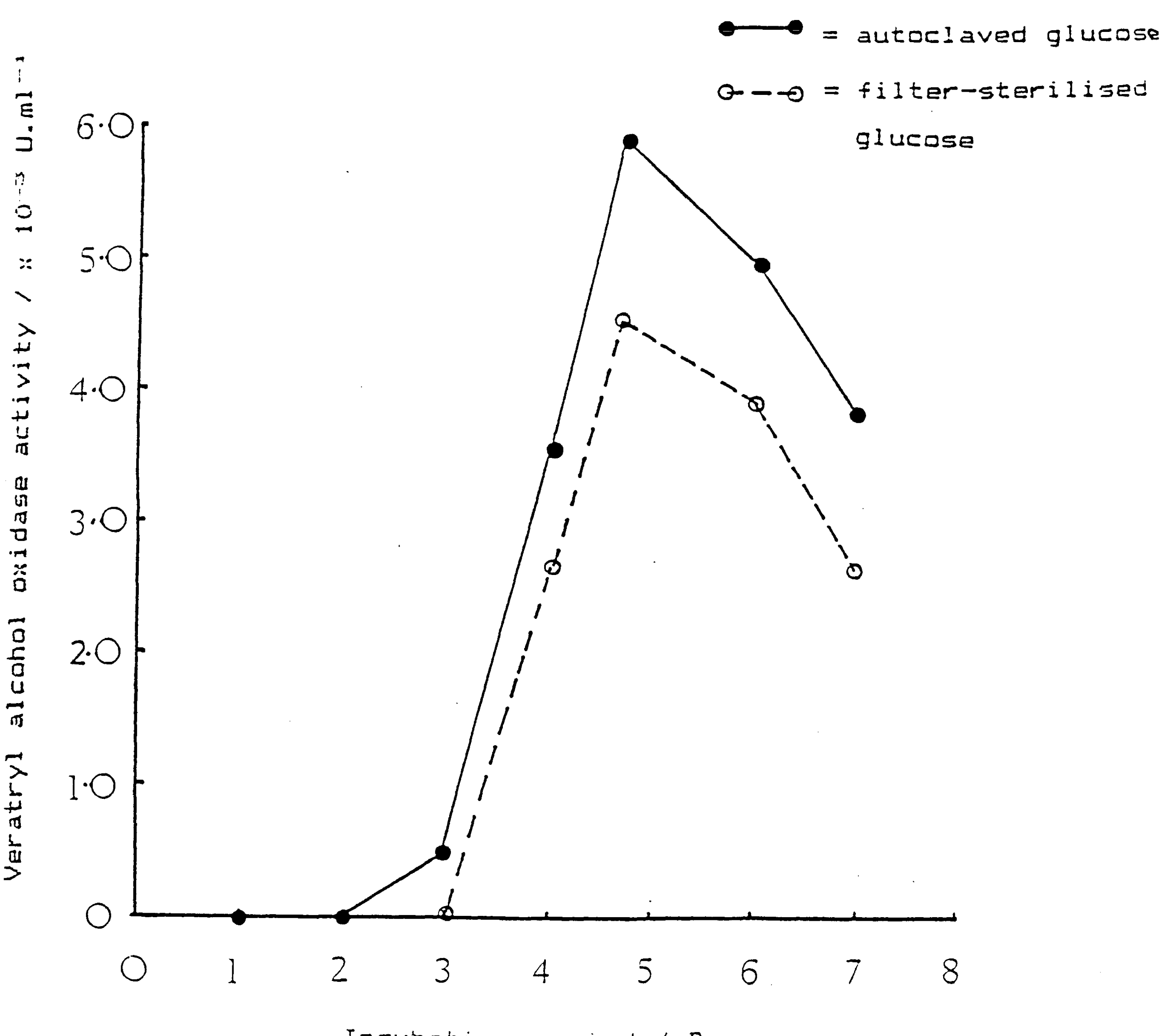


Fig. 6 Comparison of veratryl alcohol oxidase activity produced by stationary liquid cultures of *P.chrysosporium* incubated using medium containing an autoclaved or filter-sterilised source of glucose

Fig. 7 <u>Comparison of veratryl alcohol oxidase activity produced by stationary agar-immobilised cultures of *P. chrysosporium* incubated using medium containing an autoclaved or filter-sterilised source of glucose</u>

The effect of using autoclaved or filter-sterilised glucose upon the production of veratryl alcohol oxidase activity was carried out as described in Section 2.2.4. Each measurement of veratryl alcohol oxidase activity (see Section 2.2.2) represents the mean of three replicates; S.D.= ±10%.

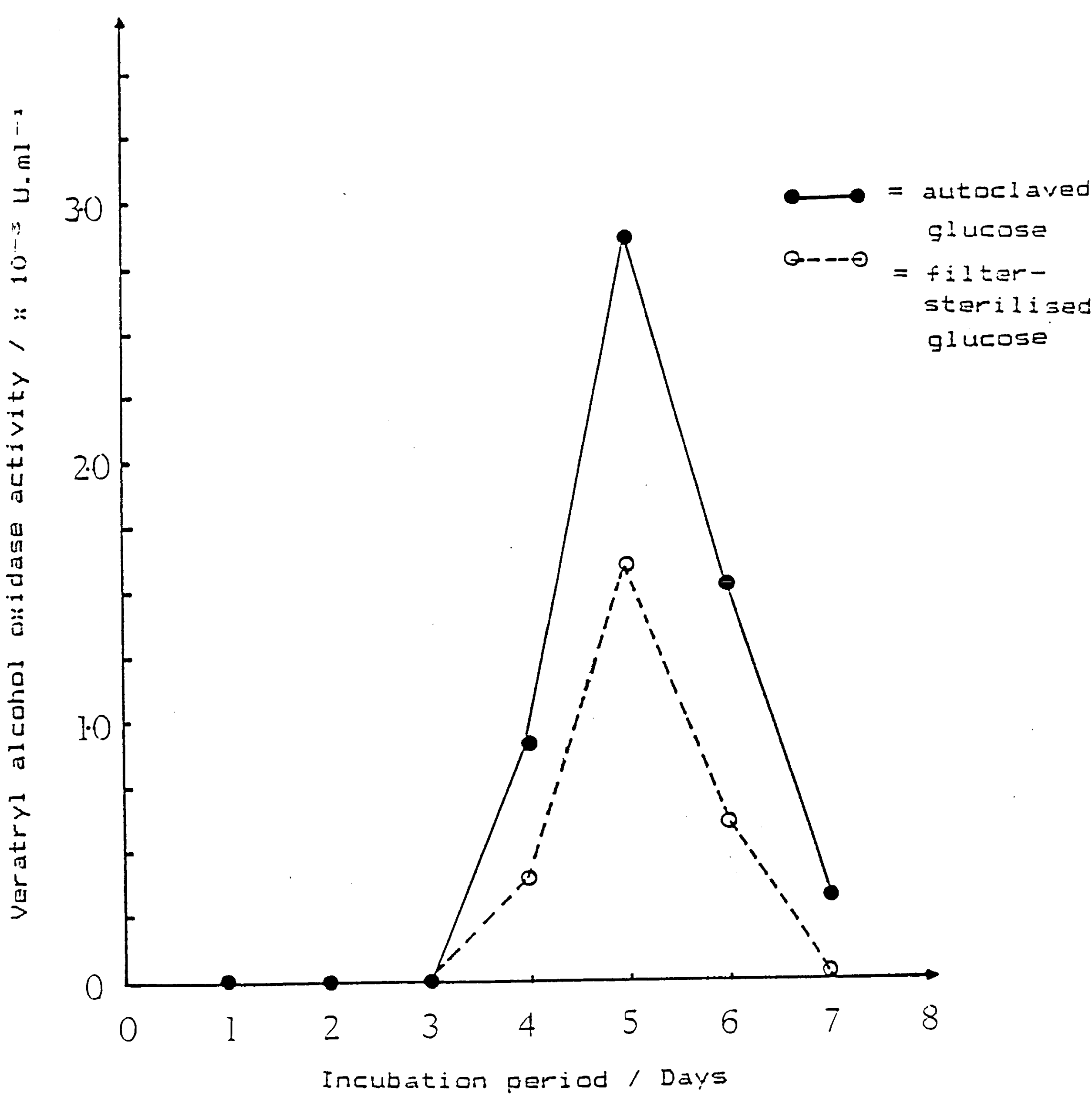


Fig. 7 <u>Comparison of veratryl alcohol oxidase activity produced by stationary agar-immobilised cultures of *P.chrysosporium* incubated using medium containing an autoclaved or filter-sterilised source of glucose</u>

As described in Section 2.2.4, to determine whether it was possible to mimic the effects of the autoclaving of the glucose, a range of concentrations of 5-hydroxy methyl furfural were added to a set of filter-sterilised glucose agar-immobilised cultures of P. chrysosporium.

The results obtained are presented in Fig.8, and show that as the concentration of 5-hydroxy methyl furfural increases, the maximum ligninase activity produced not only increases, but would also appear to reach its peak level earlier. Hence, it was concluded that increasing levels of ligninase activity were apparently being induced by increasing concentrations of 5-hydroxy methyl furfural.

In other experiments, where the final concentration of 5-hydroxy methyl furfural was greater than 50µM, the ligninase activity produced was found to be lower than the control cultures which contained no added compound, inferring that above a critical concentration, 5-hydroxy methyl furfural was in fact toxic to the fungus (Data not shown).

3.1.3 Induction of ligninase activity using organosolv lignin

The effect of organosolv on the production of ligninase was investigated as described in Section 2.2.5. The results presented in Figs. 9 and 10, illustrate that Organosolv lignin leads to higher levels of ligninase being produced in both liquid and agar-immobilised

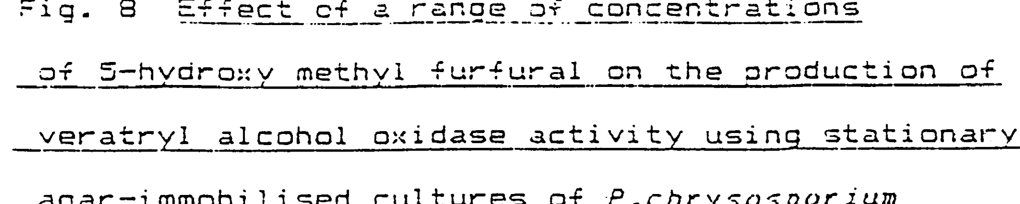
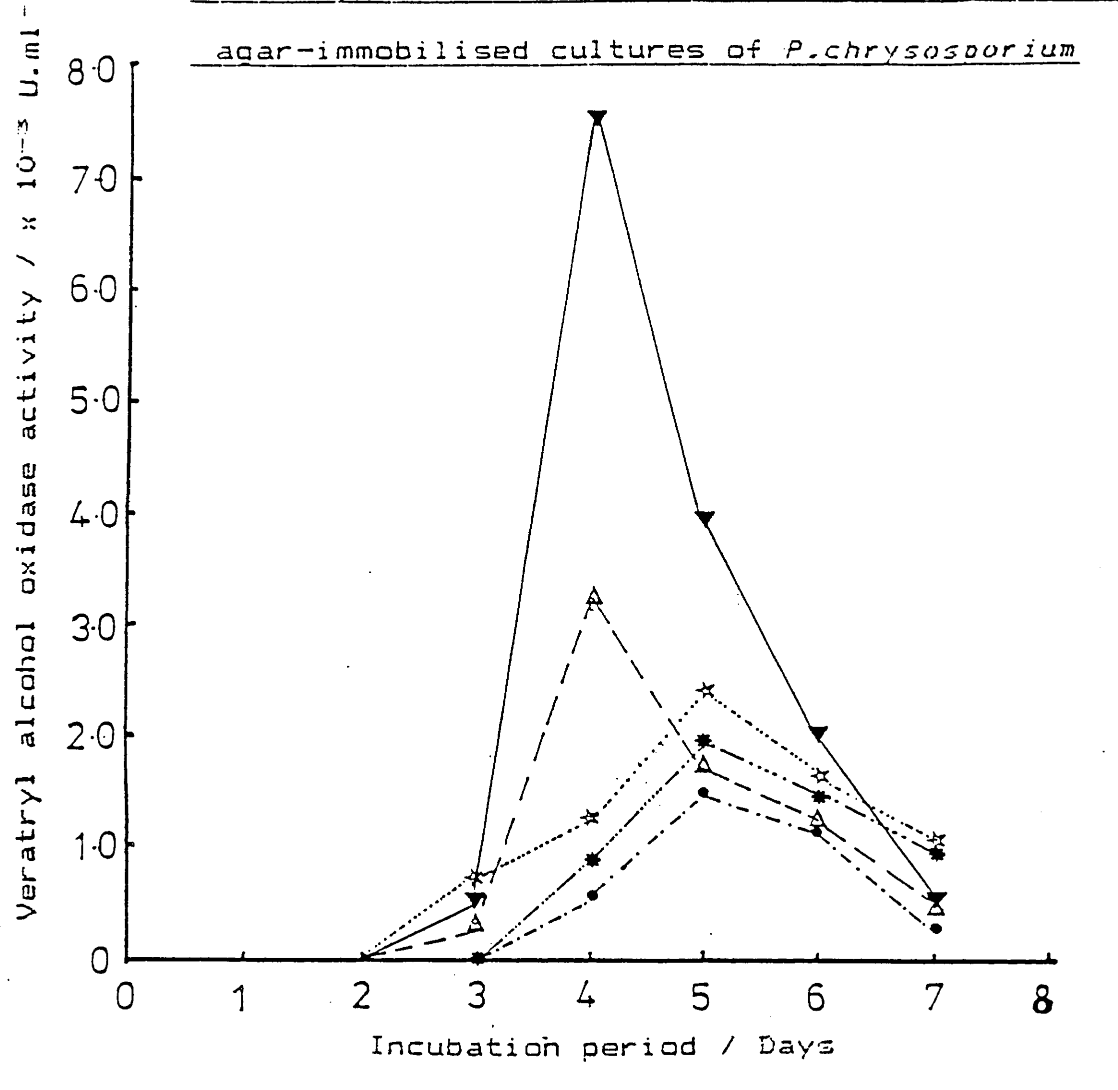


Fig. 8 Effect of a range of concentrations of 5-hydroxy methyl furfural on the production of veratryl alcohol oxidase activity using stationary agar-immobilised cultures of *P.chrysosporium*



▼——▼	10·0 μM	Final concentration of 5-hydroxy methyl furfural
△– – –△	7·5 μM	
☆·········☆	5·0 μM	
✱-·-·-✱	2·5 μM	
•-·-·-•	CONTROL (i.e. no 5-hydroxy methyl furfural)	

This experiment was carried out as described in Section 2.2.4. Each measurement of veratryl alcohol oxidase activity (see Section 2.2.2) represents the mean of three replicates; S.D.= ±10%.

Fig. 9 Induction of veratryl alcohol oxidase activity in stationary liquid cultures of *P.chrysosporium* incubated in the presence of organosolv lignin

This experiment was carried out as described in Section 2.2.5. Each measurement of veratryl alcohol oxidase activity (see Section 2.2.2) represents the mean of three replicates; S.D.= ±10%.

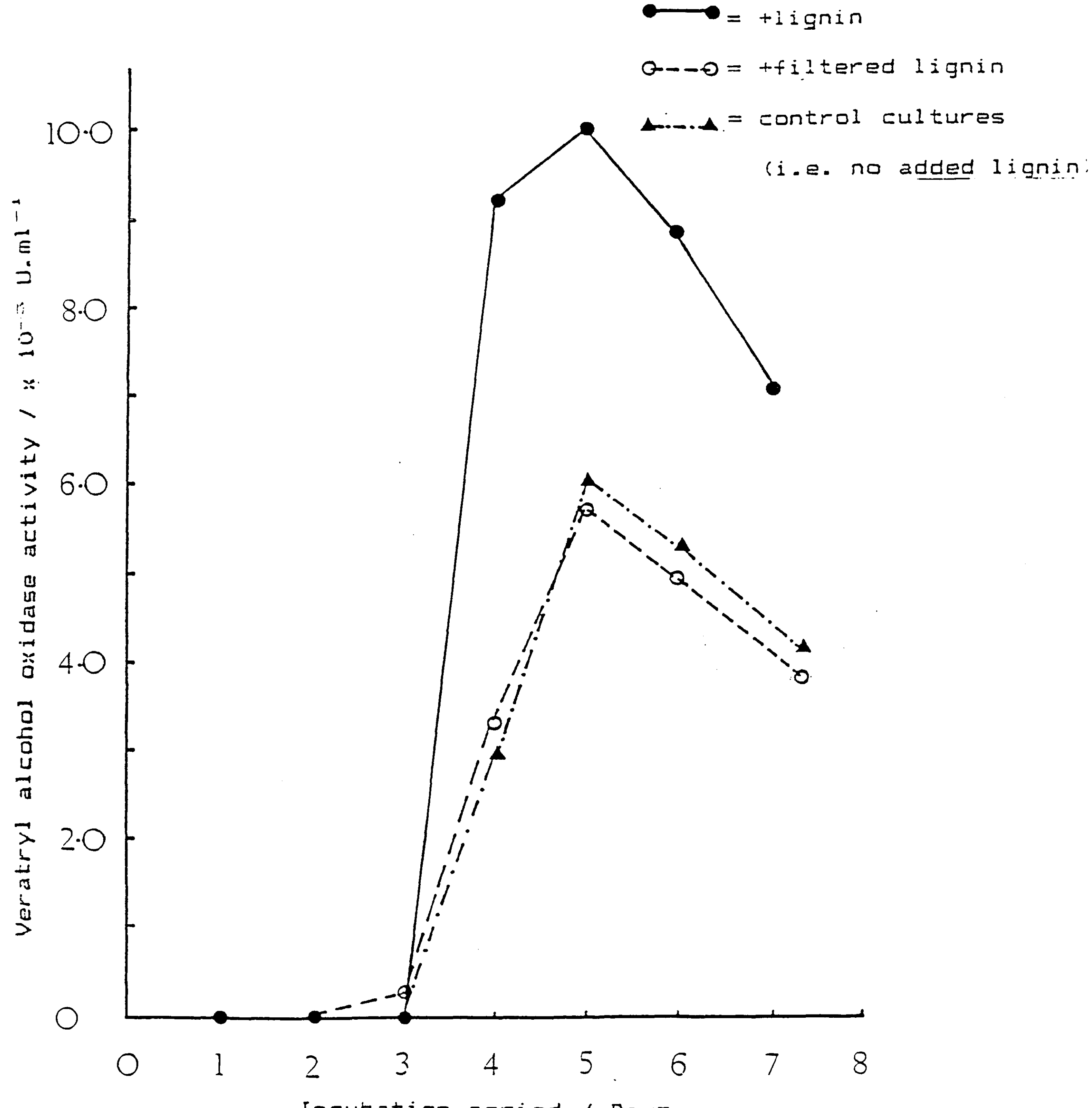


Fig. 9 Induction of veratryl alcohol oxidase activity in stationary liquid cultures of *P.chrysosporium* incubated in the presence of organosolv lignin

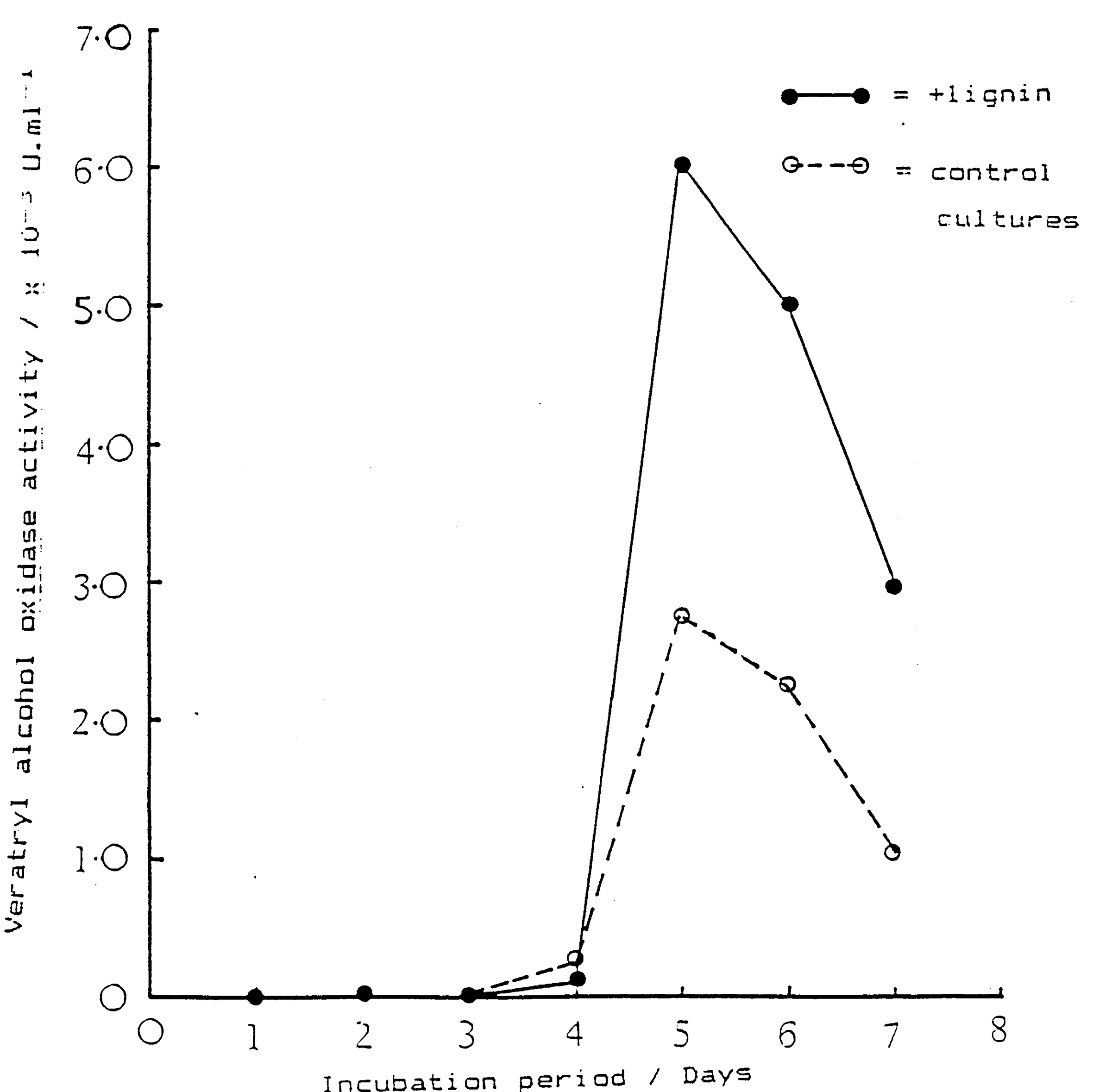


Fig. 10 Induction of veratryl alcohol oxidase activity in stationary agar-immobilised cultures of *P.chrysosporium* incubated in the presence of organosolv lignin

This experiment was carried out as described in Section 2.2.5. Each measurement of veratryl alcohol oxidase activity (see Section 2.2.2) represents the mean of three replicates; S.D.= ±10%.

cultures of *P. chrysosporium*, respectively. For both sets of cultures, the presence of the lignin resulted in an approximate doubling of the maximum level of ligninase produced. It is interesting to note that the presence of the lignin did not alter the timing of peak ligninase activity, but did lead to an apparent slight delay in its initial production.

When the particulate material present in the organosolv lignin preparation was removed by filtration, it was found that the filtrate was unable to lead to the production of increased yields of ligninase in a liquid culture experiment, suggesting that it was the insoluble material that was ultimately responsible for the elevated levels of ligninase produced in the presence of the unfiltered preparation.

3.1.4 The use of polyurethane foam as an immobilisation support for the production of ligninase by P.chrysosporium

It was shown in Section 3.1.1, that *P.chrysosporium* was apparently able to partially solubilise agar when the latter was used as an immobilisation support, and since a prerequisite of any immobilisation support is that it remains inert to that organism contained within its structure, it was concluded that agar was not suitable as an - inert - immobilisation support. In 1985, however, Thepenier et al. reported the use of polyurethane foam as an immobilisation support, and that this material was resistant to degradation by microorganisms. It was thus

decided to carry out a pilot investigation, to determine whether polyurethane foam would be a more suitable immobilisation support for P. chrysosporium than agar.

Table 3 shows the ligninase activity obtained in the presence of a range of different polyurethane foams when measured five days after inoculation, the day when the control flasks containing no foam reached maximum ligninase activity.

In addition to measuring the ligninase activity obtained in the presence of the foam, cultures were also assessed for the pattern of visible growth and the volume of extracellular culture fluid produced upon decanting the flasks, and these results are presented in Table 4.

From these results, it was concluded that of the polyurethane foams tested, that designated Code 3300A merited further investigation as an immobilisation support that might eventually be used to produce successive harvests of ligninase from the same fungal biomass.

3.2 Ligninase production using agitated cultures of P.chrysosporium

3.2.1 Production of ligninase in batch culture in the presence and absence of polyurethane foam as an immobilisation support

The production of ligninase activity in agitated cultures of P. chrysosporium incubated in the presence and absence of polyurethane foam Code 3300A was compared

Table 3 <u>Production of ligninase activity using stationary cultures of *P.chrysosporium* incubated in the presence of a range of polyurethane foams</u>

Polyurethane foam	Veratryl alcohol oxidase activity / $U.ml^{-1}$	Relative rate
Control (no foam)	5.34×10^{-3}	1.00
3300A	1.06×10^{-2}	1.99
4200A	–	–
H4200	3.61×10^{-4}	0.07
74165A'O	5.59×10^{-3}	1.04
PR 22 / 60	1.99×10^{-4}	0.04

The veratryl alcohol oxidase activities quoted above represent the means of three replicates, when the cultures were assayed five days after inoculation. Culture conditions were as described in Section 2.2.8, and veratryl alcohol oxidase activity was measured as described in Section 2.2.2. The standard deviation between replicate cultures was found to be ±10%.

Table 4 Appearance of fungal growth and recovery of extracellular medium from stationary cultures of *P.chrysosporium* incubated in the presence of a range of polyurethane foams

Polyurethane foam	General comments
3300A	Fungal growth entirely restricted to withinthe polyurethane foam, with large quantities of conidia visible on the surface of the foam. c.3.5ml of extracellular medium decanted.
4200A	No fungal growth visible outside of the foam. No visible conidiation. No extracellular medium able to be recovered.
H4200	Fungal growth entirely restricted to within the foam. No visible conidiation. Only c.1.0ml of extracellular medium able to be recovered.
74165A'O	Some visible conidiation on the surface of the foam, with fungal hyphe visible having grown outside of the foam matrix. c.3.5ml of extracellular medium able to be recovered.

Table 4 (contd.)

PR 22 / 60	Foam matrix became reduced in size upon autoclaving (by c.1.5cm across the diameter of the foam disc). Fungal hyphae visible having grown outside of the foam matrix. No visible conidiation. c.8.0ml of extracellular medium able to be recovered.

These comments refer to the properties of the cultures five days after inoculation; see Section 2.2.8. Where it has been commented that conidiation was visible, in all cases this was where the foam was slightly exposed above the surface of the liquid medium, and the fungus had grown through the entire matrix of the foam and actually above the surface of the liquid medium.

Fig. 11 Production of veratryl alcohol oxidase activity using agitated cultures of *P.chrysosporium* incubated in the presence and absence of cubes of polyurethane foam

This experiment was carried out as described in Section 2.3.3. Each measurement of veratryl alcohol oxidase activity (See Sections 2.2.2 and 2.3.2) represents the mean of three replicates; the standard deviation for each measurement is indicated on the figure.

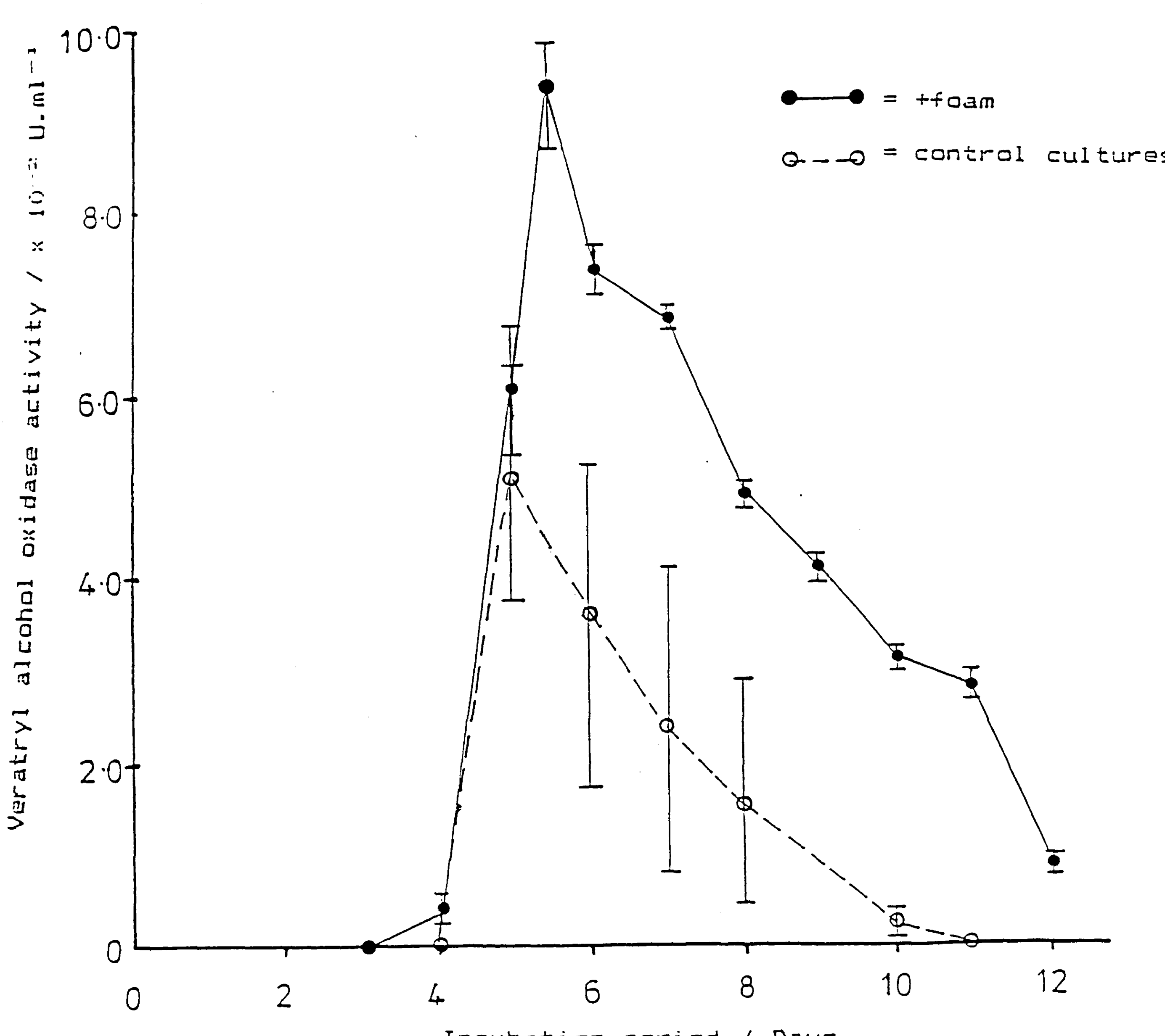


Fig. 11 Production of veratryl alcohol oxidase activity using agitated cultures of *P.chrysosporium* incubated in the presence and absence of cubes of polyurethane foam

as described in Section 2.3.3. The results presented in Fig. 11 show that in the presence of the foam, more ligninase is produced, and also, the variation between replicate flasks is very much reduced.

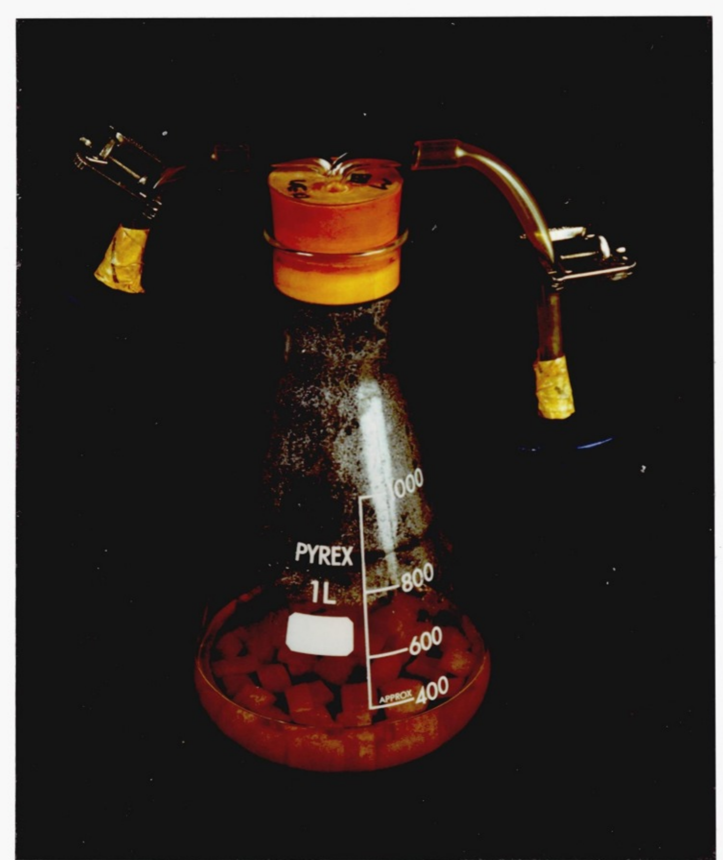
It is interesting to note that the pattern of ligninase production is the same for both stationary and agitated cultures of P.chrysosporium.

