STUDIES ON LIGNOCELLULOLOLYTIC ENZYMES PRODUCED
IN PHANEROCHAETE CHRYSPORIUM

Being a Thesis presented in accordance with the Regulations governing the Award of the Degree of Doctor of Philosophy in the University of Surrey

by

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SUMMARY

In most sources of lignocellulosic materials, the presence of lignin restricts access to the carbohydrate and hence prevents degradation. In the last few years biological pretreatment of lignocellulose has become a subject of growing commercial interest because of the possibility of converting it to a food or energy source at lower cost than present extraction processes.

An extracellular peroxidase involved in the degradation of lignin and lignin model compounds has recently been discovered in the basidiomycete Phanerochaete chrysosporium.

The scale-up of lignin peroxidase production has been hindered by phenomena such as low yields of enzyme production and inhibition of ligninolytic enzyme synthesis in agitated cultures.

In this thesis, the growth conditions for the white-rot fungus P. chrysosporium for the production of lignin peroxidase have been investigated. Various substrates for growth have been studied and the appearance of lignin peroxidase was followed. The presence of cellulolytic enzymes in the media for ligninase production has also been shown.

The production of lignin peroxidase by both free and immobilized cells of P. chrysosporium under shaken conditions, in medium containing 1% glucose or 1% glucose plus different concentrations of molasses, has been described.

Isolation and some studies of the RNA of the fungus have also been carried out.
To my parents, who in their own way ensured that I grew up having the determination, perseverance and honesty I need to pursue my dreams and achieve my ideals.

To Steve, for his kindness, love and everlasting patience.
I should like to thank my supervisors Dr. Alan Wiseman and Dr. Peter Goldfarb for their help and guidance throughout the course of this work.

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ABBREVIATIONS

\[ \mu \text{mol} = \text{mmol (mimicromoles)} \]

\[ \mu g = \text{mg (micrograms)} \]

SSC - Standard Saline Citrate
CHAPTER 1

Introduction
1 INTRODUCTION

1.1 The Structure and Composition of Lignocellulosic Materials

Lignocellulosic materials represent a major source of renewable organic matter and comprises about 95% of the earth's land-based biomass (Amer and Drew, 1980).

Lignocelluloses are basically formed by the interlinking of the three structural polymers: cellulose, hemicellulose and lignin (Janshekar et al., 1981; Kirk, 1980; Kirk, 1983; Brown, 1985 & Schlegel, 1987).

Cellulose is a basic component of all plant materials and its production exceeds that of all other natural substances. It is a linear homopolymer of glucose units linked together by $\beta$-D-1,4 glucosidic bonds (about 14000 glucose units) forming chains organised in parallel. These chains are grouped together forming microfibrils which are covered by a matrix of lignin and hemicellulose.

Hemicellulose is a heteropolymer made up largely of xylose units (xylan), but with varying amounts of other saccharides such as arabinose, glucose, mannose and galactose uronic acids and pectins. The xylan chain consists of $1\text{-}4$-glycosidically linked $\beta$-D-xylose (30 to 100 xylan units).

Lignin is distinct from cellulose and hemicellulose. It is an aromatic polymer synthesized by the oxidative polymerization of three substituted cinnamyl alcohols: $p$-coumaryl, coniferyl and synapyl alcohols. It is not uniform chemically, but it is instead a very complex compound. The complexity results from the different bonds by which the monomeric alcohols are linked. Figure 1.1 illustrates the monomers and some dimers which
compose lignin.

Figure 1.1 Monomers and Dimers of lignin
Chemical and spectrometric studies of softwood lignin (Adler, 1977 & Sakakibara, 1983) indicated that the monomeric guaiacylpropane units are connected both by ether and carbon-carbon linkages. Several substructures involved in lignin macromolecules were elucidated, and it was found that guaiacylglycerol-B-aryl ether (B-O-4) is the most abundant interphenylpropane linkage in lignin, followed by phenylcoumaran, diarylpropane, pinoresinol, biphenyl and diphenyl ether linkages. Figure 1.2 shows a structural model of softwood lignin proposed on the basis of chemical and spectrometric studies (Sakakibara, 1983).

Lignin occurs in intimate association with cell-wall polysaccharides, and it has been shown that some hemicelluloses are linked by covalent bonds with lignin (Fengel & Wegener 1983). The lignin-hemicellulose matrix surrounds cellulose fibrils which is almost impenetrable. Figure 1.3 illustrates the ultrastructure of a plant cell wall. The cell wall is organized in several layers with the microfibrils oriented in a well defined manner. The cell walls are bonded together by the middle lamella, an intercellular layer that is rich in pectin and lignin. This high degree of organization in the cell structure produces the properties of wood, rigidity, strength and high toughness.
Figure 1.2 Structural Model of Softwood Lignin
Figure 1.3 Ultrastructure of a Plant Cell Wall

M.L.: Midle Lamella; P.: Primary Wall
S1, S2, and S3: Secondary Cell Walls
1.2 The Occurrence of Lignocelluloses in Nature

Lignocelluloses derived from higher land plants represent a major source of renewable organic matter. In form and availability they occur in whole trees, forestry processing wastes, agricultural wastes such as straw and bagasse and in many forms of domestic, municipal and industrial wastes.

Of the structural components, cellulose is the most abundant, making up 35% to 50% of dry weight of most woody tissues. The hemicelluloses and lignin usually make up 20% to 40% and 15% to 35% respectively. Table 1 shows the composition of some lignocelluloses found in higher land plants (Janshekar & Fiechter, 1983)

Table 1 Composition of some lignocellulosic materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coniferous wood</td>
<td>40-50</td>
<td>20-30</td>
<td>25-35</td>
</tr>
<tr>
<td>Deciduous wood</td>
<td>40-50</td>
<td>30-40</td>
<td>15-20</td>
</tr>
<tr>
<td>Cotton</td>
<td>94</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bagasse</td>
<td>40</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Nut shells</td>
<td>25-30</td>
<td>25-30</td>
<td>30-40</td>
</tr>
<tr>
<td>Corn cobs</td>
<td>45</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Corn stalks</td>
<td>35</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>30</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>Paper</td>
<td>85-99</td>
<td>0</td>
<td>0-15</td>
</tr>
<tr>
<td>Newsprint</td>
<td>50</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>
Lignocelluloses comprise 50% of all biomass with an estimated annual production of $50 \times 10^9$ tonnes (Smith et al, 1988). It has also been estimated that around half of the total production of plant residues from agricultural and industrial processes remain unused.

Table 2 shows the amount of some organic wastes in the United Kingdom (Smith et al, 1987).

Table 2 Organic Wastes In The U.K.  

<table>
<thead>
<tr>
<th>Waste Type</th>
<th>Fresh Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood shavings, sawdust and bark</td>
<td>&gt;1 t/a</td>
</tr>
<tr>
<td>Food processing wastes, brewers grains</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Potato haulms, sugar beet tops</td>
<td>1.6</td>
</tr>
<tr>
<td>Cereal straws, surplus</td>
<td>5-7</td>
</tr>
<tr>
<td>Municipal refuse (about 50% organic matter)</td>
<td>18</td>
</tr>
<tr>
<td>Farm Manures</td>
<td>120</td>
</tr>
</tbody>
</table>

Biomass, including forest product wastes and municipal and agricultural wastes may provide solutions to some of the long term problem associated with chemical and energy from petroleum, if proper technology is developed for their utilization (Broda, 1986).
1.3 Problems Involved in the Degradation of Lignocellulosic Materials.

Although cellulose and hemicellulose can be efficiently degraded, in nature they are protected from enzymatic attack by the lignin surrounding them (Higuchi, 1985; Kirk, 1983; Kirk, 1985; Brown, 1985). Most lignin is found within the cell walls, where it is associated with hemicelluloses, forming a matrix with surrounds the orderly cellulose microfibrils (Janshekar & Fiechter, 1983).

Lignin is highly recalcitrant due to its high molecular weight and complex three dimensional structure. It performs a number of functions which are essential to the life of plants. A summary of how lignin properties affect plant characteristics is shown in Table 3. Lignin functions as a binding and encrusting material for the cell wall constituents, and gives them rigidity, protecting them from external attacks.

Table 3 Contribution of Lignin in Plant Materials

<table>
<thead>
<tr>
<th>Function of Lignin</th>
<th>Plant Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonding agent between cells</td>
<td>Resistance to mechanical stresses</td>
</tr>
<tr>
<td>Impedes penetration of enzymes and microorganisms</td>
<td>Resistance to biochemical stresses (infection and wounding)</td>
</tr>
<tr>
<td>Water proofing agent</td>
<td>Resistant to physical-chemical stresses</td>
</tr>
</tbody>
</table>
Degradation of the lignin barrier is important for the efficient conversion of cellulose and hemicellulose to fuels and chemicals (Lewis, 1983; Kirk, 1983 and Leisola & Fiechter, 1985). However, the structural features of lignin make lignocellulosic materials a complex and difficult substrate to be degraded (Chen & Chang, 1985). Hence a pretreatment of the substrate is required in order to alter significantly the structural characteristics of the lignocellulosic matrix and to increase the rate and extent of holocellulose utilization (Datta, 1981; Selvan, 1983; Lewis, 1983 and Bono et al, 1985).

1.4 Methods Used to Pretreat Lignocellulosic Materials

Pretreatment steps are essential to lignocellulose bioconversion steps. Untreated lignocellulose give very low and commercially unattractive yields (10-30%) (Datta, 1981).

In order to be effective, pretreatment techniques for enhancing the chemical and enzymatic reactivity of cellulosic materials must alleviate two major constraints: the lignin seal, which restricts enzymatic and microbiological access to the cellulose; and cellulose crystallinity, which limits the rate of all forms of attack on the cellulose. Physical and chemical process, either singly or in combination are used for lignocellulosic pretreatment (Lewis, 1983 and Toussaint & Bataille, 1985).

Physical processes like pulping, milling, steaming and electron irradiation are used to increase pore size and surface area and to reduce cellulose crystallinity. These processes demand expensive energy, and their practical adaptation hinges
on favourable results from further research and development (Dale, 1987; Eder, 1988 and Kumakura & Kaetsu, 1988).

Acid hydrolysis, alkali swelling, SO2 and solvent delignification are the principal chemical pretreatment methods (Hatakka, 1983; Wei & Cheng, 1985 and Wayman, et al, 1987). Chemical treatment with strong acids or bases, such as sulphuric acid or sodium hydroxide, also effectively increase the hydrolysis of cellulose (Fan et al, 1982). These chemicals are generally quite expensive and corrosive. They are often toxic or inhibitory to microorganisms or their enzymes and have to be completely removed from the pretreated material. These factors combine to increase the expense and difficulty of such chemical treatment methods.

Another technique of current interest, which involves both physical and chemical methods, is the steam-explosion process (Dekker et al, 1987 and Converse & Grethlein, 1987). Lignocellulosic materials are saturated with water under pressure (300-400 psig) at elevated temperatures (215 °C-260 °C). When the pressure is released, the water evaporates rapidly and the wood fibre tend to separate, increasing he surface area for subsequent hydrolysis. The technique is highly effective but involves the use of considerable energy in the form of steam. In addition, some of the sugars are degraded by the high temperatures involved in the process.

In the last few years biological pretreatment has become a subject of growing interest in order to convert lignocellulose into feed or energy source, because it may offer a lower cost process then the ones already in use, for the utilization of
such materials (Hatakka, 1983; Broda, 1986; Smith et al, 1987 and Dale, 1987).

Biodelignification by microorganisms or isolated enzymes could well become an important pretreatment for lignocelluloses, reducing or removing the lignin barrier and exposing the cellulose to biological activity (Smith et al, 1987).

1.5 Biodegradation of Lignin

Lignin degradation plays a central role in the earth's carbon cycle because of its abundance and, perhaps more significantly, because lignin physically protects most of the world's cellulose and hemicellulose from enzymatic hydrolysis.

Lignin is degraded by a narrower array of microbes than the other biological polymers, because of its complex structure (Amer & Drew, 1980; Kirk, 1983; Janshekar & Fiechter, 1983; Kirk, 1985; Brown, 1985 and Higuchi, 1985).


1.5.1 Methods Used For the Determination of Lignin Degradation

Analysis of residual lignin is an important step in the study of microbial degradation of lignin. Determination of the extent of lignin degradation as well as of the structural changes in the degraded lignin are important factors in
determining the ability of microorganisms to degrade lignin.

Some of the more common methods used to quantify lignin content in biodegraded samples are shown below (Johnson et al., 1961; Janshekar et al., 1981; Paterson, 1984; Chen & Chang, 1985; and Pometto & Crawford, 1985).

1- Sulphuric Acid (Klason's) Method:
It refers to the material (dry weight) left after treatment with 72% sulphuric acid for 3h at room temperature, followed by 4 h of boiling under reflux in 3% sulphuric acid. Soluble lignin content are not estimated.

2- Spectrophotometric Methods:
In these methods lignin is dissolved in a appropriate solvent, such as dioxane; acetyl bromide acetic acid; phosphoric acid or sulphuric acid and the absorbance of the solution measured at 280 nm.

3- Chlorine Number
This method is based on the fact that lignin is oxidised by chlorine. It is an indirect method of determining the amount of lignin in pulp samples and is defined as the weight (g) of chlorine consumed per gram of pulp under set conditions of time and temperature.

4- Permanganate (Kappa) Number
The Kappa number is expressed as the amount of 0.1 N potassium permanganate solution consumed per gram of woody material at 25°C for 10 minutes.

5- Radioisotopic Tracer Studies
This technique is based on the degradation of $^{14}$C-labeled
natural lignin (Halder & Trojanowski, 1975), and of free lignocelluloses which contain $^{14}$C only in their lignin component. The evolution of $^{14}$CO$_2$ by cultures growing on $^{14}$C-labelled lignin or the production of low molecular weight water soluble $^{14}$C-labeled intermediate compounds is monitored. It allows the study of sequential attack on lignin molecule that are $^{14}$C-labeled only in side-chains, methoxyl groups, or ring structures. Radiolabelled products can be further characterised by the use of sensitive analytical techniques such as HPLC.

1.5.2 Substrates for Lignin Degradation Studies


1- Milled wood lignin (MWL):
It is prepared by solvent (dioxane-water) extraction of finely ground wood. Only minor changes are supposed to occur in the structure of lignin during this procedure. Therefore, MWL is one of the best lignin preparation for microbial studies. Labelled plant tissues are also used for preparation of MWL. In this case, plants are fed with radioactive precursors e.g. $^{14}$C-phenylalanine or $^{14}$C-ferulic acid during growth. Mechanical breakdown, extensive solvent extraction and enzyme treatment of the plant material remove contaminating proteins and carbohydrates. [$^{14}$C-lignin]-lignocellulose is a widely used substrate for lignin degradation studies since it can be
prepared relatively easily and offers a simple and conclusive test for lignin degradation.

2- Kraft lignin and lignosulfonates:
They are products from the pulp and paper industry and are widely used, since large amounts of kraft lignin and lignosulfonates are produced annually. They contain low molecular weight degradation products of lignin. It has been suggested that an organic-soluble, ether insoluble fraction of Kraft lignin (Indulin) can be used as a standard lignin preparation in research and for analytical work (Jansshekar et al, 1982b). Its approximate molecular size is 3,000 Daltons. Although these compounds do not represent native lignins, research into their degradation is potentially useful as lignin-degrading species might be able to detoxify these by-products.

3- Synthetic Lignin:
Synthesis of a polymer resembling lignin can be achieved by oxidative polymerization of coniferyl, synapyl, and/or coumaryl alcohol using commercially available peroxidase and hydrogen peroxide. This synthetic lignin, or dehydrogenative polymerizate (DHP) is macromolecular and contains interlignol bonds similar to those found in native lignin. The main advantage of DHP over natural substrates is that it can be specifically labelled in the side chain, aromatic ring or methoxyl groups. Therefore the analysis of the degradation products can provide information about the reaction taking place.

4- Lignin Model Compounds:
These are soluble low molecular weight compounds, usually di-,
tri- or tetramers, which contain chemical structures known to exist in lignin. Such compounds are useful in determining activity against a specific bond type but they are not representative of the insoluble lignocellulose complex.

1.6 Microorganisms Involved in Lignin Biodegradation

Although a wide variety of organisms can degrade lignocellulose to some extent, degradation of the lignin component is confined to relatively few species (Crawford & Crawford, 1984; Kirk & Shimada, 1985 and Leisola & Fiechter, 1985). Biological lignin degradation is an important part of the biospheric carbon-oxygen cycle and efficient mechanisms for lignin degradation exist in nature since no accumulation seems to occur.

Complete degradation of lignin is believed to be a result of a cooperative action of various fungi and bacteria (Janshekar & Fiechter, 1983 and Leisola & Fiechter, 1985). Actually many microorganisms are known to modify lignin to some extent and metabolize low molecular weight intermediary compounds. However, the extensive degradation of lignin is thought to occur mainly through the action of a few wood degrading fungi (Kirk & Shimada, 1985 and Leisola & Fiechter, 1985). Some of the lignin degrading microorganisms are described below.

1.6.1 Anaerobic Microorganisms

Lignin is apparently not degraded anaerobically. Zeikus et al. (1982) reported that after a period of 110 days only low
molecular weight materials (<600 daltons) suffered degradation. The substrates used were an alkaline-degraded synthetic lignin, a dimeric lignin model compound and lignin related phenols, all labelled with $^{14}$C. Colberg & Young (1985) obtained similar results in studies with Douglas fir wood labelled by $[^{14}C]$-phenylalanine feeding.

Holt & Jones (1983) showed that beech wood buried in anaerobic sea water, freshwater or brackish muds were only partially degraded after 18 months.

It has been suggested that limited anaerobic metabolism of lignin by various microflora during a period of long incubation can be attributed to non lignin components or to low molecular weight materials freed by other processes, rather than microbial attack (Kirk & Farrell, 1987).

1.6.2 Aerobic Degradation by Bacteria

Bacterial lignin degradation has been most studied in actinomycetes, (Crawford et al. 1983 and McCarthy, 1987), particularly Streptomyces spp (Antai & Crawford, 1981; Pometto & Crawford, 1986; Ruttimann et al, 1987 and Crawford & Crawford, 1988). Streptomyces viridosporus and S. setonii caused losses of 32% to 44% of various lignocelluloses, as determined by chemical analyses of insoluble residues. Characterization of spruce lignin degraded by S. viridosporus indicated the presence of oxidative alterations (Crawford et al, 1984). Nevertheless, attack on fully lignified tissues has been shown to be minimal (McCarthy & Broda, 1984). Degradation of lignicelluloses by actinomycetes produces a water soluble residue termed APPL (acid precipitable polymeric lignin), which

Recent studies with other bacteria have failed to demonstrate extensive degradation of lignin. Janshekar & Fiechter (1982) isolated strains of Nocardia, Pseudomonas and Corynebacterium for the ability to grow in lignin-related phenols, but none of them were able to grow on various source of lignin. Several Pseudomonas spp. are reported to degrade extracted lignins. Decomposition of kraft lignin and milled wood lignin (MWL) by P. ovalis was also described. Several strains of Pseudomonas, Xanthomonas and Acinetobacter able to cause extensive modifications in lignin but not to release CO2 from lignin in wood, have been studied Vicuna (1988).

No aerobic bacteria have been shown to extensively degrade lignin. The size of the lignin polymer might be a limiting factor for the attack of these materials by bacteria (Kirk & Farrell, 1987).

1.6.3 Aerobic Degradation by Fungi

Lignin-degrading fungi are classified as soft-rot, brown-rot and white-rot depending on the type of decay they cause.

1- Soft-rot Fungi

Certain Ascomycetes and Fungi Imperfect attack wood extensively under conditions of high humidity, causing a softening of the surface layer of the wood (Leisola & Fiechter,
1985 and Buswell & Odier, 1987).

Soft-rot wood decay involves lignin degradation but polysaccharides are preferentially degraded (Kirk, 1984 and Kirk & Cowling, 1984). *Chaetomium piluliferum* converted 20% to 30% of $^{14}$C-labeled synthetic lignins to $^{14}$CO$_2$ in 50 days (Haider & Trojanowski, 1975).

A few species of Ascomycetes (e.g. *Xylaria*, *Libertella* and *Hypoxylon*) were reported to cause white-rot wood decay accompanied by substantial lignin losses (Kirk & Farrell, 1987). None of these fungi have been studied extensively and the mechanism of action of their ligninolytic system remains to be clarified.

2- Brown-Rot Fungi

The brown-rot fungi, comprising numerous basidiomycetes, are closely related taxonomically to white-rot fungi, and several genera (e.g. *Poria*, *Polyporus*, *Lentinus*) include members of both decay groups (Buswell & Odier, 1987 and Kirk & Farrell, 1987). Brown-rot fungi mainly decompose the polysaccharides in wood, causing a limited decrease in lignin content (Kirk & Shimada, 1985; Leisola & Fiechter, 1985). Brown-rot attack on lignin is oxidative and studies with $^{14}$C-DHP suggest that main modifications are demethylation of aromatic methoxy groups and, to a lesser extension, aromatic ring hydroxylation (Janshekar & Fiechter, 1983). These structural changes lead to the formation of o-diphenolic compounds which are though to autooxidize, producing quinone-like chromophores which give brown rotted wood its characteristic colour (Kirk & Farrell, 1987).
3- White-Rot Fungi

Fungi belonging to this group are able to degrade all the major components of wood and are considered to be the main agents of lignin decomposition in nature. The relative rates at which the lignin, hemicellulose and cellulose are attacked may vary, depending upon the fungus and the environmental conditions (Blanchette, 1984; Otjen & Blanchette, 1985 and Buswell & Odier, 1987). Some species are particularly effective at degrading lignin, which may undergo a number of oxidative changes including aromatic ring cleavages. Recent studies have shown that depolymerization occurs and a wide array of low molecular weight fragments (< 1 kd) are released (Leisola et al, 1983, Reid, 1985).

There are several hundred known species of white-rot fungi which form a heterogeneous group consisting most of basidiomycetes belonging to a number of families of Hymenomycetes (e.g. Agaricaceae, Corticiaceae, Hydnaceae, Polyporaceae and Thelephoraceae) and a few ascomycetes in the order Sphariales (Ustilina vulgaris and Xylaria polymorpha) (Buswell & Odier, 1987).

Colonisation of lignified tissues in wood begins with the invasion of the lumen of wood cells by the fungal mycelium, followed by the secretion of enzymes which bring about degradation of both lignin and non lignin components. This causes progressive thinning of the cell wall and is likely to involve cellulases (Janshekar & Fiechter, 1983; Kirk & Cowling, 1984a and 1984b; Leisola & Fiechter, 1985 and Buswell & Odier, 1987).
Of the white-rot fungi, *Phanerochaete chrysosporium* (=Sporotrichum pulverulentum), (Burdsall & Eslyn, 1974; Raeder & Broda, 1984), has been studied widely.

1.7 Culture Parameters and Physiological Features Influencing Lignin Degradation by *Phanerochaete chrysosporium*

An extensive programme of research that has been carried out by various groups, but most notably by Kirk and coworkers has helped to define many of the nutritional, physiological and environmental factors affecting lignin biodegradation in *P. chrysosporium*. It is now clear that the lignin degrading system in *P. chrysosporium* is an expression of secondary metabolism, although this does not apply to actinomycetes or to other fungi (Kirk & Fenn, 1982; Keiser et al, 1978; Bu'Lock, 1975; and Leatham & Kirk, 1983). Several features which are associated with the ligninolytic system of *P. chrysosporium* all appear after the primary phase of growth has ceased. These features and the physiological and culture conditions which trigger their appearance will be outlined below.

Although lignin is a potentially energy rich material, it has been reported that lignin can not serve as the sole source of carbon and energy for growth of *P. chrysosporium* (Kirk et al, 1978), for lignin degradation to proceed. A more readily utilizable source of carbon is necessary to be present (Kirk et al, 1978; Ulmer et al, 1983 and Leisola et al, 1984). Although glucose is most frequently used, a range of carbon cosubstrates support, to varying extents, lignin decomposition by *P. chrysosporium* (Kirk et al, 1978 and Kirk, 1980). Under certain conditions (carbon starvation), the carbon supplement can
markedly affect production of a lignin degrading enzyme. Several studies have confirmed that hemicellulose and cellulose, or added carbohydrates are metabolised with the lignin in lignocelluloses (Yang et al, 1980; Agosin & Odier, 1985 and Leisola et al, 1984). The underlying basis for this growth requirement is unclear. However, since lignin is such a stable molecule, a cosubstrate might serve to provide energy for the synthesis of enzymes and/or other components of the ligninolytic system (Tien & Kirk, 1983).

Lignin decomposition is largely an oxidative process, and increased oxygen levels considerably enhance polymer degradation by various wood decaying fungi (Bar-Lev & Kirk, 1981; Reid & Seifert, 1982; and Leisola et al, 1984).

Kirk et al (1978), found that cultures of P. chrysosporium maintained under an air atmosphere released 1% of available label as $^{14}$CO$_2$ from $^{14}$C-DHP after 35 days of incubation. By contrast, 47% and 57% of the $^{14}$C label was converted to $^{14}$CO$_2$ under 21% and 100% O$_2$, respectively.

The observed stimulatory effect of molecular oxygen on lignin biodegradation appears complex and may involve the interaction of several factors:

1- Increase in the amount of H$_2$O$_2$ produced under 100% O$_2$ compared to the very little produced under lower concentration of O (Faison & Kirk, 1983 and Faison & Kirk, 1985).

2- Increase in the titre of the ligninolytic system:

It has been suggested that enhanced H$_2$O$_2$ production, in part, accounted for the stimulatory effect of O$_2$ on the action of the ligninolytic system as a whole. More recently, a direct
correlation between $O_2$ concentration and activity of lignin peroxidases enzymes has been reported. Highest enzyme activities were recorded in cultures held under air during primary growth and then under $O_2$ atmosphere during secondary phase of growth (Faison & Kirk, 1985).

In *P. chrysosporium*, lignin is degraded only during secondary metabolism, which is triggered by limiting the cultures for nitrogen, carbon or sulphur (Jeffries et al, 1981; Keyser et al, 1978 and Reid, 1983). Lignin degradation by several other species, but not all white-rot fungi is stimulated by nitrogen starvation (Leatham & Kirk, 1983; Leatham, 1986). Nitrogen limitation reflects the condition of the natural habitat of white-rot fungi since nitrogen content in wood is very low (Cowling & Merryl, 1966). For practical purposes nitrogen starvation rather than carbon or sulphur is used in experiments with *P. chrysosporium* (Jeffries et al, 1981 & Kirk & Farrell, 1987). Lignin degradation due to limitation of sulphur is difficult to demonstrate and carbon limitation leads to autolysis and only transient degradation.

Agitation of *P. chrysosporium* cultures was originally found to strongly suppress lignin degradation as well as metabolism of dimeric models, synthesis of veratryl alcohol and formation of lignin peroxidase (Kirk et al, 1978; Enoki et al, 1980; Shimada et al, 1981; Faison & Kirk, 1985. Culture agitation results in pellet formation and the oxygen concentration within the pellet was reported to be too low for the normal synthesis and activity of the lignin degrading
system (Kirk et al, 1978).