3.2.2 Growth of P. chrysosporium in foam-immobilised cultures

It was found that when P. chrysosporium was incubated as described in Section 2.3.3, the fungus grew as discrete pellets. It was observed that as the pellets developed, they were initially free in the medium, but after an incubation period of approximately two to three days, the entire population of the pellets became embedded in the pores of the polyurethane foam. The typical appearance of foam-immobilised cultures of P.chrysosporium is shown in Figs. 12 and 13.

Using cubes of the foam which contained embedded pellets of the fungus, electron microscopy was performed as described in Section 2.3.6. The results of this work demonstrated that every available pore on the surface of the cubes of foam was occupied by a pellet; whereas, only a relatively low proportion of the pores within the cubes of foam were occupied by the fungus (c.10-15% ?). Electron micrographs showing the typical appearance of pores of the foam occupied by pellets of the fungus are presented in Figs. 14 and 15.

Fig. 12 Foam-immobilised *P.chrysosporium*

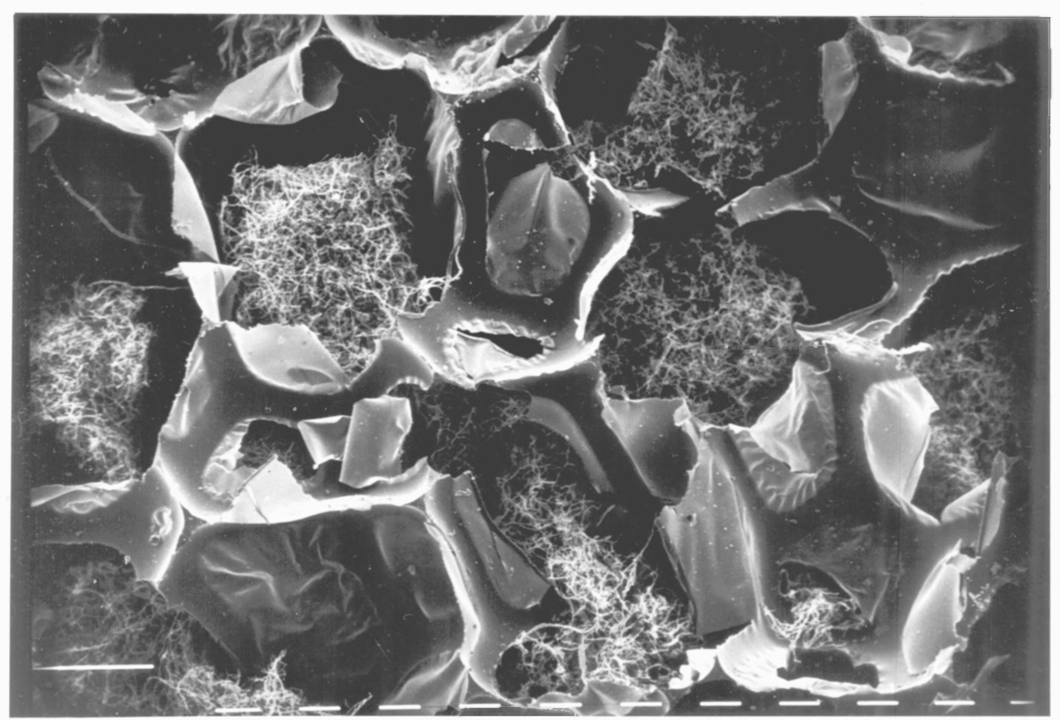


This figure shows a 1l Erlenmeyer flask containing forty five 1cm cubes of foam-immobilised *P.chrysosporium*, in 200ml of medium (see Section 2.3.3).

Fig. 13 Foam-immobilised *P.chrysosporium*

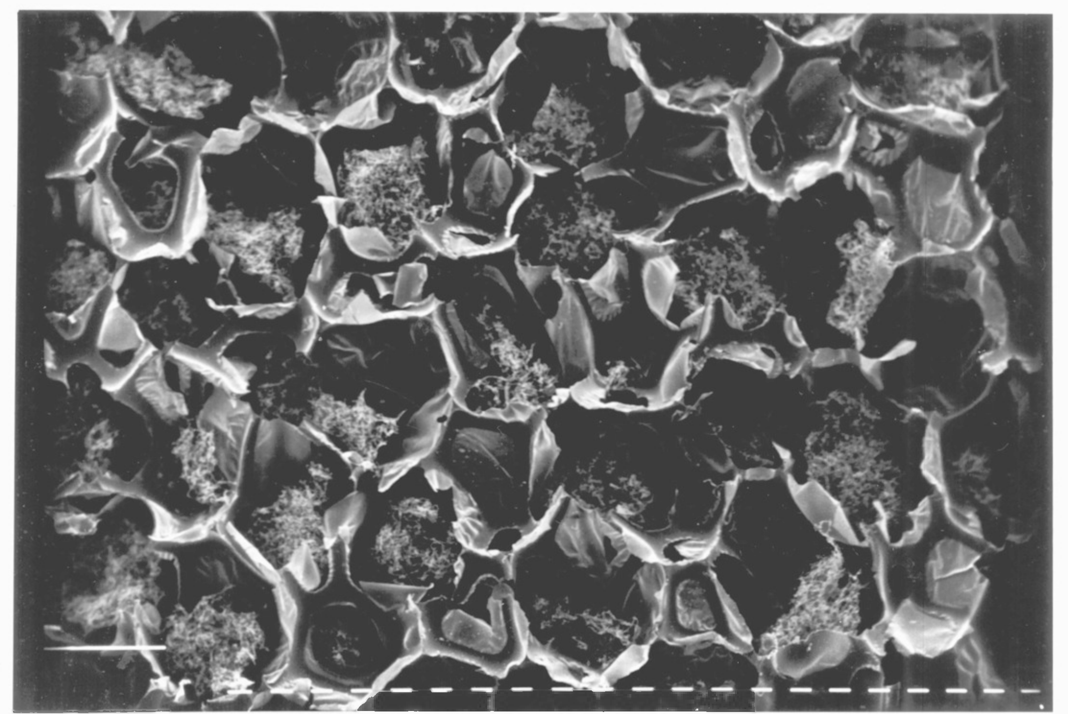
This figure shows the appearance of foam-immobilised *P.chrysosporium*. It should be noted that fungal growth is entirely restriced to the pores of polyurethane foam (1 cube = 1cm x 1cm x 1cm).

Fig. 14 Electron-micrograph of *P. chrysosporium*



Samples were prepared by critical point drying as desribed by Anderson (1951); see Section 2.3.6. (1bar = 100μm).

Fig. 15 Electron-micrograph of *P.chrysosporium*



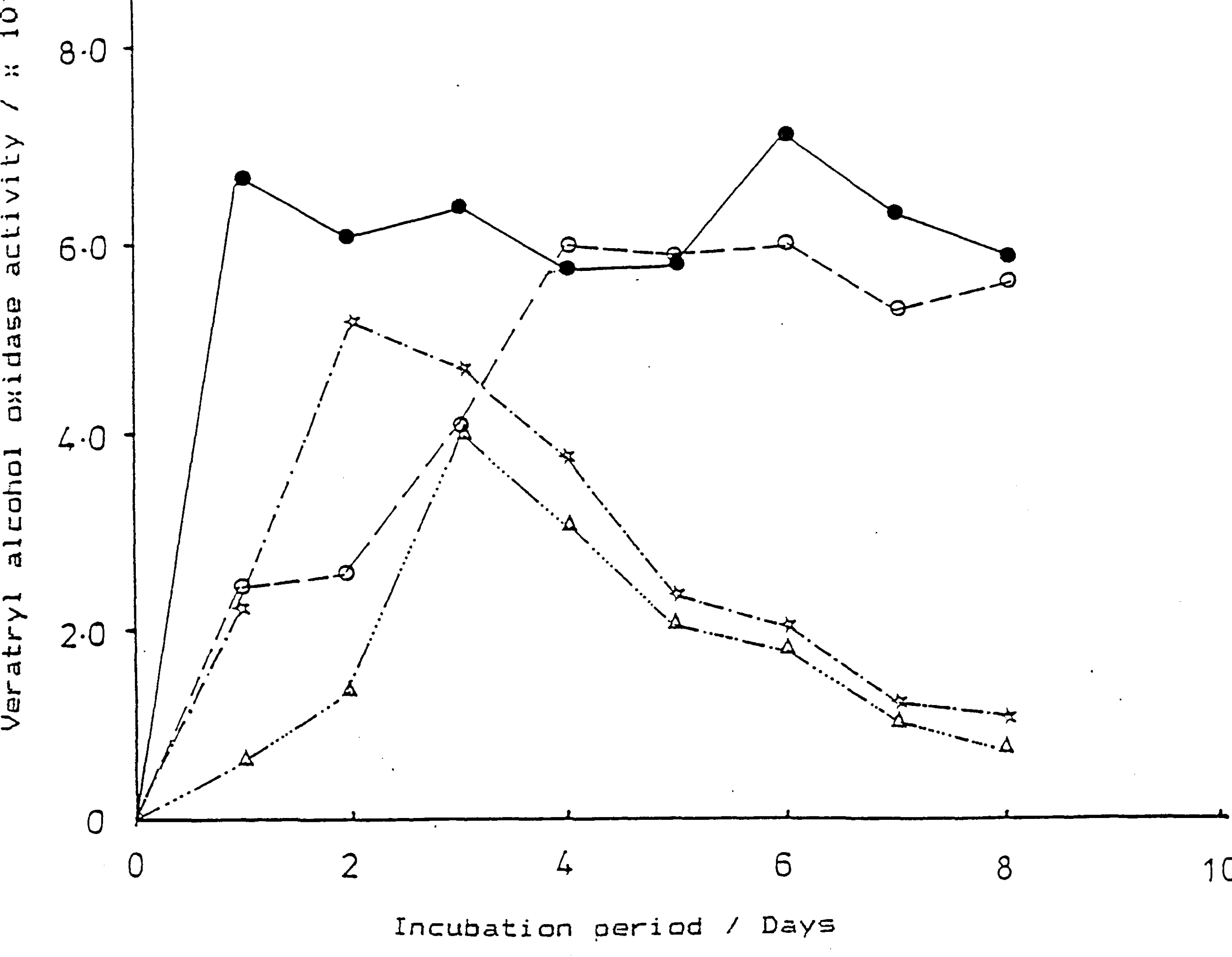
Samples were prepared by critical point drying as desribed by Anderson (1951); see Section 2.3.6. (1bar = 100 μm).

3.2.3 Production of successive harvests of ligninase using semi-continuous cultures of foam-immobilised P.chrysosporium

Using foam-immobilised cultures of P.chrysosporium, the effect of decanting the extracellular medium at peak ligninase activity, and then replacing it with a range of dilutions of the standard batch culture medium, or buffer alone, was investigated as described in Section 2.3.4. The results of this work are presented in Fig. 16. It was found that when undiluted or twofold diluted medium was added back, ligninase was produced in a single peak of activity over the eight day period studied. The peak produced in the presence of the twofold diluted medium was larger than that produced in the presence of buffer alone, and, in addition, was produced one day earlier. However, when fivefold diluted medium or buffer alone was added back, the ligninase activity produced did not show a single rise and fall, but fluctuated at approximately the same level; the fivefold diluted medium and the buffer alone reaching an apparent plateau in the production of ligninase activity one day and four days, respectively, after the replacement of the medium.

From these results, an experiment was carried out to determine if it would be possible to produce harvests of ligninase on a semi-continuous basis, using fivefold diluted medium as the standard replacement medium. It was found that using this dilution of medium, successive harvests of ligninase were able to be produced from the same fungal biomass every 24 to 48 hours. This method

Fig. 16 Effect of dilution of the batch culture medium on the production of a second harvest of veratryl alcohol oxidase activity using agitated cultures of foam-immobilised *P.chrysosporium*



⊖----⊖ = + buffer alone

●——● = + fivefold diluted medium

☆-·-·☆ = + twofold diluted medium

△·····△ = + undiluted medium

This experiment was carried out as described in Section 2.3.4. Each measurement of veratryl alcohol oxidase activity (See Sections 2.2.2 and 2.3.2) represents the mean of three replicates; S.D.= <5%.

was consequently adopted as the standard method for the semi-continuous production of ligninase. A typical set of results is shown in Table 5. It can be seen that the first three to four harvests were obtained every 24 hours and were approximately of the same magnitude, whereas subsequent harvests decrease in magnitude, albeit to more or less the same extent, and are only obtained every 48 hours.

Using fivefold diluted medium as the standard replacement medium, it was then shown that once _P. chrysosporium_ was immobilised in the cubes of polyurethane foam, an increase in the agitation rate from 60 r.p.m. to 150 r.p.m. made no difference to the semi-continuous production of ligninase, see Table 6; and also, that when veratryl alcohol was omitted during the semi-continuous culture, there was a rapid tailing off in the production of successive harvests of ligninase, see Table 7. In addition, it can be seen in Table 7, that when the cubes of foam-immobilised _P. chrysosporium_ were washed with distilled water (See Section 2.3.4) and veratryl alcohol omitted as described above, then no further harvests of ligninase were produced during semi-continuous culture.

Finally, in Section 2.3.4, it was also described how two separate approaches were adopted to determine whether increasing the biomass within individual flasks would lead to the production of increased titres of ligninase. It can be seen from the results presented in

Table 5 Successive harvests of ligninase produced using semi-continuous cultures of foam-immobilised *Phanerochaete chrysosporium*

Incubation Period/hours	Harvest number	Yield of ligninase/$U.l^{-1}$
120	1	93.0
+24	2	92.5
+24	3	100.0
+24	4	74.0
+48	5	48.0
+48	6	62.0
+48	7	55.0

These results are for a typical set of successive harvests of ligninase produced using foam-immobilised cultures of *P.chrysosporium*. Culture conditions were as described in Section 3.2.3, and ligninase activity was measured as described in Sections 2.2.2 and 2.3.2.

Table 6 Effect of increasing the agitation rate on the production of ligninase using foam-immobilised *P.chrysosporium*

Incubation period / hours	Ligninase activity / x 10^{-2} U.ml^{-1}	
	60 r.p.m.	150 r.p.m.
+48	6.23	5.80
+24	9.54	9.23
+24	8.26	8.41
+48	6.35	5.84
+48	5.07	5.12

This experiment was carried out as described in Section 2.3.4, with the ligninase activity being measured as described in Sections 2.2.2 and 2.3.2.

Table 7 Effect of omitting veratryl alcohol from the replacement media of foam-immobilised cultures of *P.chrysosporium*

Incubation period / hours	Ligninase activity / x 10^{-2} U.ml^{-1}		
	No. 1	No. 2	No.3
+48	5.63	6.37	6.12
+24	9.37	2.04	0.00
+24	8.76	0.00	0.00

This experiment was carried out as described in Section 2.3.4. Culture No. 1 was the control culture, in which veratryl alcohol was present in each replacement medium; in culture No. 2, the veratryl alcohol was omitted from the replacement medium used to produce the second harvest of ligninase; and in culture No. 3, the cubes of foam-immobilised *P.chrysosporium* were washed with distilled before introducing the second batch of replacement medium which also had the veratryl alcohol omitted from it. The ligninase activity was measured as described in Sections 2.2.2 and 2.3.2.

Fig. 17 and Table 8, that both of these approaches failed to result in a significant increase in the production of ligninase; suggesting that ligninase production was being limited by some other factor when the cultures were incubated as described for both of these methods.

3.2.4 Reactivation of foam-immobilised cultures of P. chrysosporium after storage at 4°C

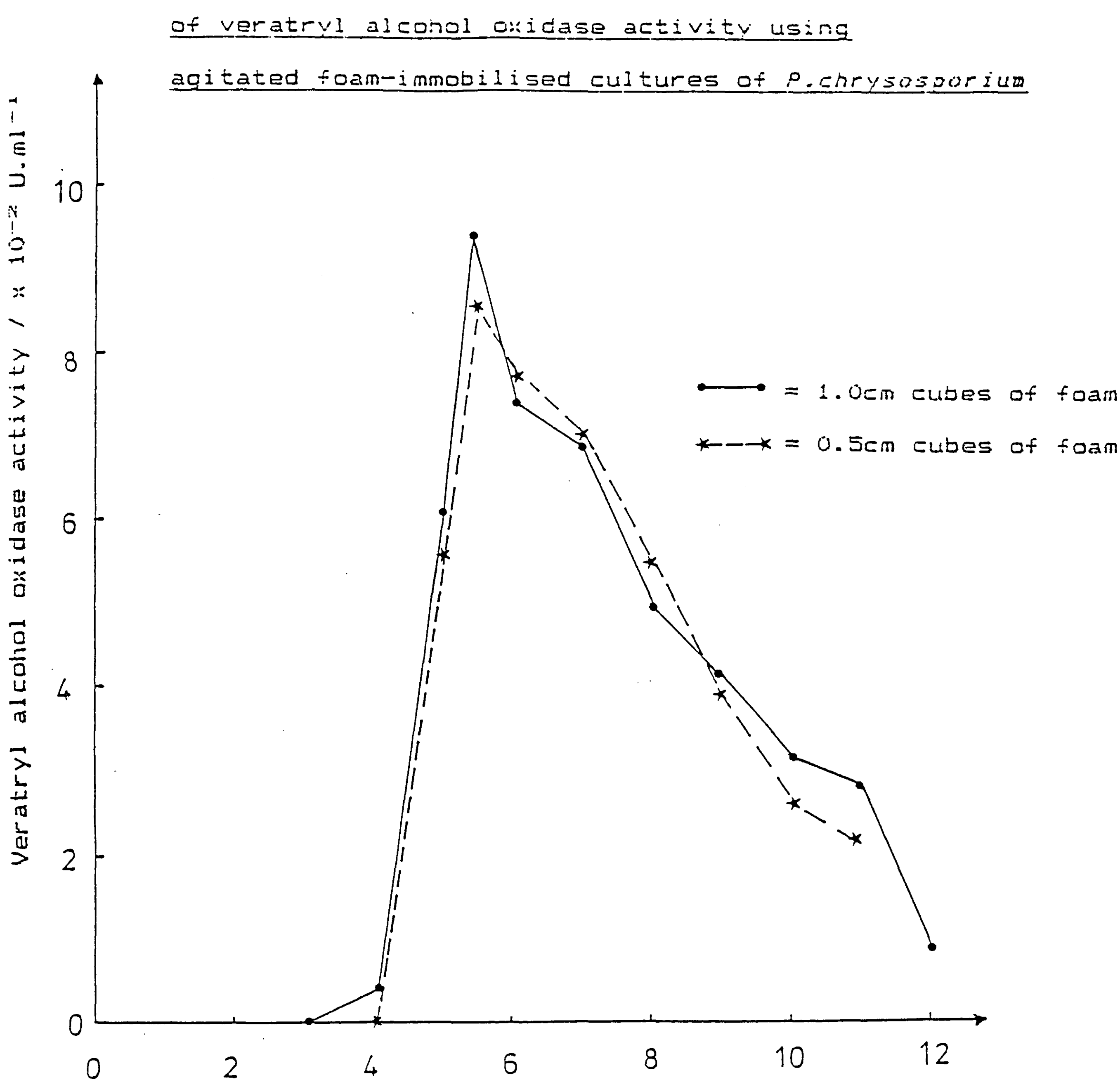
A typical set of results obtained when foam-immobilised cultures of P. chrysosporium were reactivated as described in Section 2.3.5, is presented in Fig. 18. After a lag period of at least 24 hours, the ligninase activity in the cultures increased to a given level, and then continued to fluctuate about that level over a period of several days. If these results are compared to the results presented in Fig. 16 when fivefold diluted medium was also added back to cultures, except without any storage period, it can be seen that the only difference that storage at 4°C seems to make is to delay the onset of the production of ligninase activity by a period of approximately 24 hours.

3.3 **Purification of the isozymes of ligninase**

3.3.1 Anion-exchange chromatography using Tris-acryl DEAE sepharose

The elution profile produced when the isozymes of ligninase were purified by tris-acryl DEAE sepharose anion-exchange chromatography (see Section 2.4.2) is shown in Fig. 19. This shows that when the absorbance at

Fig. 17 Effect of cube size upon the production of veratryl alcohol oxidase activity using agitated foam-immobilised cultures of *P.chrysosporium*



This experiment was carried out as described in Section 2.3.4. Each measurement of veratryl alcohol oxidase activity (See Sections 2.2.2 and 2.3.2) represents the mean of three replicates; S.D.= <5%.

Table 8 Effect of increasing the number of cubes of foam-immobilised *P.chrysosporium* on the production of ligninase

Incubation period / hours	Ligninase activity / x 10^{-2} U.ml^{-1}	
	Controls	2 x Cubes
+120	10.13	9.63
+24	9.98	9.27
+24	8.72	9.00
+24	9.33	8.42
+48	6.89	6.14

This experiment was carried out as described in Section 2.3.4, with the ligninase activity being measured as described in Sections 2.2.2 and 2.3.2.

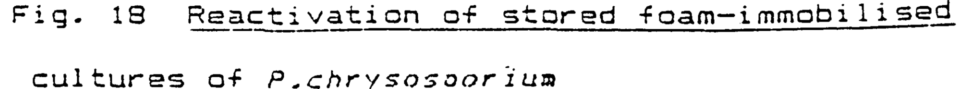


Fig. 18 Reactivation of stored foam-immobilised cultures of *P.chrysosporium*

Veratryl alcohol oxidase activity / x 10^{-3} U.ml^{-1}

10·0
8·0
6·0
4·0
2·0
0

0 1 2 3 4 5 6 7 8

Incubation period / Days

The storage and reactivation of foam-immobilised cultures was carried out as described in Section 2.3.5. Each measurement of veratryl alcohol oxidase activity (See Sections 2.2.2 and 2.3.2) represents the mean of three replicates; S.D.= <5%.

Fig. 19 Purification of the isozymes of ligninase by tris-acryl DEAE sepharose chromatography

The initial purification of the isozymes of ligninase was carried out using the method described in Section 2.4.2. The preparation of ligninase used for this experiment was prepared by pooling the first five concentrated harvests of ligninase produced using foam-immobilised *P.chrysosporium*; see text. This purification resulted in the separation of five fractions designated Fractions I, II, III, IV and V; the horizontal bar lines on the figure indicating which tubes were pooled for each fraction.

The veratryl alcohol oxidase activity of the eluate off the column was measured as described in the text (see Section 2.4.2).

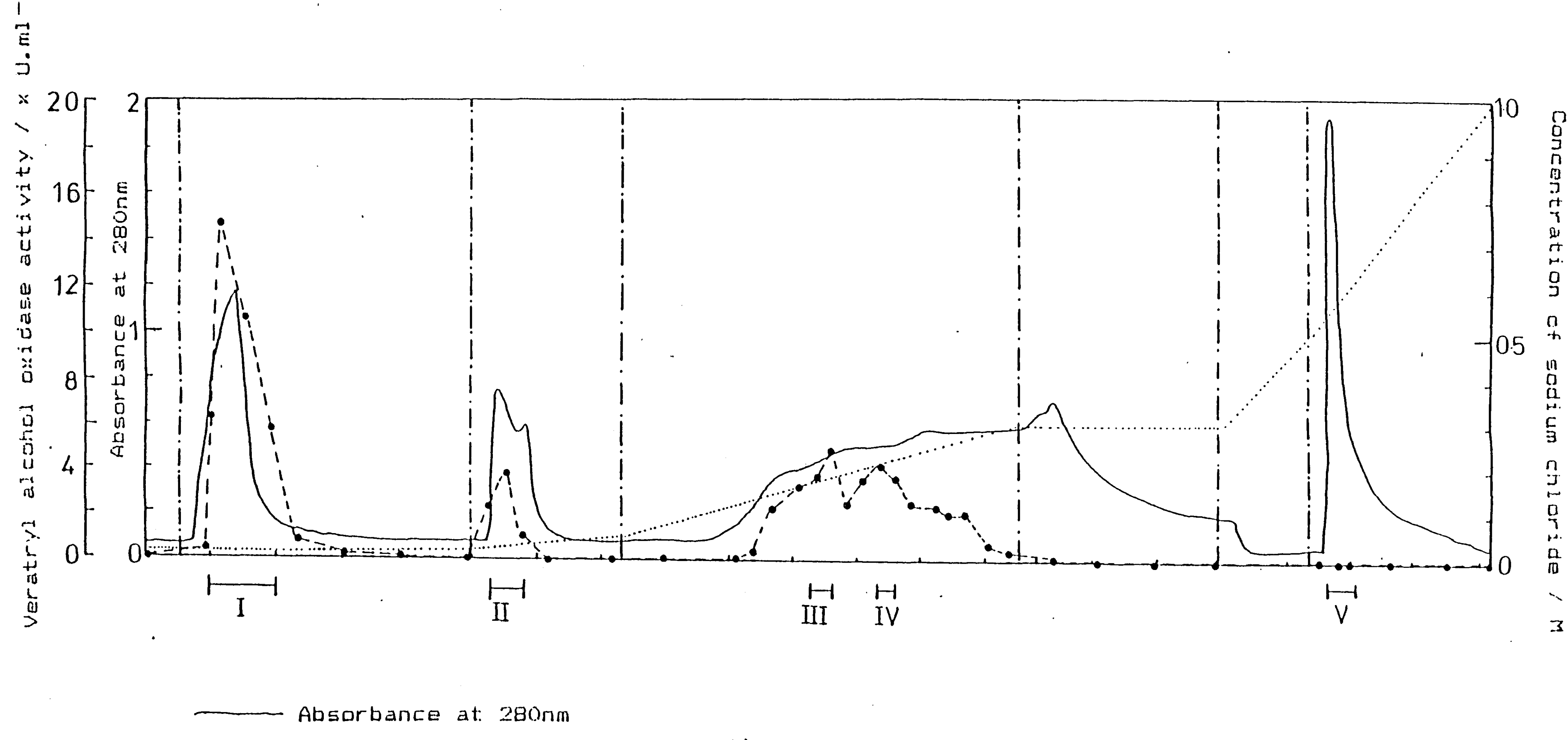


Fig. 19 Purification of the isozymes of ligninase by tris-acryl DEAE sepharose chromatography

280nm and the veratryl alcohol oxidase activity at pH 3.0 were monitored (See also Section 3.5.3), four peaks that contained ligninase activity were able to be separated. These were subsequently referred to as Fractions I, II, III and IV. In addition, during the final washing stage (see Section 2.4.2), one further peak was obtained which had a strong absorbance at 280nm but did not show any ligninase activity, and this was subsequently referred to as Fraction V. It should be pointed out that before the final washing stage, there was a diffuse band of brown material half way down the column, and that this material was eluted in Fraction V; all the other fractions were colourless.

A summary of the results of the purification of the isozymes of ligninase by this method is presented in Table 9. It can be seen that of the original ligninase activity, c.65% was lost during the concentration procedure, with c.15% of the activity being recovered in Fraction I and c.2.5% being recovered in each of Fractions II, III and IV. The measurement of protein concentration, and its resultant effect on the determination of specific activities is dealt with in The Discussion to this thesis.

3.3.2 Anion-exchange chromatography using Mono-Q FPLC

The isozymes of ligninase purified by tris-acryl DEAE sepharose anion-exchange chromatography were assessed for purity as described in Sections 2.6.3 and 2.6.4. The results obtained (see Sections 3.4.2 and

Table 9 Summary of the purification of the isozymes of ligninase

Sample	Volume/ml	Total Units	%Recovery
Extracellular Medium(5 Harvests)	5x3600	1740 in toto	100.0
Ultrafiltrate/ 10Kd cut-off (5 Harvests)	5x25	625 in toto	35.9
Acetone precipitate (Pooled material)	20	600	34.9
DEAE-sepharose			
Fraction I	12	260	14.9
Fraction II	14	48	2.8
Fraction III	6	44	2.5
Fraction IV	7	46	2.6
FPLC Mono-Q			
Fraction I'	4	156	9.0
Fraction II'	ND	ND	ND
Fraction III'	4	8	0.5
Fraction IV'	4	12	0.7

The units referred to above are units of veratryl alcohol oxidase activity, which was measured essentially as described in Section 2.2.2, except that all assays were carried out at pH 3.0 (see Section 2.4.2). It should be noted that Fractions I', III' and IV' represent Isozymes I, III and IV, respectively.

3.4.3) showed that the isozymes required further purification, and this was carried out using Mono-Q FPLC anion-exchange chromatography, as described in Section 2.4.3. During the assessment of purity, it was shown that Fraction II obtained during the first phase of anion-exchange chromatography (See Section 3.3.1) was apparently composed of a mixture of Fraction I, III and IV, and was consequently not further purified; see Section 3.5.1. Since Fractions I, III and IV were shown to consist primarily of one main protein, and each fraction showed ligninase activity as determined by the oxidation of veratryl alcohol, Fractions I, III and IV were subsequently referred to as Isozymes I, III and IV ("Isozyme II" was not used so as to avoid confusion with the original system of nomenclature).

The elution profiles obtained for Isozymes I, III and IV, are presented in Figs. 20, 21 and 22, respectively; see also Table 9.

It is interesting to note that, at least for Isozyme I, it was possible to isolate a compound that was tentatively identified as veratryl alcohol from the isozyme preparation. This was eluted from the Mono-Q column after a retention time of less than one minute, and was shown to have a similar UV spectrum to authentic veratryl alcohol (see Fig. 23), and also, to yield an increase in absorbance at 310nm when the compound was substituted for authentic veratryl alcohol in a standard veratryl alcohol oxidase assay (see Section

Fig. 20 Purification of Isozyme I by FPLC Mono Q anion-exchange chromatography

After the preliminary purification of Isozyme I by tris-acryl DEAE sepharose anion-exchange chromatography (see Section 2.4.2), the isozyme was further purified by FPLC Mono Q anion-exchange chromatography. Running conditions were as described in Section 2.4.3.

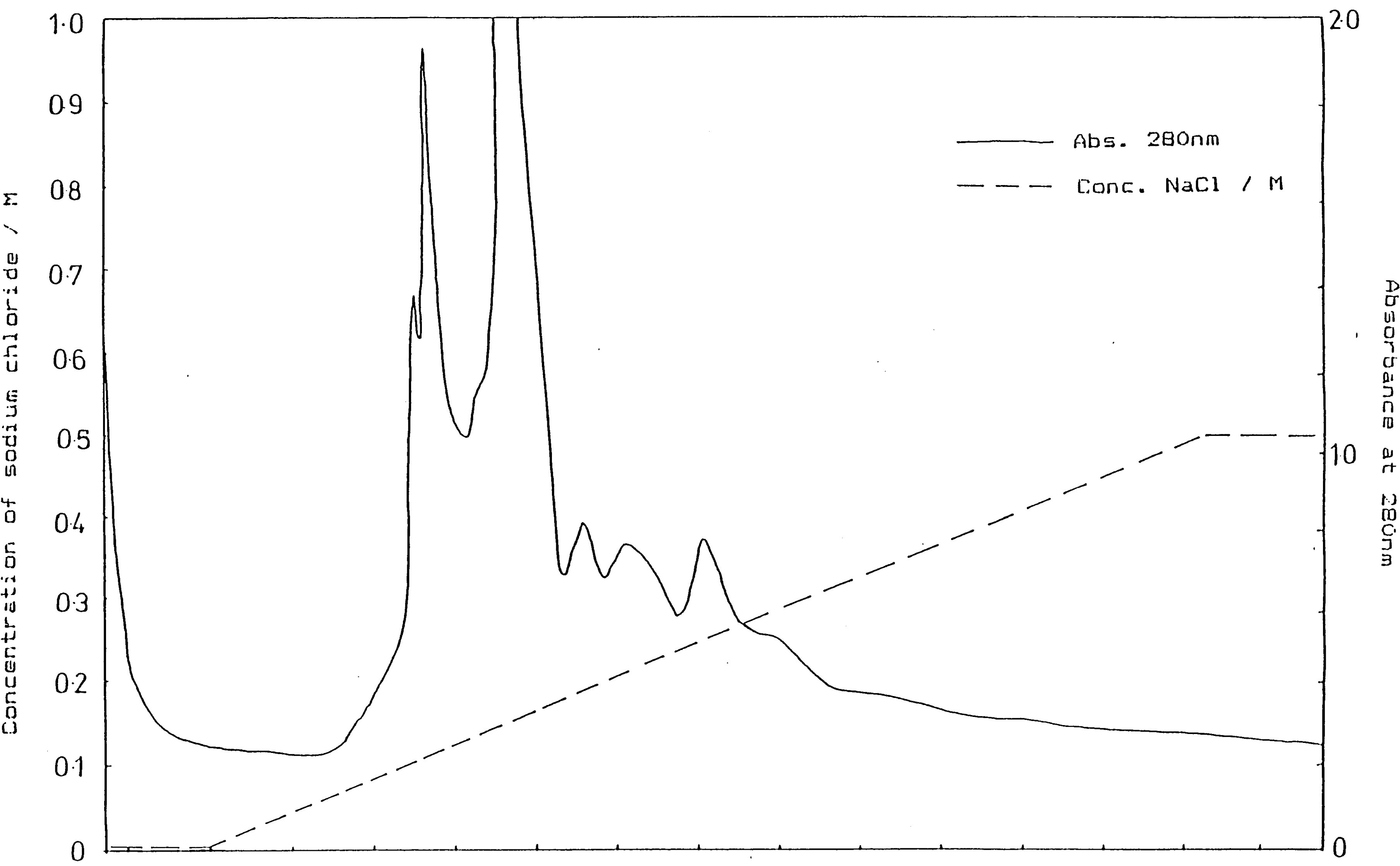


Fig. 20 Purification of Isozyme I by FPLC Mono Q anion-exchange chromatography

Fig. 21 Purification of Isozyme III by FPLC Mono Q anion-exchange chromatography

After the preliminary purification of Isozyme III by tris-acryl DEAE sepharose anion-exchange chromatography (see Section 2.4.2), the isozyme was further purified by FPLC Mono Q anion-exchange chromatography. Running conditions were as described in Section 2.4.3.

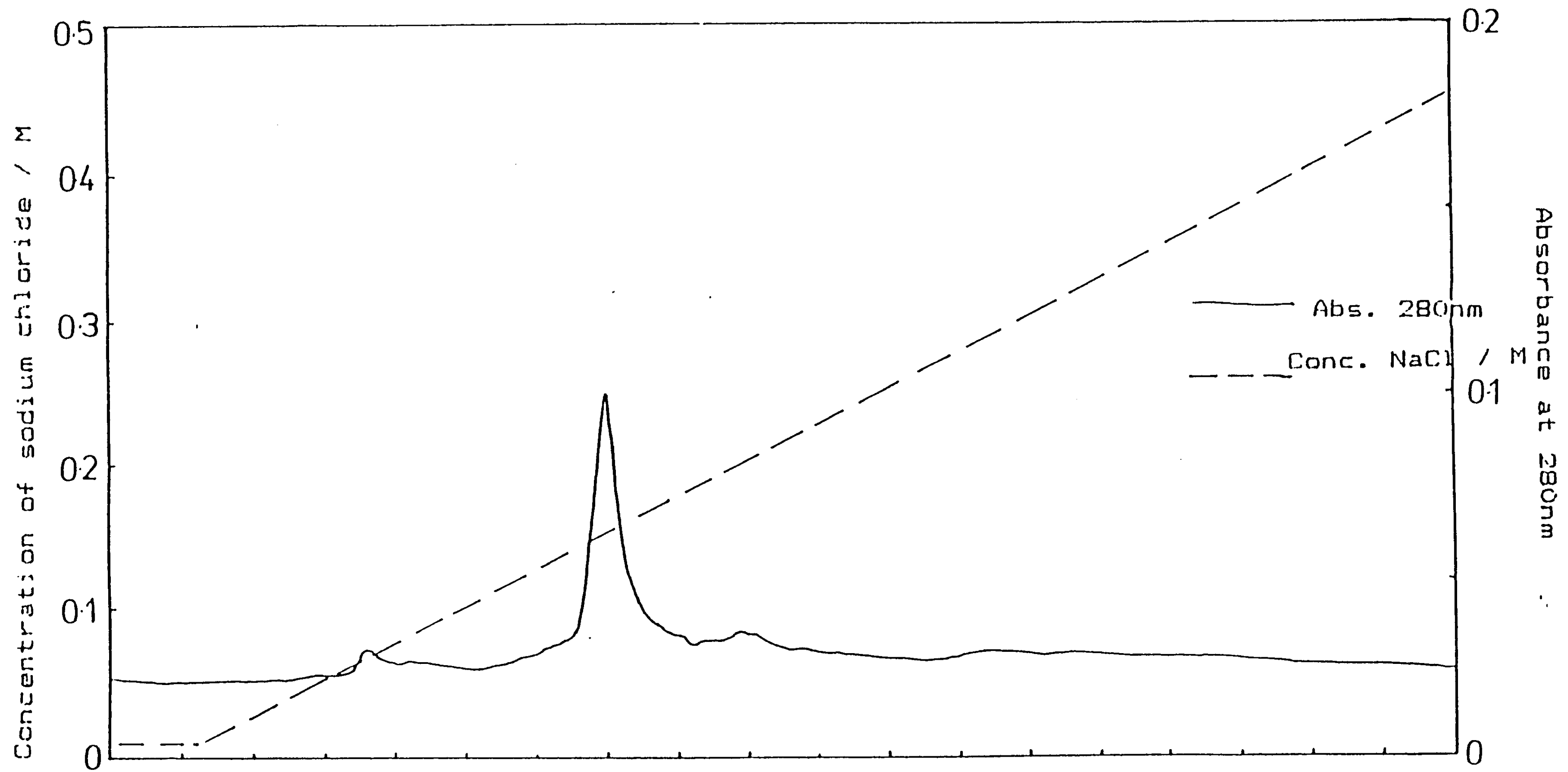


Fig. 21 Purification of Isozyme III by FPLC Mono Q anion-exchange chromatography

Fig. 22 Purification of Isozyme IV by FPLC Mono Q anion-exchange chromatography

After the preliminary purification of Isozyme IV by tris-acryl DEAE sepharose anion-exchange chromatography (see Section 2.4.2), the isozyme was further purified by FPLC Mono Q anion-exchange chromatography. Running conditions were as described in Section 2.4.3.

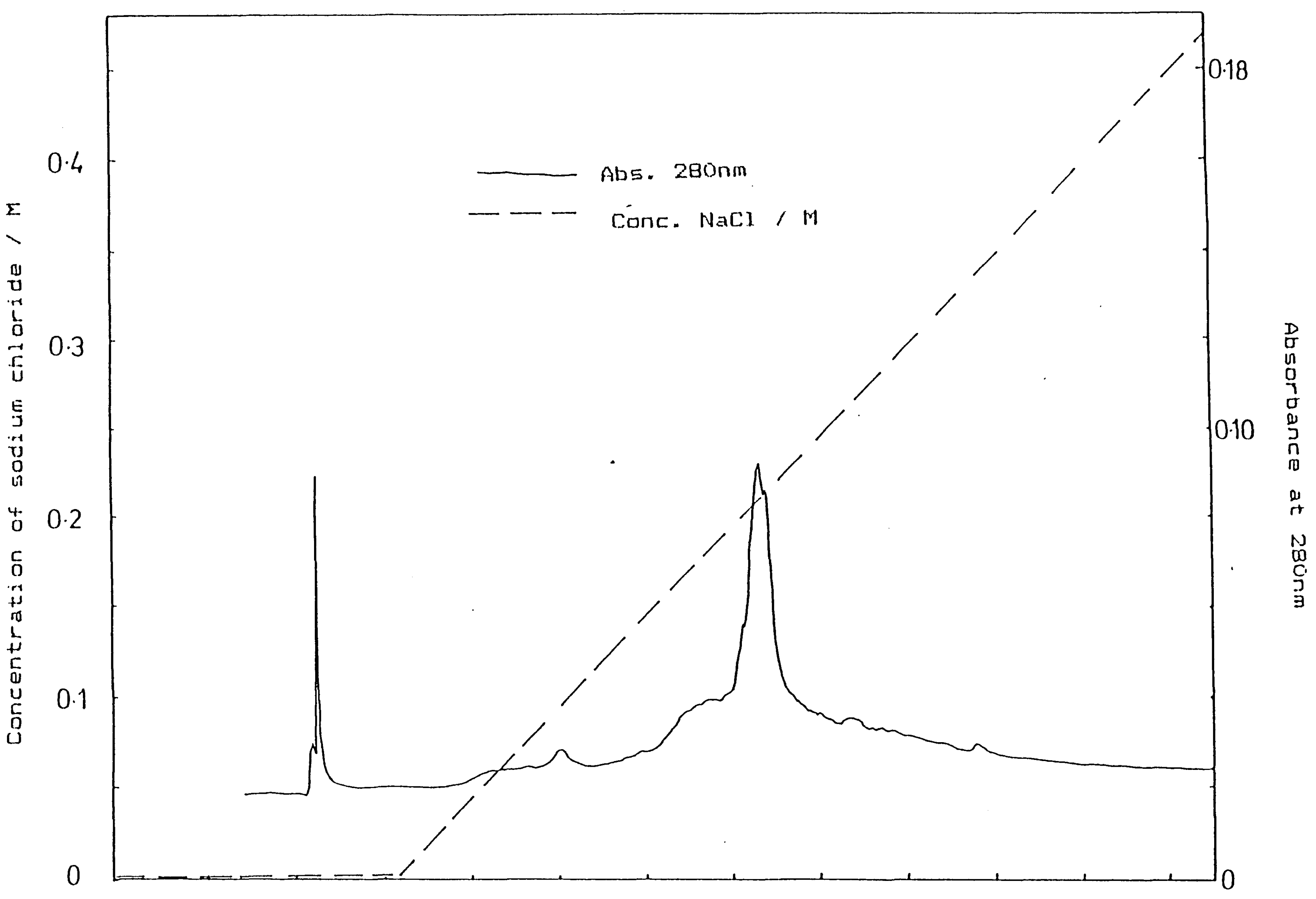


Fig. 22 Purification of Isozyme IV by FPLC Mono Q anion-exchange chromatography

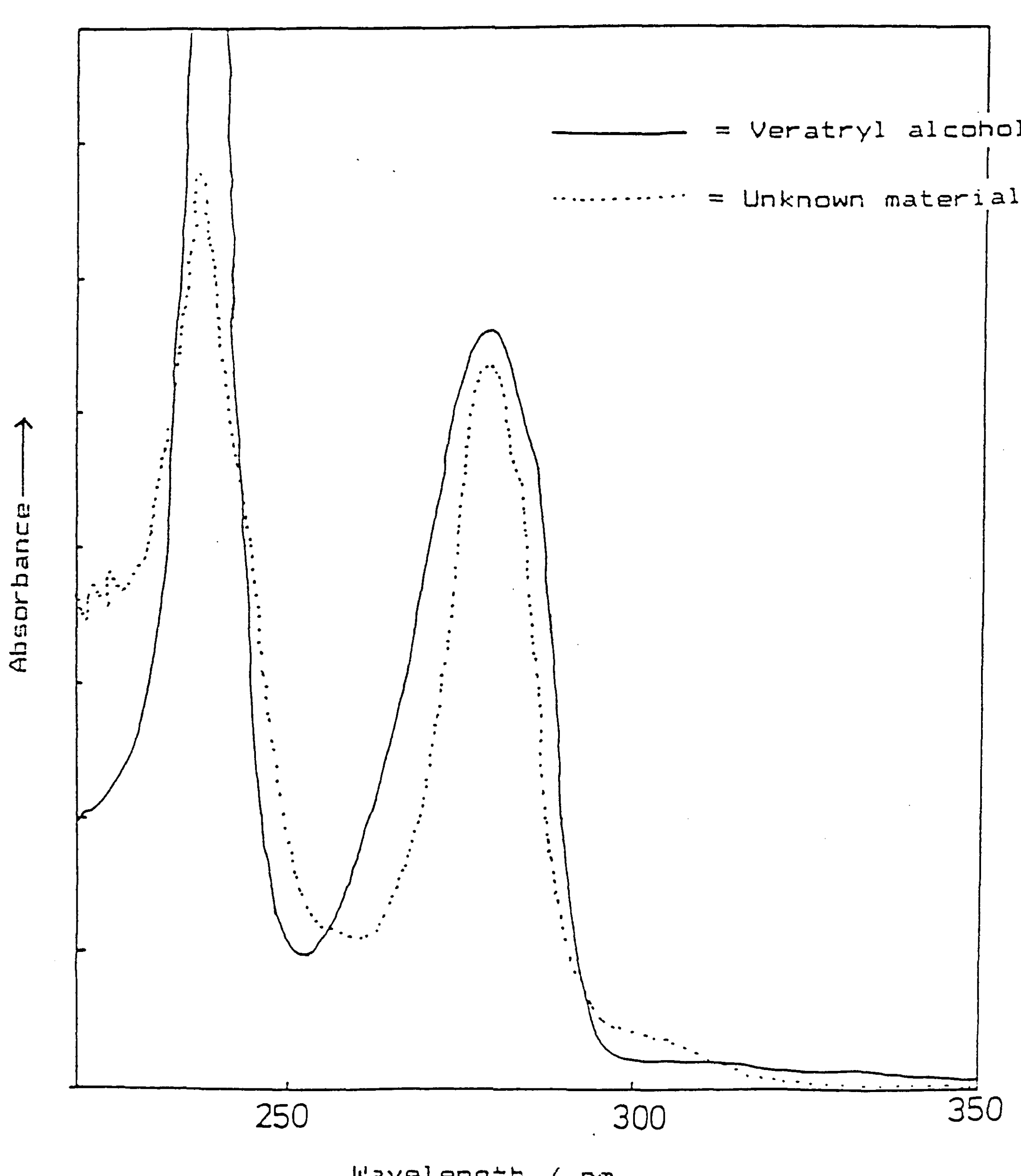


Fig. 23 Comparison of the UV spectrum of the first fraction collected during the purification of Isozyme I by FPLC Mono Q chromatography with that of veratryl alcohol

2.2.2), and the assay mixture then sequentially scanned every 60 seconds between 250nm and 350nm, see Fig. 24.

3.4 A comparison of the properties of a crude preparation of ligninase with those of the main isozymes of ligninase purified by anion-exchange chromatography

3.4.1 Introduction

In this section, the properties of crude ligninase are compared with those of the three main isozymes of ligninase that were purified from the same preparation. The crude preparation of ligninase was obtained by pooling the first five successive harvests of ligninase produced using foam-immobilised P. chrysosporium; each harvest having been individually concentrated by ultrafiltration, and then lyophilisation and resuspension in a smaller volume of solution, and then, having pooled each of these preparations, the resultant preparation was then further concentrated by acetone precipitation (see Section 2.4.1). The purified isozymes of ligninase were obtained by the purification of the pooled concentrated crude preparation described above. The initial method of purification was by tris-acryl DEAE sepharose anion-exchange chromatography, and then each of the main isozymes of ligninase separated by this method, was then further purified by FPLC Mono Q anion-exchange chromatography (see Sections 2.4.2 and 2.4.3).

Fig. 24 Sequential alteration of the UV spectrum of the "unknown material" collected during the purification of Isozyme I when substituted for veratryl alcohol in a standard veratryl alcohol oxidase assay

The "unknown material" was collected as the first fraction when Isozyme I was further purified by FPLC Mono Q anion-exchange chromatography (see Section 2.4.3). Assay conditions were as described in Section 2.2.2, with scans being repeated every 60 seconds. The unknown material was used without dilution relative to when it was eluted from the Mono Q column, with 100µl of this material being substituted for authentic veratryl alcohol in an assay in which the total volume was 1.0ml.

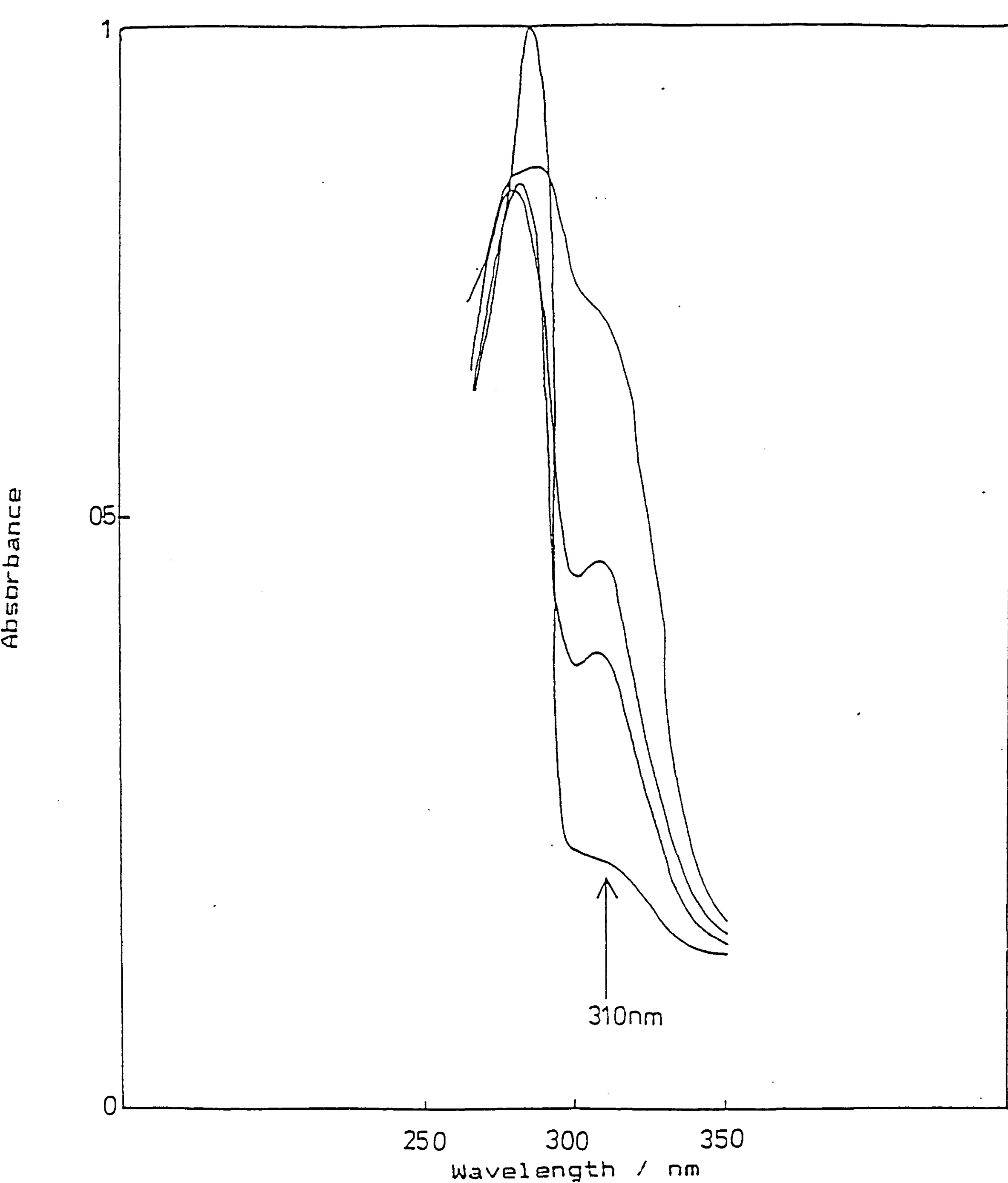


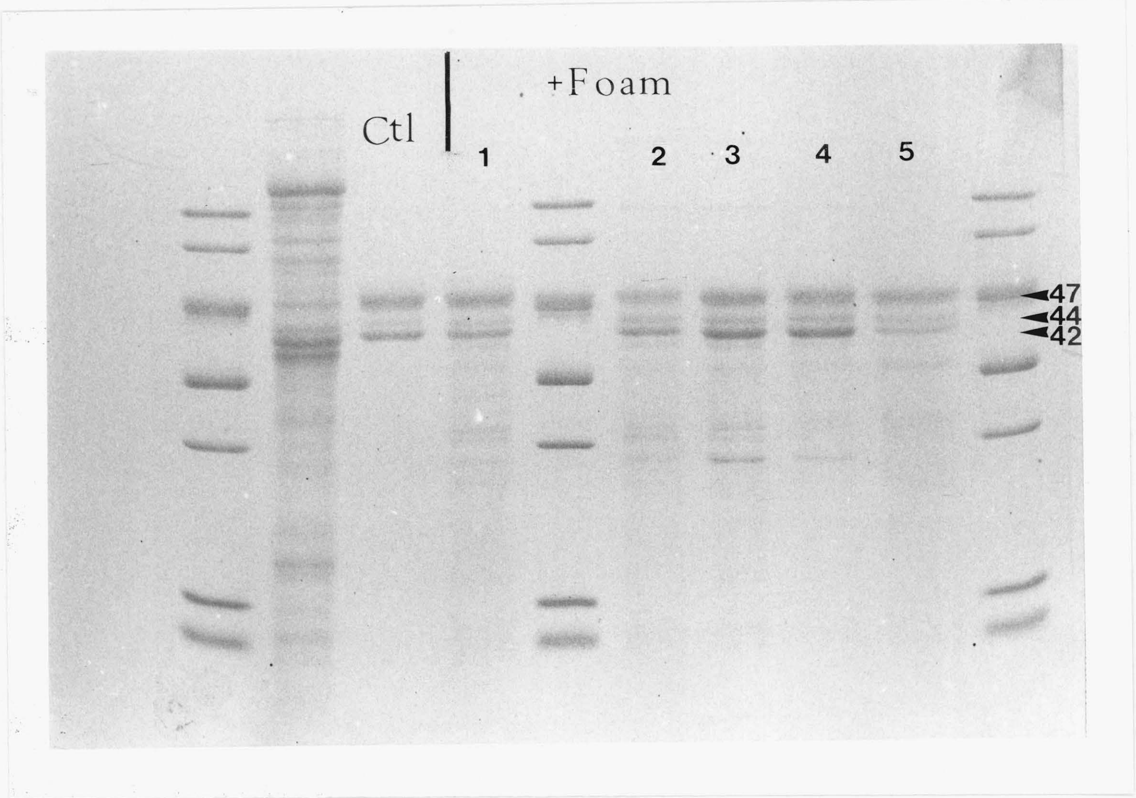
Fig. 24 Sequential alteration of the UV spectrum of the "unknown material" collected during the purification of Isozyme I when substituted for veratryl alcohol in a standard veratryl alcohol oxidase assay

3.4.2 SDS-polyacrylamide gel electrophoresis

Before pooling the individual successive harvests of concentrated ligninase produced using foam-immobilised P.chrysosporium, the extracellular proteins produced in each successive harvest were first compared by SDS-polyacrylamide gel electrophoresis to establish that each harvest contained the same set of proteins. This was carried out as described in Section 2.5.1. From the results presented in Fig. 25, it can be seen that both the control cultures (i.e. those that contained no cubes of polyurethane foam) and also each of the five successive harvests produced by the foam-immobilised cultures, all showed the presence of three main bands with molecular weights between c.42-47Kd. In addition to these bands, the successive harvests produced by the foam-immobilised cultures also showed the presence of a number of lower molecular weight bands, the latter appearing less intensely stained with Coomassie Blue than the three higher molecular weight bands. From Fig. 25, it might also be tentatively concluded that one of these bands which had an apparent molecular weight of about 24Kd was produced in relatively larger quantities in the third successive harvest; however, this might simply have been due to differences in the loading of the gel.

From the calibration curve obtained by plotting Log_{10} Molecular weight of the markers (see Appendix II) against the distance migrated through the gel (of which a typical example is shown in Fig. 26), the molecular weights of the

Fig. 25 A comparison of the SDS-polyacrylamide gel electrophoresis of the extracellular proteins produced by *P.chrysosporium* in the presence and absence of cubes of polyurethane foam



This figures shows the SDS-polyacrylamide gel electrophoresis of the extracellular proteins produced by control cultures of *P.chrysosporium* (i.e. no foam), and also of the first five successive harvests produced by foam-immobilised *P.chrysosporium*. SDS-polyacrylamide gel electrophoresis was carried out as described in Section 2.5.1, and culture conditions were as described in Sections 2.3.3 and 3.2.3.

(Ctl = control flasks (i.e.no foam); +Foam 1 to 5 = 5 successive harvests produced using foam-immobilised cultures; numbers on far right are molecular weights in kilodaltons)

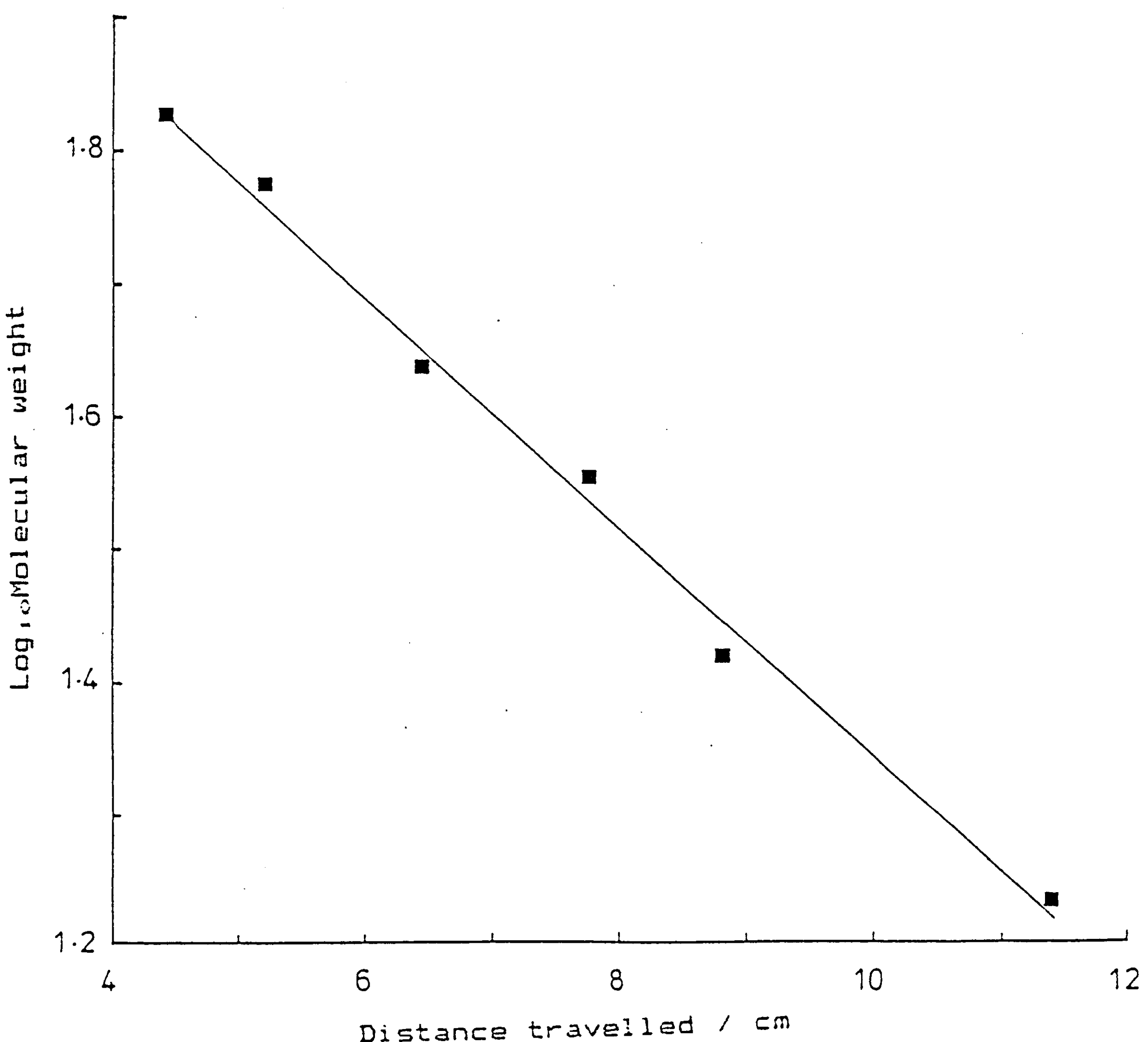


Fig. 26 Typical calibration curve showing $\log_{10}$molecular weight of a range of markers against distance travelled when separated by SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out as described in Section 2.5.1. For details of the markers, see Appendix II.

three main bands were determined to be c.42Kd, c.44Kd and c.47Kd, respectively.

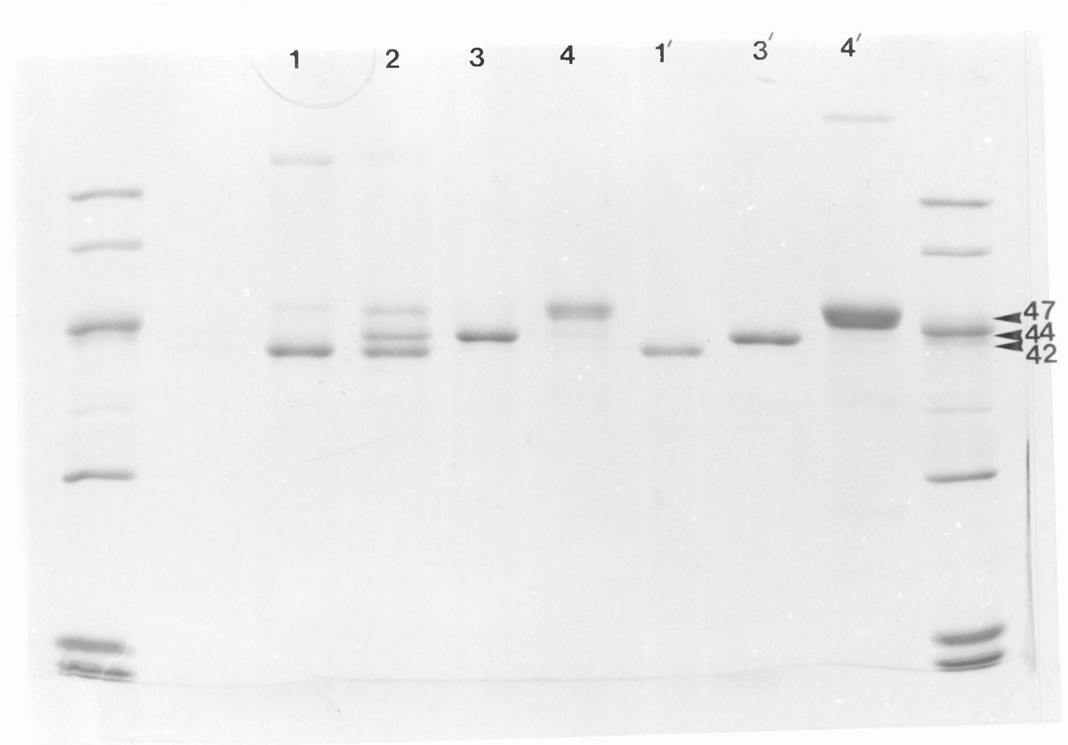
From the SDS-polyacrylamide gel electrophoresis of the purified isozymes of ligninase, it was possible to determine the precise molecular weight of each of the three main isozymes of ligninase, and at the same time, to assess their relative degree of purification.

The appearance of the gel after staining is shown in Fig. 27. From this figure, it can be seen that of the four fractions separated by tris-acryl DEAE sepharose chromatography, Fraction II was apparently composed of a mixture of Fractions I, III and IV, and consequently, this fraction was not further purified.

It can also be concluded from Fig. 27, that after the second phase of purification, the highest molecular weight isozyme, i.e. Isozyme IV (See Section 3.3.2 for explanation of nomenclature of the isozymes), was still relatively impure compared to the other two main isozymes. In fact, it would appear that Isozyme IV might actually have consisted of two distinct major proteins; each having a very similar molecular weight (see also The Discussion to this thesis). Unfortunately, during the course of this thesis, it was not possible to purify these isozymes further.

The molecular weights of the three main isozymes of ligninase produced by the foam-immobilised cultures of <u>P. chrysosporium</u> were 41.7Kd, 43.7Kd and 46.7Kd, respectively

Fig. 27 SDS-polyacrylamide gel electrophoresis of the isozymes of ligninase



This figure shows the SDS-polyacrylamide gel electrophoresis of the different fractions initially separated by tris-acryl DEAE sepharose anion-exchange chromatography (see Section 2.4.2), which were then further purified by FPLC Mono Q anion-exchange chromatography (see Section 2.4.3).

(Numbers 1, 2, 3 and 4 refer to Fractions I, II, III and IV, respectively, see Section 3.3.1; numbers 1′, 3′ and 4′ refer to Isozymes I, III and IV, respectively, see Section 3.3.2; numbers on far right are molecular weights in kilodaltons)

(see Fig. 26 for a typical example of a calibration curve used to determine the molecular weight of unknown proteins).