More recently, however, degradation of lignin by submerged pellets in agitated cultures has been reported by use of mutant strain, or by adding detergent, veratryl alcohol or benzyl alcohol to the cultures (Asther et al, 1987; Tonon, 1987; Kirkpatrick & Palmer, 1987a and b; and Colombie et al, 1988). This process of induction has not been explained.

Lignin degradation by *P. chrysosporium* is strongly influenced by the culture pH. Maximum ligninolytic activity was observed when the pH of cultures was initially set at 4.5 (Kirk et al, 1978). The choice of buffer in the culture medium can affect lignin degradation significantly. It has been reported that cultures buffered with polyacrylic acid, mineralized lignosulfonates more rapidly than those buffered with 2,2-dimethylsuccinate, which is widely used (Kern, 1983 and Kirk & Farrell, 1987).

The balance of trace elements provided is important. Increased amounts of trace elements enhance the production of ligninolytic enzymes (Jeffries et al, 1981 and Kirk et al, 1986). Thiamine is needed for growth and for ligninolytic enzymes production. The addition of some fatty acids and triglycerides also leads to an increase in the titre of the ligninolytic system (Asther et al, 1987).

The initiation of ligninolytic activity after cessation of the primary growth is accompanied by a number of physiological changes. There is an increase in the level of cyclic AMP (cAMP) and addition of a nitrogen source such as glutamate reduces cAMP levels and delays the onset of ligninolytic activity.
(Wallace et al, 1983; MacDonald et al, 1984; MacDonald et al, 1985 and Faison & Kirk, 1985). The role of cAMP in the regulation of lignin degradation is not fully understood. cAMP has been shown to be important in secondary metabolism in other fungi (Bu'Lock, 1975).

Formation of an extracellular glucan and de novo synthesis of the secondary metabolite, veratryl (3,4-dimethoxybenzyl) alcohol are also manifestations of the secondary metabolism of nitrogen limited cultures of P. chrysosporium (Kirk & Fenn, 1982; Leisola et al, 1982; Tien & Kirk, 1984; Lundquist & Kirk, 1978 and Shimada et al, 1981).

Although the ligninolytic system in P. chrysosporium appears in the absence of lignin, the presence of lignin related compounds such as veratryl alcohol enhances lignin degradation (Ulmer et al, 1984; Faison & Kirk, 1985 and Faison et al, 1986). Leisola et al (1984), proposed that veratryl alcohol, which is produced by the fungus, is a natural inducer of the ligninolytic system.

1.8 Biochemistry of Lignin Biodegradation

The structural nature of the lignin polymer requires a biodegradative system which is extracellular, non-specific and oxidative. Until recently, information concerning the mechanism of lignin degradation was obtained by studying chemical changes in the lignin polymer after microbial attack, analysis of low molecular intermediate products or analysis of the microbial metabolism of selected lignin model compounds. These studies have clearly shown that degradation is an oxidative process and many degradation reactions like $\text{C}_\alpha-\text{C}_3$ cleavage, $\text{C}_\alpha-$
oxidation, aromatic ring cleavage and possibly some reductive reaction seemed to occur (Kirk & Chang, 1975; Chang et al, 1980; Tai et al, 1983; and Gold et al, 1984).

In 1983, Tien & Kirk and Gold et al, reported the isolation of a protein from cultures of *P. chrysosporium* which could degrade lignin model dimers and polymeric lignin, both synthetic DHP-lignin and milled wood lignin. It was the first biochemical demonstration of a lignin-degrading enzyme. The enzyme was shown to have a molecular weight of 42 kd, to contain a protohaem group and to be glycosylated. It needs hydrogen peroxide for activity, but takes oxygen from air for oxidation reactions. Its pH optimum is 2.5-3.0. The enzyme catalyzed the oxidation of veratrylalcohol (3,4 dimethoxybenzyl alcohol) to veratrylaldehyde, in the presence of catalytic amounts of hydrogen peroxide. Veratrylaldehyde could be measured by its absorbance at 310 nm, providing a suitable assay to monitor the enzyme activity and hence lignin degrading activity (Tien & Kirk, 1984).

During the last few years after its discovery, the enzyme has been called a lignin degrading enzyme, lignin degrading H₂O₂-dependent oxygenase, diaryl propane oxygenase and ligninase. Recently this enzyme was shown to be a peroxidase which oxidizes different aromatic substrates by a one-electron transfer mechanism (Kuila et al, 1985). Several isomeric forms of this lignin peroxidase have now been isolated and separated by SDS-gel electrophoresis, iso-electric focusing techniques and HPLC. All the isomers have a molecular weight between 39 and 49 kD, are glycosylated, contain one protohaem

The mechanism of reaction for the lignin peroxidase is characteristic of peroxidase in general with the first step in the reaction involving the formation of a radical cation in the substrate molecule (Shoemaker, 1985; Kersten, 1985; Evans, 1987; Kirk, 1988). Some of the reactions catalysed by lignin peroxidase are illustrated in figure 1.4. Lignin peroxidase catalyzes the cleavage of Cα and Cβ carbons in dimeric lignin model compounds of the B-O-4-aryl ether. This reaction was observed in ligninolytic cultures of P. chrysosporium (Kirk & Nakatsubo, 1983 and Kirk & Farrell, 1987). Since B-ether linkages accounts for 48% of the total intermonomeric bonds in lignin (Crawford, 1981), cleavage of these bonds might lead to extensive depolymerization. Other reactions of this enzyme include aromatic ring cleavage, aryl-Cα cleavage, phenolic oxidation and demethoxylation (Leisola et al, 1985; Umezawa et al, 1986; Miki et al, 1987; Kawai et al, 1987; Shimada et al, 1987 and Miki et al, 1988).

The oxidation of aromatic substrates in lignin degradation are complex and diverse. Kersten et al (1985), and Hammel et al (1986), reported that oxidation of these substrates by lignin peroxidase occurred via one electron oxidation of susceptible aromatic nuclei, leading to the formation of unstable cation radicals. These cation radicals promote a variety of nonezymatic reactions. Shoemaker et al (1985), suggested a
Figure 1.4 Reactions Catalysed by Lignin Peroxidase

**Oxidation of veratryl alcohol**

\[
\begin{align*}
\text{CH}_3\text{OH} & \quad \rightarrow \\
\text{OCH}_3 \\
\text{OCH}_3 \\
\text{OCH}_3
\end{align*}
\]

**Aromatic ring cleavage**

\[
\begin{align*}
\text{CH}_3\text{OH} & \\
\text{OCH}_3 \\
\text{OCH}_3 \\
\text{OCH}_3
\end{align*}
\]

**Cα-Cβ cleavage**

\[
\begin{align*}
\text{CH}_2\text{OH} \\
\text{HCOH} \\
\text{OCH}_3 \\
\text{OCH}_3 \\
\text{OCH}_3
\end{align*}
\]

**Cβ-O-4 cleavage**

\[
\begin{align*}
\text{CH}_2\text{OH} \\
\text{HCOH} \\
\text{OCH}_3 \\
\text{OCH}_3 \\
\text{OC}_2\text{H}_5
\end{align*}
\]

**Demethoxylation**

\[
\begin{align*}
\text{Lignin} & \quad \text{via radical cation} \\
\text{OCH}_3 & \quad + \text{H}_2\text{O} \\
\text{OH}
\end{align*}
\]

**Phenolic oxidation**

\[
\begin{align*}
\text{Lignin} & \quad \text{via radical cation} \\
\text{OH} & \quad + \text{H}_2\text{O}
\end{align*}
\]
cation-radical mechanism based on some of the reactions catalysed by lignin peroxidase. Harvey et al (1985), showed that chemical one-electron oxidation of lignin-related compounds gives the same products as lignin peroxidase. Figure 1.5 illustrates the degradation of a lignin related compound by the mechanism described above.

The oxidation of the natural secondary metabolite by lignin peroxidase might have special significance. Haemmerly et al (1986), reported stimulation of lignin peroxidase oxidation of benzo(a)pyrene by the addition of veratryl alcohol. Harvey et al (1986), showed that veratryl alcohol increases the rate of oxidation of anisyl substrates by lignin peroxidase, which are only partially oxidized in its absence, and suggested that veratryl alcohol act as a mediator (cation radical) promoting oxidation of the substrate, as shown in figure 1.6. However, Tien et al (1986), seem to disagree with this postulate. It is suggested that veratryl alcohol simply protect the enzyme from inactivation by anisyl substrates. It has also been suggested that veratryl alcohol might act as an electron relay at the enzyme active site or it might alter the conformation of the enzyme. Wariishi & Gold (1989) reported that veratryl alcohol stabilises the enzyme in the presence of excess of hydrogen peroxide.
Figure 1.5 Degradation of a Lignin Model Compound by Enzymatic/Chemical Action

Lignin model compound

\[ \text{Ligninase or Fe(phen)}_3^+ \]

\[ \text{C-C cleavage and loss of } H^+ \]

\[ \text{Peroxy-radical} \]

\[ \text{Atmospheric } O_2 \]

\[ \text{Cation} \]

\[ \text{Ligninase} \]

\[ \text{More radical cations} \]
Tien et al (1986), carried out a detailed study of the oxidation of veratryl alcohol by lignin peroxidase. With excess of veratryl alcohol they observed a stoichiometry of one mole of veratryldehyde formed per mole of hydrogen peroxide used. Steady-state kinetic indicated a ping-pong mechanism (Km = 29 uM for H₂O₂ and 72 uM for veratryl alcohol) in which the enzyme first reacts with hydrogen peroxide, and then the oxidized enzyme reacts with veratryl alcohol.

In addition to lignin-related compounds, polycyclic aromatics and certain dibenzodioxins are oxidized by lignin peroxidase, producing quinones and unidentified products (Haemmerly, 1986; Hammel, 1986; Sanglard, 1986; and Bumpus, 1987). These reaction are also consistent with the cation radical mechanism described for lignin-related compounds.

Intact cultures of P. chrysosporium are able to degrade a variety of chlorinated aromatic compounds, showing once more the non specificity of the lignin degrading system and opening the possibility of application of the microorganism and its

Lignin peroxidase activity has recently been detected in other white-rot fungi, including *Phlebia radiata*, *Panus tigrin*, *Coriolus versicolor*, *Pleurotus ostreatus* and *Bjerkandera adusta*. (Dodson et al, 1987; Kantelinen et al, 1988).

**Manganese Peroxidase Enzymes:**

Kuwahara et al (1984), reported the characterization of a second extracellular hydrogen-dependent oxidase. This peroxidase catalysed the oxidation of a variety of dyes and also the decarboxylation of vanillic acid, but only in the presence of Mn2+. Similarly, Glenn et al (1985 and 1987) have described the purification and properties of a novel *P. chrysosporium* peroxidase. The enzyme is a haem protein with a molecular weight of 46 kD. It is dependent on hydrogen peroxide and manganese (II) for activity, and it is stimulated by hydroxy acids and protein (gelatine, albumin). The enzyme catalyses the oxidation of manganese (II) and also of a variety of organic compounds, including NADH and some polymeric dyes. Also Paszczynski et al (1986), have described a second type of extracellular peroxidase from *P. chrysosporium*. The enzyme oxidizes manganese (II) to manganese (III), which acts as a diffusible catalyst, oxidising phenolic compounds, similar to phenol oxidizing enzymes such as laccase. Leisola et al (1987) separated six Mn peroxidases from the extracellular fluid of *P. chrysosporium* and Kirk & Farrell (1987) separated four Mn
peroxidases. Manganese peroxidases appear to function as phenol-oxidizing enzymes and perhaps participate in H2O2 production.

Phenoloxidases:

Most white-rot fungi produce extracellular phenoloxidases, namely laccase (p-diphenol:oxygen oxidoreductase; E.C.1.10.3.7), which catalyses the one electron oxidation of phenols to phenoxy radicals Reinhammer (1984). The resultant free radicals undergo a variety of non enzymatic reactions including polymerization via oxidative coupling, C-C oxidation, alkyl-phenyl cleavage and demethylation. Coupling/polymerization reaction is the predominant reaction when lignin related phenol and isolated lignins are used. Polymerization of phenols is also a consequence of lignin peroxidase oxidation and other phenoloxidizing enzymes and the in vivo mechanism which limits polymerization is yet to be elucidated.

_P. chrysosporium_ belongs to a minority of white-rot fungi that apparently produce no detectable laccase (Kirk & Farrell, 1987).

H2O2-Producing Enzymes:

Several authors have shown the close relationship between H2O2 production and ligninolytic activity in cultures of _P. chrysosporium_. Hydrogen peroxide is produced in microbodies located beneath the cell wall in the hyphae (Forney et al, 1982). Glucose oxidase activity is associated with these microbodies. Kelly & Reddy (1986a and 1986b) showed this to be glucose-1-oxidase and mutants lacking this enzyme activity were unable to degrade labelled lignin. Wild type strains and
revertant mutant strains showed normal ligninolytic activity. Because the activity is produced during growth on various sugars and is associated only with ligninolytic cultures, Kelly & Reddy (1986c) concluded that glucose oxidase is the primary source of hydrogen peroxide. Eriksson et al (1986), identified an intracellular glucose-2-oxidase that which could be responsible for supplying hydrogen peroxide. Greene & Gold (1984), attributed its production to fatty acyl CoA. Paszczynski et al (1985) showed that Mn peroxidases oxidizes various substrates, including glutathione, NADPH and dihydroxymaleic acid with the coupled reduction of oxygen to hydrogen peroxide. This is a interesting observation since extracellular NAD(H) and NADP(H) were found to be present in N-starved cultures ligninolytic cultures (Kuwahara et al 1984 and Asada et al, 1987). More recently, a glyoxal oxidase has been identified by Kersten & Kirk (1987), which also have hydrogen peroxide as a product of reaction. As can be concluded, hydrogen peroxide necessary for ligninolytic activity may be produced by several different enzymes.