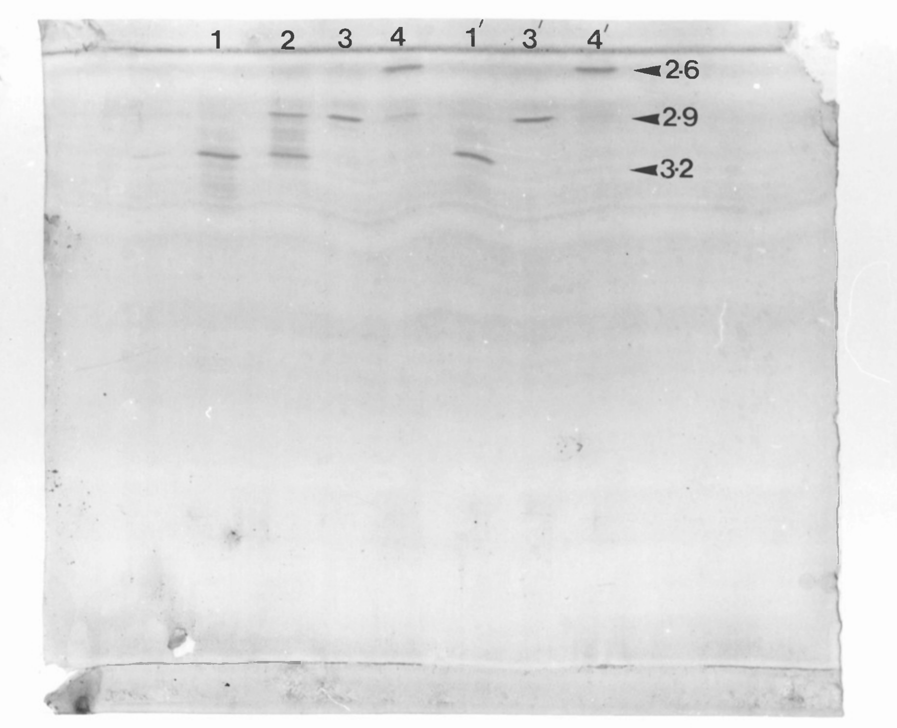
3.4.3 Determination of the isoelectric points of the isozymes of ligninase by isoelectric focusing

This was carried out as described in Section 2.6.6. Unfortunately, the track loaded with the crude preparation of ligninase was underloaded, and consequently did not show any bands after staining; see Fig. 28. However, by reference to the pH gradient across the gel; see Fig.29, it can be concluded that the isoelectric points of Isozymes I, III and IV were at pH 2.6, 2.9 and 3.2, respectively.

3.4.4 Spectral properties

It was found that the UV/visible spectra for concentrated preparations of ligninase prepared from both control cultures containing no cubes of foam, and also for each of the successive harvests obtained from foam-immobilised cultures, had essentially the same appearance. A typical UV/visible spectrum of a crude preparation of ligninase is shown in Fig. 30. It can be seen that the spectrum has a Soret band at c.410nm. However, other than this, it is not possible to accurately pick out any other features of the spectrum, although it might tentatively be concluded that there is at least one visible band occurring at c.640nm.

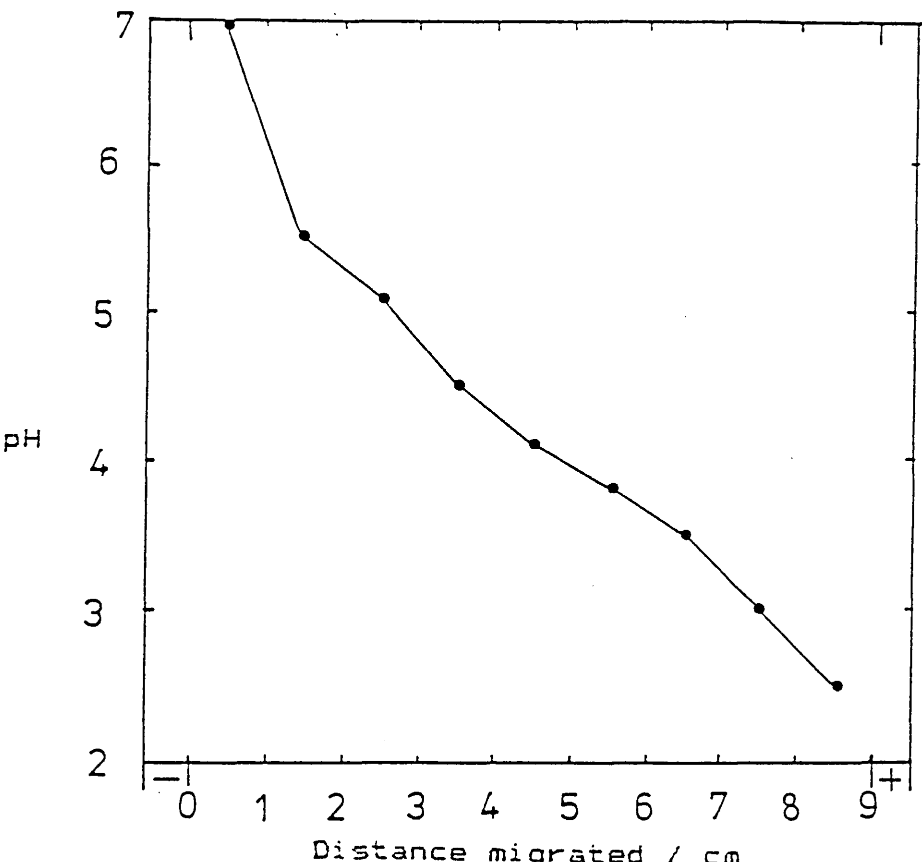
Fig. 28 Isoelelcric focusing of the isozymes of ligninase



This figure shows the isoelectric focusing of the isozymes of ligninase separated initially by tris-acryl DEAE sepharose anion-exchange chromatography (see Section 2.4.2), and then further purified by FPLC Mono Q anion-exchange chromatography (see Section 2.4.3).

(Numbers 1, 2, 3 and 4 refer to Fractions I, II, III and IV, respectively, see Section 3.3.1; numbers 1′, 3′ and 4′ refer to Isozymes I, III and IV, respectively, see Section 3.3.2; numbers on far right refer to isoelectric points)

Fig. 29 pH gradient across the isoelectric focusing gel used to determine the isoelectric points of the isozymes of ligninase



The isoelectric focusing of the isozymes of ligninase, and also the determination of the pH gradient across the isoelectric focusing gel, was carried out as described in Section 2.6.4.

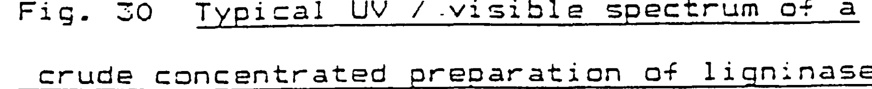


Fig. 30 Typical UV / visible spectrum of a crude concentrated preparation of ligninase

This spectrum was obtained using a crude preparation of ligninase obtained by pooling the first five harvests of ligninase produced using foam-immobilised *P.chrysosporium*; see Section 2.4.2. The preparation of enzyme used was diluted such that it contained 4.0 $U.ml^{-1}$ when assayed as described in Section 2.2.2. All spectra were performed as described in Section 2.5.2.

By comparison, the UV/visible spectra of the three main isozymes of ligninase produced by foam-immobilised cultures of P. chrysosporium, i.e. Isozymes I, III and IV, are much clearer, see Figs. 31, 32 and 33, respectively; and from these, it can be seen that for each isozyme, there is a Soret band occurring at c.408nm, and that there are also at least three visible bands occurring at c.500nm, c.590nm and c.630nm.

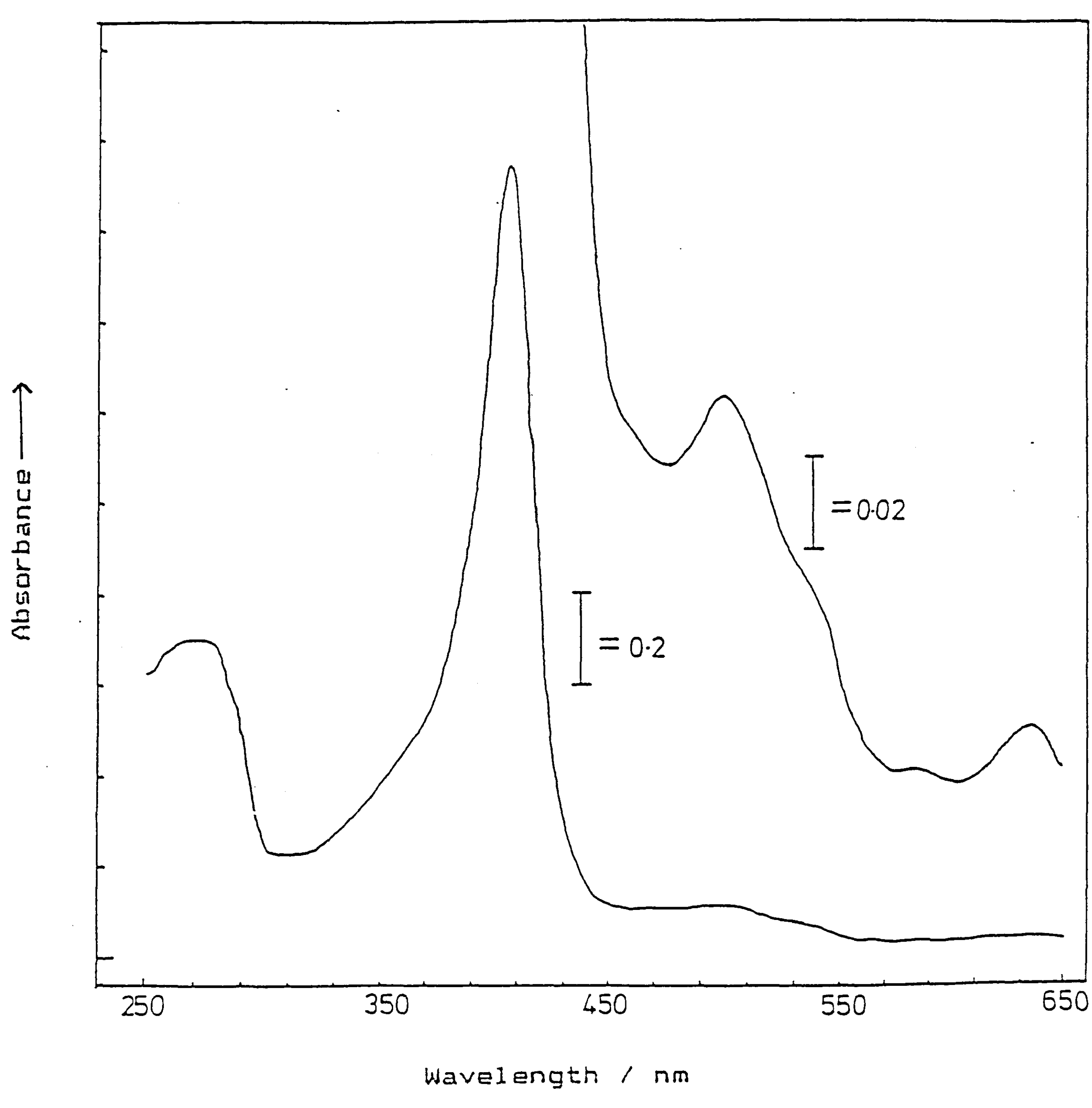
3.4.5 pH optima

The determination of the pH optimum of veratryl alcohol oxidase activity in concentrated preparations of ligninase was carried out as described in Section 2.5.3. It was found that the pH optimum of crude ligninase was pH 3.1; see Fig. 34. It can also be seen that this pH curve is not symmetrical and that there is an apparent shoulder to the curve at about pH 2.5.

By comparison, it was found that for the three main isozymes of ligninase, i.e. Isozymes I, III and IV, the pH optimum of veratryl alcohol oxidase activity was at pH 2.2, pH 2.8 and pH 2.0, respectively, see Figs. 35, 36 and 37.

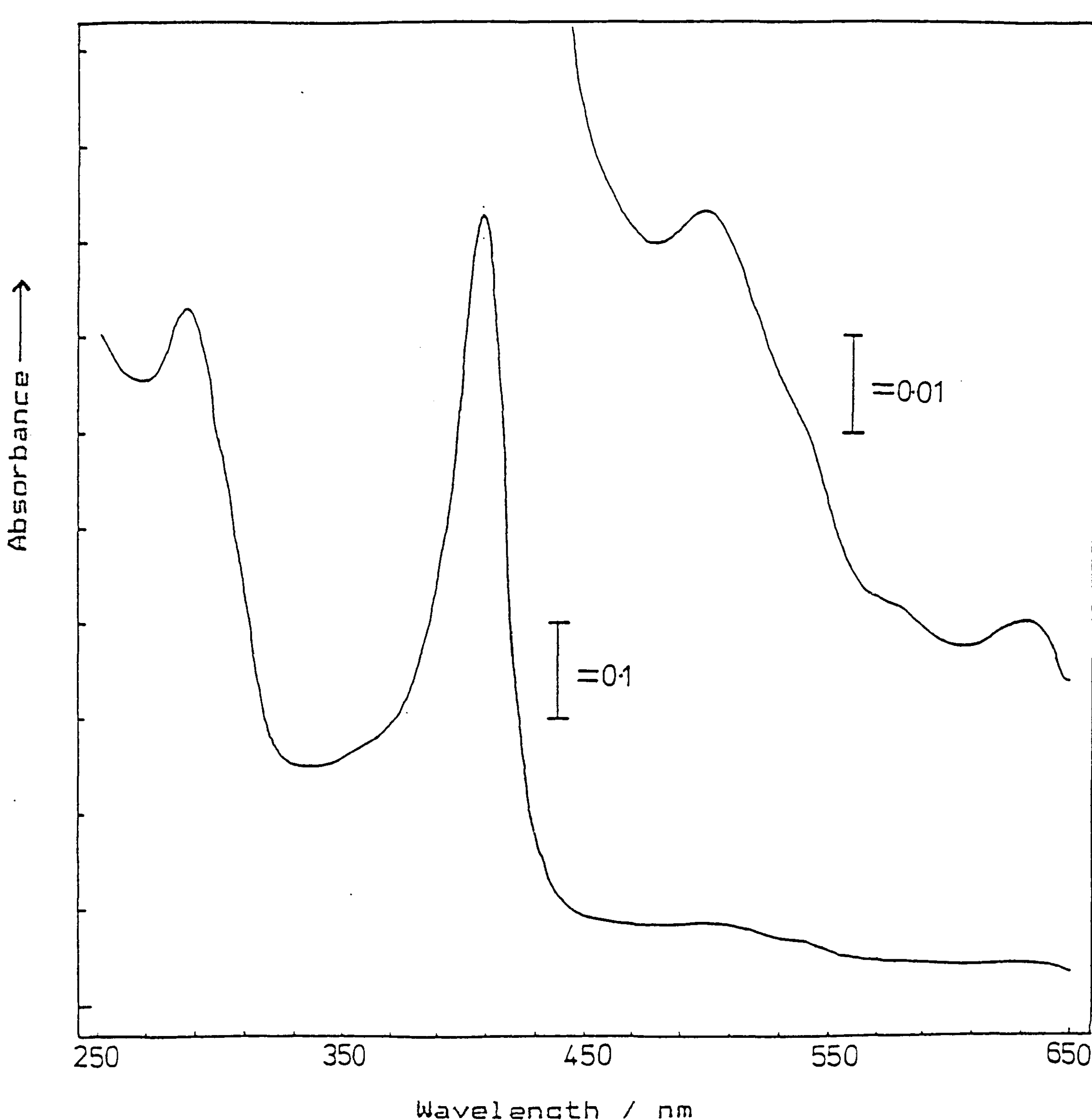
It was also found for Isozyme I, the use of 20mM glycine/HCl buffers instead of 20mM phosphate buffers, did not alter the appearance of the pH curve (data not shown).

Fig. 31 UV / visible spectrum of Isozyme I



This spectrum was performed as described in Section 2.2.2, using a dilution of Isozyme I that contained 25.0 $U.ml^{-1}$ when assayed as described in Section 2.2.2.

Fig. 32 UV / visible spectrum of Isozyme III



This spectrum was performed as described in Section 2.2.2, using a dilution of Isozyme III that contained 9.0 $U.ml^{-1}$ when assayed as described in Section 2.2.2.

Fig. 33 UV / visible spectrum of Isozyme IV

=0·01

=0·1

Absorbance →

250 350 450 550 650

Wavelength / nm

This spectrum was performed as described in Section 2.2.2, using a dilution of Isozyme IV that contained 5.0 U.ml^{-1} when assayed as described in Section 2.2.2.

Fig. 34 Determination of the pH optimum of a crude concentrated preparation of ligninase

The crude preparation of ligninase used in this experiment was obtained by pooling the first five harvests using foam-immobilised *P.chrysosporium* as described in Section 2.4.2. The method used to determine the pH optimum of the veratryl alcohol oxidase activity of this enzyme preparation was as described in Section 2.5.3, with the alteration of the pH curve in the presence of magnesium ions being observed when magnesium ions were used at a final concentration of 1.0mM; see Section 2.5.4.

Fig. 34 Determination of the pH optimum of a crude concentrated preparation of ligninase

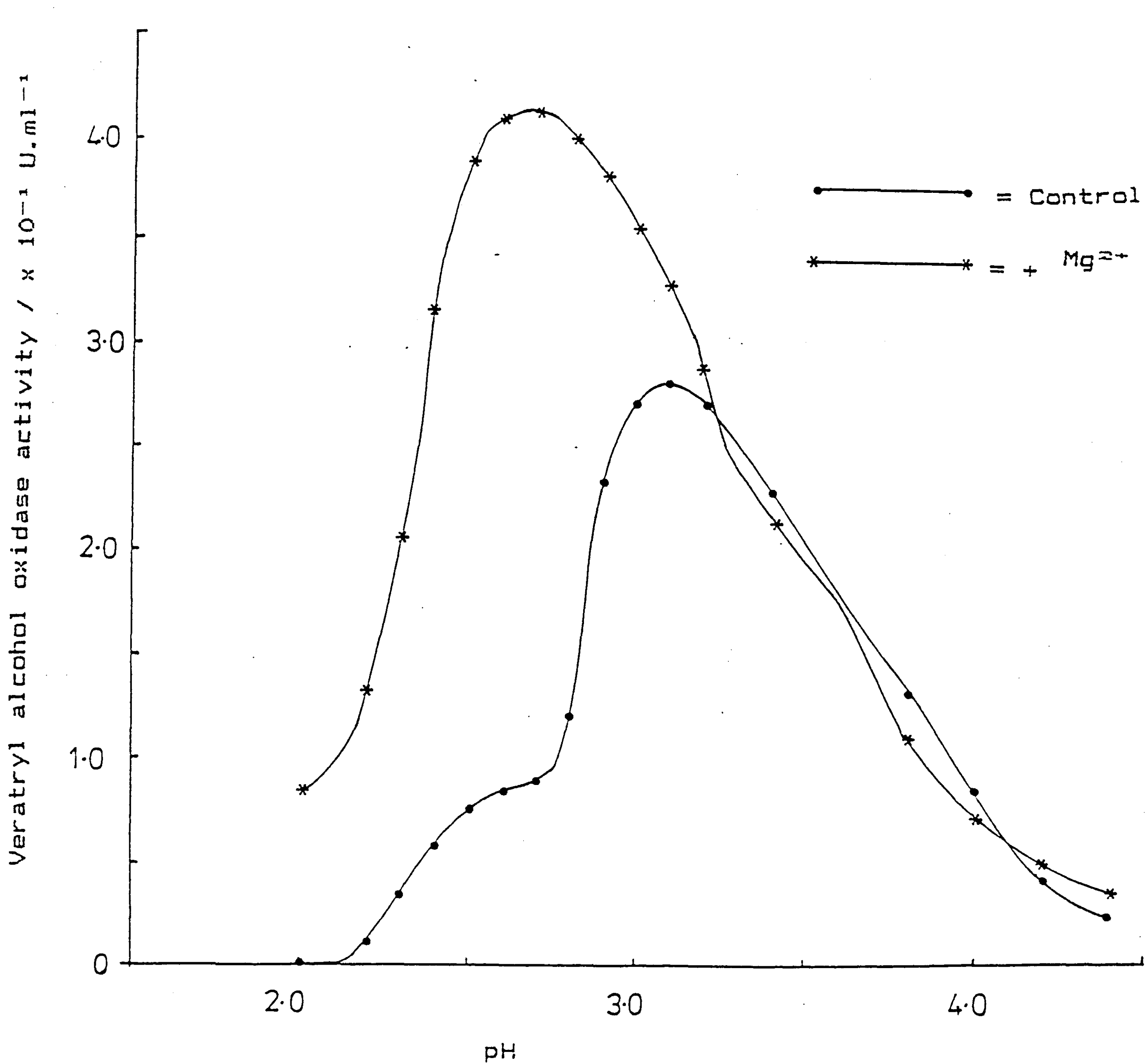
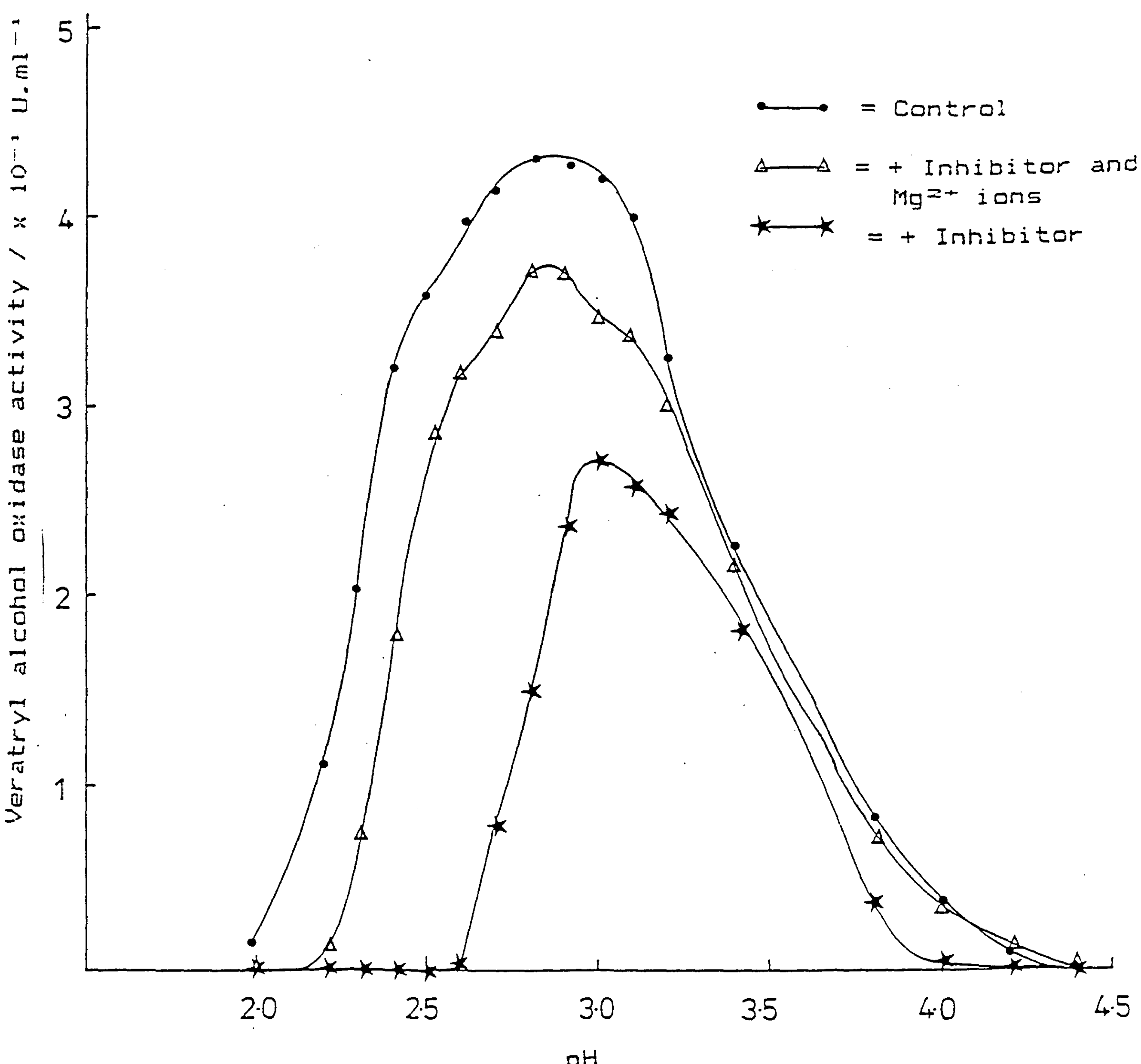


Fig. 35 Determination of the pH optimum of Isozyme I

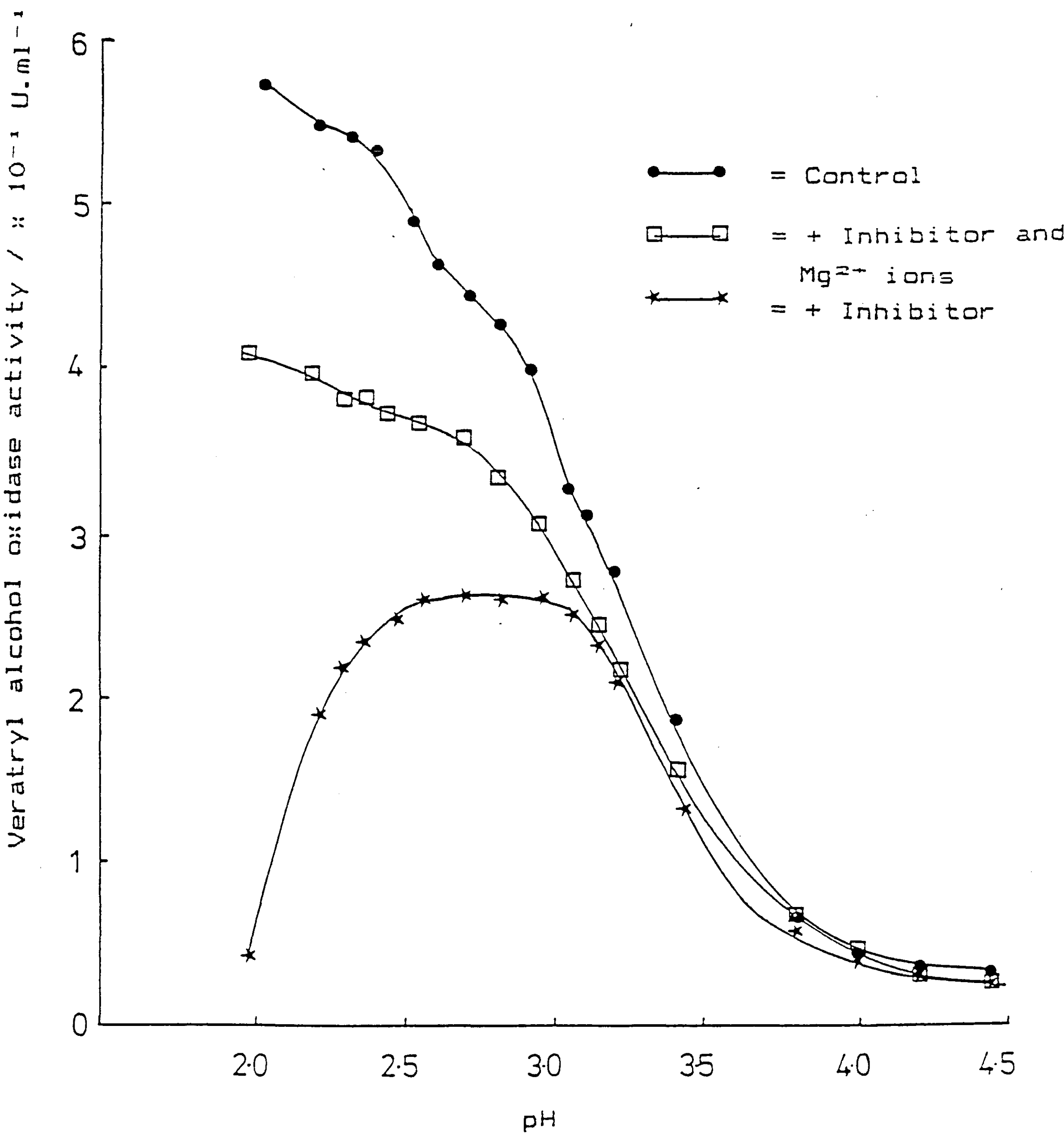
This experiment was carried out using a preparation of Isozyme I purified as described in Sections 2.4.2 and 2.4.3. The effect of an aliquot of the inhibitor and also of the inhibitor and magnesium ions on the pH optimum of Isozyme I were carried out as described in Section 2.7.3.

Fig. 36 <u>Determination of the pH optimum of Isozyme III</u>



This experiment was carried out using a preparation of Isozyme III purified as described in Sections 2.4.2 and 2.4.3. The effect of an aliquot of the inhibitor and also of the inhibitor and magnesium ions on the pH optimum of Isozyme I were carried out as described in Section 2.7.3.

Fig. 37 Determination of the pH optimum of Isozyme IV



This experiment was carried out using a preparation of Isozyme IV purified as described in Sections 2.4.2 and 2.4.3. The effect of an aliquot of the inhibitor and also of the inhibitor and magnesium ions on the pH optimum of Isozyme I were carried out as described in Section 2.7.3.

3.4.6 Determination of the % carbohydrate present relative to protein in crude and purified preparations of ligninase

The results presented in Table 10 show that when the concentration of carbohydrate and protein were determined in the crude preparation of ligninase as described in Sections 2.2.7 and 2.5.7, respectively, it was found that for an accurate determination of the relative % of carbohydrate actually present as part of the ligninase molecules, it was necessary to separate the proteins present from the large amount of free carbohydrate that was also present. This was achieved by precipitating the proteins out of solution as described in Section 2.5.8.

It was found that for the crude preparation of ligninase used in this thesis, the relative % carbohydrate present was 22.2%, and that for Isozymes I and III, the relative % carbohydrate present were 21.9% and 23.0%, respectively. Unfortunately, because of the sensitivities of the protein and carbohydrate assay methods used in this thesis, and also since TCA precipitation is a destructive technique that results in the denaturation of proteins, it was not possible to provide sufficient quantities of Isozyme IV to obtain an accurate figure for the % carbohydrate present relative to protein for this isozyme.

In addition to showing the % carbohydrate present as part of the proteins in the concentrated ligninase preparation, the results presented in Table 10 also illustrate that when the proteins present were separated from the excess carbohydrate by the TCA precipitation,

Table 10 Summary of the protein and carbohydrate concentrations of crude and purified ligninase

Before TCA precipitation	Protein (mg.ml⁻¹)	Carbohydrate (mg.ml⁻¹)	% carbohydrate relative to protein	Ligninase (U.ml⁻¹)	Sp. Ac (U.mg⁻¹)
Crude enzyme	0.67	49.95	>7000	29.9	44.6
Isozyme I	2.07	2.82	136.2	52.5	25.4
Isozyme III	0.12	1.22	>1000	3.5	29.2
Isozyme IV	0.11	3.00	>2000	5.7	51.8
After TCA precipitation					
Crude enzyme	4.36	0.97	22.2	29.9	6.9
Isozyme I	2.94	0.64	21.9	52.5	17.9
Isozyme III	0.221	0.051	23.0	3.5	15.8
Isozyme IV	ND	ND	ND	5.7	ND

The results presented above for the protein and carbohydrate concentrations of crude and purified ligninase, show that after TCA precipitation, the concentration of protein in the samples apparently increases, thus leading to the conclusion that the presence of contaminating carbohydrate leads to the "masking" of the protein present and thus gives an over-estimate of the true concentration of protein. The significance of this observation to the calculation of specific activities is disussed in The Discussion to this thesis.

then there was apparently a greater concentration of protein present than was originally able to be detected, hence making previous assessments of specific activities completely unreliable, see also The Discussion to this thesis.

To determine whether the high concentration of carbohydrate present relative to that of protein was a feature restricted to use of foam-immobilised cultures of P. chrysosporium, the relative concentrations of carbohydrate and protein present in concentrated preparations of ligninase produced using control cultures containing no cubes of foam were compared to those levels of carbohydrate and protein present in each of the first five successive harvests of concentrated produced using foam-immobilised P.chrysosporium; see Table 11.

From this table, it can be seen that for all of these preparations, the carbohydrate concentration was in much the same magnitude and was also significantly lower than that present in the pooled concentrated ligninase preparation used for the results obtained in Table 10. This probably accounts for the apparently lower specific activities of the ligninase in these preparations (see also The Discussion to this thesis).. In addition, it can be seen that although the concentration of carbohydrate was indeed initially greater in the preparations of ligninase produced using the foam-immobilised cultures, it did show a steady decrease as continued successive harvests of ligninase were produced from the same biomass of the fungus.

Table 11 Carbohydrate and protein concentrations of harvests of ligninase produced in the presence and absence of polyurethane foam as an immobilisation support

Sample	Ligninase ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Sp.Act. ($U.mg^{-1}$)	Carbohydrate ($mg.ml^{-1}$)	Relative % Carbohydrate to protein
Control (no foam)	0.938	0.223	4.20	3.14	>1400
+ Foam / 1	1.180	0.341	3.46	8.97	>2600
+ Foam / 2	1.200	0.336	3.57	6.53	>1900
+ Foam / 3	0.710	0.307	2.31	5.22	>1700
+ Foam / 4	0.280	0.172	1.63	3.83	>2200
+ Foam / 5	0.315	0.169	1.86	2.03	>1200

Foam / 1 to 5 refers to five successive harvests of ligninase produced using foam-immobilised *P.chrysosporium*. Carbohydrate and protein determinations were carried out as described in Sections 2.2.7 and 2.5.7, respectively.

3.4.7 Amino acid analysis of the isozymes of ligninase

This was carried out as described in Section 2.6.5. The results obtained are presented in Table 12, and show that the amino acid composition of Isozyme I is typical of that of an acidic glycoprotein (see The Discussion to this thesis).

3.4.8 Effect of metal ions on ligninase activity

After the concentration and pooling of the crude successive harvests of ligninase produced using the foam-immobilised *P. chrysosporium*, it became apparent that during the concentration procedure, there was a significant loss in the total number of units of ligninase that was originally thought to have been present. In an attempt to determine the reasons for this loss of enzyme activity, an experiment was carried out in which an aliquot of unconcentrated spent medium was added back to the crude concentrated preparation of ligninase. When it was found that this led to a stimulation of veratryl alcohol oxidase activity, it was then decided to repeat this experiment using fresh uninoculated medium. It was found that this also led to a stimulation of the veratryl alcohol oxidase activity, and consequently, it was then decided to investigate each component in the growth medium individually for its ability to stimulate the veratryl alcohol oxidase activity (see Appendix I). From this experiment, it was demonstrated that the component that was responsible for the stimulation of the veratryl alcohol oxidase activity, was the mineral solution.

Table 12 Amino acid analysis of Isozyme I

Amino Acid	No. moles per molecule
Aspartate	27
Threonine	24
Serine	32
Glutamate	29
Proline	24
Glycine	26
Alanine	32
Cysteine	20
Valine	25
Methionine	5
Isoleucine	15
Leucine	18
Tyrosine	2
Phenylalanine	21
Lysine	8
Histidine	8
Arginine	9

The amino acid of Isozyme I was carried out as described in Section 2.6.5. The above calculations were made assuming a molecular weight of 41700Kd and a carbohydrate content of 21.9%.

A range of experiments were then carried out to determine which of the metal ions in the mineral solution were capable of stimulating the veratryl alcohol oxidase activity, and also to determine the differences in the kinetics of veratryl alcohol oxidase acivity when "stimulated" compared to "non-stimulated", and it was hoped that this series of experiments would help elucidate the precise nature of how veratryl alcohol oxidase activity was being stimulated. These experiments are outlined in Section 2.5.4.

In the first experiment, the effect of a range of metal ions, each at 1.0mM concentration (unless stated otherwise), on the veratryl alcohol oxidase activity of the crude preparation of ligninase produced using foam-immobilised P. chrysosporium (see Section 2.4.2) was examined; the results obtained are presented in Table 13, where it can be seen that the stimulation of the veratryl alcohol oxidase activity of the crude preparation of ligninase tested was apparently a general phenomenon, and was not restricted to specific ions only.

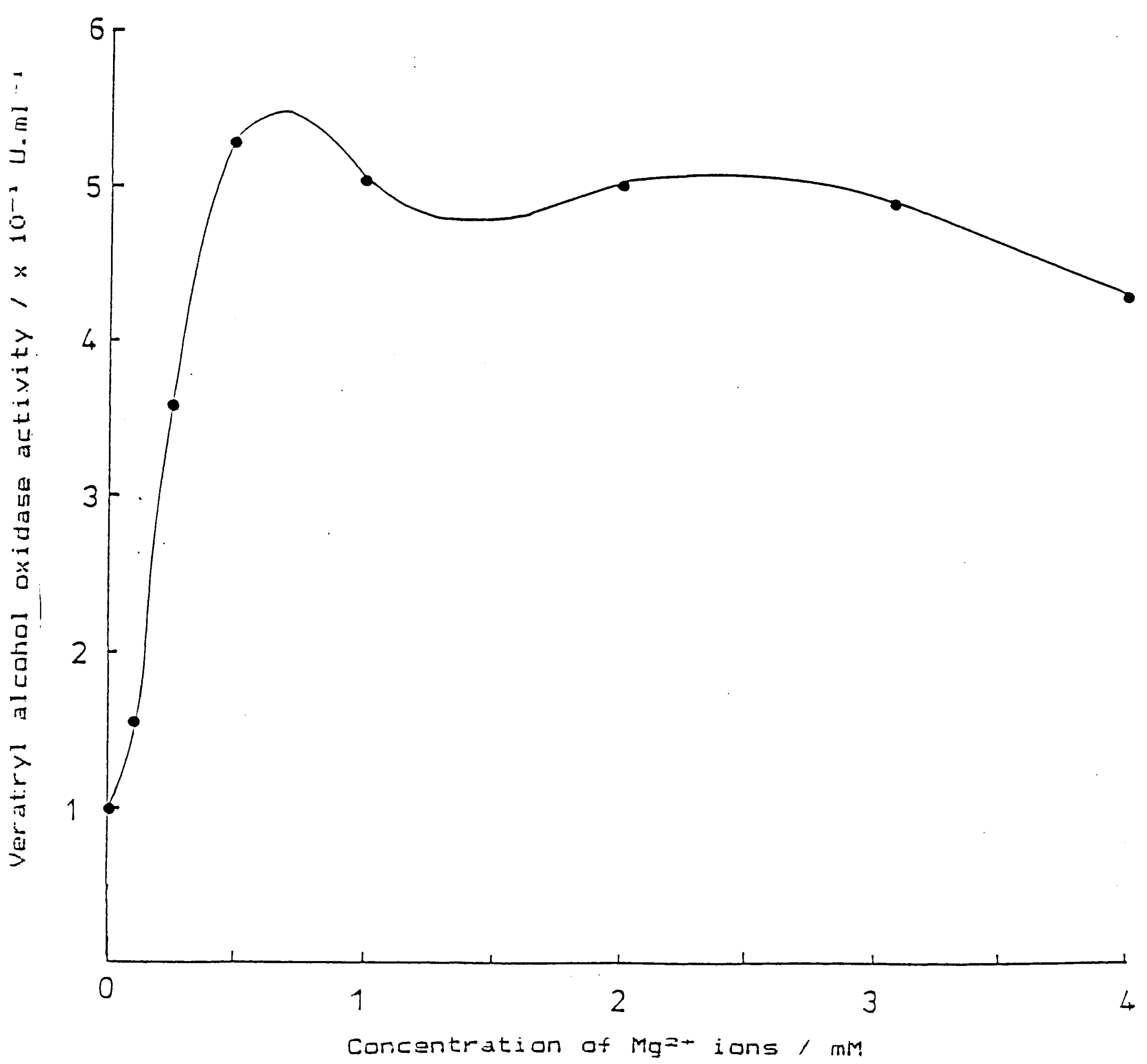
From the results presented in Table 13, it was decided to select one divalent metal cation, and then to examine the effect of a range of concentrations of this ion on the veratryl alcohol oxidase activity of the crude preparation of ligninase. The metal ion chosen was Mg^{2+}, and the results obtained are presented in Fig. 38. It can be seen that under the assay conditions used, the maximum stimulation of ligninase activity was produced at concentrations of Mg^{2+} of 1.0mM or above.

Table 13 Effect of a range of cations on the veratryl alcohol oxidase activity of a crude preparation of ligninase

Cation	Concentration / mM	Relative increase in rate of activity
Mg^{2+}	1.0	x 6.4
Mn^{2+}	1.0	x 5.8
Ca^{2+}	1.0	x 5.7
Zn^{2+}	1.0	x 6.2
Co^{2+}	1.0	x 4.7
La^{3+}	1.0	x 5.5
Na^{+}	1.0	x 1.0
Na^{+}	10.0	x 1.0
Na^{+}	50.0	x 2.5
Decamethonium	1.0	x 1.0

The above set of results were obtained using a crude preparation of ligninase that had been produced by pooling the first five harvests produced using foam-immobilised *P.chrysosporium* (see Section 2.5.4). Veratryl alcohol oxidase was assayed as describe in Section 2.2.2.

Fig. 38 Effect of concentration of Mg^{2+} ions on the veratryl alcohol oxidase activity of a crude preparation of ligninase



This experiment was carried out using a crude preparation of ligninase obtained by pooling the first five harvests of ligninase produced using foam-immobilised *P.chrysosporium*; see Section 2.4.2. The effect of Mg^{2+} on the veratryl alcohol oxdase activity of this preparation was carried out as described in Section 2.5.4.

The next experiment to be carried out was to determine whether the stimulation of veratryl alcohol oxidase activity was also observed for concentrated preparations of ligninase produced in cultures containing no cubes of foam, and also for concentrated preparations of ligninase produced at each successive harvest using foam-immobilised cultures of P. chrysosporium.

The results obtained are presented in Table 14, and show that the stimulation of veratryl alcohol oxidase activity did not occur for a crude concentrated preparation of ligninase produced in the absence of cubes of polyurethane foam, and that it was only significant for the later harvests of ligninase produced using the foam-immobilised cultures; see also The Discussion to this thesis.

When the determination of the pH optimum was repeated in the presence of Magnesium ions (1mM), using the concentrated crude preparation of veratryl alcohol oxidase, it was observed that there was an increase in the rate of veratryl alcohol oxidase activity at pH values lower than pH 3.1, and that there was also an apparent shift in the pH optimum from pH 3.1 in the absence of added Mg^{2+} to pH 2.7 in the presence of added Mg^{2+}, see Fig. 34; see also The Discussion to this thesis.

Table 14 Effect of 1.0mM Mg^{2+} on the veratryl alcohol oxidase activity of different preparations of ligninase produced in the presence and absence of polyurethane foam as an immobilisation support

Sample	Relative increase in activity
Control (no foam)	x 1.04
Foam / 1	x 1.06
Foam / 2	x 1.05
Foam / 3	x 1.44
Foam / 4	x 1.56
Foam / 5	x 10.64
Foam / 6	x 17.43

Foam / 1 to 6 refers to the first six successive of ligninase produced using foam-immobilised *P.chrysosporium* (see Section 2.5.4). Veratryl alcohol oxidase activity was measured as described in setion 2.2.2

Having shown that the stimulation of veratryl alcohol oxidase activity was only observed for the "later" harvests of ligninase produced using foam-immobilised cultures of P. chrysosporium, and also that it was apparently pH-dependent, it was then decided to investigate the effect of one metal ion on the kinetics of veratryl alcohol oxidase activity. From these experiments, it was found that for both H_2O_2 and veratryl alcohol, the presence of 1.0mM Mg^{2+} ions led to an increase in the V_{max} of veratryl alcohol oxidase activity, but did not alter the K_m for either H_2O_2 or veratryl alcohol, see Figs. 39 and 40, respectively (see also The discussion to this thesis). A decrease in the V_{max} with no alteration of the K_m is indicative of noncompetitive inhibition (Rudolph 1983), and thus at this point, it was tentatively concluded that the apparent stimulation of veratryl alcohol oxidase activity by a range of metal ions might actually have been due to an apparent release from a state of inhibition, rather than a true stimulation of the veratryl alcohol oxidase activity.

To investigate this hypothesis further, and also to determine whether the stimulation of veratryl alcohol oxidase activity was still observed for the purified isozymes of ligninase, it was decided to assay the isozymes of ligninase after purification by tris-acryl DEAE sepharose anion-exchange column (see Section 2.4.2) at both pH 2.5 and pH 3.0, and also in the presence and absence of added metal ions at both pH values, since from

Fig. 39 Lineweaver-Burk plot to determine the K_m for hydrogen peroxide and the V_{max} of veratryl alcohol oxidase activity in the presence and absence of Mg^{2+} ions

The effect of 1.0mM Mg^{2+} ions on the K_m for hydrogen peroxide and the V_{max} of veratryl alcohol oxidase activity of a crude preparation of ligninase obtained by pooling the first five harvests of ligninase produced using foam-immobilised *P.chrysosporium* (see Section 2.4.2) was carried out as described in Section 2.5.5.

	$+Mg^{2+}$	No Mg^{2+}
K_m / µM	40.6	39.7
V_{max} / U	5.99	1.29

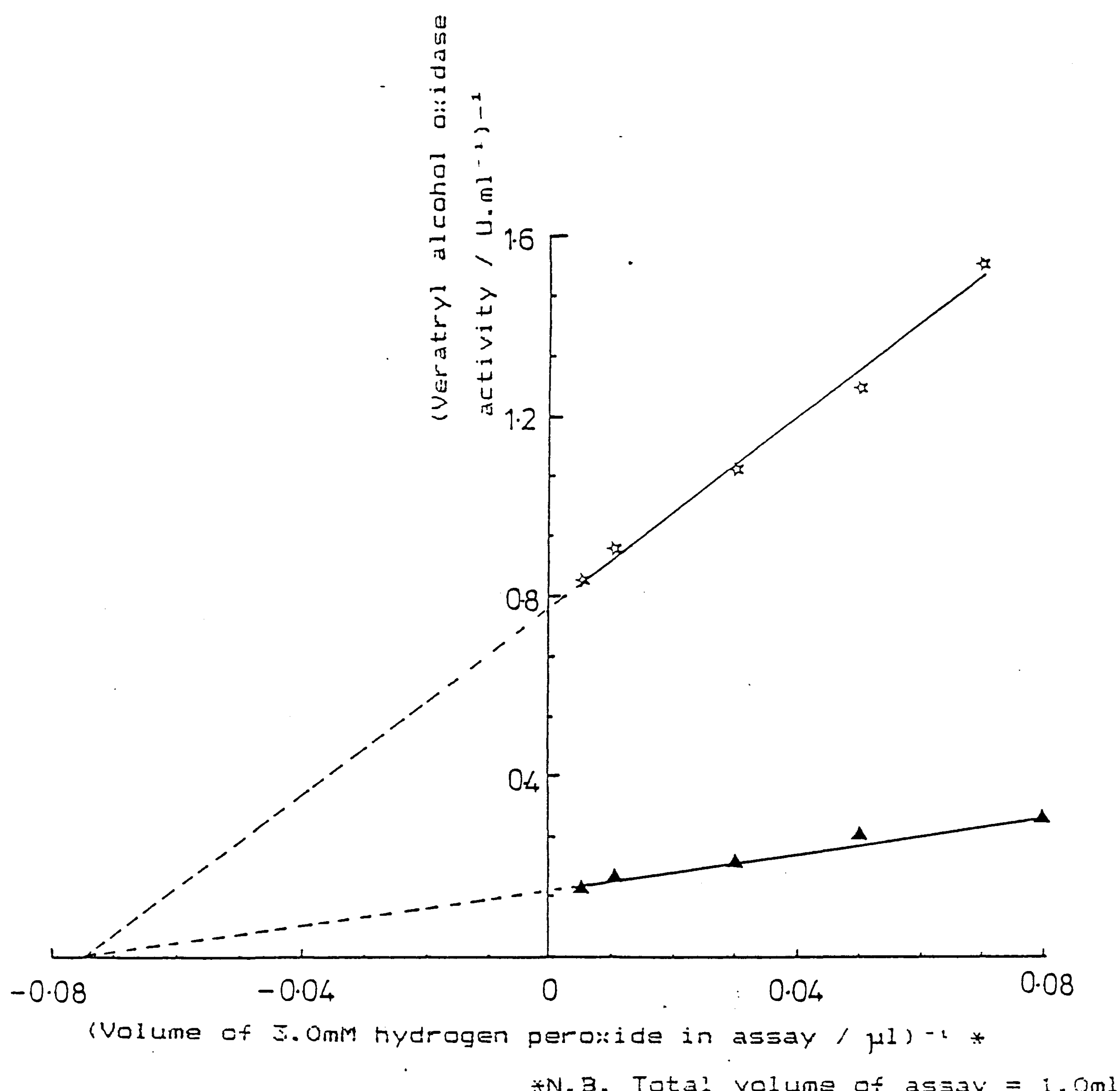
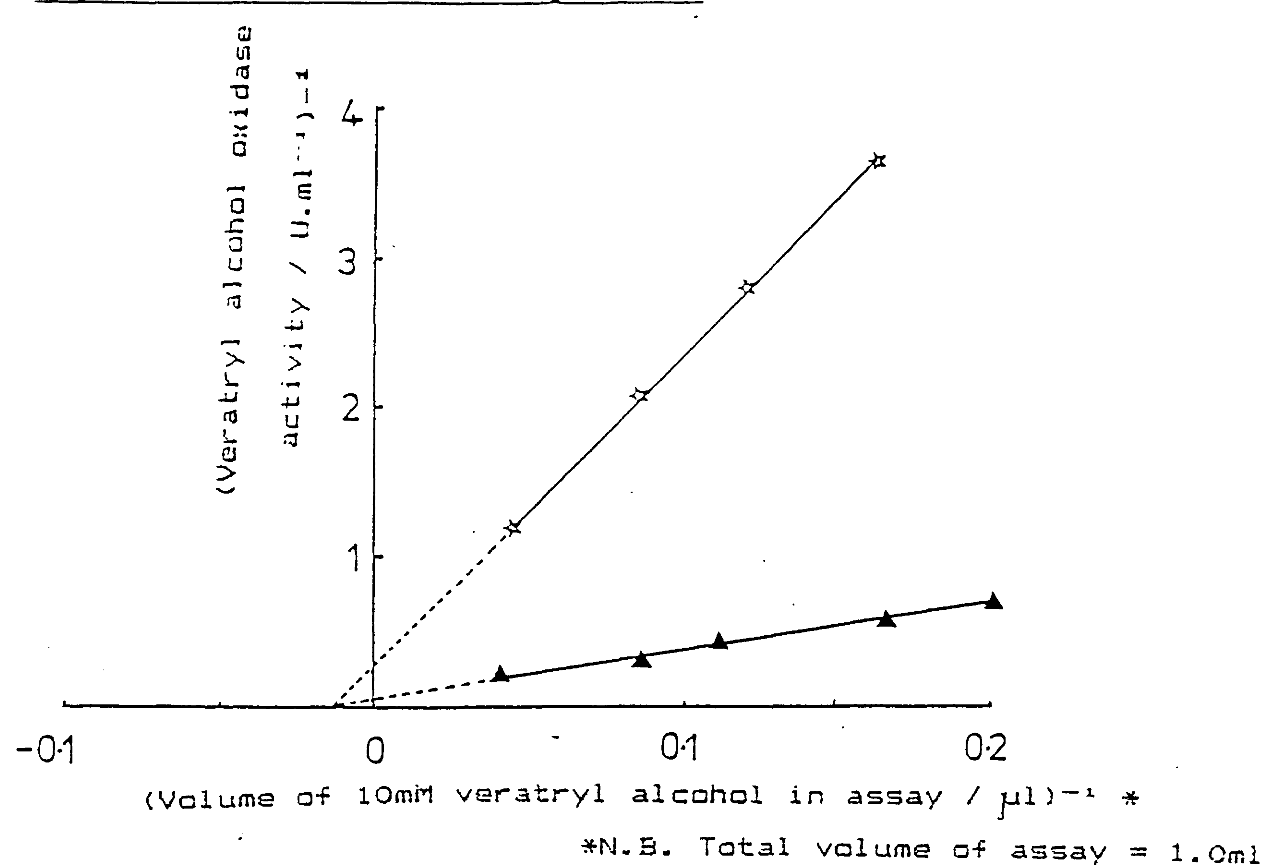


Fig. 39 Lineweaver-Burk plot to determine the K_m for hydrogen peroxide and the V_{max} of veratryl alcohol oxidase activity in the presence and absence of Mg^{2+} ions

Fig. 40 Lineweaver-Burk plot to determine the K_m for veratryl alcohol and the V_{max} of veratryl alcohol oxidase activity in the presence and absence of Mg^{2+} ions



The effect of 1.0mM Mg^{2+} ions on the K_m for veratryl alcohol and the V_{max} of veratryl alcohol oxidase activity of a crude preparation of ligninase obtained by pooling the first five harvests of ligninase produced using foam-immobilised *P.chrysosporium* (see Section 2.4.2) was carried out as described in Section 2.5.6.

	$+Mg^{2+}$	No Mg^{2+}
K_m / µM	290.0	293.7
V_{max} / U	9.90	1.61

the results presented above, it was evident that the stimulation of veratryl alcohol oxidase activity by added metal ions was significantly greater at pH 2.5 than it was at pH 3.0, see Fig.34.

The elution profile produced when the isozymes of ligninase were purified by tris-acryl DEAE sepharose chromatography (see Section 2.4.2) is illustrated in Fig. 19, and shows the absorbance at 280nm and also the veratryl alcohol oxidase activity when assayed at pH 3.0. The data presented in Table 15 shows the results of assaying each of the four main peaks of ligninase activity separated by this purification, under the different assay conditions described above, and from these results, it can be observed that the purification of the isozymes of ligninase resulted in an apparent loss of the ability of added metals to stimulate veratryl alcohol oxidase activity.

The reason for this loss of veratryl alcohol oxidase activity stimulation, and also the explanation for all the observations described above, is made clear in Section 3.5.

3.5 Properties of the inhibitor of ligninase

3.5.1 Introduction

From the results presented in Fig. 19, it can be seen that the final fraction eluted during the purification of the isozymes of ligninase (i.e. Fraction V) had

Table 15 Veratryl alcohol oxidase activity of the four fractions of ligninase separated by anion-exchange chromatography when measured at pH 2.5 and pH 3.0, in the presence and absence of 1.0mM Mg^{2+}

	Veratryl alcohol oxidase activity ($U.ml^{-1}$)			
	No Mg^{2+}		$+Mg^{2+}$	
	pH 2.5	pH 3.0	pH 2.5	pH 3.0
Fraction I	18.45	14.65	18.25	14.75
Fraction II	3.70	3.85	3.65	3.85
Fraction III	3.80	4.55	3.90	4.45
Fraction IV	5.25	3.80	5.45	3.90

The separation of the four fractions was by tris-acryl DEAE sepharose anion-exchange chromatography which was carried out as decribed in Section 2.4.2; see also Section 2.5.2.

a strong absorbance at 280nm but did not show any veratryl alcohol oxidase activity. In this section, it will be shown how this fraction was able to bring about a pH-dependent inhibition of the isozymes of ligninase which was rendered largely ineffective in the presence of added metal ions, thus providing an explanation of all the observations described in Section 3.4.8. In addition, the results of a preliminary analysis of the component(s) present in Fraction V will be presented, from which a tentative identification of the inhibitor is made, and which is backed up by a similar pattern of inhibition of veratryl alcohol oxidase activity produced using a test compound belonging to the same family of compounds as to that which the inhibitor has been proposed to belong to.

3.5.2 Effect of pH on the inhibition of ligninase in the presence and absence of 1.0mM Mg^{2+}

To determine whether the component(s) in Fraction V were able to help explain the loss of the ability of Mg^{2+} to stimulate the ligninase activity of the isozymes of ligninase activity as described in Section 3.4.8, it was decided to assay the isozymes in the presence of an aliquot of Fraction V.

Initially, the effect of a range of concentrations of Fraction V on the ligninase activity of Isozyme I was investigated as described in Section 2.7.2. From the results presented in Fig. 41, it can be seen that the addition of an aliquot of Fraction V to the assay mixture resulted in an inhibition of the veratryl alcohol oxidase

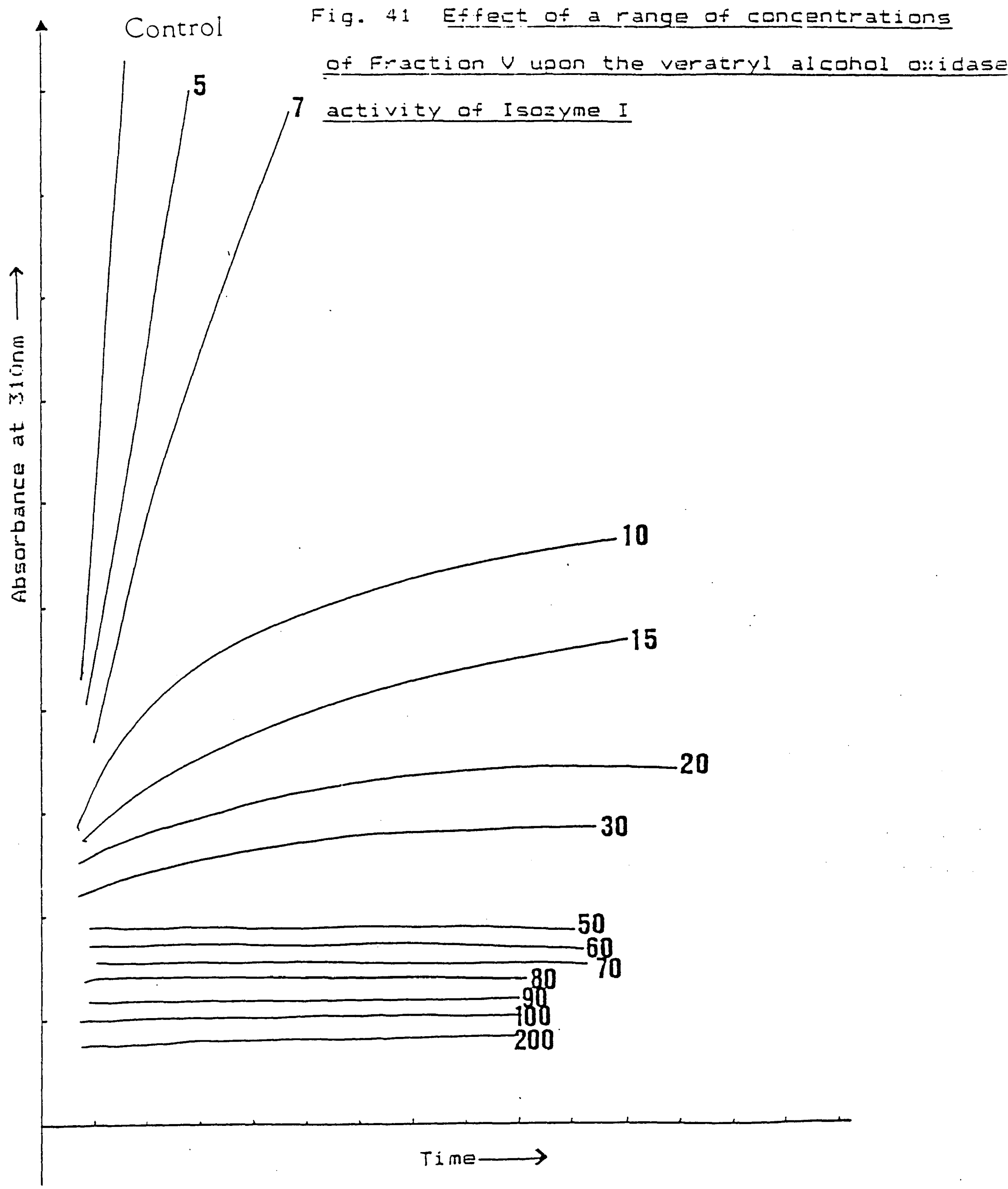


Fig. 41 Effect of a range of concentrations of Fraction V upon the veratryl alcohol oxidase activity of Isozyme I

The inhibition of the veratryl alcohol oxidase activity of Isozyme I was demonstrated by assaying the enzyme in a range of concentrations of Fraction V. All assays were carried out as described in Section 2.2.2; with the numbers on the figure referring to the volume of undiluted Fraction V (i.e. relative to when it was eluted off the column; see Section 3.3.1) present in each assay / µl. The total volume of the assays was 1.0ml.

activity of Isozyme I, and that the inhibition of the veratryl alcohol oxidase activity was apparently directly related to the concentration of the Fraction V that was present.

In Section 3.4.5, it was shown that the apparent stimulation of ligninase activity of the concentrated preparation of ligninase by 1.0mM Mg^{2+} was pH-dependent. Consequently, using a dilution of Fraction V which had been previously shown to only partially inhibit that the veratryl alcohol oxidase activity of Isozyme I when assayed at pH 2.75, the inhibition of the veratryl alcohol oxidase activity of each of the isozymes of ligninase was then investigated over a range of pH values as described in Section 2.7.3. In addition, to determine whether any inhibition of the isozymes of ligninase was rendered ineffective in the presence of Mg^{2+} ions, the determination of the pH optimum of the isozymes of ligninase when assayed in the presence of an aliquot of Fraction V, was also repeated in the presence of 1.0mM Mg^{2+}; see also Section 2.7.3. The results obtained for Isozymes I, III and IV are summarised in Figs. 35, 36 and 37, respectively, where it can be broadly concluded that for each of the three isozymes, the inhibition of ligninase was pH-dependent, and was partially reversed in the presence of 1.0mM Mg^{2+}.

3.5.3 Spectral properties

The UV/visible spectrum of Fraction V is shown in Fig. 42. It can be seen that between 600nm and c.320nm, the spectrum shows no discernible features, and that below c.320nm, the absorbance increases steadily as the wavelength tends towards 200nm.

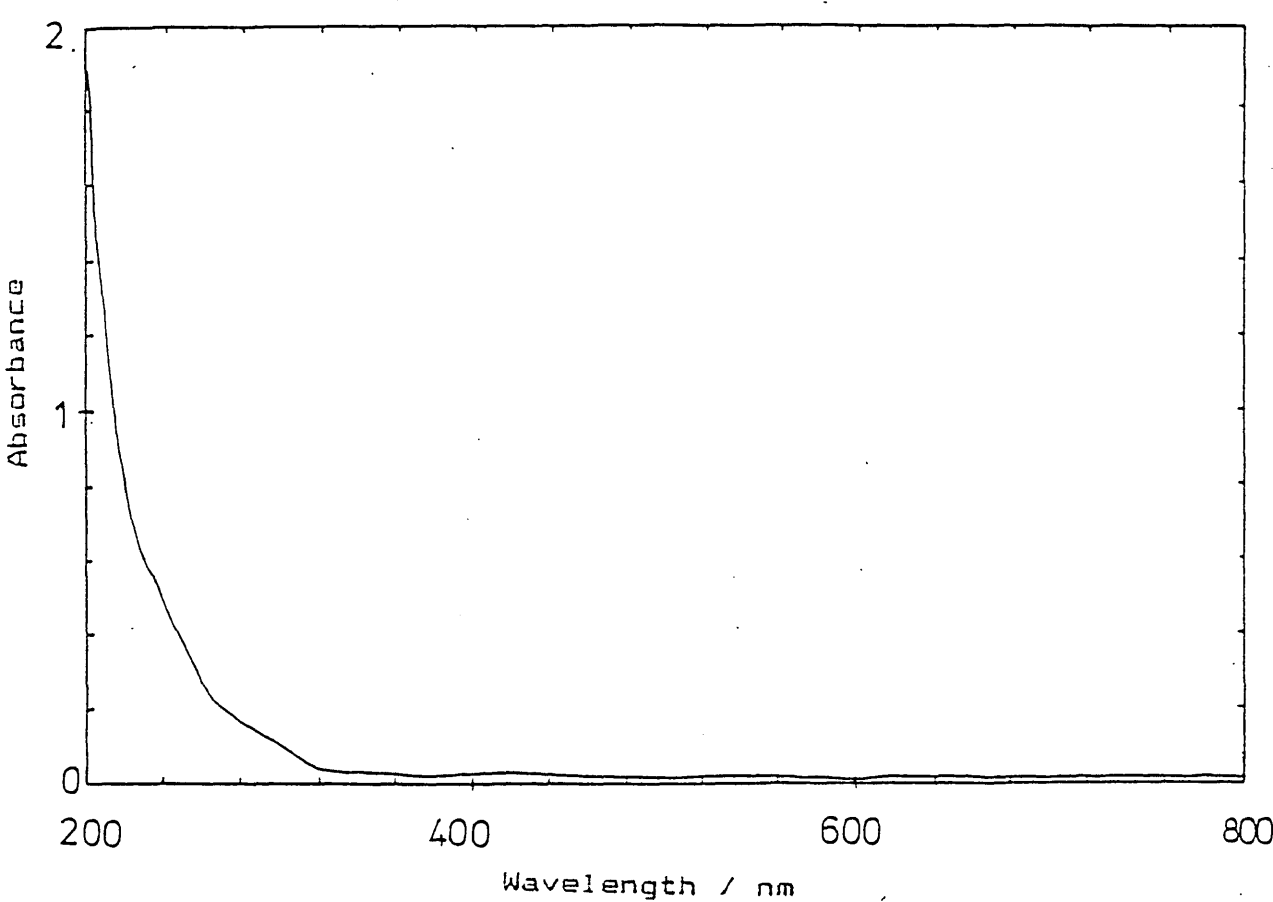
3.5.4 Determination of the presence of protein and carbohydrate in Fraction V

When Fraction V was assayed to determine whether it contained any protein, it was found that no protein was able to be detected; see Section 2.5.7. However, when Fraction V was assayed to determine whether it contained any carbohydrate, it was found that using a standard of D-glucose, the concentration of carbohydrate present in Fraction V as it was eluted from the column, was $0.12mg.ml^{-1}$. The maximum absorbance of the colour developed in the carbohydrate assay was 480nm, see Fig. 43. Thus, it was concluded that the predominant component of the carbohydrate present in Fraction V was either a pentose sugar or a uronic acid (Dubois et al. 1956).