Other Enzymes:

In white-rot decay of wood cell walls lignin degradation takes place in parallel with cellulose hydrolysis. Cellulolytic enzymes which are linked to phenol metabolism have been identified in P. chrysosporium cultures and include cellobiose quinone oxido-reductase and cellobiose oxidase (Morpeth & Jones, 1986). When cellobiose is oxidized to glucose, quinones are reduced to phenols, suggesting a mechanism which would prevent polymerization of phenoxy radicals. Recently, it has
been found that cellobiose quinone oxidoreductase does not prevent *in vitro* polymerization of phenols (Odier et al, 1988).

1.9 **Enzymes and Microorganisms Involved in the Breakdown of Cellulose**

Microorganisms which can grow on cellulose include true bacteria, actinomycetes and higher fungi. Those which can utilise native cellulose rather than only soluble derivatives are termed truly cellulolytic. The fungi play a significant part in the degradation of cellulose under aerobic conditions. They are more successful than bacteria in acid soils and in the degradation of cellulose embedded in lignin (Schlegel, 1987).

Among the best characterised cellulase systems are those of the fungi *Trichoderma reesei*, *Trichoderma koningii*, *Fusarium solani*, *Penicillium funiculosum*, *Talaromyces emersonii* and *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*). *Neocallimastix frontalis*, an anaerobic fungus found in bovine rumen, produces an extracellular cellulase system that catalyzes extensive hydrolysis of cellulose (Tsao & Chiang, 1983; Schlegel, 1987; and Coughlan & Ljungdahl, 1988).

Among aerobic cellulolytic bacteria are *Cellulomonas* sp., *Cellvibrio* sp., *Microbyspora bispora* and *Thermomonospora* sp. Examples of anaerobic bacteria are *Acetovibrio cellulolytics*, *Clostridium thermocellum* and *Ruminococcus albus* (Schlegel, 1987 and Coughlan & Ljungdahl, 1988).

The basic model of cellulase action is based specially on fungal systems. Cellulase is a multicomponent enzyme system generally considered to be composed of three major components: (Tsao & Chiang, 1983; Crueger & Crueger, 1984; Eveleigh, 1987
Endo-B-1,4-glucanase (endo-1,4-B-D-glucan 4-glucanohydrolase, EC 3.2.1.4), also called endocellulase, carboxymethylcellulase or Cx cellulase.

Exo-B-1,4-glucanase (1,4-B-D-glucan cellobiohydrolase, EC 3.2.1.9), also called cellobiohydrolase, avicelase or Cl cellulase.

B-glucosidase or cellobiase (B-D-glucohydrolase EC 3.2.1.21)

The first enzyme acts on the interior of the polymer to generate new chain ends. Its activity is assayed by reactivity towards soluble cellulose as shown by increase in reducing sugar or by decrease in viscosity. The endoglucanases are generally found to be inactive against crystalline cellulose, but hydrolyse amorphous cellulose and soluble derivatives such as CM-cellulose.

Exoglucanases degrade amorphous cellulose by consecutive removal of cellobiose units from the non-reducing end of the substrate. They are considered to be inactive against crystalline cellulose, when acting in isolation. Endoglucanases and exoglucanases act cooperatively and sometimes synergistically in the hydrolysis of crystalline cellulosic substrates.

The B-glucosidases complete the hydrolytic process by converting the resultant cellobiose to glucose or by removing glucose residues from the non-reducing end of short cellooligosaccharides.

Oxidative enzymes also participate in cellulose degradation by white-rot fungi (Ericksson et al, 1974 Morpeth
1985 and Coughlan & Ljungdahl, 1988). Cellobiose oxidase, a flavin/ cytchrome b protein, oxidizes cellobiose and celldextrins. One function of this enzyme may be to oxidize the reducing end group formed when a B-1,4-glycosidic linkage is cleaved by an endoglucanase (Ericksson & Wood, 1985). This would prevent the occurrence of relinkage. The cellobiose: quinone oxidoreductases, of which at least two are produced by P. chrysosporium, are simple flavoproteins. They utilise quinone as electron acceptor, are produced by several white-rot fungi and may act as a link between the processes of lignin and cellulose biodegradation.

Figure 1.7 illustrates the action of cellulolytic and oxidative enzymes in cellulose degradation by P. chrysosporium (Kirk & Cowling, 1984).

1.9 Hemicellulase and microorganisms producers

Xylanases (endo-1,4-B-xylanase, EC 3.2.1.8) are widespread in nature among microorganisms that decompose lignocelluloses materials and the fungus Schizophyllum commune is a particular strong producer of xylanases (Jurasek & Pace, 1988). Xylanases belong to a broader group of enzymes, hemicellulases, responsible for the conversion of cellulose to soluble sugars. Separation by gel permeation and ion exchange chromatography has revealed the existence of at least 15 different enzymes in the xylanase complex (Magee & Kosaric, 1985).

John and Schmidt (1988) have identified two different xylanases from cultures of Trichoderma lignorum, a endoxylanase and a B-xylosidase (B-xyloside xylohydrolase EC 3.2.1.37). The endoxylanase is responsible or the initial breakdown of the
Figure 1.7 Action of Cellulolytic and Oxidative Enzymes on the Degradation of Cellulose
xylan chain and the formation of oligosaccharides and the B-xylosidase causes fragmentation of the oligosaccharides leading finally to xylose. Studies on these enzymes revealed the xylanase has a high glycosyltransferase activity and that B-xylosidase is a glycoprotein and shows high thermal stability.

Several other fungi have been shown to produce xylanase enzymes, such as *Trichoderma reesei*, *Aspergillus foetidus*, *Aspergillus niger* and others (Magee & Kosaric, 1985 and Matsui & Yasui, 1988).

Xylanases are also produced by bacteria such as *Bacillus pumillus* and *Streptomyces* sp. (Okada & Shinmyo, 1988 and Yasui et al, 1988).

1.10 Immobilization of *P. chrysosporium* for the Production of Lignin Peroxidase

The high cost of enzyme production makes the use of repeated or continuous use desirable in most of the cases where biocatalysis is used. However, it is generally impractical to reuse suspension of enzymes or other biocatalysts such as organelles or cells, because their recovery from the reaction mixture is difficult and uneconomical. On the other hand, immobilization allows both reuse of biocatalysts and their application in continuous processes (Hartmeier, 1985 and Webb, 1987).

For whole cells or cell organelles, physical entrapment in polymeric matrices is the most widely used technique, whereas single enzymes are most often bound to carriers or crosslinked by bi- or multifunctional reagents like glutaraldehyde and hexamethylene diisocyanate (Cheettham, 1985, Hatmeier, 1985 and
Webb, 1987).

The immobilization of *P. chrysosporium* in cubes of polyurethane foam has allowed production of lignin peroxidase in a semi-continuous basis (Palmer, 1987, personal communication and Kirkpatrick & Palmer, 1987). At each successive harvest, cultures were purged with oxygen and lignin peroxidase activity induced with veratryl alcohol. Compared to the non immobilized cells, higher levels of lignin peroxidase were obtained by the foam immobilised microorganism.

Kirk et al, (1986) have described an alternative immobilization procedure for the continuous production of lignin. In their method, the fungus is grown in the roughened interior walls of a disc fermenter. However, the fungus is not entrapped by this method. The yield of lignin peroxidase reported was significantly less then the foam immobilized microorganism.

More recently, Linko (1988a and 1988b) has reported the production of lignin peroxidase by nylon-web immobilized *P. chrysosporium* in a modified Biostat E bioreactor. Veratryl alcohol was used as inducer for lignin peroxidase. A maximum activity of 730 U/l was obtained after 130 hours, and the bioreactor produced a total of 6200 U of lignin peroxidase activity after a period 5.5 days.

1.11 The Application of Some Techniques of Molecular Biology to Lignin Biodegradation.

In natural environments, lignocellulosic materials are broken down by mixed populations of organisms. The contribution of
each microorganism and/or enzyme is difficult to analyse because of the complexity of their interrelationships. Isolation of the components of degradation, using genetic methods affords the best opportunity for detailed understanding of the process. Understanding of the ligninolytic system is necessary for its effective use and for the possibility of targeted manipulation of particular functions such as increased lignin solubilization or reduced cellulolytic activity (Johnsrud & Ericksson, 1985).

Ligninolytic activity in *P. chrysosporium* occurs in the stationary phase of growth, as a secondary metabolite, in response to nutrient nitrogen starvation. It does not occur in the primary phase of growth, under conditions of nitrogen sufficiency. The possibility of distinguishing between the primary metabolic phase and the secondary metabolic (ligninolytic) phases offers several experimental approaches for investigation of the switch mechanism, the isolation of mutants and the cloning of genes. Comparison of the primary and secondary metabolites showed changes in the population of total protein (Wallace et al., 1984) and messenger RNA (mRNA) (Hayloch et al., 1985). Genomic libraries of *P. chrysosporium* were screened with complementary DNA (cDNA) probes derived from RNA of the primary and secondary metabolic stage. In this way a small number of clones containing genes expressed only or mainly in secondary metabolic stage were identified (Raeder et al., 1987).

Tien & Tu (1987) constructed a cDNA library with mRNA isolated from a 6-days old culture of *P. chrysosporium*
consisted of over 250,000 clones. One of these clones, which hybridized to an oligonucleotide probe synthesised according to a sequence of amino acids of the purified lignin peroxidase H8 (Kirk et al, 1987) was sequenced. The sequenced cDNA demonstrated that lignin peroxidase shows homology with other known peroxidases and that expression of lignin peroxidase is regulated at mRNA levels.

Raeder & Broda (1988), have described a set of DNA related experiments used to characterise basic genomic and genetic properties of the ligninolytic basidiomycete *P. chrysosporium*. DNA preparation and construction of genomic libraries; genome size estimation by dot-blot hybridization; and use of CsCl-bisbenzimide gradients to separate mitochondrial, ribosomal and chromosomal DNA and to estimate %CG were described. The genome size of *P. chrysosporium* was calculated as 44,000 kb, which is similar to that of other basidiomycetes. The %CG estimated was 59% for chromosomal DNA; 52% for ribosomal DNA and 33% for mitochondrial DNA. The high %CG for chromosomal DNA and low %CG for mitochondrial DNA are typical for basidiomycete DNA.

Studies to isolate and characterise the cDNA clones for lignin peroxidase were also carried out by Zhang and Reddy (1988). Construction of a cDNA library using poly(A) RNA from 6 days old culture; isolation of cDNA clones specific for 6 day old culture using differential hybridization; synthesis of oligonucleotide probes, deduced from partial amino acid sequence of lignin peroxidase and use of these probes to screen, isolate and identify the lignin peroxidase clones from the cDNA library were employed.
Holzbaur & Tien (1988), have isolated and sequenced the gene for the lignin peroxidase isoyme H8. The gene was shown to be split into nine exons and eight introns. The promoter was shown to contain the eukaryotic consensus elements: a TATA box at -78 and a ACAAT box at -106. Transcription initiates downstream from these sequences. Induction of lignin peroxidases isoymes were compared by analyses of RNA and protein and the results indicated that the lignin peroxidase isoymes are differentially regulated in response to environmental stress.

1.12 Aims of This Thesis

When work for this thesis began, the characterization and production of ligninolytic enzymes were in its early stages. The lignin peroxidase discovered in P. chrysosporium by Tien & Kirk (1983) and Glenn et al (1983), was produced under stationary conditions and low yields were obtained. However, the same organism was able to produce good yields of cellulases in suitable conditions (Ericksson & Wood, 1985). The scale-up of lignin peroxidase production was hampered by several phenomena such as the association of ligninolytic activity with secondary metabolism and low yields of enzyme production. In addition, ligninolytic enzymes synthesis was inhibited under shaken conditions. Therefore, the primary aim of this work was to develop conditions that would allow higher production of ligninolytic enzymes and also the scale-up of the process. In order to do this, growth conditions were investigated and optimization of the culture parameters was sought. Cellulases and hemicellulases were monitored under conditions suitable for
lignin peroxidase production, in order to determine their role in the degradation of lignocelluloses and also to find out if it was possible to develop media where good yields of lignocellulases would be obtained. With a view to obtaining a clearer overall picture of the establishment of the ligninolytic system in *P. chrysosporium*, some studies in the RNA extracted from non ligninolytic and ligninolytic cultures, was carried out. The combined results of the studies carried out here have offered further insight into production of lignocellulases.
CHAPTER 2

General Methods
2 GENERAL METHODS

2.1 Enzyme Source

2.1.1 Extracellular Extract

Extracellular extract was collected by filtering the entire content of flasks grown with *P. chrysosporium* in low nitrogen medium for the production of lignocellulases, as described in chapter 3 section 3.2.2, through two layers of muslin and one layer of glass wool. The supernatant was then used for the assay of extracellular lignocellulases enzymes, sugar determination and protein determination. When necessary the supernatant was stored at -20°C before using.

2.1.2 Intracellular Extract

The microorganism collected in muslin layers, as described above, was washed with excess of cold distilled water, which was then removed by gentle compression. The microorganism was stored at -20°C and then disrupted by grinding in a mortar with a pestle. Cold 50 mM acetate buffer pH 5.0 was added to it and the mixture was made as homogeneous as possible. Centrifugation at 4000rpm at 4°C was then carried out for 15 minutes. The resultant supernatant was used as the intracellular enzyme source.