3.5.5 Determination of the presence of uronic acids

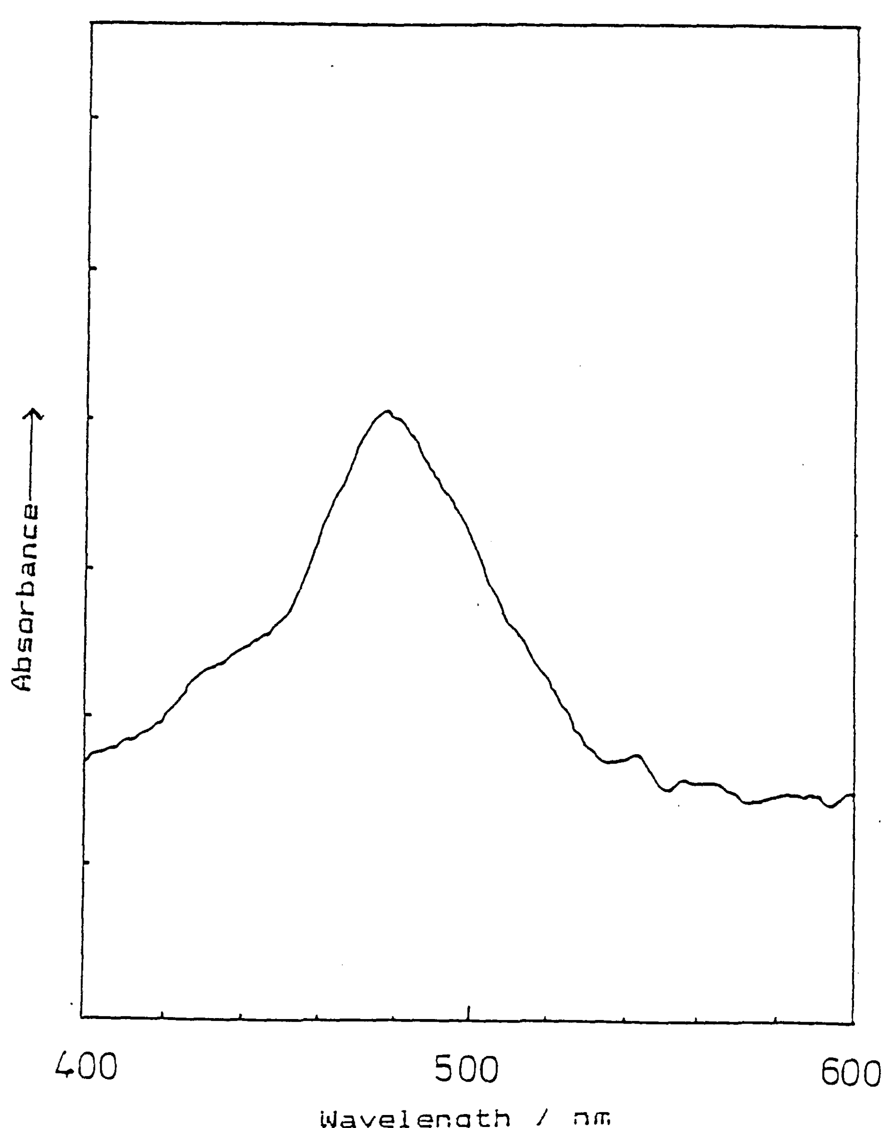
It was found that when Fraction V was assayed as described in Section 2.7.4, the presence of borate ions resulted in a significant difference of the spectrum of the assay solution, with the resultant generation of a chromophore having a maximum absorbance at 450nm, see

Fig. 42 UV / visible spectrum of Fraction V



The UV / visible spectrum of Fraction V was performed using ten times diluted material (relative to when it was eluted off the column; see Section 3.3.1), using quartz cuvettes with a 1cm light path in a Perkin Elmer PE-555 spectrophotometer against a water blank.

Fig. 43 Spectrum of the coloured solution produced when Fraction V was assayed for the presence of carbohydrate by the phenol sulphuric method



The concentration of carbohydrate in undiluted Fraction V (relative to when it was eluted off the column; see Section 3.3.1) was carried out using the phenol sulphuric acid method as described in Section 2.2.7.

Fig. 44. This result infers that Fraction V contained uronic acid(s) with unsubstituted threo hydroxyl groups at their C-3 - C-4 positions (Scott 1979).

Unfortunately, this assay was only used to determine whether or not uronic acid was present in Fraction V, and was not used to estimate the precise concentration of the uronic acid(s) that was detected; however, if the uronic acid component detected above showed a similar degree of reactivity as D-galacturonic acid, then it might be estimated that the concentration of uronic acid present was c.80ug.ml^{-1} (Scott 1979; see also The Discussion to this thesis).

3.5.6 HPLC analysis of Fraction V

The results presented in Fig. 45, show that when Fraction V was analysed using the HPLC system described in Section 2.7.5, there were apparently two components present that were eluted very close together, and with a relatively short retention time; thus, inferring that they were both very polar in nature.

3.5.7 Comparison of the inhibition of ligninase in the presence of heparan sulphate with the inhibition of ligninase in the presence of Fraction V

It was found that when Isozyme I was assayed as described in Section 2.2.2, except in the presence of 20mM phosphate buffer pH 2.5, and in the presence of a range of concentrations of heparan sulphate (Sigma), a similar pattern of ligninase inhibition was observed

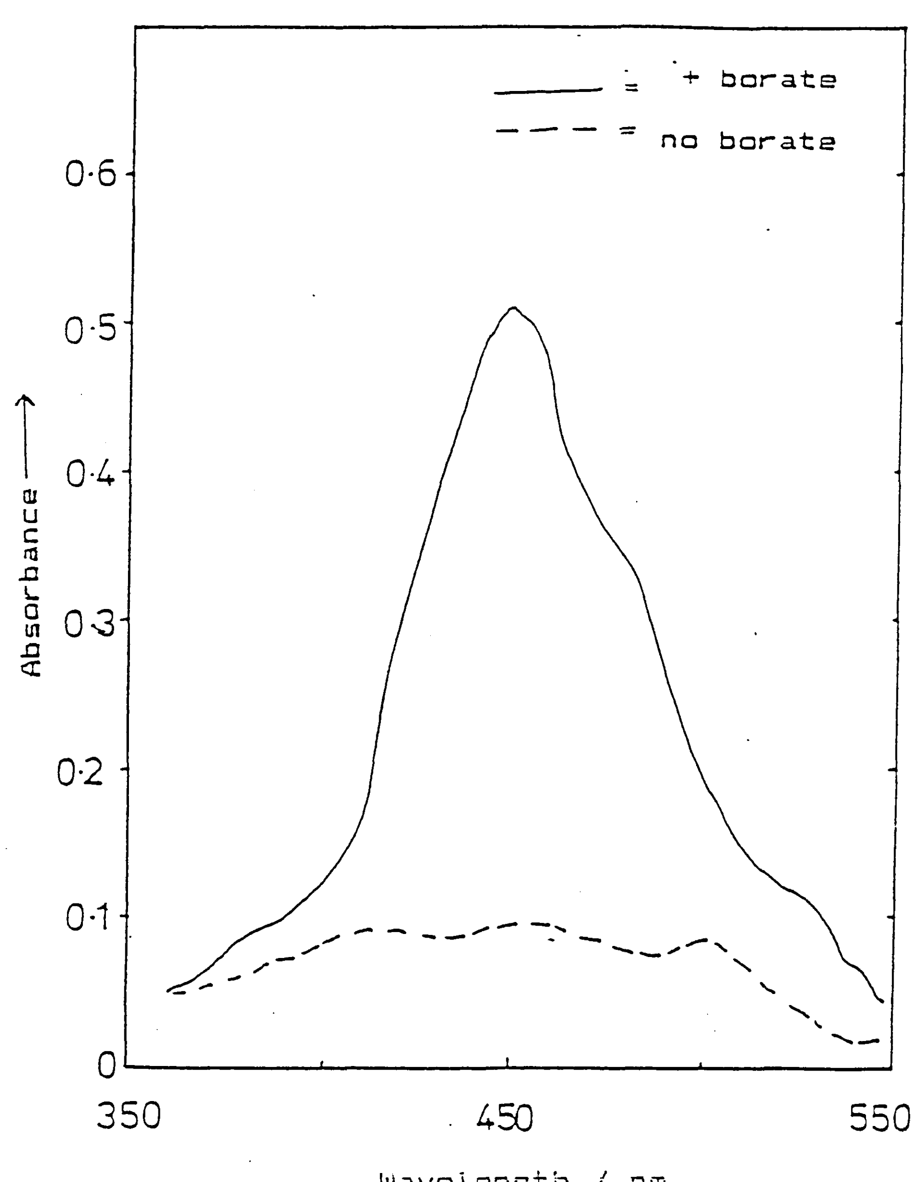
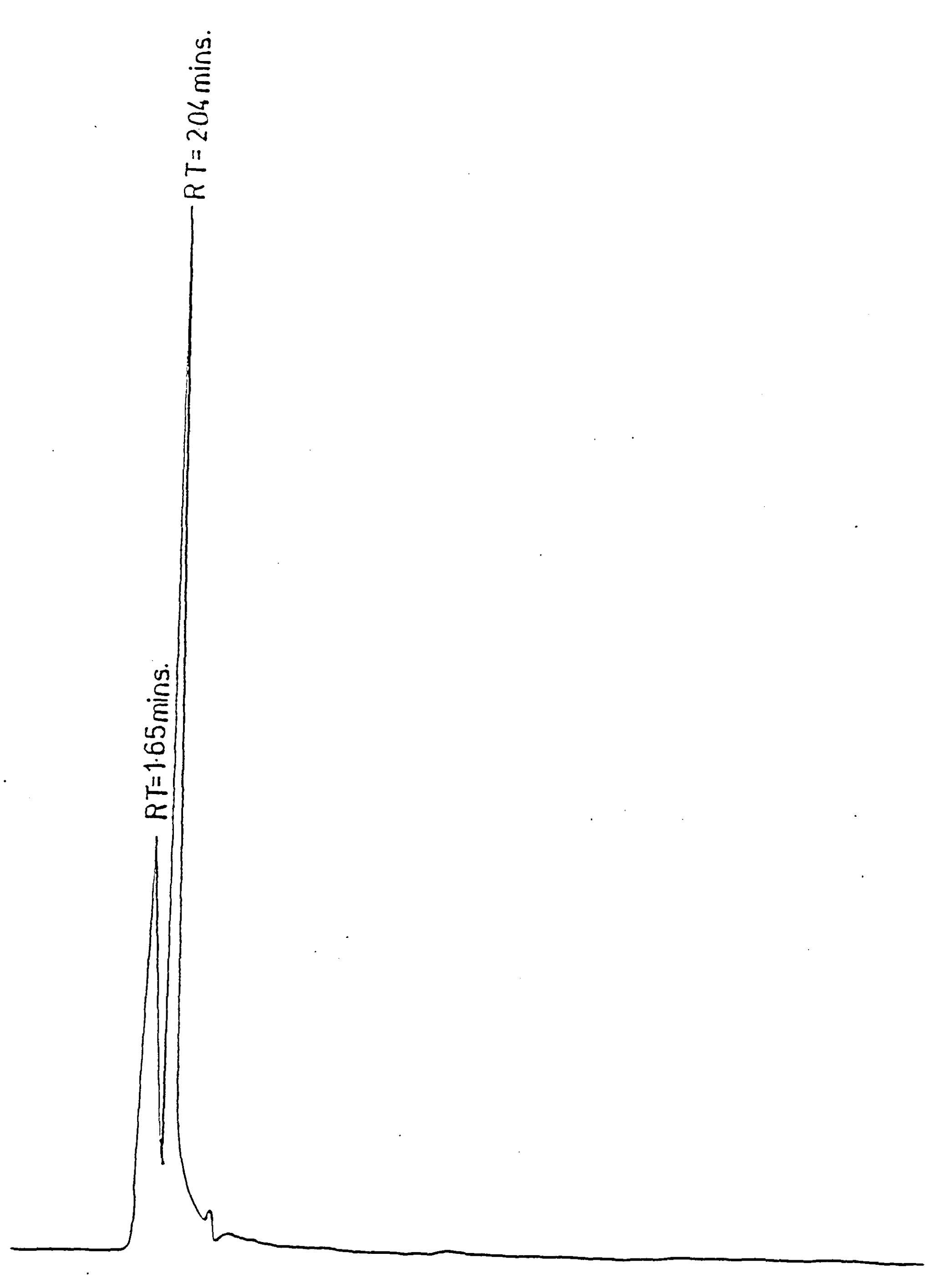


Fig. 44 Effect of borate ions on the spectrum of Fraction v when assayed for the presence of uronic acids by the Scott method

The presence of uronic acids in undiluted Fraction V (relative to when it was eluted off the column; see Section 3.3.1) was carried out using the Scott method as described in Section 2.7.4.

Fig. 45 HPLC analysis of Fraction V



Undiluted Fraction V (relative to when it was eluted off the column; see Section 3.3.1) was filterd through a 45μm Millipore filter, and then analysed using the HPLC system described in Section 2.7.5. The components separated were monitored at 230nm.

compared to the inhibition brought about by a range of dilutions of Fraction V; see Figs. 41 and 46.

In addition, it was also found that the inhibition of the veratryl alcohol oxidase activity of Isozyme I in the presence of heparan sulphate, was very much reduced at pH 3.0 compared to pH 2.5; however, unlike the inhibition of ligninase brought about by Fraction V, there was no significant reduction in the inhibition in the presence of 1.0mM Mg^{2+}; compare Fig. 35 and Table 16.

3.5.8 Effect of low concentrations of the inhibitor on the K_m for veratryl alcohol

This was carried out as described in Section 2.7.7. It can be seen from the results presented in Figs. 47 and 48 that increasing concentrations of the inhibitor led to a corresponding increase in the K_m, and a simultaneous decrease in the V_{max}; thus, these results do not clearly show the precise nature of the inhibition of ligninase (Rudolph, 1983; see also The Discussion to this thesis).

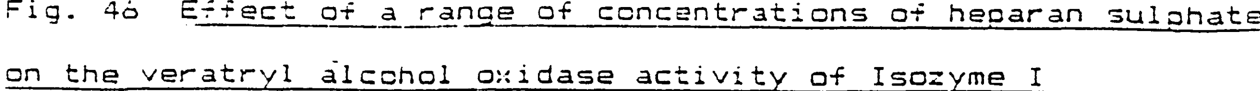


Fig. 46 Effect of a range of concentrations of heparan sulphate on the veratryl alcohol oxidase activity of Isozyme I

Control

10^{-4}

10^{-3}

10^{-2}

10^{-1}

"Neat"

Absorbance ⟶

Time ⟶

The inhibition of the veratryl alcohol oxidase activity of Isozyme I was determined using a range of dilutions of a $5.0 mg.ml^{-1}$ stock solution of heparan sulphate as described in section 2.7.6. The numbers on the figure refer to to the dilution of the stock solution of heparan sulphate.

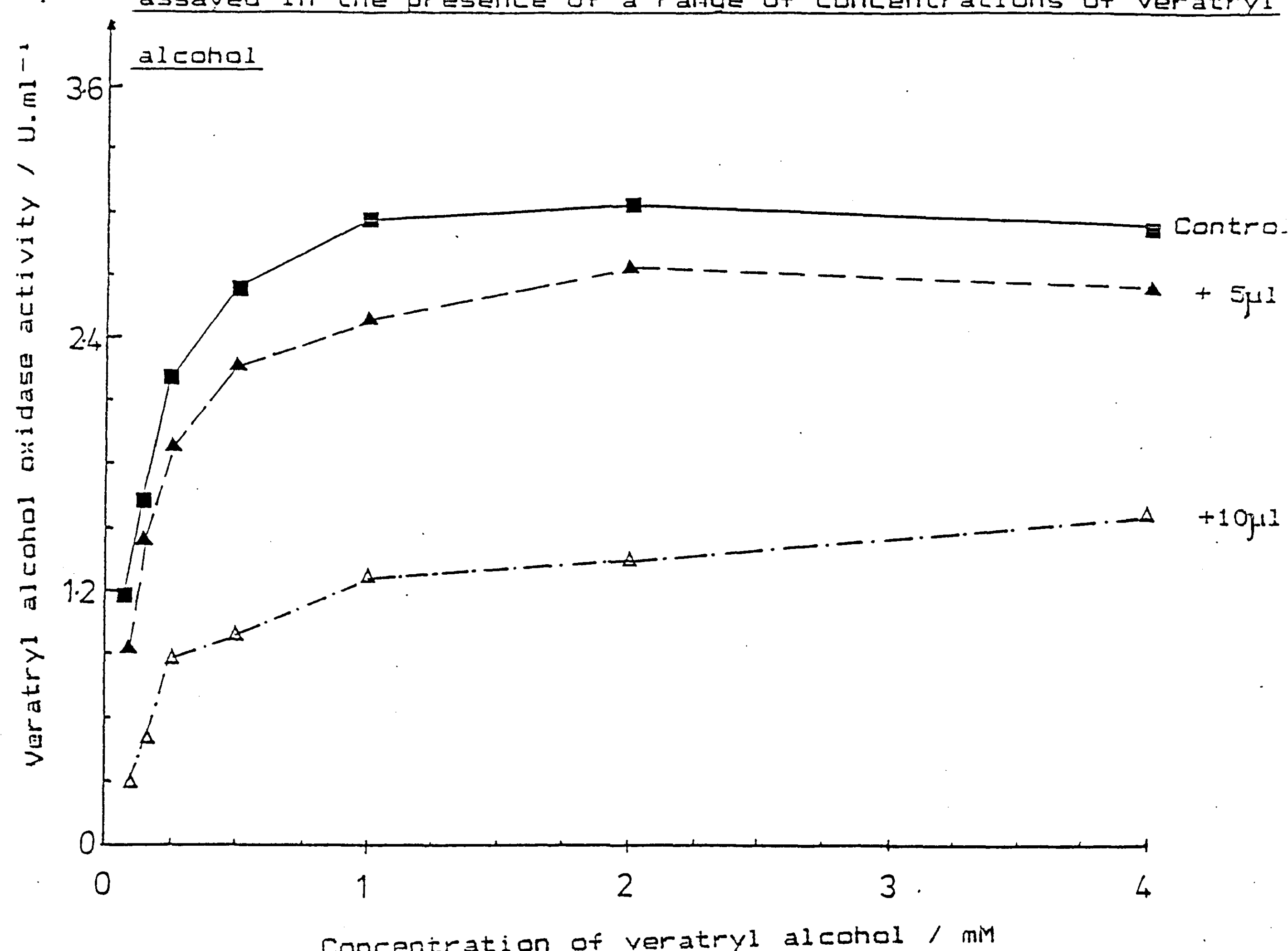
Table 16 Effect of pH and 1.0mM Mg^{2+} ions on the inhibition of the veratryl alcohol oxidase activity of Isozyme I by heparan sulphate

	% Inhibition of veratryl alcohol oxidase activity
Control (no h.p.*, no Mg^{2+})	
pH 2.5	0
pH 3.0	0
+ h.p.*, no Mg^{2+}	
pH 2.5	82
pH 3.0	15
+ h.p.*, + Mg^{2+}	
pH 2.5	86
pH 3.0	16

(Where * = heparan sulphate)

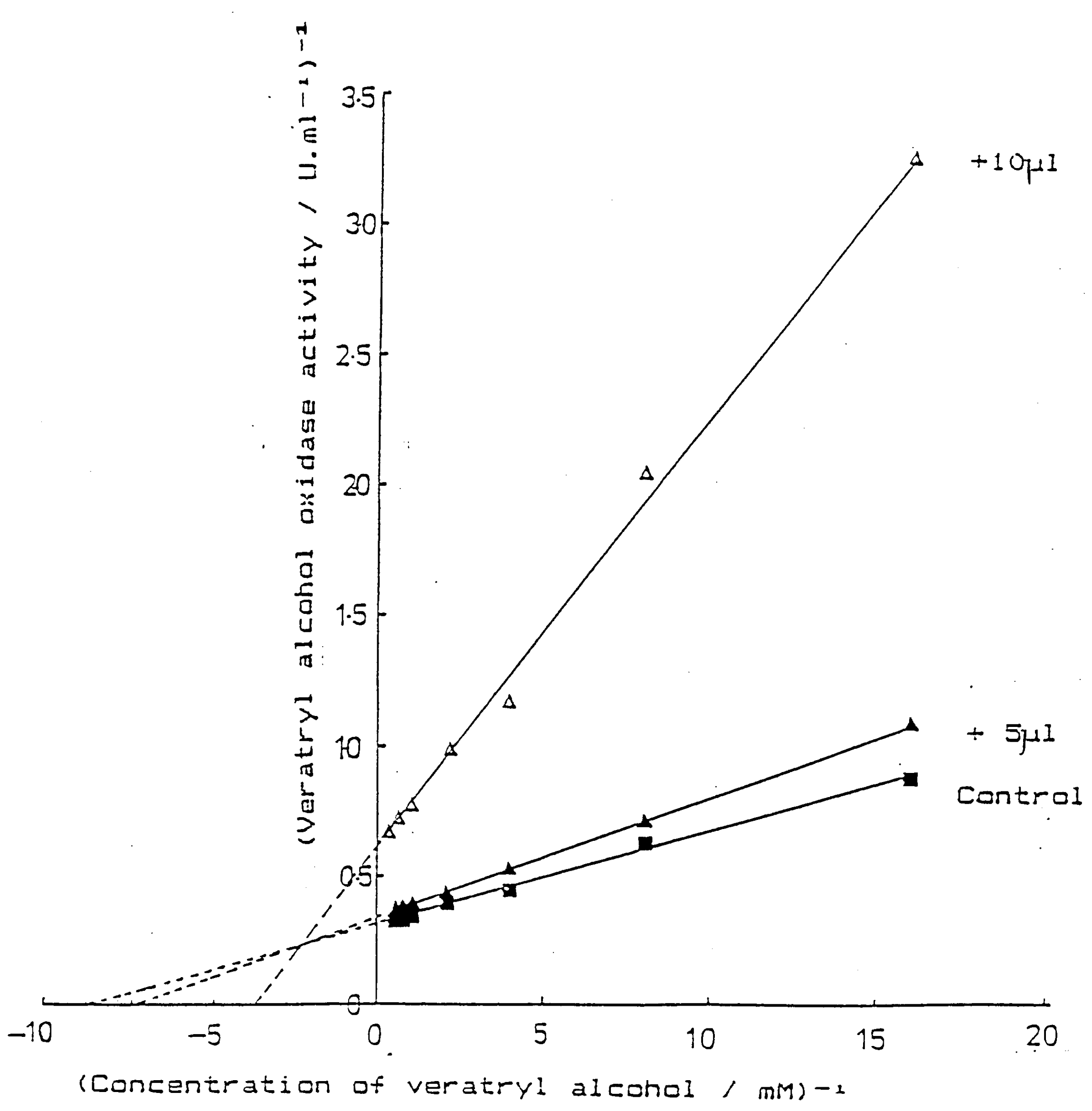
The above set of results were obtained when heparan sulphate was used at a final oncentration of 0.5 $\mu g.ml^{-1}$; the control enzyme activity was 1.8 $U.ml^{-1}$ at pH 2.5, and 1.5 $U.ml^{-1}$ at pH 3.0 (see Section).

Fig. 47 Effect of two concentrations of Fraction V on the intial rate of veratryl alcohol oxidase activity of Isozyme I when assayed in the presence of a range of concentrations of veratryl alcohol



This was carried out as described in Section 2.7.7. The two concentrations of Fraction V were 5ul and 10ul of 100 times diluted Fraction V (relative to when it was eluted off the column; see Section 3.3.1).

Fig. 48 Effect of two concentrations of Fraction V on the determination of the K_m for veratryl alcohol and the V_{max} of veratryl alcohol oxidase acivity of Isozyme 1



This experment was carried out as described in Secton 2.7.7. The two concentrations of Fraction V were 5µl and 10µl of 100 times diluted Fraction V (relative to when it was eluted off the column; see Section 3.3.1).

	Control	+ 5µl	+ 10µl
K_m / µM	107.8	121.4	269.0
V_{max} / U	3.14	2.74	1.62

4. DISCUSSION

4.1 Production of ligninase using stationary and agitated cultures of *P.chrysosporium*

4.1.1 Stationary cultures

In the Introduction to this thesis, it was described that ligninase was first produced in low volume non-induced stationary submerged cultures of *P. chrysosporium* (Tien and Kirk, 1983; Glenn et al., 1983). These culture conditions had in fact been reached through the optimisation of the complete ligninolytic system (Kirk et al., 1978); however, since ligninase was shown to exhibit the two main criteria by which lignin degradation had been previously assessed, i.e. to degrade lignin model compounds and also to partially degrade ^{14}C-labelled lignin (see Section 1.3.2.1), it did not seem unreasonable to assume that the optimal culture conditions for the production of ligninase should not be significantly different, or would improve drastically through further optimisation, to those conditions used for the production of the complete ligninolytic system.

In this thesis, it is described how stationary cultures of *P. chrysosporium* were used to demonstrate that not only was ligninase activity apparently inducible, but that it was also able to be produced using agar- or foam-immobilised cultures.

The induction of ligninase was first observed when it was noticed that autoclaved glucose produced a higher titre of ligninase than filter-sterilised glucose. Since it is known that the heating of glucose, especially in acidic environments, leads to the generation of small quantities of 5-hydroxy methyl furfural (Haworth and Jones, 1944), it was decided to test whether the addition of this compound to filter-sterilised glucose would mimic the effect of the autoclaving. It was found that increasing titres of ligninase were produced with increasing concentrations of the furfural compound; and hence that the production of ligninase was apparently inducible by 5-hydroxy methyl furfural. In fact, using "furfural-induced" cultures of P. chrysosporium, a higher titre of ligninase was produced than with the standard autoclaved glucose cultures; the furfural compound also resulting in peak ligninase activity being reached 24 hours earlier.

It was also shown that organosolv lignin led to the induction of ligninase, and that the induction itself was caused by insoluble particulate material present in the organosolv preparation. These results were apparently in contrast to the findings of Keyser et al. (1978) who were unable to demonstrate that the presence of lignin led to increased ligninolytic activity, and hence concluded that the ligninolytic system was non-inducible. The induction of ligninase by a variety of lignin-related compounds was subsequently published by Faison and Kirk (1985).

During the course of writing this thesis, data has recently been presented by Tonon et al. (1987) which showed that veratryl alcohol may act more as a "protector" rather than an "inducer". The results of their studies demonstrated that in the presence of veratryl alcohol, the isozyme of ligninase having the highest specific activity, was apparently protected from the deleterious effects of hydrogen peroxide, whereas, other isozymes were not protected and thus became more rapidly inactivated.

The immobilisation of P. chrysosporium was investigated as a first step towards the eventual use of the same fungal biomass of P. chrysosporium for the production of successive harvests of ligninase. In the Results section of this thesis, however, it was demonstrated that P. chrysosporium was able to partially solubilise the agar immobilisation support during its primary phase of growth; and hence an alternative immobilisation support that would remain unaffected by the presence of the fungus was desired. This led to the use of polyurethane foam as an alternative - inert - immobilisation support (Thepenier et al., 1985).

It is interesting to note that the solubilisation of the agar by P. chrysosporium occurred during primary metabolism, and that it has been reported that the fungus is able to use other polysaccharides, such as cellulose, as growth substrates also during primary metabolism (Kirk et al., 1976). In contrast to these results, however, Linko et al. (1986), using the same strain of fungus,

describe the use of agar beads as an immobilisation support without reporting any problems of agar solubilisation. This apparent difference in results may either have been due to genetic differences in the two strains of fungi that might have occurred during continued serial sub-culturing, or simply due to the differences in culture conditions; for example, Linko et al. (1987) grew their fungus under conditions of carbon limitation, and with culture agitation, whereas, in this thesis, agar-immobilised cultures of P. chrysosporium were grown in nitrogen-limited, stationary cultures.

The agar-immobilised cultures of P. chrysosporium were only ever shown to produce approximately half the titre of ligninase compared to control cultures. This was undoubtedly due to the dilution effect of the sterile distilled water top layer used in this technique. However, during the preliminary assessment of polyurethane foam as an immobilisation support, it was evident that at least one of the foams investigated was able to lead to the production of approximately twice the titre of ligninase compared to the control cultures. The use of polyurethane foam as an immobilisation support is discussed further in Section 4.1.2.

4.1.2 Agitated cultures

Stationary cultures of P. chrysosporium grow as mycelial mats, whereas the agitation of cultures results in the formation of discrete mycelial pellets. In early

investigations, it was reported that the formation of mycelial pellets led to a suppression of lignin degradation and also of the breakdown of lignin model compounds (Kirk et al., 1978; Weinstein et al., 1980; Goldsby et al., 1980). However, subsequent investigations have shown that lignin degradation is equally as effective in agitated cultures (Reid et al., 1985), and also that it is possible to use the agitation of cultures to facilitate the scale up of production of ligninase (see Section 1.3.2.1).

The results of this thesis have shown that the immobilisation of P. chrysosporium in cubes of polyurethane foam leads to the production of increased yields of ligninase compared to control cultures, and that the presence of the foam facilitates the production of successive harvests of ligninase using the same fungal biomass. In addition, it was also shown that foam-immobilised pellets of P. chrysosporium were able to be stored for periods of at least two months at 4°C with no apparent loss in the ability to produce fresh harvests of ligninase after culture reactivation.

Leisola et al. (1985a) have shown that in nitrogen limited cultures, the production of ligninase is directly proportional to pellet concentration and that the optimal fungal pellet size was 1.0 to 2.0 mm diameter. Using the foam immobilisation technique, the fungal pellets become embedded in the open pores of the polyurethane foam, and thus the diameter of the fungal pellets is determined by

the pore size of the foam. In this case, it was found that the foam which yielded the highest titre of ligninase had a pore size, and hence gave a fungal pellet size, of 0.8 to 1.0mm diameter. In the absence of the foam, it was found that pellets were of variable size and that they would sometimes fuse together forming large clumps (Reid et al., 1985); however, in the presence of the foam, the formation of clumps of pellets was avoided. Thus it can be concluded that the presence of the foam leads to a controlled fungal pellet size which at the same time as preventing the formation of clumps of pellets, allows there to be more individual pellets per unit volume of medium, and also helps maintain a more uniform diffusion of oxygen into those pellets which have developed (Miura et al., 1975; Schugerl et al., 1983; see also Leisola et al., 1983b). Hence, in this way, the presence of the foam ultimately leads to a more rigid control over the production of ligninase in agitated batch cultures which thus results in the production of higher yields of ligninase compared to control cultures, with reduced variation between replicate flasks, see Fig. 11.

In comparison to the results of other groups using the same "wild type" strain of P. chrysosporium in agitated nitrogen limited cultures, the levels of ligninase reported in this thesis compare favourably (see Leisola et al., 1985a; Kirk et al., 1986c; Jager et al., 1985). The highest ligninase activity that Leisola et al. (1985a) obtained was $118U.l^{-1}$. However, this was produced

in 50ml of medium in 1l Erlenmeyer flasks, and hence does not represent such a high yield in terms of total number of units obtained per flask (see Section 3.2.1).

Kirk et al. (1986c) also obtained c.118U.l^{-1}, although this was for low volume stationary cultures incubated in the presence of added veratryl alcohol and an increased concentration of mineral salts; nevertheless, this represents a considerable improvement compared to the typical levels of ligninase previously obtained in low volume - non-induced - stationary cultures, which was only c.5U.l^{-1}(see Section 3.1).

Jager et al. (1985) obtained a maximum ligninase activity of c.134U.l^{-1} when cultures were incubated in the presence of the detergent Tween 80. In this paper, it is stated that similar results were obtained when culture volumes and flasks sizes were increased proportionately; however, the figures quoted only refer to 30ml medium in 125ml Erlenmeyer flasks.

It is appropriate to point out at this point, that an alternative approach to the scaling up the production of ligninase is to use mutants of the wild type fungus. For example, Buswell et al. (1984) isolated a mutant capable of producing over 400U.l^{-1} when grown in nitrogen limited low volume stationary cultures with glycerol as the carbon source; and Kirk et al. (1986c) used a mutant strain in a disk fermentor containing 2.5l of medium which was able to yield c.115U.l^{-1}.

Undoubtedly the easiest method of facilitating the scale up of the production of ligninase, was illustrated by Leisola et al. (1985a) who compared the effect of carbon- versus nitrogen-limitation upon the production of ligninase by the wild type strain of P. chrysosporium. They found that in carbon limited cultures, the yield of ligninase was $670U.l^{-1}$ compared to $118U.l^{-1}$ produced in nitrogen limited cultures. However, it is interesting to note that this observation was in contrast to the previous observations for the effect of carbon limitation upon ligninolytic activity (Kirk et al., 1978; Faison and Kirk, 1985), and also upon ligninase activity when produced in the absence of inducer molecules such as veratryl alcohol and in stationary cultures (Faison and Kirk, 1985).

In this thesis, the effect of carbon-limitation upon the production of ligninase in foam-immobilised cultures did not constitute a major part of the investigations carried out. However, using the techniques developed in this thesis, Dodson and Kamal (Unpublished data) found that carbon limitation did lead to the production of higher yields of ligninase, although not to the extent that Leisola et al. have reported (1985a). This may have been due to the increased levels of iron used in the medium by Leisola et al. which was not mentioned in their manuscript (Leisola, personal communication).

As mentioned above, the use of polyurethane foam facilitates the production of successive harvests of ligninase from the same fungal biomass. Fresh harvests of ligninase are produced by simply decanting the extra-cellular medium and then replacing it with an appropriate dilution of fresh medium, and since all the fungal pellets are embedded in the cubes of foam and also the cubes of foam are saturated with liquid medium, they have a greater density than the liquid medium, and thus remain in the flasks during the aseptic replacement of the medium. Other groups have also reported the use of immobilisation systems for the production of successive harvests of ligninase. For example, Paszczynski et al. (1985 and 1986) have grown P. chrysosporium on the roughened interior walls of a 20l carboy. The latter contained 1l of medium and was purged with oxygen and then slowly rotated in a water bath. This system is relatively bulky compared to the foam immobilisation technique, and is only able to lead to the production of relatively very poor harvests of ligninase. In another report, Linko et al. (1986) immobilised P. chrysosporium in agar or agarose gel beads. As discussed above (see Section 4.1.1), during the early investigations of this thesis, it was observed that P. chrysosporium was apparently able to partially solubilise agar, whereas, Linko et al. do not appear to have suffered from this problem. Using carbon limited medium with the agar- or agarose-immobilised fungus, they report the production of ligninase in repeated batch cultures; and, in addition, using free mycelial pellets, they also report

the production of ligninase in continuously run horizontal and vertical reactors. Using the repeated batch culture technique, i.e. the same approach as the semi-continuous culture method devised in this thesis, Linko et al. obtained a maximum activity of $245U.l^{-1}$ with successive harvests being produced every 3 to 4 days. These results are greater than the ligninase activities quoted in this thesis for nitrogen limited cultures. However, using the techniques developed in this thesis, as mentioned above, Dodson and Kamal used the foam immobilisation technique under conditions of carbon limitation, and found that not only did it lead to the production of higher yields of ligninase in batch culture, but also that when adapted for the semi-continuous method of ligninase production, it led to the production of harvests of ligninase with a similar magnitude to those reported by Linko et al. (1986), with activities being produced between 150 and $300U.l^{-1}$, and with harvests being produced every 24 hours. It was also found that after six harvests when the experiment was stopped, there were no indications that the production of successive harvests was about to tail off. Obviously, the production of ligninase using carbon limited foam immobilised cultures of P. chrysosporium merits further investigation.

The continuous production of ligninase reported by Linko et al. (1986) showed that ligninase was apparently produced in a number of successive cycles. This probably indicates that the medium composition used in their study

still required to be optimised. In addition, since no data was given for the flow rate of medium used, it is not possible to critically assess the total number of units produced by this approach.

One feature of the foam immobilisation technique that has not been reported in any other study is that it can be used to facilitate the storage of pellets of P. chrysosporium at 4°C, with no apparent loss in their ability to produce fresh harvests of ligninase after culture reactivation.

Thus it can be concluded that the use of polyurethane foam as an immobilisation support represents a cheap and very simple modification to existing methods of ligninase production, resulting in a more uniform production of ligninase in batch culture, and also facilitating the production of successive harvests of ligninase from the same fungal biomass. In addition, foam immobilised fungal pellets can be stored at 4°C for periods of at least two months before being used to produce fresh successive harvests of ligninase; thus eliminating the time consuming and skilled preparation that is required to establish fresh ligninolytic cultures. From an economic point of view, the production of ligninase using foam immobilised P. chrysosporium is also attractive. For example, as mentioned above, polyurethane foam is cheap and does not require the purchase of any other equipment to adapt its use to existing systems of ligninase production; but in addition to these advantages,

it also leads to a significant long term saving on the cost of chemicals since during the production of successive harvests of ligninase, medium components are used at one fifth of the concentration used in the original batch culture.

4.2 Properties of ligninase before and after purification

4.2.1 Introduction

In this thesis, the examination of the effect of pH upon the ligninase activity of enzyme prepared using the foam immobilisation technique, was shown to lead to the eventual discovery of a pH-dependent inhibitor of ligninase activity that was apparently rendered ineffective in the presence of divalent metal ions. Consequently, the effect of pH and of metal ions upon ligninase activity and also the inhibitor itself, will be discussed separately, and the rest of this section will deal with a general discussion of some of the other properties of ligninase determined in this thesis compared to the results of other research groups.

4.2.2 General properties of ligninase

The purification of the isozymes of ligninase by a combination of tris-acryl DEAE and Mono Q anion-exchange chromatography was shown to lead to the purification of three isozymes of ligninase that differed in molecular weight, isoelectric point, pH optimum (see Section 4.2.3), and relative % carbohydrate present compared to protein.

The separation and purification of more than one isozyme of ligninase produced by P. chrysosporium has been previously well documented (see Section 1.3.2.1); the number of isozymes reported varying from two to fifteen. The results presented in this thesis are consistent with the results of Renganathan et al. (1985) and Kirk et al. (1986c), who both demonstrated that using anion-exchange column chromatography, three predominant isozymes of ligninase were produced in nitrogen limited cultures of P. chrysosporium. It should be pointed out, however, that Kirk et al. (1986c) were also able to show that the total number of proteins showing the ability to oxidize veratryl alcohol was actually six; whereas, Leisola et al. (1987), using analytical isoelectric focusing, reported that the total number of proteins showing the same apparent ability was ten for nitrogen limited cultures and seven for carbon limited cultures. It has been suggested that the reasons for this apparent difference in the number of isozymes of ligninase able to be separated is due to the "incomplete separation and/or inactivation of minor peroxidase components during purification" (see also Tonon et al., 1987), with the number of isozymes present varying for different times of harvesting and also for different nutrient limitations (Leisola et al., 1987).

It is interesting to note that the main isozymes of ligninase separated by Renganathan et al. (1985) had a molecular weight of c.41Kd, and that the molecular

weights of the other two isozymes were c.39Kd and c.43Kd. Similarly, Leisola et al. (1985b) also showed that of the three main isozymes separated by anion-exchange chromatography, the molecular weight of the main isozyme was c.42Kd, and that the molecular weights of the other two isozymes were c.39Kd and c.46Kd. In contrast to these results, although the main isozyme of ligninase produced throughout the course of this thesis, was also shown to have a molecular weight of c.42Kd, the molecular weights of the other two proteins were both greater than this, having a molecular weight of c.44Kd and c.47Kd. It is not known why the molecular weights of the less abundant isozymes should differ in this respect to the results of other groups; this may reflect differences in culture conditions, or post-harvesting processing, or it may even reflect that after a number sub-cultures, the stock culture of P. chrysosporium used in this study, may have mutated such that as a result, it differs with respect to the supposedly same "wild type" strain as used by other groups, in that property which gives the isozymes their differences in molecular weight.

It should be pointed out that as was mentioned in Section 3.4.2, Isozyme IV may actually have consisted of two isozymes (or unrelated proteins) of very similar molecular weight. If this was the case, then it seems likely that the c.47Kd isozyme characterised in this thesis may have been contaminated with the Mn-dependent peroxidase reported by Kuwahara et al. (1984) and

Paszczynski et al. (1985) (see Section 1.3.2.2) which has been shown to have a molecular weight of c.46Kd. Unfortunately, this possibility was not investigated further.

The isoelectric points of the three main isozymes of ligninase characterised in this thesis were at pH 3.2, pH 2.9 and pH 2.6. These values are slightly more acidic than the results obtained by Leisola et al. (1985b and 1987). From an analysis of the amino acid composition of three isozymes of ligninase, Leisola et al. (1987) concluded that the isoelectric points of the isozymes of ligninase were not directly related to the relative proportion of the acidic amino acid residues present, and since the amino acid analysis of the main isozyme of ligninase characterised in this thesis, see Table 12, showed a similar composition as to those reported by Leisola et al. (1987), it might be tentatively concluded that the differences in the isoelectric points (and molecular weights) of different isozymes of ligninase is due to differences in the nature (and number of residues) of the carbohydrate moieties of different isozymes.

This conclusion is backed up to a certain extent by the data presented in this thesis, which showed that the % carbohydrate present relative to protein revealed for two of the three main isozymes of ligninase, Isozymes I and III, (see Section 3.3.2), was 21.9% and 23.0%, respectively, (unfortunately, it was not possible to obtain a figure for Isozyme IV; this was due to the

limited quantities of this isozyme that were available; see Section 3.4.6) and thus, it can be calculated that the relative distribution of molecular weight between the carbohydrate moiety and the rest of the isozyme molecule, was 9150Kd + 32550Kd (= 41700) Kd for Isozyme I, indicating the presence of c.51 sugar residues per molecule; and 10050Kd + 33650Kd (= 43700) Kd for Isozyme III, indicating the presence of c.56 sugar residues per molecule. This is the first report of the % carbohydrate present for an isozyme other than that of the most predominant isozyme, and shows that at least for the two isozymes compared in this study, their differences in molecular weight and also in isoelectric point, are in part related to their relative proportion of sugar residues. However, it is fair to point out that from the data presented in this thesis, this would not appear to be the sole reason accounting for their different properties.

It should be also noted that not all isozymes of ligninase which differ with respect to their isoelectric points, also differ with respect to their molecular weights. For example, Leisola et al. (1985b) reported the separation of three isozymes of ligninase that differed in their relative isoelectric points, but which each had the same apparent molecular weight of c.42Kd. From the data obtained in this thesis, it might tentatively be concluded that this was also the case for Fraction II (see Section 3.4.2). For example, when this

fraction was characterised by SDS-polyacrylamide gel electrophoresis, it appeared to contain only material apparently identical in molecular weight material to that present in Fractions I, III and IV, and was, perhaps mistakenly, not further purified. In fact, if Fig. 28 is examined carefully, it can be seen that in terms of isoelectric points, Fraction II did indeed contain the two major proteins also present in Fractions I and III, but that in addition to these, it also contained a number of other proteins that had isoelectric points between those of the main proteins in Fractions I and III, and that were present in a greater relative quantities than the apparently identical isozymes also present in Fractions I and III.

It is interesting to note that although differences between the nature of the carbohydrate portions of glycoprotein isozymes is well documented (Sharon and Lis, 1982; Kornfeld and Kornfeld, 1980), the characterisation of the isozymes of horseradish peroxidase has shown that several of these isozymes differed not only in respect of their carbohydrate composition, but also in terms of their amino acid composition (Shannon et al., 1966).

In comparison to the results of other groups, the % carbohydrate relative to protein for the main 41-42Kd isozyme has been variously reported to be 6%, 13% and 21% (Renganathan et al., 1985; Tien and Kirk, 1984; and Paszczynski et al., 1986, respectively). Hence, although the results presented in this thesis show a close

similarity to the results of Paszczynski et al. (1986), this does not explain why the % carbohydrate figures for different groups should vary so significantly.

However, one likely explanation for these differences, is the accuracy of the methods used to determine the concentrations of protein and carbohydrate. The estimation of the protein content of acidic glycoproteins, such as ligninase, is particularly unreliable, since these proteins contain only a low proportion of the aromatic residues that are required for an accurate determination of the real protein concentration (Paszczynski et al., 1986). In this study (see Section 2.5.7), the determination of the protein concentration was carried out by the Bradford method (Bradford, 1976); however, for each measurement of protein concentration, the results obtained were "adjusted" by a correction factor previously determined by comparing the protein concentration of a pure isozyme of ligninase as predicted by the Bradford method using a standard of bovine serum albumin, with its real concentration as determined from its amino acid analysis. The results of this experiment showed that by the Bradford method, the protein concentration was $0.74mg.ml^{-1}$, whereas, from its amino acid analysis, the actual protein concentration was $1.62mg.ml^{-1}$ (i.e. x 2.2). This correction factor was then used for all determinations of the protein concentration of ligninases, and assumed that for each preparation of ligninase, the predominant protein(s) present was

ligninase and that all isozymes of ligninase should be corrected by the same relative amount. These assumptions may or may not have been fully justified, but nevertheless the use of the correction factor probably resulted in a more accurate determination of the real protein concentration than had it not been used at all.

Another problem which became evident in this thesis, was the apparently high levels of contaminating carbohydrate present in both crude and purified preparations of ligninase. This problem was overcome by precipitating the proteins out of solution, and then determining the protein and carbohydrate concentrations of the precipitated material after its separation from the contaminating carbohydrate (see Section 2.5.8). During the latter procedure, it also became evident that the presence of contaminating carbohydrate was resulting in an under estimate of the concentration of protein that was present, since after the precipitation of the proteins, there was apparently more protein present than had been originally predicted; see Table 10.

A consequence of these difficulties in measuring the true concentration of protein, has meant that the determination of the specific activity of ligninase preparaions has produced results which cannot be considered to be wholly reliable. For example, from Table 10, it can be seen that before TCA precipitation, the specific activity of a crude preparation of ligninase was apparently $44.6U.mg^{-1}$, whereas, after TCA precipitation,

the concentration of protein detected had apparently increased by more than six times, and consequently, the specific activity of the same preparation of ligninase decreased to only $6.9U.mg^{-1}$.

Similarly, it can be seen that for at least two of the three main isozymes of ligninase purified during the course of this thesis, there is a similar decrease in specific activity after TCA precipitation. However, more importantly, it can be seen that the relative decrease in specific activity is different for each of these two isozymes, and that whereas before TCA precipitation, the specific acivity of the most abundant isozyme of ligninase (i.e. Isozyme I) was less than that of the other isozyme (i.e. Isozyme III), after TCA precipitation, the specific activity of Isozyme I is apparently greater than that of Isozyme III. This is an extremely significant result, since in all other reports on the specific activities of the isozymes of ligninase, it has always been the most abundant isozyme of ligninase which has been shown to have the highest specific activity (e.g. Renganathan et al., 1985). It should be pointed out that for all calculations of specific activity, it was assumed that the activity of a given aliquot of a ligninase preparation was the same before and after TCA precipitation. This assumption was made since proteins are denatured by TCA precipitation, and thus can no longer be assayed to determine how their activity might have been affected by the presence of the "excess" carbohydrate. However, it should be noted that

the presence of high levels of polysaccharide has also been shown to inhibit the degradation of lignin (Leisola et al., 1982) and consequently, the assumption made above may have been misleading (see also Section 4.2.4).

The K_m for H_2O_2 was originally reported to be c.30uM (Tien and Kirk, 1984). In a later study, however, Tien et al. (1986) showed that the K_m for H_2O_2 was actually pH-dependent, and this undoubtedly explains why in this study the K_m for H_2O_2 was found to be higher than that originally reported with a value of c.40uM.

In contrast to the K_m for H_2O_2, the K_m for veratryl alcohol reported by Tien et al. (1986) was said to be unaffected by changes of pH and to not be significantly different for different preparations of enzyme; remaining at a steady value of c.55 - 70µM. However, in this thesis, it was found that for a crude preparation of ligninase, the K_m for veratryl alcohol was c.300µM, and for a pure isozyme, it was c.110µM. Similarly, Renganathan et al. (1985) found that the K_m for veratryl alcohol for the three main isozymes of ligninase were 95, 71, and 55µM, respectively. This suggests that the K_m for veratryl alcohol does indeed differ for different preparations of ligninase. These differences in the K_m for veratryl alcohol may either have been related to differences in culture conditions (e.g. Tien et al., (1986) used stationary low volume cultures, and Renganathan et al. (1985) used agitated increased volume cultures), or in the preparation (and purification) of the ligninase used in these studies, or even to a mutation of the fungus.

At least one property of ligninase which is consistent in all reports, is the UV/visible spectrum of pure preparations of ligninase; for example, in addition to the data presented in this thesis, see Tien and Kirk (1984), Gold et al. (1984), Renganathan et al. (1985), Tien et al. (1986), Renganathan and Gold (1986) and Paszczynski et al. (1986). For general comments on the UV/visible spectra of ligninase, see Section 3.4.4.

4.2.3 pH optimum

The pH optimum of ligninase (as determined by the oxidation of veratryl alcohol to veratraldehyde, Tien and Kirk 1984) was originally reported to be at or near pH 3.0 (Tien and Kirk, 1984; Kuwahara et al., 1984). However, in a number of reports, ligninase activity was measured at pH 2.5 without detailing the reason for this apparent shift from the pH optimum (e.g. Jager et al., 1985; Kirk et al., 1986c). More recently, Tien et al. (1986) carried out a detailed study of the kinetics of veratryl alcohol oxidation using a purified isozyme of ligninase, and demonstrated that the pH optimum was in fact pH 2.0, although the enzyme was unstable at this pH.

In this thesis, the pH optimum of ligninase (veratryl alcohol oxidase) was determined before and after purification. It was found that for a crude preparation of ligninase produced by pooling the concentrated extracellular medium from five successive harvests of foam-immobilised P. chrysosporium, the pH

optimum became more acidic in the presence of added Mg^{2+} ions. In the absence of added Mg^{2+} ions, the pH optimum was pH 3.1, and in the presence of added Mg^{2+} ions, the pH optimum was pH 2.7. This finding was consistent with the previous observation that when using the same preparation of enzyme, there was a substantial increase in the rate of veratryl oxidation when ligninase was assayed at pH 2.75 in the presence of a variety of metal ions.

The purification of the isozymes of ligninase resulted in the separation of three isozymes in a relative ratio of 90:5:5. It was found that the pH optima of the three isozymes were at pH 2.2, pH 2.7 and pH 2.0, respectively. It was also found that after the purification procedure, added metal ions were no longer able to increase the rate of veratryl alcohol oxidation. The effect of metal ions on the oxidation of veratryl alcohol and its apparent pH-dependence will be discussed in more detail in Section 4.2.4.

Tien et al. (1986) suggest that the decrease in ligninase activity observed at pH values above pH 2.0 is probably due to the deprotonation of a group required for catalysis. It may be hypothesized that the more acidic pH optima observed after the purification of two of the isozymes of ligninase, was perhaps due to the removal of impurities which may themselves be able to function as buffers and thus lessen the deprotonation of a vital group required for catalysis. Alternatively, the

purification may in some way result in improved accessibility to the active site of the enzyme, and provide a greater general stability in more acidic conditions. Unfortunately, the differences in the pH optima of the three isozymes of ligninase were not able to be simply correlated to differences in their iso-electric points, molecular weights or relative carbohydrate contents, and it is not clear why the pH optimum of one of the isozymes of ligninase was not so acidic as the other two isozymes after purification.

The differences in the pH optima of crude and purified ligninase preparations reported in this thesis may well explain the apparently inconsistent pH optima reported by other laboratories.

It is interesting to note that the pH optimum of lignin degradation is actually pH 4.5 (i.e. the same pH as that of the medium in which the fungus is routinely grown - see Appendix I), and that at pH values of pH 3.5 and pH 5.5, lignin degradation is substantially reduced (Kirk et al., 1978). The observation that the pH optimum of veratryl alcohol oxidation is clearly more acidic than that of lignin degradation may simply be due to the fact that the rate limiting step in the degradation of lignin is caused by an activity other than that of ligninase. However, the precise role of ligninase in the degradation of lignin is certainly an area that requires further investigation. For example, both crude and purified preparations of ligninases were shown to actually

polymerise lignins when assayed at pH 4.0 (Haemmerli et al., 1986a); and Kirk et al., (1986a), using a range of mutants of P. chrysosporium, were unable to demonstrate that there was any correlation between the degradation of lignin and the production of ligninase.

4.2.4 Effect of metal ions and the isolation of an inhibitor of ligninase

In Section 4.2.3, it was described that when using a crude preparation of ligninase obtained by pooling the concentrated extracellular medium from five successive harvests of foam-immobilised P. chrysosporium, Mg^{2+} ions were able to stimulate the rate of veratryl alcohol oxidation in the more acidic pH region, at the same time leading to an apparent shift of the pH optimum from pH 3.1 to pH 2.7, and that after the purification of the isozymes of ligninase, this effect was no longer observed. To determine whether this phenomenon was peculiar to the use of foam-immobilised cultures of P. chrysosporium, it was decided to examine the effect of Mg^{2+} ions on the rate of veratryl alcohol oxidation by a crude preparation of concentrated ligninase obtained without the use of polyurethane foam as an immobilisation support. This was initially investigated at a single pH value, where it was predicted that if there was any effect, it should be possible to observe it. When it was found that Mg^{2+} ions were not able to alter the rate of veratryl alcohol oxidation of this preparation, it was then decided to examine the effect of Mg^{2+} ions on the

rate of veratryl alcohol oxidation of each individual concentrated harvest produced by a foam-immobilised culture of P. chrysosporium. The results of this experiment demonstrated that the first two successive harvests were largely unaffected by the presence of Mg^{2+} ions, and that for the next two harvests there was a marginal increase in the rate of veratryl alcohol oxidation, but that for the fifth and also the sixth harvests, there was a greater than tenfold increase in the rate of veratryl alcohol oxidation. Indeed, it can be concluded from these results that the general trend was for Mg^{2+} ions to stimulate the veratryl alcohol oxidase activity of each successive harvest of concentrated ligninase produced by the foam-immobilised P. chrysosporium by an increasing relative amount for each fresh successive harvest.

It was also shown in this thesis that this apparent stimulation of the rate of veratryl alcohol oxidation by Mg^{2+} ions was able to be explained by the production of an inhibitor of ligninase which was rendered largely ineffective in the presence of the Mg^{2+} ions. Hence, it is more accurate to describe the effect of Mg^{2+} not as a "stimulation" but more as a "reactivation" or a "restoration".

This is the first report of a metal-ion inactivated inhibitor of ligninase, and has probably not been observed before mainly because most research groups are still using a standard batch culture method of ligninase production

where it is unlikely to be produced in sufficient quantities to permit its detection, but also because when ligninase is assayed at pH 3.0, there is no reason to suspect that the ligninase activity might indeed be inhibited, and finally because the inhibitor is "lost" during anion-exchange chromatography. The production of this inhibitor may be a major contributory factor for the decline in the magnitude of successive harvests of ligninase produced using the foam immobilisation technique. It is interesting to note that Dodson and Kamal (Unpublished data) did not observe any apparent stimulation of the rate of veratryl alcohol oxidation by Mg^{2+} ions when they used the foam immobilisation technique devised in this thesis under conditions of carbon limitation, and thus, it would appear that under these conditions, the inhibitor is not produced.

Unfortunately, it was not possible to determine the precise identification of the inhibitor(s) of ligninase. However, from the results obtained in this thesis, it can be concluded that the inhibitor has a molecular weight of greater than 10Kd, and that by implication from its isolation by anion-exchange chromatography and also its retention time when subjected to C-18 HPLC analysis, it is is extremely polar in nature. It can also be concluded that it is not a protein but that it is most likely a carbohydrate. A comparison of its UV/visible spectrum (see Fig. 42) with that of hyaluronic acid (see Ogston and Stanier 1950) showed that

there was a great deal of similarity, and it was then found that when the inhibitor-containing fraction (i.e. Fraction V; see Section 3.3.1) was specifically assayed for the presence of uronic acids, a uronic acid with unsubstituted threo hydroxyl groups at the C-3 - C-4 positions was able to be detected, see Section 3.5.5. When a polymeric uronic acid, in this case heparan sulphate, was tested for its ability to inhibit ligninase, it was found that a similar pH-dependent inhibition of the oxidation of veratryl alcohol was produced to that observed for the isolated inhibitor; however, this inhibition was unaffected by the presence of added Mg^{2+} ions, see Section 3.5.7. When gas chromatography/mass spectrometry was performed on the isolated inhibitor, it was found that there was insufficient material present to permit the identification of any of the molecular structures present in the inhibitor fraction (data not shown in this thesis).

From the evidence presented in this thesis, it may be hypothesized that the inhibitor of ligninase activity is a polymeric uronic acid that in the presence of added metal ions is no longer able to inhibit the action of ligninase. Although the mechanism whereby metal ions render the inhibitor ineffective has not been elucidated, it could be imagined that the metal ions are able to chelate the inhibitor, thus preventing it from binding to the enzyme.

From the kinetic studies carried out using the inhibitor, it was apparent that for a purified isozyme of ligninase, increasing concentrations of the inhibitor resulted in a decrease in the V_{max} and a simultaneous increase in the K_m, and hence, was indicative of a mixed pattern of inhibition (Rudolph, 1983). In contrast to these results, it was found that using the crude preparation of ligninase from which the inhibitor was isolated, a comparison of the kinetic properties in the presence and absence of Mg^{2+} ions revealed that for both veratryl alcohol and H_2O_2, the presence of the Mg^{2+} ions resulted in a definite increase in the V_{max} with no alteration in the K_m. This implies that for the crude enzyme preparation, the nature of the inhibition, which is presumably "masked" in presence of the Mg^{2+} ions, is a classic example of noncompetitive inhibition (Rudolph, 1983). It is not known why the nature of the inhibition is not so clearly defined for the pure isozyme of ligninase, but it should be pointed out that for the crude preparation of ligninase, the effect being studied is the apparent release from a state of partial inhibition in a fixed amount of inhibitor; whereas, for the purified isozyme of ligninase, the effect being studied is the pattern of inhibition in the presence of increasing concentrations of inhibitor, and thus the two sets of experiments are not directly comparable. This observation probably accounts for why the two sets of results do not produce the same results, and with hind-sight, it can be concluded that a better experiment to

have carried out, would have been to study the effect of metal ions on the pattern of inhibition of the purified isozyme of ligninase in a fixed concentration of the isolated inhibitor fraction.

In a recent report on the analysis of the polysaccharide material produced by a continuous culture of P. chrysosporium, Buchala and Leisola (1987) showed that after acid hydrolysis, this material contained glucose, mannose, galactose, fucose, and xylose, but that it did not contain uronic acids. The inability to detect the presence of uronic acids may have been due to the fact that Buchala and Leisola (1987) used an assay method which is reported to be less sensitive and less selective than that used in this thesis (see Scott, 1979). An alternative explanation for inability of Buchala and Leisola (1987) to detect the presence of uronic acids may have been due to the entirely different methods of culture, but perhaps more importantly it is not described whether the continuous cultures of P. chrysosporium used for the isolation of the polysaccharide material were ligninolytic or whether they were still actively growing. However, in the original paper which describes the continuous culture of P. chrysosporium, it was concluded by Leisola et al. (1982) that the production of polysaccharide resulted in the inhibition of lignin degradation, although the authors were unable to define the precise nature of this inhibition.

Finally, it useful to summarise what has been learnt from the results obtained in this thesis, and what directions subsequent research should follow to unravel some of the observations made in this thesis, but which have not been able to be fully resolved. First, it should be stated that from the results presented and discussed in this thesis, the culture conditions which lead to the highest levels of ligninase production are observed when using agitated, induced cultures incubated under conditions of carbon limitation, and in the presence of an immobilisation support to facilitate the production of successive harvests of ligninase from the same fungal biomass. Although the use of carbon limited cultures for the production of ligninase had only just been reported after much of the developmental microbiology had already been carried out in this thesis, the results of preliminary studies using carbon limited foam-immobilised cultures of P. chrysosporium, and certainly the results of Linko et al. (1987) which have been discussed above (see Section 4.1), have both shown that higher levels of ligninase are produced under conditions of carbon limitation, and also that the continued production of successive harvests of ligninase using foam, or agar/agarose immobilised cultures, is significantly better under conditions of carbon limitation since there is no apparent decrease in the magnitude of successive harvests of ligninase. Having described the optimal conditions of ligninase production that exists to-date, it is worth considering that perhaps the next

leap forward in the production of ligninase by *P. chrysosporium* will be to adapt the use of immobilised cultures of the fungus to the production of ligninase in a fully continuous system. Linko et al. (1987) have already presented some sketchy data in this respect, but it is obvious that there are still many problems that are required to be sorted out. For example, which immobilisation system? What fermentor design? Composition of medium? Use of inducers or protectors? Strain improvement and how to select for a better strain? In addition, in terms of the microbiology, there is also the cloning of ligninase first mentioned in Section 1.3.3. One of the problems that requires to be sorted out with this approach is the incorporation - and stability - of the haem group; and for that matter, how is it best to improve the haem group? Having mentioned haem groups, there is also some interesting work coming out of the laboratories of David Dolphin, UBC, Vancouver, Canada and Mike Shimada, Kyoto University, Japan, who are using artificial haem groups to carry out some of the transformations mediated by ligninase.

Having described some of the possible objectives that exist in the field of microbiology, it should also be stated that in the field of biochemistry, the regulation of ligninase in the breakdown of lignin *in vivo* has yet to be fully sorted out; for example, what is it that prevents ligninase from polymerising lignin when the enzyme is functioning in the presence of the fungus, and

conversely, why does ligninase polymerise lignin when isolated away from the fungus? And also, returning to the observations described in this thesis, why is it that an organism capable of degrading lignin, and incubated under ligninolytic conditions, produces an inhibitor of ligninase? In the introduction to this thesis, it was described that wood is likely to be a nitrogen limited substrate, and so consequently, it may not be totally inconceivable that this inhibitor is produced *in vivo*. If so, does it act as a regulator of ligninase activity? Is the role of metal ions in wood perhaps related to the inactivation of this inhibitor; for example, is this the explanation for the accumulation of manganese ions in degraded wood (Blanchette, 1984), or the observation that inorganic salts can be used to act as wood preservatives (Nicholas and Preston, 1984), or the observation by Leatham (1986) that the ligninolytic activity of *P. chrysosporium* is apparently stimulated in the presence of a range of inorganic metals? The average pH of wood is reported to be between pH 3.0 and pH 5.5 (Stamm, 1964) and yet although the ligninolytic fungus studied by most researchers, i.e. *P.chrysosporium*, will grow optimally at pH 4.5, why is it that at this pH, it produces an enzyme that has a pH optimum of anything between pH 2.0 and pH 3.1? And finally what is the role of the other enzymes such as the manganese-dependent peroxidase described in Section 1.3.2.2?

These are just some of the many issues that have yet to be sorted out, and which together help maintain a large interest in the field of lignin degradation, both in industry and in academia.

Appendix I

This appendix describes the composition of the medium used in all stationary and agitated batch culture experiments. Routinely, five times concentrated stock solutions of the medium components were autoclaved at 121°c for 15 minutes and were then stored at 4°c until required.

A.1.1 Standard culture medium

	Concentration / $g.l^{-1}$
Glucose	10.0
Ammonium tartrate	0.22
Potassium dihydrogen phosphate	2.0
Magnesium sulphate	0.5
Calcium chloride	0.1
Thiamine.HCl	1.0×10^{-3}
Sodium tartrate buffer, pH 4.5	10.0mM
Mineral solution	10.0ml *

(* See below - Please turn over)

A.1.2 Mineral solution

	Concentration / $g.l^{-1}$
Nitrilotriacetate	1.5
Magnesium sulphate	3.0
Manganese sulphate	0.5
Sodium chloride	1.0
Iron (II) sulphate	0.1
Cobalt sulphate	0.1
Calcium chloride	0.11
Zinc sulphate	0.1
Copper (II) sulphate	0.01
Aluminium potassium sulphate	0.01
Boric acid	0.01
Sodium molybdate	0.01

Appendix II

A.2.1 Markers for SDS-polyacrylamide gel electrophoresis

Protein	Molecular Weight (Kd)	Log_{10} Molecular weight
Bovine serum albumin	67.0	1.820
Catalase	60.0	1.778
Ovalbumin	43.0	1.633
Peroxidase	40.0	1.602
Lactate dehydrogenase	36.0	1.556
Chymotrypsin	25.7	1.410
Myoglobin	17.2	1.23
Cytochrome c oxidase	12.3	1.090

REFERENCES

Adler, E. (1977) Lignin Chemistry. Past, present and future. Wood Sci. Technol. 11:169-218.

Ander, P. and Eriksson, K.E. (1975) Influence of carbohydrates on lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Sven. Papperstid. 78:643-652.

Ander, P. and Eriksson, K.E. (1976) The importance of phenoloxidase activity in lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Arch. Microbiol. 109:1-8.

Ander, P. and Eriksson, K.E. (1978) Lignin degradation and utilization by micro-organisms. Prog. Ind. Microbiol. 14:1-58.

Anderson, T.F. (1951) Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. Trans. N.Y. Acd. Sci. 13:130-134.

Andersson, L.A., Renganathan, V., Chiu, A.A., Loehr, T.M. and Gold, M.H. (1985) Spectral characterization of diarylpropane oxygenase, a novel peroxide-dependent, lignin-degrading heme enzyme. J. Biol. Chem. 260:6080-6087.

Antai, S.P. and Crawford, D.L. (1981) Degradation of softwood, hardwood, and grass lignocelluloses by two Streptomyces strains. Appl. Env. Microbiol. 42:378-380.

Bellamy, W.D. (1972a) Chem. Eng. News. 50:14.

Bellamy, W.D. (1972b) The use of thermophilic for the recycling of cellulosic waste. Proc. Am. Inst. Chem. Eng., Meeting, Aug. 28, 1972.

Bellamy, W.D. (1974) Single cell protein from cellulosic wastes. Biotechnol. Bioeng. 16:869-880.

Benner, R., MacCubbin, A.E. and Hodson, R.E. (1984) Preparation, characterization, and microbial degradation of specifically radiolabelled (^{14}C)lignocelluloses from marine and freshwater macrophytes. Appl. Env. Microbiol. 47:381-389.

Blanchette, R.A. (1984) Manganese accumulation in wood decayed by white rot fungi. Phytopathology 74:725-730.

Blanchette, R.A. and Reid, I.D. (1986) Ultrastructural aspects of wood delignification by Phlebia (Merulius) tremellosus. Appl. Env. Microbiol. 52:239-245.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ana. Biochem. 72:248-254.

Brandt, D.L., Hartz, L. and Mandels, M. (1972) Engineering aspects of the enzymatic conversion of waste cellulose to glucose. Proc. Am. Chem. Eng., Meeting, Aug. 28, 1972.

Buchala, A.J. and Leisola, M.S.A. (1987) Structure of the beta-glucan secreted by Phanerochaete chrysosporium in continuous culture. Carbohydrate Chemistry (in press).

Burdsall, H.H. and Eslyn,W.E. (1974) A new Phanerochaete with a chrysosporium imperfect state. Mycotaxon. 1:123-133.