2.2 Dry Weight Determination

The microorganism collected in muslin layers and washed and compressed as described in section 2.1.2 was spread over three layers of filter paper Whatman n.1 previously weighed and then stored at 80°C for 48 hours. The resultant dried material was weighed and the dry weight for the microorganism was calculated.
2.3 Lignin Peroxidase Assay

Lignin peroxidase activity was determined using veratryl alcohol (3,4-dimethoxybenzyl alcohol), a simple lignin model substrate (Tien & Kirk, 1984). Veratryl alcohol is oxidized to the corresponding aldehyde in the presence of hydrogen peroxide. The formation of veratryl aldehyde was monitored by the associated increase in absorbance at 310 nm. The enzyme assay mixture was as below:

Veratryl alcohol - 0.4 mM
335 µl of enzyme source
Hydrogen Peroxide - 0.15 mM

Veratryl alcohol and hydrogen peroxide solutions were prepared in 100 mM tartrate buffer pH 3.0

Final volume of the reaction - 0.5 ml

The veratryl alcohol used was grade purum and was obtained from Fluka AG, Switzerland.

2.4 Determination of Glucose

2.4.1 Somogy-Nelson Assay for the Determination of Glucose

Solution A: 28 g of disodium phosphate and 40 g of Rochelle salt were dissolved in 700 ml of distilled water. 100 ml of 1N NaOH was added to it, followed by 8 g of copper sulphate (with stirring). Finally, 180 g of sodium sulphate was added and the volume was made up to 1 litre. The solution was allowed to stand at room temperature for 24 hours, before using.

Solution B: Arsenomolybdate colour reagent - 25 g of ammonium molybdate was dissolved in 450 ml of distilled water and 21 ml of concentrated sulphuric acid was added to it,
followed by 3 g of Na$_2$HAsO$_4$·7H$_2$O previously dissolved in 25 ml of distilled water. The mixture was mixed and placed in a incubator at 37°C for 48 hours.

Reaction: 1 ml of sample was mixed with 2 ml of solution A, and the mixture was heated for 10 minutes in a boiling water bath. The mixture was then cooled in ice and 2 ml of solution B was added and mixed. The volume was then completed to 25 ml with distilled water and absorbance was read at 540 nm. A glucose standard curve was prepared (figure 1) and used to calculate the sugar concentration of the samples (Somogy, 1945).

2.4.2 GOD-Perid Method for the Determination of Glucose

This kit was obtained from Boehringer Mannheim GmbH Diagnostica.

Test Principle:

GOD

\[
\text{glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{gluconate} + \text{H}_2\text{O}.
\]

POD

\[
\text{H}_2\text{O} + \text{ABTS} \rightarrow \text{coloured complex} + \text{H}_2\text{O}.
\]

Reagents: 1- Standard Glucose - 9.1 mg/100ml

2- Phosphate buffer - 100 mmol/l, pH 7.0

Peroxidase (POD) - 0.8 U/ml

Glucose Oxidase (GOD) - 10 U/ml

ABTS (di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) - 1.0 mg/ml

Reaction: 0.2 ml of sample was mixed with 5.0 ml of reagent 2 and allowed to stand at room temperature for 30 min. Absorbance was read at 600 nm. The concentration of glucose in the standard glucose solution (reagent 1) was determined in the same way as for the samples and it was used to calculate the
2.5 Determination of Protein

Protein concentrations were determined by the method of Lowry et al (1951), using bovine serum albumin as a standard.

Reagents:
- 2% (w/v) sodium carbonate in 0.1 M NaOH
- 1% (w/v) hydrated copper sulphate
- 2% (w/v) sodium potassium tartrate

Copper sulphate, sodium potassium tartrate and sodium carbonate/hydroxide were mixed in the ratio (1:1:100) immediately before use.

0.5 ml of sample was mixed with 0.5 ml of 1 M NaOH and 5 ml of the copper solution described above and allowed to stand at room temperature for 10 minutes. 0.5 ml of Folin Ciocalteau phenol reagent diluted 1:1 with distilled water, was then added, mixed and left for 30 minutes at room temperature. The absorbance at 700 nm was recorded.

The bovine serum albumine (BSA) standard curve shown in figure 2 was used to calculate the protein concentration of the samples.

2.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

The method of Laemmli (1970) was used for analysis of intracellular proteins. The proteins samples were initially solubilized with sodium dodecyl sulphate and then separated on polyacrylamide using a discontinuous buffer system.

Reagents:
- Gel Stock: 30% (w/v) acrylamide containing 0.8% (w/v) N,N'-methylenebis-acrylamide
Figure 2.1 Standard Curve for Glucose

Figure 2.2 Standard Curve for BSA Protein
Buffer 1: 1.5 M Tris-HCl (pH 8.8) containing 0.4% (w/v) SDS (Sodium Dodecyl Sulphate).

Buffer 2: 500 mM Tris-HCl (pH 6.8) containing 0.4% (w/v) SDS.

Buffer 3: 25 mM Tris-HCl (pH 8.3) containing 192 mM glycine and 0.1% (w/v) SDS

Buffer 4: 62.5 mM Tris-HCl (pH 6.8) containing 2.3% (w/v) SDS, 15% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 0.001% bromophenol blue

Method:

A vertical slab gel apparatus made in the workshop of the Biochemistry Department was used. The gel was contained in a glass and perspex cuvette, with internal dimensions of 120 mm x 100 mm x 1.5 mm. Electrophoresis was carried out at room temperature.

The glass plates of the gel cuvette were washed in detergent, rinsed with tap water, distilled water and acetone, and allowed to dry. The cuvette was assembled and sealed with 2% (w/v) molten agar and then clamped in a vertical position. The lower (running) gel was prepared by mixing Buffer 1 (10ml), gel stock (13.3 ml), and water (16.6 ml), and polymerization was started by addition of N,N'-tetramethylenediamine (20 ul) and 240 ul of freshly prepared ammonium persulphate solution 10% (w/v). The solution was poured into the glass cuvette to a height of 80 mm. A layer of distilled water was introduced above the gel mixture to ensure a flat interface between the separating gel and stacking gel after polymerization. When polymerization was complete the water layer was removed and the
upper (stacking) gel, consisting of a mixture of buffer 2 (2.5 ml), gel stock (1.0 ml), water (6.5 ml), ammonium persulphate solution (10% (w/v), 60 ul) and TEMED (20 ul) was added to the cuvette above the running gel. A perspex comb was introduced into the stacking gel before polymerization, to form the sample wells. The comb was removed when polymerization was complete and a small amount of buffer 3 was introduced into the sample wells to keep them separated. The lower spacer was removed from the cuvette, and the cuvette was placed in the electrophoresis tank. Buffer 3 was added to the upper and lower reservoirs, and air bubbles trapped underneath the gel were removed using a syringe.

Protein samples were diluted with buffer 4 and placed in boiling water for 3 minutes. The samples were cooled and applied to the stacking gel (wells). A constant current of 20 mA was used until the bromophenol blue entered the separating gel, and then this was increased to a constant current of 40 mA until the bromophenol blue came within 5 mm of the end of the gel. The cuvette was removed from the tank when electrophoresis was complete. The glass plates were separated, the stacking gel removed and the gel stained as described below.

2.6.1 Detection of Protein on Polyacrylamide Gels

The proteins were detected using the Coomassie Blue Stain. Reagents:

Stain Solution: Propa^-2-ol/acetic acid/water (5:2:13 by volume) containing 0.05% (w/v) Coomassie Blue G-250.

Destaining solution: Propan-2-ol/acetic acid/water (1:1:8 by volume).
Method:

Staining of the proteins was achieved by overnight immersion of the gel in staining solution. The gel background was destained by immersing in progressive changes of the destaining solution. The gels were stored in 3% (v/v) glycerol at 4°C once the background was cleared.
CHAPTER 3

Studies on Culture Conditions and Growth of
P. chrysosporium for the Production of
Lignin Peroxidase
3 STUDIES ON CULTURE CONDITIONS AND GROWTH OF P. chrysosporium FOR THE PRODUCTION OF LIGNIN PEROXIDASE

3.1 Introduction


Correct growth conditions and lignin peroxidase production are strongly associated. The size of inoculum, temperature, pH of the medium, oxygenation and agitation are important factors that can cause a physiologically heterogenous culture leading to poor lignin peroxidase production.

Synthesis of the ligninolytic enzyme system follows a physiological shift from primary to secondary metabolism and throughout these experiments this shift was promoted by nutrient nitrogen starvation. The strain used was Phanerochaete chrysosporium ATCC 24725. It was a gift from Prof. H. Dalton, Department of Biological Sciences, University of Warwick.

3.2 Methods

3.2.1 Growth Media

The composition and preparation of the low nitrogen growth medium was as given below:

Basal medium contained per litre of distilled water:

$KH_2PO_4$ 0.2g
MgSO₄·7H₂O 0.05g
CaCl₂ 0.01g
Mineral solution 1.0 ml
Vitamin solution 0.5 ml

Additions to this medium included nutrient nitrogen, buffer and growth substrate (carbon source). The various carbon sources used are described below.

Carbon sources: 1% glucose
0.5% xylan plus 0.5% glucose
0.5% xylan only
1% cellobiose
1% lactose
1% glucose plus various molasses concentrations

The compositions of the mineral and the vitamin solutions are described in appendix 1.

Minerals and vitamins without sterilization were added aseptically to sterile distilled water. Carbon source solution was sterilized at 121°C for 10 minutes and added to the buffer solution that had previously been sterilized for 20 minutes at 121°C, to give the desired final concentration.

3.2.2 Culture Parameters

The microorganism was grown under two different conditions, viz stationary culture and shaken culture. In the case of stationary cultures 10 ml of growth media was added to each 100 ml conical flask, inoculated and incubated in a vertical incubator without agitation. For shaken cultures, 250ml of growth medium was added to each 1 litre conical
flask, inoculated and incubated in a orbital shaker at 140 rpm for three days and then decreased to 70 rpm.

In both culture conditions incubation was at the optimum temperature of 39 - 40 °C.

3.2.3 Inoculum Preparation

3.2.3.1 Inoculum Preparation For Stationary Cultures

A portion containing mycelium and spores of *P. chrysosporium* grown on 2% malt agar slope was cultivated in 250 ml conical flasks containing 50ml of a medium composed of (w/v): glucose 2%, peptone 2% and yeast extract 1% for 36 hours at 39 °C without agitation. The mycelium was then filtered off and washed free of culture medium with sterile distilled water. The circular area 1.5 cm away from the edge of the flask was equally divided in small pieces and placed into 100 ml conical flasks containing 10 ml of the nitrogen limited medium.

If the inoculum prepared from the microorganism maintained in 2% (w/v) malt agar slope failed to produce good yields after proper growth for lignin peroxidase production, it was discarded. The whole culture of those flasks which showed good lignin peroxidase activity was then used as a starter for the inoculum preparation.

3.2.3.2 Inoculum Preparation For Shaken Cultures

250 ml conical flasks with dextrose potato agar slopes were inoculated with spores and mycelium of *P. chrysosporium* and incubated at 39 °C for eight days. Inoculum for lignin peroxidase production was prepared by scraping the surface of the agar and washing off the spores with sterile distilled
water. The spore solution was filtered through a layer of muslin and then through a layer of glass wool and muslin. The final spore solution was counted on a haemocytometer and added to the 1 litre flasks to give a final concentration of 10 spores/ml.

3.2.4 Choice of Buffer

The optimum pH for growth of P. chrysosporium is 4.0-4.5. Initially the growth medium was buffered with dimethyl succinate (DMS). Concentrations of 5 mM, 10 mM and 20 mM were used. This buffer was later replaced by 10 mM sodium tartrate buffer.

3.2.5 Oxygenation

3.2.5.1 Oxygenation of the 100 ml Conical Flasks

Oxygen was flushed for 1 min, into a 100 ml flask sealed with a rubber bung, using a wide needle connected via rubber tubing to an oxygen cylinder.

3.2.5.2 Oxygenation of the 1 Litre Conical Flasks

1 litre conical flasks containing medium and microorganism were allowed to settle and concentrated down to 100 ml by aseptically pouring off 150 ml to 200 ml of the medium. Flasks were then stoppered with rubber bungs and purged with oxygen for 10 minutes through a needle connected via tubing to an oxygen cylinder.

In both cases, 100 ml flasks (small scale and stationary condition) and 1 litre flasks (large scale and shaken condition) oxygenation was carried out on the third day after
inoculation. Controls where no oxygenation was carried out were also run. Large scale flasks were stoppered with rubber bungs and small scale flasks were stoppered with either rubber bungs or cotton stoppers.

3.3 Results and Discussion

3.3.1 Variation of pH During Growth

The influence of pH on fungal growth and metabolism is complex. The optimum pH for the growth of fungi varies with the strain or species and the nutritional environment. Fungi are tolerant of acidic conditions, but for optimal growth require a pH of between 5.0 and 7.0.

Besides being affected by the pH of the medium, fungi also have a marked effect on the pH of the medium. Differential uptake of anions and cations, the excretion of organic acids or the release of ammonia can all produce rapid changes in the pH of the medium (Berry, 1975 and Garraway & Evans, 1984). In this way, the prevailing pH and buffering capacity of the culture medium can influence fungal growth and product formation.

The optimum pH for growth of *P. chrysosporium* is between 4.5 and 5.0. The efficiency of dimethyl succinate (DMS) and tartrate buffers were investigated.

In figure 3.1 the variation of pH when 5 mM DMS buffer was used, is shown. The initial pH was corrected to 4.3 by the addition of sodium hydroxide. 5 mM DMS buffer showed a very poor buffering capacity and lignin peroxidase production was very low under these conditions, indicating that low pH (< 3.7) has a negative effect on the production of lignin peroxidase.
Figure 3.1 Variation of the pH of the Growth Medium With Time of Culture - 5 mM DMS Buffer
Figure 3.2 Variation of the pH of the Growth Medium With Time of Culture - 20 mM DMS Buffer (initial pH not corrected)
Figure 3.3 Variation of the pH of the Growth Medium With Time of Culture - 20 mM DMS Buffer (initial pH corrected)
Figures 3.2 and 3.3 show the variation of pH for 20 mM DMS buffer without correction of the initial pH, and with correction of the initial pH from 5.1 to 4.5, respectively. Higher variation occurred when 20 mM DMS had its initial pH adjusted to 4.5, perhaps indicating a weaker buffering capacity than the 20 mM DMS buffer without correction of initial pH.

In figure 3.4 the variation of pH during growth under oxygenation, and without oxygenation, is shown using 10 mM tartrate buffer. It is interesting to note that oxygenation might have had an indirect effect on the pH, whose behaviour under the two conditions was distinctly different. Oxygenation is necessary for the establishment of ligninolytic activity. After oxygenation, the microorganism might have undergone physiological changes which affected the pH of the medium in a different way to that of culture without oxygenation.

Figures 3.5, 3.6 and 3.7 show the variation of lignin peroxidase activity with pH in various flasks on the same day of culturing. Cultures grown in 20 mM DMS buffer (both, with initial pH corrected and not corrected), showed a higher variation of pH and lignin peroxidase activity among flasks of the same day of culturing when compared to 10 mM tartrate buffer. Later, it was found that P. chrysosporium was able to grow on DMS as the only source of carbohydrate and produce some lignin peroxidase. According to Berry (1975) esters are readily assimilated by fungi as intermediates of the TCA (tricarboxylic acid cycle). This could explain the variation in pH and lignin peroxidase activity when DMS buffer was used and also the appearance of lignin peroxidase on DMS as the only
Figure 3.4 Variation of the pH of the Growth Medium With Time of Culture - 10 mM Tartrate Buffer

A : Culture With Oxygenation on the 3rd Day
B : Culture Without Oxygenation on the 3rd Day
Figure 3.5 Variation of Lignin Peroxidase with pH of the Growth Medium - Stationary Culture - 20 mM DMS Buffer (Initial pH of Medium Not Corrected)

- : pH of Medium
- : Lignin Peroxidase Activity (umol/ml/min)
Figure 3.6 Variation of Lignin Peroxidase With pH of the Growth Medium - Stationary Culture - 20 mM DMS Buffer (Initial pH of Medium Corrected)

- : pH of Medium
- : Lignin Peroxidase Activity (umol/ml/min)
Figure 3.7 Variation of Lignin Peroxidase With pH of the Growth Medium - Stationary Culture - 10 mM Tartrate Buffer
carbon source. Being an ester of an intermediary compound of the tricarboxylic acid cycle (succinic acid), DMS might be metabolized by _P. chrysosporium_, providing an alternative source of energy for growth and bringing about changes that would not occur when a non assimilated buffer was used.

All the experiments in this section were carried out using 10 ml of medium in 100 ml conical flasks. Tartrate buffer was chosen as the buffer for the experiments under shaken conditions. This particular buffer also has the advantage of being cheaper than DMS, which is a factor to be considered in scaling up the process.

3.3.2 The Inocula Used

The standardization of the inoculum is a very important factor affecting any growth experiment.

Consistency of results between experiments requires an inoculum, either spore solution or mycelium, that has the same viability, the same physiological state and that may be added to the medium in the same concentration each time.

Initially, before it was decided to use the inoculum prepared as described in section 3.2.3.1, the inoculum used was a piece of mycelium cut from microorganisms cultivated on a 2% malt agar slope. This led to a high discrepancy of lignin peroxidase activity among flasks and reasons for this could include non-uniformity in the distribution of spores in the one agar slope used, and also from slope to slope. Furthermore, the filamentous growth of the microorganism produces cells irregularly aged, or in different physiological
states. The preparation of a inoculum as described in section 3.2.3.1 was carried out in order to try to minimize physiological differences within the culture.

### 3.3.3 Time Course of Growth and Utilization of Growth Substrate

Batch culture is an example of a closed culture system which contains an initial, limited amount of nutrient. When this type of culture is inoculated and growth plotted over a period of time, the typical sigmoidal curve that results can be divided into four phases of different physiological properties (Stanbury & Whitaker, 1984). These are the lag phase (period after inoculation during which no growth seems to take place); the exponential phase; deceleration phase, and stationary phase.

The growth of many filamentous fungi also exhibits a sigmoidal growth curve (Righelato, 1975 and Garraway & Evans, 1984). The capacity of filamentous fungi to form branches results in new hyphal tips, which enable the colony to grow exponentially.

Figures 3.8, 3.9 and 3.10 show the time course for growth of *P. chrysosporium* grown in 100 ml conical flasks, when various carbon sources were used. As can be seen, the growth pattern was the same, independent of the carbon source used. Most of the growth took place during the two days after inoculation, but a lower increase in dry weight was observed up to the 4th day of growth. Higher growth (grams of dry weight), was observed in 1% glucose as the carbon source using 20 mM DMS
Figure 3.8 Time Course for Growth of *P. chrysosporium* in 10ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 1% Glucose (20 mM DMS Buffer)
B : Carbon Source - 1% Glucose (10 mM Tartrate Buffer)
C : Carbon Source - 20 mM DMS (No Glucose Added)
Figure 3.9 Time Course for Growth of *P. chrysosporium* in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 0.5% Xylan + 0.5% Glucose

B : Carbon Source - 0.5% Xylan
Figure 3.10 Time Course for Growth of *P. chrysosporium* in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 1% Cellobiose
B : Carbon Source - 1% Lactose
Figure 3.11 Phases of Growth of *P. chrysosporium* in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 1% Glucose (Tartrate Buffer)
B : Carbon Source - 1% Glucose (DMS Buffer)
Figure 3.12 Phases of Growth of *P. chrysosporium* in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 0.5% Xylan + 0.5% Glucose

B : Carbon Source - 0.5% Xylan

A : Carbon Source - 0.5% Xylan + 0.5% Glucose

B : Carbon Source - 0.5% Xylan
Figure 3.13 Phases of Growth of *P. chrysosporium* in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 1% Cellobiose

B : Carbon Source - 1% Lactose
Figure 3.14 Consumption of Glucose During Growth of P. chrysosporium in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Carbon Source - 1% Glucose (DMS Buffer)
B : Carbon Source - 1% Glucose (Tartrate Buffer)
C : Carbon Source - 0.5% Xylan + 0.5% Glucose
as the buffer. This suggests that DMS and not only glucose was used for growth. When only DMS was used for growth, about 50% of the growth in glucose plus DMS was observed. Similar growth (grams of dry weight) was observed for 1% glucose and for 0.5% xylan plus 0.5% glucose in tartrate buffer. However, only about 50% of this growth was obtained when 0.5% xylan was used as the carbon source. The lowest growth was obtained in 1% lactose as the carbon source, suggesting that the fungus was unable to efficiently metabolize this disaccharide.

Figures 3.11, 3.12 and 3.13 show the respective phases of growth for the fungus grown in the same conditions as mentioned above. The "lag" phase is not shown in these figures, since no growth measurements were taken at preliminary stages of growth. But, it was observed that growth would start from 3 to 4 hours after incubation. The exponential phase lasted until the first day of growth, followed by the deceleration phase up to the 4th day of growth. The stationary phase can then be seen from the 4th day on.

The consumption of glucose during growth of P. chrysosporium under stationary conditions is illustrated in figure 3.14. Most of the glucose was consumed by the 4th day of growth and by the 7th day only traces of glucose were detected in the medium.

When P. chrysosporium was grown under stationary conditions in a medium containing 1% cellobiose, 0.5% xylan or 1% lactose as the carbon source, formation of monosaccharides was observed after the 1st day of growth. As can be seen in
Figure 3.15 Glucose Formation and Consumption by *P. chrysosporium* in 10 ml Medium - 100 ml Flask

A : Carbon Source - 1% Cellobiose
B : Carbon Source - 0.5% Xylan
C : Carbon Source - 1% Lactose
figure 3.15, this formation was more accentuated in 1% cellobiose medium, followed by 0.5% xylan and 1% lactose. Release of monosaccharides from the respective di- and polysaccharides was observed up to the 5th day of growth. These sugars were subsequently utilized by the microorganism.

Figure 3.16 shows the time course of \textit{P. chrysosporium} grown under shaken conditions and when various growth substrates were used. The growth pattern under shaken conditions was similar to that found under stationary conditions. The maximum growth rate occurred during the first three days, and a plateau of constant dry weight was reached by about the 6th day.

Glucose consumption and pH variation for \textit{P. chrysosporium} grown under shaken conditions in various sources of carbon are shown in figures 3.17 to 3.20. Glucose consumption and pH variation under shaken conditions were lower than under stationary conditions.

3.3.4 The Morphology of \textit{P. chrysosporium} During Growth

When filamentous fungi are grown in submerged culture the type of growth varies from the filamentous form in which the hyphae constitute a homogeneous suspension dispersed through the medium, to a pelleted form consisting of interwoven mycelia (Whitaker and Long, 1973; Whitaker, 1987). More than one growth form may be present in a defined set of conditions during a growth cycle.

When grown under stationary conditions \textit{P. chrysosporium} assumed the filamentous form, growing radially and forming a
Figure 3.16 Time Course for Growth of *P. chrysosporium* in 1 Litre Flask (Shaken Conditions)

- **A**: Carbon Source - 1% Glucose
- **B**: Carbon Source - 1% Glucose + 0.6% Molasses
- **C**: Carbon Source - 1% Glucose + 0.8% Molasses
- **D**: Carbon Source - 1% Glucose + 1% Molasses
Figure 3.17 Variation of the pH of the Growth Medium and Glucose Consumption With Time of Culture - Carbon Source: 1% Glucose (Shaken Conditions)

A: Glucose Concentration (mg/ml)

B: pH of Culture Medium
Figure 3.18 Variation of the pH of the Growth Medium and Glucose Consumption With Time of Culture - Carbon Source: 1% Glucose + 0.6% Molasses (Shaken Conditions)

A : Glucose Concentration (mg/ml)

B : pH of Culture Medium
Figure 3.19 Variation of the pH of the Growth Medium and Glucose Consumption With Time of Culture - Carbon Source: 1% Glucose + 0.8% Molasses (Shaken Conditions)

A : Glucose Concentration (mg/ml)
B : pH of Culture Medium
Figure 3.20 Variation of the pH of the Growth Medium and Glucose Consumption With Time of Culture - Carbon Source: 1% Glucose + 1% Molasses (Shaken Conditions)

A : Glucose Concentration (mg/ml)

B : pH of Culture Medium
homogeneous suspension.

Under agitation conditions the fungi were either compact smooth pellets or pellets with a hairy surface. After concentration of the growth medium, oxygenation and change of agitation rate from 240 rpm to 70 rpm, all carried out on the third day of growth, the hairy pellets started to aggregate and by the fifth day clumps had been formed. On the other hand, the compact pellets remained unaltered. The former exhibited good lignin peroxidase activity, whereas lignin peroxidase was never detected in the latter. Haemmel (1988), also observed this connection between pellets with hairy surfaces and lignin peroxidase activity. He stated that the presence of these pellets seemed to be important for lignin peroxidase production, but no further explanation was given. Solomons (1975) stated that from changes in morphology a whole range of biochemical changes result, some due to inherent changes within the cell which merely reflect the changes of form in filamentous fungi.

3.4 Summary

Tartrate buffer was found to have better buffering capacity than DMS buffer. 5 mM DMS solution was a very poor buffer, and pH under this condition decreased to levels that did not support lignin peroxidase production. 20 mM DMS buffer, either with initial pH corrected or not corrected to 4.5, exhibited a high variation in both lignin peroxidase activity and pH between different flasks. DMS was found to be metabolized by P. chrysosporium, since growth and some lignin
peroxidase activity were observed when it was used as the only carbon source.

Oxygenation was found necessary for good levels of lignin peroxidase production. Culture grown in 10 mM tartrate buffer and oxygenated on the 3rd day of growth, showed different variation in pH when compared to culture with no oxygenation.

Under both stationary and shaken conditions, *P. chrysosporium* exhibited a linear growth curve, with the four phases of growth, "lag" phase, exponential phase, deceleration and stationary phase. In both cases, lag phase lasted from 3 to 4 hours.

*See chapter 4*
CHAPTER 4

Studies on Lignin Peroxidase Production
4 STUDIES ON LIGNIN PEROXIDASE PRODUCTION

4.1 INTRODUCTION

The white-rot fungus P. chrysosporium produces a variety of extracellular enzymes which are involved in the degradation of wood components (Kirk, 1987; Kirk & Farrell, 1987; Buswell & Odier, 1987; Kirk, 1985). An enzyme identified as peroxidase and designated "ligninase" or "lignin peroxidase", has been discovered in this fungus (Glenn et al, 1983 and Tien & Kirk, 1983).

Practical applications of ligninolytic enzymes have been limited by the unavailability of lignin peroxidase in commercial quantities.


Lignin peroxidase catalyses the one-electron oxidation of various aromatic compounds in the presence of hydrogen peroxide and its activity is increased by adding lignins or related low molecular weight aromatic compounds, including veratryl alcohol, to the cultures (Faison et al, 1986; Buswell & Odier, 1987; Kirk & Farrell, 1987 and Tonon & Odier, 1988).

It has been reported that the scale-up of lignin peroxidase production is hampered by inhibition of the ligninolytic enzyme synthesis in agitated cultures (Ulmer et al, 1983; Faison & Kirk, 1985). Recently it has been shown that the addition of certain compounds such as veratryl alcohol and oleic acid alone or emulsified with sorbitan
polyoxyethylene monooleate (Tween 80) favoured the production of lignin peroxidase under mild agitation (Kirk et al., 1986; Asther and Corrieu, 1987 and Colombie et al., 1988).