Buswell, J.A., Mollet, B. and Odier, E. (1984) Ligninolytic enzyme production by Phanerochaete chrysosporium under conditions of nitrogen sufficiency. FEMS Microbiol. Lett. 25:295-299.

Chalel, D.S., Moo-Young,M. and Dhillon,D.S. (1979) Bio-conversion of wheat straw components into single cell protein. Can. J. Microbiol. 25:793-797.

Chandra, S. and Jackson, M.G. (1971) A study of various chemical treatments to remove lignin from coarse roughages and increase their digestibility. J. Agric. Sci., Camb. 77:11-17.

Chen, C.L. and Chang, H.M. (1985) Chemistry of lignin biodegradation. Academic Press, San Diego, C.A, 535-556.

Chen, C.L., Chang, H.M. and Kirk, T.K. (1982) Aromatic acids produced during degradation of lignin in spruce wood by Phanerochaete chrysosporium. Holzforshung 36:3-9.

Chen, C.L., Chang, H.M. and Kirk, T.K. (1983) Carboxylic acids produced through oxidative cleavage of aromatic rings during degradation of lignin in spruce wood by Phanerochaete chrysosporium. J. Wood Chem. Technol. 3:35-57.

Chua, M.G.S. Chen, C.L., Chang, H.M. and Kirk, T.K. (1982) ^{13}C NMR spectroscopic study of spruce lignin degraded by Phanerochaete chrysosporium. I. New structures. Holzforschung 36:165-172.

Chua, M.G.S., Choi, S. and Kirk, T.K. (1983) Mycelium binding and depolymerization of synthetic ^{14}C-labelled lignin during decomposition by Phanerochaete chrysosporium. Holzforschung 37:55-61.

Clausen, E.C., Sitton, O.C. and Gaddy, J.L. (1977) Bio-conversion of crop materials to methane. Process Biochem. 12:5-8.

Colberg, P.J. and Young, L.Y. (1985) Anaerobic degradation of soluble fractions of (^{14}C)lignocellulose. Appl. Env. Microbiol. 49:345-349.

Commoner, B. (1979) The economics of methane. Environ-ment. 21:29-33.

Cowling, E.B. and Merrill, W. (1966) Nitrogen in wood and its role in wood deterioration. Can. J. Bot. 44:1539-1554.

Crawford, D.L., Barder, M.J., Pometto, A.L. and Crawford, R.L. (1982) Chemistry of softwood degradation by Streptomyces viridiosporus. Arch. Microbiol. 131:140-145.

Crawford, D.L. and Crawford, R.L. (1976) Microbial degradation of lignocellulose:the lignin component. App. Env. Microbiol. 31:714-717.

Crawford, D.L., Pometto, A.L. and Crawford, R.L. (1983) Lignin degradation by Streptomyces virdiosporus: Isolation and characterization of a new polymeric lignin degradation intermediate. Appl. Env. Microbiol. 45:898-904.

Crawford, R.L. (1981) Lignin biodegradation and transformation. New York:Wiley and Sons.

Daugulis, A.J. and Bone, D.H. (1977) Submerged cultivation of edible white-rot fungi on tree bark. Eur. J. Appl. Microbiol. 4:159-166.

Davis, B.D. (1980) In "Microbiology", Harper and Row Publishers Inc., eds. B.D.Davis, R.Dulbecco, H.M.Eisen, H.S.Ginsberg, pp. 67-68.

Dekker, F.H. and Richards, G.N. (1973) Effect of delignification on the *in vitro* rumen digestion of polysaccharides of bagasse. J. Sci. Fed. Agri. 24:375-379.

Deschamps, A.M., Mahoudreau, G. and Lebeault, J.M. (1980) Fast degradation of kraft lignin by bacteria. Eur. J. Appl. Microbiol. 9:45-51.

Dhawan, S. and Gupta, K. (1977) Enzymic hydrolysis of common cellulosic wastes by cellulase. J. Gen. Appl. Microbiol. 23:155-161.

Drew, S.W. and Kadam, K.L. (1979) Lignin metabolism by *Aspergillus fumigatus* and white-rot fungi. Dev. Ind. Microbiol. 20:153-161.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for the determination of sugars and related substances. Anal. Chem. 28:350-356.

Eaton, D.C. (1985) Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: a ligninolytic fungus. Enz. Microbiol. Technol. 7:194-196.

Erickson, M. and Miksche, G.E. (1974a) Two dibenzofurans obtained on oxidative degradation of the moss *Polytrichum communa* Hedw. Acta Chem. Scand. B 28:109-113.

Erickson, M. and Miksche, G.E. (1974b) Characterization of gymnosperm lignins by oxidative degradation. Holzforschung 28:135-138.

Eriksson, K.E., Johnsrud, S.C. and Vallander, L. (1983) Degradation of lignin and lignin model compounds by variouus of the white-rot fungus, *Sporotrichum pulverulentum*. Arch. Microbiol. 135:161-168.

Eriksson, K.E., Pettersson, B., Volc, J. and Musilek, V. (1986) Formation and partial characterization of glucose-2-oxidase, a H_2O_2 producing enzyme in *Phanerochaete chrysosporium*. Appl. Microbiol. Biotech. 23:257-262.

Evans, W.C. (1977) Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17-22.

Faison, B.D. and Kirk, T.K. (1983) Relationship betweeen lignin degradation and production of reduced oxygen secies by *Phanerochaete chrysosporium*. Appl. Env. Microbiol. 46:1140-1145.

Faison, B.D. and Kirk, T.K. (1985) Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Appl.Env. Microbiol. 49:299-304.

Faix, O., Mozuch, M.D. and Kirk, T.K. (1985) Degradation of gymnosperm (guaiacyl) vs. angiosperm (syringyl/ guaiacyl) lignins by *Phanerochaete chrysosporium*. Holzforschung 39:203-208.

Farnham, R.S. (1978) Energy from peat: subcommittee 8 report to the Minnesota Energy Agency. Alternative Energy Research and Development Policy Formulation Project. St. Paul, Minn.

Ford, C.W. (1978) Effect of partial delignification on the in vitro digestibility of cell wall polysaccharides in Digitaria decumbens (Pangola grass). Aust. J. Agric. Res. 29:1157-1166.

Forney, L.J., Reddy, C.A. and Pankratz, H.S. (1982a) Ultrastructural localization of hydrogen peroxide production in ligninolytic Phanerochaete chrysosporium cells. Appl. Env. Microbiol. 44:732-736.

Forney, L.J., Reddy, C.A., Tien, M. and Aust, S.D. (1982b) The involvement of the hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white-rot fungus Phanerochaete chrysosporium. J. Biol. Chem. 257:11455-11462.

Ghosh, S. and Klass, D.L. (1979) Biomethanation of Minnesota reed sludge peat. Rec. Recov. Conservat. 4:115-139.

Glenn, J.K. and Gold, M.H. (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete Phanerochaete chrysosporium. Arch. Biochem. Biophys. 242:329-341.

Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. and Gold, M.H. (1983) An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete Phanerochaete chrysosporium. Biochem. Biophys. Res. Comm. 114:1077-1083.

Gold, M.H. and Cheng, T.M. (1979) Conditions for fruiting body formation in the white-rot basidiomycete Phanerochaete chrysosporium. Arch. Microbiol. 121:37-41.

Gold, M.H., Kuwahara, M., Chiu, A.A. and Glenn, J.K. (1984) Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white-rot basidiomycete, Phanerochaete chrysosporium. Arch. Biochem. Biophys. 234:353-362.

Goldsby, G.P., Enoki, A. and Gold, M.H. (1980) Alkyl-phenyl cleavage of the lignin model compound guaiacylglycol and glcerol-B-guaiacyl ether by Phanerochaete chrysosporium. Arch. Microbiol. 128:190-195.

Goldstein, I.S. (1975) Potential for converting wood into plastics. Science 189:847-852.

Greene, R.V. and Gould, J.M. (1984) Fatty acyl-coenzyme A oxidase activity and H_2O_2 production in Phanerochaete chrysosporium. Biochem. Biophys. Res. Comm. 118:437-443.

Haars, A. and Huttermann, A. (1980) Macromolecular mechanism of lignin degradation by Fomes annosus. Naturwissenschaften 67:39-40.

Haemerli, S.D., Leisola, M.S.A. and Fiechter, A. (1986a) Polymerisation of lignins by ligninases from Phanerochaete chrysosporium. FEMS Microbiol. Lett. 35:33-36.

Haemerli, S.D., Leisola, M.S.A., Sangland, D. and Fiechter, A. (1986b) Oxidation of benzo(a)pyrene by extracellular ligninases of Phanerochaete chrysosporium. J. Biol. Chem. 261:6900-6903.

Haider, K. and Trojanowski, J. (1975) Decomposition of specifically ^{14}C-labelled phenols and dehydropolymers of coniferyl alcohol as models for lignin degradation by soft and white rot fungi. Arch. Microbiol. 105:33-41.

Hajny, G.J., Gardner, C.H. and Ritter, G.J. (1951) Thermophilic fermentation of cellulose and lignocellulose materials. Ind. Eng. Chem. 43:1384-1389.

Hammel, K.E., Kalyanaraman, B. and Kirk, T.K. (1986a) Substrate free radicals are intermediates in lignin catalysis. Proc. Nat. Acad. Sci. USA 83:3708-3712.

Hammel, K.E., Kalyanaraman, B. and Kirk, T.K. (1986b) Oxidation of polycyclic aromatic hydrocarbons and dibenzo(p)-dioxins by Phanerochaete chrysosporium ligninase. J. Biol. Chem. 261:16948-16952.

Hammel, K.E., Kalyanaraman, B. and Kirk, T.K. (1987) Oxidation of aromatic pollutants by Phanerochaete chrysosporium ligninase. Proceedings "International Seminar on lignin enzymic and microbial degradation". In press.

Hammel, K.E., Tien, M, Kalyanaraman, B. and Kirk, T.K. (1985) Mechanism of oxidative C-alpha - C-beta cleavage of a lignin model dimer by Phanerochaete chrysosporium: stoichiometry and involvement of free radicals. J. Biol. Chem. 260:8348-8353.

Harkin, J.M., Crawford, D.L. and McCoy, E. (1974) Bacterial protein from pulps and papar millsludge. Tappi 57:131-134.

Harvey, P.J., Schoemaker, H.E., Bowen, R.M. and Palmer, J.M. (1985a) Single-electron transfer processes and the reaction mechanism of enzymic lignin degradation. FEBS Lett. 183:13-16.

Harvey, P.J., Schoemaker, H.E. and Palmer, J.M. (1985b) Enzymic lignin degradation of lignin and its potential to supply chemicals. In "Plant products and the new technology" eds. K.W. Fuller, J.R. Gallon. Ann. Proc. Phytochem. Soc. Eur. 26:249-266. Oxford: Clarendon Press.

Harvey, P.J., Schoemaker, H.E. and Palmer, J.M. (1986) Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by Phanerochaete chrysosporium. FEBS Lett. 195:242-246.

Hashimoto, K. (1975) Oxidation of phenols by yeast. II Oxidation of cresols by Candida tropicalis. J. Gen. Appl. Microbiol. 19:171-187.

Hatakka, A. (1986) Degradation and conversion of lignin, lignin-related aromatic compounds and ligno-cellulose by selected white-rot fungi. Ph.D thesis. Univ. Helsinki, Finland. p97.

Hatakka, A.I., Buswell, J.A., Pirhonen, T.I. and Uusi-Rauva, A.K. (1983) Degradation of ^{14}C-labelled lignins by white-rot fungi. In "Recent advances in lignin biodegradation research", Tokyo: Uni Publishers, pp.176-185.

Haworth, W.N., Jones, W.G.M. (1944) The conversion of sucrose into furan compounds. Part 1. 5-hydroxymethylfuraldehyde and some derivatives. J. Chem. Soc., pp.667-670.

Higuchi, T. (1987) Catabolic pathways and role of ligninase for the degradation of lignin sub-structure model compounds by white-rot fungi. Wood Research (Kyoto Univ.). In press.

Higuchi, T., Shimada, M., Nakatsubo, F. and Tanahashi, M. (1977) Differences in biosynthesis of guaiacyl and syringyl lignins in wood. Wood Sci. Technol. 11:153-167.

Holt, D.M. and Jones, E.B.G. (1983) Bacterial degradation of lignified wood cell walls in anaerobic aquatic habitats. Appl. Env. Microbiol. 46:722-727.

Huynh, V.B. and Crawford, R.L. (1985) Novel extra-cellular enzymes (ligninases) of Phanerochaete chrysosporium. FEMS Microbiol. Lett. 28:119-123.

Jager, A., Croan, S. and Kirk, T.K. (1985) Production of ligninases and degradation of lignin in submerged cultures of Phanerochaete chrysosporium. Appl. Env. Microbiol. 50:1274-1278.

Jeffries, T.W., Choi, S. and Kirk, T.K. (1981) Nutritional regulation of lignin degradation by Phanerochaete chrysosporium. Appl. Env. Microbiol. 42:290-296.

Johnsrud, S.C. and Eriksson, K.E. (1985) Cross-breeding of selected and mutated homokaryotic strains of Phanerochaete chrysosporium K-3: New cellulase-defficient strains with increased ability to degrade lignin. Appl. Microbiol. Biotech. 21:320-327.

Kamaya, Y. and Higuchi, T. (1983) Degradation of d,I-syringaresinol and its derivatives, B,B'-linked substructure models, by Phanerochaete chrysosporium. Mokuzai Gakk. 29:789-794.

Kawai, S., Umezawa, T. and Higuchi, T. (1987) De novo synthesis of veratryl alcohol by Coriolus versicolor. Wood Res. (Kyoto Univ.) 73:18-21.

Kelley, R.L. and Reddy, C.A. (1986a) Purification and characterization of glucose oxidase from ligninolytic cultures of Phanerochaete chrysosporium. J. Bact. 166:269-274.

Kelley, R.L. and Reddy, C.A. (1986b) Identification of glucose oxidase activity as the primary source of hydrogen peroxide in ligninolytic cultures of Phanerochaete chrysosporium. Arch. Microbiol. 144:248-253.

Kersten, P.J. and Kirk, T.K. (1987) Involvement of glyoxal, methyl glyoxal and extracellular oxidases in H_2O_2 producton by Phanerochaete chrysosporium. In press.

Kersten, P.J., Tien, M., Kalyanaraman, B. and Kirk, T.K. (1985) The ligninase of Phanerochaete chrysosporium generates cation radicals from methoxybenzenes. J. Biol. Chem. 260:2609-2612.

Keyser, P., Kirk, T.K. and Zeikus, J.G. (1978) Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bact. 135:790-797.

Kirk, T.K. (1984) Degradation of lignin. In "Microbial degradation of organic compounds". ed. D.T. Gibson. pp.399-437. New York: Marcel Dekker.

Kirk, T.K. and Adler, E. (1970) Methoxyl-defficient structural elements in lignin sweetgum decayed by a brown-rot fungus. Acta Chem. Scand. 24:3379-3390.

Kirk, T.K., Connors, W.J., Bleam, R.D., Hackett, W.F. and Zeikus, J.G. (1975) Preparation and microbial degradation of synthetic ^{14}C-lignins. Proc. Nat. Acad. Sci. USA 72:2515-2519.

Kirk, T.K., Connors, W.J. and Zeikus, J.G. (1976) Requirement for a growth substrate during lignin decomposition by two white-rotting fungi. Appl. Env. Microbiol. 32:192-194.

Kirk, T.K. and Cowling, E.B. (1984) Biological decomposition of solid wood. In "Chemistry of solid wood". ed.R.M.Rowell. Adv. Chem. Ser. 207. pp.455-487. Washington: Am. Chem. Soc. Press.

Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E. and Farrell, R.L. (1986c) Production of multiple ligninases by Phanerochaete chrysosporium: effect of selected growth conditions and use of a mutant strain. Enz. Microbiol. Technol. 8:27-32.

Kirk, T.K. and Farrell, R.L. (1987) "Enzymatic combustion": The microbial degradation of lignin. Ann. Rev. Microbial. In press.

Kirk, T.K. and Fenn, P. (1982) Formation and action of the ligninolytic system in basidiomycetes. In "Decomposer basidiomycetes" eds. M.J. Swift, J. Frankland, J.N. Hedger. British Mycol. Soc. 4, pp67-90. Cambridge: Cambridge Univ. Press.

Kirk, T.K., Mozuch, M.D. and Tien, T. (1985) Free hydroxyl radical is not involved in an important reaction of lignin degradation by Phanerochaete chrysosporium Burds. Biochem. J. 226:455-460.

Kirk, T.K., Nakatsubo, F. and Reid, I.D. (1983) Further study discounts role for singlet oxygen in fungal degradation of lignin model compounds. Biochem. Biophys. Res. Comm. 111:200-204.

Kirk, T.K., Schultz, E., Connors, W.J., Lorenz, L.F. and Zeikus, J.G. (1978) Influence of culture parameters on lignin metabolism by Phanerochaete chrysosporium. Arch. Microbial. 117:277-285.

Kirk, T.K. and Shimada, M. (1985) Lignin biodegradation: The microorganisms involved and the physiology and biochemistry of degradation by white-rot fungi. In "Biosynthesis and Biodegradation of wood components". ed. T. Higuchi. San Diego, Academic Press, pp.233-245.

Kirk, T.K., Tien, M., Johnsrud, S.C. and Eriksson, K.E. (1986a) Lignin-degrading activity of Phanerochaete chrysosporium Burds.: Comparisons of cellulase-negative and other strains. Enz. Microbial. Technol. 8:75-80.

Kirk, T.K., Tien, M., Kersten, P.J., Mozuch, M.D. and Kalyanaraman, B. (1986b) Ligninase of Phanerochaete chrysosporium. Mechanism of its degradation of the non-phenolic arylglycerol B-aryl ether substructure of lignin. Biochem. J. 236:279-287.

Kuila, D., Tien, M., Fee, J.A. and Ondrias, M.R. (1985) Resonance raman spectra of extracellular ligninase: evidence for a heme active site similar to those of peroxidases. Biochemistry 24:3394-3397.

Kornfeld, R. and Kornfeld, S. (1980) In "The biochemistry of glcoproteins and proteoglycans". ed. W.J. Lennarz, Plenum, New York, pp.1-34.

Kuwahara, M., Glenn, J.K., Morgan, M.A. and Gold, M.H. (1984) Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of Phanerochaete chrysosporium. FEBS Lett. 169:247-250.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of baceriophage T4. Nature 227:680-685.

Leatham, G.F. (1986) The ligninolytic activities of Lentinus edodes and Phanerochaete chrysosporium. Appl. Microbiol. Biotech. 24:51-58.

Leatham, G.F. and Kirk, T.K. (1983) Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. FEMS Microbial. Lett. 16:65-67.

Leisola, M.S.A., Brown, C., Laurila, M., Ulmer, D. and Fiechter, A. (1982) Polysaccharide synthesis by Phanerochaete chrysosporium during degradation of kraft lignin. Appl. Microbiol. Biotech. 15:180-184.

Leisola, M.S.A., Haemmerli, S. Smit, J.D.G., Troller, J., Waldner, R., Schoemaker, H.E. and Schmidt, H. (1987b) Phanerochaete chrysosporium - characterisation of ligninolytic enzymes and metabolism of veratryl alcohol. Proceedings "International Seminar on lignin enzymic and microbial degradation". In press.

Leisola, M.S.A., Kozulic, B., Muessdoerffer, F. and Fiechter, A. (1987a) Homology among multiple extracellular peroxidases from Phanerochaete chrysosporium. J. Biol. Chem. In press.

Leisola, M.S.A., Muessdoerffer, F., Waldner, R. and Fiechter, A. (1985b) Production and identification of extracellular oxidases of Phanrochaete chrysosporium. J. Biotech. 2:267-270.

Leisola, M.S.A., Thanei-Wyss, U. and Fiechter, A. (1985a) Strategies for production of high ligninase activities by Phanerochaete chrysosporium. J. Biotech. 3:97-107.

Leisola, M.S.A., Ulmer, D.C. and Fiechter, A. (1983b) Problem of oxygen transfer during degradation of lignin by Phanerochaete chrysosporium. Appl. Microbiol. Biotech. 17:113-116.

Leisola, M.S.A., Ulmer, D.C., Haltmeier, T. and Feichter, A. (1983a) Rapid solubilization and depolymerization of purified kraft lignin by thin layers of Phanerochaete chrysosporium. Appl. Microbiol. Biotech. 17:117-120.

Leisola, M.S.A., Ulmer, D.C., Waldner, R. and Fiechter, A. (1984) Role of veratryl alcohol in lignin degradation by Phanerochaete chrysosporium. J. Biotech. 1:331-339.

Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56:658-666.

Linko, Y.Y., Leisola, M.S.A., Lindholm, N., Troller, J., Linko, P. and Fiechter, A. (1986) Continuous production of lignin peroxidase by Phanerochaete chrysosporium. J. Biotech. 4:283-291.

Lobarzewski, J., Trojanowski, J. and Wojtas-Wasilewska, M. (1982) The effects of fungal peroxidase on Na-lignosulfonates. Holzforschung 36:173-176.

Lundquist, K. and Kirk, T.K. (1978) De novo synthesis and decomposition of veratryl alcohol by a lignin-degrading basidiomycete. Phytochemistry 17:1676.

Maione, T.E., Javaherian, K., Belew, M.A., Gomez, L.E. and Farrell, R.L. (1987) Activities of recombinant ligninase. Proceedings "International Seminar on lignin enzymic and microbial degradation". In press.

Mandels, M., Kostick, J. and Darizek, R. (1971) The use of adsorbed cellulose in the continuous conversion of cellulose to glucose. J. Polym. Sci. Part C 36:445-459.

McCarthy, A.J., MacDonald, M.J., Paterson, A. and Broda, P. (1984) Screening for lignin-degrading actinomycetes and characterization of their activity against (^{14}C)-labelled wheat lignocellulose. J. Gen. Microbiol. 130:1023-1030.

McCarthy, A.J., Paterson, A. and Broda, P. (1986) Lignin solubilization by Thermomonospora mesophila. Appl. Microbiol. Biotech. 24:347-352.

Mellenberger, R.W., Stratter, L.D., Millett, M.D. and Baker, M.D. (1971) Digestion of aspen, alkali-treated aspen, and aspen bark by goats. J. Anim. Sci. 32:756-763.

Miksche, G.E. and Yasuda, S. (1978) Lignin of "giant" mosses and some related species. Phytochemistry 17:503-504.

Millett, M.A., Baker, A.J., Feist, W.E., Mellenberger, R.W. and Stratter, L.D. (1970) Modifying wood to increase its in vitro digestibility. J. Anim. Sci. 31:781-788.

Miura, Y., Miyamoto, K., Kanamorio, T., Teramoto, M. and Ohira, N. (1975) Oxygen transfer within fungal pellet. J. Chem. Eng., Japan. 8:300-304.

Moo-Young, M., Daugulis, A.J., Chalel, D.S. and MacDonald, D. (1979) The Waterloo Process for SCP production from waste biomass. Proc. Biochem. 14:38-40.

Muranaka, M., Kinoshita, S., Yamada, Y. and Okada, H. (1976) Decomposition of lignin model compound, 3-(2-methoxy-4-formylphenoxy)-1,2-propanediol by bacteria. J. Ferm. Technol. 54:635-639.

Nicholas, D.D. and Preston, A.F. (1984) Interaction of preservatives with wood. In "Chemistry of solid wood". ed. R.M.Rowell, Advances in chemistry series, pp.307-322.

Nimz, H. (1974) Beech lignin-proposal of a constitutional scheme. Angew. Chem. 86:336-344.

Odier, E. and Monties, B. (1983) Absence of microbial mineralization of lignin in anaerobic enrichment cultures. Appl. Env. Microbiol. 46:661-665.

Odier, E. and Roch, P. (1983) Factors controlling bio-degradation of lignin in wood by various white-rot fungi. In "Recent advances in lignin bio-degradation research" eds. T. Higuchi, N.M. Chang, T.K. Kirk. Tokyo:Uni Publishers, pp.188-194.

Ogston, A.G. and Stanier, J.E. (1950) On the state of hyaluronic acid in synovial fluid. Biochem. J. 46:364-376.

Otjen, L. and Blanchette, R.A. (1986) A discussion of microstructural changes in wood during decomposition by white rot basidiomycetes. Can. J. Bot. 75:1005-1010.

Palmer, J.M., Harvey, P.J. and Schoemaker, H.E. (1987) The role of peroxidases, radical cations and oxygen in the degradation of lignin. Phil. Trans. R. Soc. Lond. In press.

Pamment, N., Robinson, C.W. and Moo-Young, M. (1979) Pulp and paper mill solid wastes for single-cell protein production. Biotechnol. Bioeng. 21:561-573.

Paszczynski, A., Huynh, V.B. and Crawford, R.L. (1985) Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. 29:37-41.

Paszczynski, A., Huynh, V.B. and Crawford, R.L. (1986) Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. 244:750-765.

Peitersen, N. (1975) Cellulase and protein production from mixed culture of *Trichoderma viride* and a yeast. Biotechnol. Bioeng. 17:1291-1299.

Petty, T.M. and Crawford, D.L. (1985) Characterization of acid-precipitable, polymeric lignin (APPL) produced by *Streptomyces viridiosporus* and protoplast fusion recombinant *Streptomyces* strains. Biotech. Bioeng. Symp. Ser. 15:179-190.

Quierzy, P., Therien, N. and Leduy, A. (1979) Production of *Candida utilis* protein from peat extracts. Biotechnol. Bioeng. 21:1175-1190.

Raeder, U. and Broda, P. (1984) Comparison of the lignin-degrading white-rot fungi *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* at the DNA level. Curr. Gen. 8:499-506.

Ramasay, K., Prakasam, K., Bevers, J. and Verachtert, H. (1979) Production of bacterial proteins from cellulosic materials. J. Appl. Bacteriol. 46:117-124.

Reddy, C.A., Forney, L.J. and Kelley, R.L. (1983) Involvement of hydrogen peroxide-derived hydroxyl radical in lignin degradation by the white-rot fungus Phanerochaete chrysosporium. In "Recent advances in lignin biodegradation research" eds. T. Higuchi, N.M. Chang, T.K. Kirk. Tokyo:Uni Publishers, pp.153-163.

Reddy, C.A. and Kelley, R.L. (1986) The central role of hydrogen peroxide in lignin degradation by Phanerochaete chrysosporium, pp.535-542. In "Biodeterioration 6". eds. S. Barry, D.R. Houghton, D.C. Llewellyn, C.E. O'Rear,. Slough, U.K.: Commonwealth Agricultural Bureau.

Reid, I.D. (1985) Biological delignification of aspen wood by solid-state fermentation with the white-rot fungus Merulius tremellosus. Appl. Env. Microbiol. 50:133-139.

Reid, I.D., Abrams, G.D. and Pepper, J.M. (1982) Water-soluble products from the degradation of aspen lignin by Phanerochaete chrysosporium. Can. J. Bot. 60:2357-2364.

Reid, I.D., Chao, E.E. and Dawson, P.S.S. (1985) Lignin degradation by Phanerochaete chrysosporium in agitated cultures. Can. J. Microbiol. 31:88-90.

Reinhammer, B. (1984) Laccase. In "Copper proeins and copper enzymes". ed.R.Lontie, pp.1-35. Boca Raton, FL:CRC Press.

Renganathan, V. and Gold, M.H. (1986) Spectral characterization of the oxidized states of lignin peroxidase, an extracellular enzyme from the white rot basidiomycete Phanerochaete chrysosporium. Biochemistry 25:1626-1631.

Renganathan, V., Miki, K. and Gold, M.H. (1985) Multiple molecular forms of diarylpropane oxygenase, an H_2O_2-requiring, lignin-degrading enzyme from Phanerochaete chrysosporium. Arch. Biochem. Biophys. 342:304-314.

Robbins, J.E., Armhold, M.T. and Lacher, S.L. (1979) Methane production from cattle waste and delignified straw. Appl. Env. Microbiol. 38:175-177.

Rudolph, F.B. (1983) Product inhibition and abortive complex formation. In "Contemporary enzyme kinetics and mechanism". ed. D.L. Purich, New York, Academic Press, pp.207-232.

Sarkanen, K.V. and Ludwig, C.H. (1971) Lignins. Occurence, formation, structure and reactions. New York: Wiley-Interscience. p.916.

Schmid, L.A. (1975) Feedlot wastes to useful energy - fact or fiction. J. Env. Eng. Div., Proc. Am. Soc. Civil Eng. 101:787-794.

Schoemaker, H.E., Harvey, P.J. Bowen, R.M. and Palmer, J.M. (1985) On the mechanism of enzymatic lignin breakdown. FEBS Lett. 183:7-12.

Schugerl, K., Wittler, R. and Lorenz, T. (1983) The use of molds in pellet form. Trends Biotechnol. 1:120-123.

Scott, R.W. (1979) Colorimetric determination of hexuronic acids in plant materials. Anal. Chem. 51:936-941.

Scott, W.W., Fred, E.B. and Peterson, W.H. (1930) Products of the thermophilic fermentation of cellulose. Ind. Eng. Chem. 22:731-735.

Shannon, L.M., Kay, E. and Lew, J.Y. (1966) Peroxidase isozymes from horseradish roots. J. Biol. Chem. 241:2166-2172.

Sharon, N. and Lis, H. (1982) In "The Proteins" eds. H. Neurath, R.L. Hill, Academic Press, New York, 5:1-124.

Smith, A.M., Morrison, W.L. and Milham, P.J. (1982) Oxidation of indole-3-acetic acid by peroxidase: involvement of reduced peroxidase and compound III with superoxide as product. Biochemistry 21:4414-4419.

Stamm, A.J. (1964) In "Wood and cellulose science". The Ronald Press Co.:New York.

Stutzenberger, F.J. (1971) Cellulase production by Thermomonospora curvata isolated from municipal solid waste compost. Appl. Microbiol. 22:147-152.

Stutzenberger, F. (1979) Degradation of cellulosic substances by Thermomonospora curvata. Biotechnol. Bioeng. 21:909-913.

Stutzenberger, F.J., Kaufman, A.J. and Lossin, R.D. (1970) Cellulolytic activity in municipal solid waste composting. Can. J. Microbiol. 16:553-560.

Tai, D., Terasawa, M., Chen, C.L., Chang, H.M. and Kirk, T.K. (1983) Biodegradation of guaiacyl and guaiacyl-syringyl lignins in wood by Phanerochaete chrysosporium. In "Recent advances in lignin biodegradation research" eds. T. Higuchi, N.M. Chang, T.K. Kirk. Tokyo:Uni Publishers, pp.44-63.

Terasawa, M., Tai, D., Chen, C.L., Chang, H.M. and Kirk, T.K. (1983) Identification of the constituents of low molecular weight fractions obtained from birch wood degraded by Phanerochaete chrysosporium. Proc. Int. Symp. Wood Pulping Chem. 4:150-155.

Thepenier, C., Gudin, C. and Thomas, D. (1985) Immobilization of Porphyridium cruentum in polyurethane foams for the production of polysaccharide. Biomass 7:225-240.

Tien, M. and Kirk, T.K. (1983) Lignin-degrading enzyme from the hymenomycete Phanerochaete chrysosporium Burds. Science 221:661-663.

Tien, M. and Kirk, T.K. (1984) Lignin-degrading enzyme from Phanerochaete chrysosporium. Purification, characterization, and catalytic properies of a unique H_2O_2-requiring oxygenase. Proc. Nat. Acad. Sci. USA 81:2280-2284.

Tien, M., Kirk, T.K., Bull, C. and Fee, J.A. (1986) Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninase of Phanerochaete chrysosporium. J. Biol. Chem. 261:1687-1693.

Tonon, F., Odier, E., Asther, M., Lesage, L. and Corrieu, G. (1987) Influence of medium additives for the production of active ligninase by Phanerochaete chrysosporium. Proc. "International seminar on lignin enzymic and microbial degradation". April, 1987. (In press).

Ulmer, D.C., Leisola, M.S.A. and Fiechter, A. (1984) Possible induction of the ligninolytic system of Phanerochaete chrysosporium. J. Biotech. 1:13-24.

Updegaff, D.M. (1971) Utilization of cellulose from waste paper by Myrothecium verrucaria. Biotechnol. Bioeng. 13:77-79.

Weinstein, D.A., Krisnangkura, K., Mayfield, M.B. and Gold, M.H. (1980) Metabolism of radiolabelled B-guaiacyl ether linked lignin dimeric compounds by Phanerochaete chrysosporium. Appl. Env. Microbiol. 39:535-554.

Westermark, U. and Eriksson, K.E. (1974) Cellobiose: quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. Acta Chem. Scand. B. 28:209-214.

Winter, A., Ek, K. and Andersson, U.B. (1977) Analytical electrofocusing in thin layers of polyacrylamide gels. LKP Application note, LKP-Produkter AB, Broma, Sweden.

Yamazaki, I. and Yokota, K.N. (1973) Oxidation states of peroxidase. Mol. Cell Biochem. 2:39-52.

Yeck, R. (1979) Methane for biomass. Environment 21:28-29.

Zadrazil, F. (1977) The conversion of straw into feed for basidiomycetes. Eur. J. Appl. Microbiol. 4:273-281.

Zeikus, J.G., Wellstein, A.L. and Kirk, T.K. (1982) Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Microbiol. Lett. 15:193-197.