In this chapter the production of lignin peroxidase under stationary conditions is described, when different carbon sources were used. Production under agitated conditions (large scale) in medium containing glucose only, glucose plus xylan and glucose plus sugar cane molasses are described. Molasses is a by-product of either manufacture or refining of raw cane sugar. It is a dark, heavy, viscous liquid from which no further sugar can be crystallized by normal methods. Some studies on the decolorization of the molasses pigments during lignin peroxidase production were also carried out. The kinetic constants $V_{\text{max}}$ and $K_m$ for hydrogen peroxide and veratryl alcohol in the lignin peroxidase reaction were also determined.

4.2 Methods

4.2.1 Time Course For Lignin Peroxidase Production

After inoculation the flasks were incubated either under stationary conditions (100 ml flasks containing 10 ml medium), or under shaken conditions (1 litre flasks containing 250 ml medium). Every 24 hours, sample aliquots were collected and lignin peroxidase was assayed on the basis of $H_2O_2$-dependent oxidation of veratryl alcohol to veratryl aldehyde, determined by veratryl aldehyde absorbance at 310 nm as described in chapter 2 (section 2.3).

4.2.2 Kinetics Studies

The mechanism of the $H_2O_2$-dependent oxidation of veratryl
alcohol by lignin peroxidase was investigated using the assay described section 2.3. The activity of the enzyme was determined over a range of substrate concentrations while the concentration of the hydrogen peroxide was kept constant. The Km and Vmax for veratryl alcohol were then determined using the Lineweaver-Burk plot (Palmer, 1985). The enzyme activity was then determined over a range of hydrogen peroxide concentrations while the concentration of veratryl alcohol was kept constant. The constants Km and Vmax for hydrogen peroxide were calculated using the same plot as mentioned above.

4.2.3 Lignin Peroxidase Production on Glucose Only and on Glucose plus Molasses

Lignin peroxidase was produced under agitation in 1 litre flasks as described in chapter 3 (sections 3.2.2; 3.2.3.2 and 3.2.5.2). The carbon sources used were 1% glucose; 1% glucose plus 0.6% molasses (w/v) and 1% glucose plus 0.6% of dialysed molasses. (Dialysis of molasses: dialysis tubing containing 10% molasses (w/v) was dialysed overnight with constant agitation against distilled water, which was changed several times during the process). This solution was then used to prepare the medium mentioned above.

4.2.4 Effect of the Concentration of Molasses on the Activity of Lignin Peroxidase

Molasses was added to the low nitrogen growth medium described in section 3.2.1 (containing 1% glucose), to give final concentrations of 0.2%; 0.4%; 0.6%; 0.8%; 1.0%; 1.5% and 2% (w/v). These media were then used for the production of
lignin peroxidase under shaken conditions as described in section 3.2.2.

4.2.5 The Effect of Size of Inoculum on the Production of Lignin Peroxidase.

Inoculum solution was prepared for large scale production as described in section 3.2.3. This solution was then added to 1 litre conical flasks containing 250 ml of the growth medium described in section 3.2.1, where the carbon source was 1% glucose plus 0.6% molasses to give a final concentration of 10^4 - 10^6 spores in the range of 10 to 10^6 per ml. Lignin peroxidase production was followed, and activity assayed as described in chapter 2 (section 2.3).

4.2.6 Decolorization Studies

The decolorization of molasses' pigments during lignin peroxidase appearance was followed by changes in the spectra (250 nm to 500 nm).

In order to detect degradation of the pigments, gel filtration chromatography on a column of Sephadex G-25 (2.5 x 60 cm) was carried out. The column was equilibrated with 0.01 M phosphate buffer pH 6.0 and eluted with the same buffer. The eluent was collected (5 ml per tube) and the absorbance at various wavelengths was determined. The sample contained molasses 0.6% (from molasses plus glucose medium), which was decolorized by the lignin peroxidase produced in the culture. The controls were from a medium containing 0.6% molasses only, and a medium containing 0.6% molasses plus glucose but without oxygenation being carried out. Both controls and sample were
inoculated and incubated in the same way as is described in chapter 3 (sections 3.2.3.2 and 3.2.2).

4.2.7 Production of Lignin Peroxidase Under Agitation in a Medium Containing Xylan.

A medium containing 0.5% xylan plus 0.5% glucose as the carbon source was used for the production of lignin peroxidase under agitation conditions.

A 2% (w/v) xylan solution was prepared by boiling xylan in water for 30 minutes with constant agitation. This solution was sterilized at 121°C for 10 minutes and added to the growth medium described in section 3.2.1 to give a final concentration of 0.5%.

4.2.8 Effect of Temperature on Lignin Peroxidase Stability

Enzyme solution was incubated at the following temperatures, 30°C, 40°C, 50°C and 60°C. Samples were taken every 15 minutes during incubation, cooled to room temperature and lignin peroxidase activity assayed as described in chapter 2 (section 2.3). The enzyme solution was also left at room temperature for a period of six days. Activity was measured every day and expressed as residual activity.
4.3 Results and Discussion

4.3.1 Time Course for Lignin Peroxidase Production

The ligninolytic system of \textit{P. chrysosporium} is part of the secondary metabolism of the fungi and throughout these experiments it has been triggered by nitrogen starvation. Primary growth ceased by the third day and lignin peroxidase activity appeared after a lag phase, the length of which depended on the carbon source added to the growth medium, and the conditions of growth (stationary or shaken).

Figure 4.1 shows the time course for the production of lignin peroxidase by the fungus grown on 1\% glucose on 1\% glucose plus 0.6\% molasses as the carbon sources and under shaken conditions. As can be seen, when molasses was added lignin peroxidase production started earlier (24 hours), than in glucose-only medium. Maximum activity for molasses plus glucose medium was about 60\% higher than for glucose only medium. It is clear that the presence of molasses promoted an enhancement in lignin peroxidase production. However, the mechanism through which molasses stimulate lignin peroxidase production is not clearly understood. Elevated levels of lignin peroxidase and, in some cases, of extracellular hydrogen peroxide production have been detected in cultures where veratryl alcohol or other lignin related compounds had been added (Faison et al, 1986; Kirk & Farrell, 1987 and Tonon & Odier, 1988). The possible role of veratryl alcohol in lignin degradation by \textit{P. chrysosporium} is still unclear (Tonon & Odier, 1988). It might is possible that a similarity exists in the mechanism of "induction" of lignin peroxidase production by
Figure 4.1 Time Course for the Production of Lignin Peroxidase Activity

A: Carbon Source - 1% Glucose (Shaken Conditions)
B: Carbon Source - 1% Glucose + 0.6% Molasses (Shaken Conditions)
veratryl alcohol and molasses.

Lignin peroxidase production in 1% glucose under shaken and stationary conditions is shown in figure 4.2. Lignin peroxidase production was lower under shaken conditions and a sharp decrease in activity was observed after the peak of maximum activity on the 7th day. On the other hand, under stationary conditions maximum activity was observed from the 7th to the 8th day and there was a slower decrease in activity towards the 9th day. It could be concluded that if no additives, such as veratryl alcohol, molasses or lignin related compounds are added to the growth medium, stationary growth provides the best conditions for the production of lignin peroxidase.

The total lignin peroxidase activity appearing in 100 ml flasks containing 10 ml of growth medium having 1% glucose as carbon source is shown in figure 4.3. This compares the activity in cultures that were subjected to oxygenation on the third day, with cultures that did not receive any oxygen and were kept closed with cotton stoppers. Under oxygenated conditions lignin peroxidase activity appeared 48 hours earlier than without oxygenation and maximum activity was about 60% higher than the maximum activity observed under non oxygenated conditions. As can be seen, oxygenation is important for the establishment and production of lignin peroxidase. It is worth mentioning here that flasks which were stoppered with rubber bungs, providing an air tight system, and which did not receive any oxygenation, did not exhibit any lignin peroxidase activity. Cotton stoppers might have allowed some exchange
Figure 4.2 Time Course for the Production of Lignin Peroxidase Activity

A : Carbon Source - 1% Glucose (Stationary Conditions)
B : Carbon Source - 1% Glucose (Shaken Conditions)
Figure 4.3 Time Course of the Total Lignin Peroxidase Activity in 10 ml Medium - 100 ml Flask (Stationary Conditions)

A : Culture With Oxygenation on the 3rd Day
   Carbon Source - 1% Glucose

B : Culture Without Oxygenation on the 3rd Day
   Carbon Source - 1% Glucose
with oxygen in the air and hence promoted the appearance of some lignin peroxidase activity. This is in agreement with the earlier observation that molecular oxygen can be crucial in determining the rate of lignin degradation by P. chrysosporium (Bar-Lev & Kirk, 1981 and Leisola et al, 1983a). Increasing the oxygen levels in the medium leads to an increase in the titre of the ligninolytic system and the hydrogen-producing systems (Faison & Kirk, 1983 and Faison & Kirk, 1985). Hydrogen production was not determined in these experiments, but it would be expected that those flasks which received no oxygen would have produced very little or no hydrogen peroxide.

4.3.2 Kinetics Studies

Figures 4.4 and 4.5 show the Km values for hydrogen peroxide and veratryl alcohol respectively. The Km for hydrogen peroxide is 48.8 uM and for veratryl alcohol is 75.2 uM. This compares well with the figures published by Tien et al (1984), where values of Km for hydrogen peroxide and veratryl alcohol observed were 29 uM and 72 uM respectively. Tien et al (1984), suggested that a mechanism in which hydrogen peroxide reacts with the enzyme to form an intermediate that subsequently reacts with the veratryl alcohol to return the enzyme to the resting state might occur.
Figure 4.4 Lineweaver-Burk Plot of Hydrogen Peroxide and Lignin Peroxidase

Line of best fit - computed generated
Figure 4.5 Lineeweaver-Burk Plot of Veratryl Alcohol and Lignin Peroxidase

Line of best fit - computer generated
4.3.3 The Effect of Sugar Cane Molasses on Lignin Peroxidase Activity

Table 1 shows the activity of lignin peroxidase when *P. chrysosporium* was grown under shaken conditions in medium containing 1% glucose only, 1% glucose plus 0.6% molasses and 1% glucose plus 0.6% dialysed molasses.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TIME (DAYS OF CULTURE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>1% GLUCOSE ONLY</td>
<td>0.86</td>
</tr>
<tr>
<td>1% GLUCOSE + 0.6% MOLASSES</td>
<td>4.90</td>
</tr>
<tr>
<td>1% GLUCOSE + 0.6% DIALYSED MOLASSES</td>
<td>1.35</td>
</tr>
</tbody>
</table>

It can be seen that the highest yields of lignin peroxidase were obtained in glucose plus molasses medium and also that the maximum lignin peroxidase activity appeared in this medium earlier than in glucose only medium or glucose plus dialysed molasses. Maximum activity in glucose-only medium and glucose plus dialysed molasses was observed at the same time of growth. However, lignin peroxidase activity in dialysed
molasses plus glucose medium was about 40% higher than the activity observed in glucose-only medium.

The main non-sugar components of liquid sugars from cane molasses have been identified as phenolic and phenylpropane-triolic glucosides coming from the lignin material of cane during juice extraction and processing (Palla, 1983). The presence of such lignin derived compounds might be responsible for the enhancement and earlier appearance of lignin peroxidase activity and also for the stimulation of its production under mild agitation conditions in medium containing molasses. It is interesting to note that dialysis of the molasses resulted in a delay in the peak of lignin peroxidase activity, perhaps due to the loss of some low molecular weight substances.

The effect of molasses concentration on the activity of lignin peroxidase during large scale production is shown in figure 4.6. As can be seen, good yields of lignin peroxidase were obtained using molasses concentrations of 0.4-0.6%. Concentrations higher than 0.6% had a negative effect on lignin peroxidase production. It might be possible that the enhancement or induction of lignin peroxidase activity by molasses' components is related to some of the initial steps of the ligninolytic system and that different stages in the overall ligninolytic system may be subject to induction by different concentrations of "inducer".

4.3.4 Decolorization of Molasses Pigments

The brown color of the molasses pigments started to disappear at the same time as the appearance of lignin
Figure 4.6  The Effect of Molasses Concentration on Lignin Peroxidase Activity (Shaken Conditions)
peroxidase. This colour removal was proportional to the enzyme activity, which means that decolorization was maximum when lignin peroxidase activity was maximum. Conditions that avoided lignin peroxidase production, such as no addition of oxygen on the third day of growth and also growth on molasses only as the carbon source, also prevented the decolorization.

Unfortunately, it was not possible to determine whether there was a breakdown of the pigment, when gel filtration on Sephadex G-25 was carried out. A resin with a smaller pore size or another method of analysis may resolve this issue. Palla (1982), used fractionation on Sephadex G-10 and also gas chromatography and mass spectrometry to analyse the coloured components of molasses.

Figures 4.7 and 4.8 show the spectra for decolorized 0.6% molasses and dialysed molasses with the respective controls. As can be seen, samples which exhibited high lignin peroxidase activity and were highly decolorized showed a much lower absorbance than the respective control. A shift in the maximum absorbance wavelength was also observed. In the case of 0.6% molasses, the control had maximum absorbance at wavelength 305-325 nm; a sample with very poor lignin peroxidase activity and consequently, very poor decolorization, showed maximum absorbance at about 310 nm. Two samples which exhibited high lignin peroxidase and high decolorization showed maximum absorbance at 295-300 nm. In the case of dialysed molasses, maximum absorbance for the control was at 245 nm and for the decolorized sample was at 235 nm. It is interesting to note the difference in maximum absorbance wavelengths for dialysed
Figure 4.7 The Spectrum of Molasses' Pigments

A: Control (Sample without lignin peroxidase activity)
B: Sample Showing Poor Lignin Peroxidase Activity (< 10%)
C: Lignin Peroxidase Activity = 100%
D: Lignin Peroxidase Activity = 70% of sample C

The pigments were decolorized during lignin peroxidase production under shaken conditions.
Figure 4.8 The Spectrum of Dialyzed Molasses

A: Control

B: Lignin Peroxidase Activity (sample) = 5.60 umol/ml/min
Figure 4.9 The Differential Spectra of Molasses' Pigments

A : Absorbance(control) - Absorbance(sample)
Lignin Peroxidase Activity (sample) = 70% of sample B

B : Absorbance(control) - Absorbance(sample)
Lignin Peroxidase Activity(sample) = 100%

C : Absorbance(control) - Absorbance(sample)
Sample showing very poor lignin peroxidase activity
molasses and non-dialysed molasses. It possibly indicates that some of the compounds responsible for stimulation of lignin peroxidase have maximum absorbance at wavelengths higher than 300 nm and they were lost through dialysis of the molasses.

The differential spectrum for 0.6% decolorized molasses and the control is shown in figure 4.9. The differential spectrum was obtained by calculating: (Absorbance of control - Absorbance of the samples) at various wavelengths. The various degrees of decolorization, which are dependent on the levels of lignin peroxidase activity in each sample, can be observed.

4.3.5 Effect of the Size of Inoculum

The variation of lignin peroxidase activity with the size of inoculum is shown in figure 4.10. There was not much variation in activity for concentrations of $10^4$ and $10^5$ spores/ml. But $10^6$ spores/ml exhibited the highest levels of lignin peroxidase activity. When a high concentration of $10^8$ inoculum was used (higher than $10^6$), no lignin peroxidase was produced. Variation of lignin peroxidase dependent on the size of inoculum used and might be related to the time required to reach nutrient limitation as well as with oxygen concentration. Brown & Zainudeen (1978), have reported that in experiments carried out with Trichoderma reesei in batch culture, the time required to reach nutrient limitation was reduced with the increase in the size of inoculum and at high inoculum concentrations the oxygen uptake was dictated by the oxygen supply rate. It could be that when high inoculum sizes were used here, the concentration of oxygen was too low to allow
Figure 4.10 Variation of Lignin Peroxidase Activity With Size of Inoculum (Shaken Conditions)
lignin peroxidase production.

4.3.6 Use of Cellobiose, Lactose and Xylan as Carbon Source for Ligninase Production

When cellobiose, lactose, xylan only or xylan plus glucose were used as carbon sources for lignin peroxidase production under stationary conditions, low yields of enzyme were obtained (as shown in Table 2). The results indicate that the carbon source might be an important factor in the production of lignin peroxidase. It could be possible that when di- and polysaccharides are used, a state of slow feeding is established, i.e., these saccharides are metabolized at slower rates than glucose, resulting in a system where all the conditions necessary for the production of higher yields of lignin peroxidase might not be present.

TABLE 2 Production of Lignin Peroxidase in Medium Containing Lactose, Cellobiose or Xylan as Carbon Source.

<table>
<thead>
<tr>
<th>CARBON SOURCE</th>
<th>TIME (DAYS OF CULTURE)</th>
<th>ACTIVITY (umol/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>LACTOSE</td>
<td>NONE</td>
<td>NONE</td>
</tr>
<tr>
<td>CELLOBIOSE</td>
<td>0.80</td>
<td>2.70</td>
</tr>
<tr>
<td>XYLAN ONLY</td>
<td>NONE</td>
<td>1.57</td>
</tr>
<tr>
<td>XYLAN + GLUCOSE</td>
<td>0.31</td>
<td>2.75</td>
</tr>
</tbody>
</table>
Figure 4.11 Lignin Peroxidase Activity With Time of Culture:
Carbon Source - 0.5% Xylan + 0.5% Glucose (Shaken Conditions)
When a combination of 0.5% xylan and 0.5% glucose was used as the carbon source under agitation, the yield of lignin peroxidase was high, with a peak of activity on the sixth day of culture, as shown in figure 4.11. Here, better oxygenation conditions due to agitation and the presence of xylan might be the reasons for the lignin peroxidase activity obtained.

4.3.7 Effect of Temperature

After six days at room temperature the residual activity for lignin peroxidase was about 70% of its original activity. This indicates that the enzyme is stable and might not contain high amounts of proteolytic activity. These are qualities that make possible its application to long bioprocesses.

One hour of incubation at 40°C did not affect the activity of the enzyme. However, after 1 hour at 50°C or 1 hour at 60°C the remaining activities were 74% and 70% respectively.

The thermal stability of lignin peroxidase is an important factor when considering bioprocessing applications, because high thermal stability is a desirable feature of useful commercial enzymes.

4.4 Summary

Lignin peroxidase has been produced under stationary and shaken conditions using various sources of carbon. After primary growth had ceased and oxygenation was carried out (3rd day of growth) there was a lag phase, the duration of which depended on the culture parameters, and beyond this lignin peroxidase activity appeared.
Oxygenation was found to be important and non-oxygenated cultures which were stoppered with cotton plugs, allowing some air exchange to occur, had only about 40% of the activity of oxygenated cultures. Activity in non-oxygenated culture started 48 hours later than in oxygenated cultures.

The addition of molasses to the growth medium for lignin peroxidase production under shaken conditions promoted an enhancement in lignin peroxidase activity and an earlier peak of activity. Dialysis of the molasses resulted in a delay of the peak of activity. Lignin derived compounds present in the molasses might be responsible for the enhancement of lignin peroxidase production under shaken conditions. Decolorization of the molasses pigment occurred simultaneously with lignin peroxidase appearance and the degree of decolorization was proportional to the activity of the enzyme. Conditions that suppressed lignin peroxidase production, such as no oxygenation and no addition of glucose, also prevented decolorization.

The use of di- and polysaccharides as carbon sources, under stationary conditions led to lower levels of lignin peroxidase production. These saccharides might have been assimilated at lower rates than glucose and might not have provided the establishment of all the necessary conditions for production of high yields of lignin peroxidase.

The size of inoculum was shown to affect lignin peroxidase levels and a concentration of 10 spores/ml produced the highest yields of lignin peroxidase.

The Km for hydrogen peroxide and for veratryl alcohol have been determined and are 48.8 uM and 75.2 uM, respectively.
Lignin peroxidase was shown to be thermostable, losing only 30% of its activity after one hour of incubation at 60 °C and after been kept at room temperature for six days.
CHAPTER 5

Immobilization Studies
5 IMMobilization Studies

5.1 Introduction

Immobilization is the process where biocatalysts, (enzymes or cells), are bound to carriers or to each other. Alternatively, they may be physically confined in a defined volume by entrapment or encapsulation. The cost of enzyme production and their use in biotechnology makes the application of repeated or continuous operation desirable in most cases. It is generally impractical to reuse suspensions of biocatalysts because their recovery from the reaction mixture is difficult and uneconomic. Immobilization allows both reuse and re-application of enzymes or organelles in continuous processes. However, the future increase in use of immobilized biocatalysts will be dictated by the selection of a suitable support or carrier, if the process is sufficiently cheap and convenient to use on a large scale. In this chapter the immobilization of lignin peroxidase and P. chrysosporium is examined with a view to their application in an industrial process.

5.2 Methods

5.2.1 Immobilization of Lignin Peroxidase on Alginate Gel

Lignin peroxidase was first immobilized in calcium alginate (method as described by Azari & Wiseman, 1982). 5 ml of the enzyme solution were mixed with 15 ml of 1% sodium alginate and the total solution was slowly extruded into a 0.05 M CaCl₂ solution containing 10% glucose using, a 5 ml syringe fitted with a needle. The beads formed were separated from the solution by filtration. The assay for lignin
peroxidase was carried out as described in section 2.3, after which the beads were washed and stored in the calcium chloride solution at 4°C.

Lignin peroxidase was also immobilized in calcium alginate using the method described by Kierstan and Bucke (1977), where equal amounts of enzyme solution and 4% sodium alginate were mixed. The mixture was extruded dropwise via a 10 ml syringe fitted with needle into 0.2 M calcium chloride solution (ice cold), and left to harden for a period of one hour at 4°C. Lignin peroxidase was assayed as described in section 2.3, and the beads were washed and stored in the calcium chloride solution at 4°C.

In both cases air bubbles were removed by vacuum suction, before the mixture was extruded into the respective calcium chloride solutions.

5.2.2 Immobilization of Lignin Peroxidase in AffiGel (BioRad Ltd)

The gel was transferred to a Buchner funnel and washed with three volumes of cold deionised water. 5.0 ml of affigel was mixed with 5.0 ml of enzyme solution containing calcium chloride to give a final concentration of 80 mM. Gentle agitation was carried out for a period of one hour at room temperature. A column using a 10 ml pipette was then set up. A solution containing veratryl alcohol plus hydrogen peroxide was run through the column at a rate of 1.0 ml/10 minute and the increase in absorbance at 310 nm was monitored. The column was washed with solution containing 80 mM calcium chloride and stored at 4°C.
5.2.3 Immobilization of *P. chrysosporium* in Sodium Alginate

The microorganism was prepared as described in chapter 3 section 3.2.3.1 and after being washed in sterile distilled water it was transferred to a mortar where it was ground with a pestle to give an homogeneous suspension. This suspension was immobilized in sterile calcium alginate following the procedure of Kierstan & Bucke (1977) described in section 5.2.2. The mixture was extruded into the cold sterile calcium chloride solution with a 5.0 ml syringe without needle. The beads formed were stored overnight at 4 °C in the calcium chloride solution, and used to inoculate both the 100 ml flasks (stationary conditions) and the 1.0 litre flasks (shaken conditions).

5.2.4 Immobilization of *P. chrysosporium* in Polyurethane Foam

Spores of *P. chrysosporium* were grown and prepared as described in chapter 3 section 3.2.3.2 and immobilized in polyurethane foam as described by Palmer (1987, personal communication).

Foam cubes of length 0.5 cm to 0.8 cm were washed several times with distilled water, autoclaved for 20 min, washed and pressed to remove the water and sterilised for 20 min.

1 litre conical flasks containing 250 ml of the growth medium described in section 3.2.1, and 1% glucose plus 0.6% molasses as the carbon source, were inoculated with spore suspension followed by the addition of 50 to 60 foam cubes. The flasks were incubated at 140 rpm for 70 hours. After this period, 150 ml of medium were poured off, the flasks sealed and oxygen was flushed in as described in section 3.2.5.2.
Incubation was then continued at 70 rpm and 40 °C. The appearance of lignin peroxidase was followed by visual observation of colour disappearance and also by the hydrogen peroxide-dependent oxidation of veratryl alcohol as described in chapter 2 (section 2.3).

5.2.5 Reutilization of the Foam Immobilized Microorganism

Following the protocol described above, after lignin peroxidase activity declined (7th or 8th day of culturing), the foam cubes containing spores were allowed to settle and the liquid medium was poured off. Some of the flasks were stored at 4 °C for a period up to three month while others were re-used straight away for the production of lignin peroxidase. The flasks stored at 4 °C were reactivated every 30 days. During the reactivation process 100 ml of growth medium containing 0.6% molasses and 20% of the original glucose concentration were added to each flask, which were sealed and oxygen was flushed in for 10 min. as described in section 3.2.5.2. Lignin peroxidase was again followed as described above. As a control, free cells were also reactivated as described above and lignin peroxidase assayed as before.

5.2.6 Column with Foam Immobilized Microorganism

The microorganism was immobilized and lignin peroxidase followed as described in section 5.2.4. After the peak of lignin peroxidase activity was reached the microorganism was transferred to a column (4.5 cm x 60 cm), that had been previously sterilized for 30 minutes at 121 °C. The column was filled with foam immobilized microorganism (column up to 30 cm
length) and the top was closed with a rubber bung which had two Pasteur pipettes passing through it. These pipettes were either used for the addition of medium to the column or for oxygenation. Addition of medium was followed by 10 to 15 minutes of oxygenation. About 60 ml of effluent was collected per hour and lignin peroxidase assayed as described in section 2.3.

5.3 Results and Discussion

When the enzyme solution was immobilized either in alginate or affigel no lignin peroxidase was present after overnight storage at 4 °C. In the case of alginate, the low pH of the reaction (pH=3.0) and perhaps the presence of hydrogen peroxide provoked breakage of the beads. This would explain the presence of lignin peroxidase when the beads were first used. The enzyme was then washed away before the beads were stored at 4 °C, exhibiting no lignin peroxidase when assayed again.

The affigel used in the experiment was affigel 10 which according to the manufacturer's catalogue (BioRad) is recommended for neutral or basic proteins whereas for acidic proteins (such as lignin peroxidase) affigel 15 is recommended. Even though the gel was treated with 80 mM CaCl2, as recommended for coupling acidic protein to affigel 10, no binding seems to have occurred. When the affigel-enzyme column was first used lignin peroxidase was observed, as assayed according to the method described in section 2.3. However, after the first use and overnight storage at 4 °C, no lignin peroxidase activity was detected. Here again the enzyme might
have been washed out after the first use. Had the performance of affigel been better, its high cost would probably represent a drawback on its industrial application.

Leakage of the fungus suspension was also observed when the microorganism itself was immobilized in calcium alginate. After inoculation the microorganism grew, forming a mat when incubated under non-shaken conditions and forming clumps under shaken conditions. It might be possible that the microorganism was able to utilize the alginate for growth, to some extent and/or able to produce enzymes that promote the breakdown of this support. Lignin peroxidase activity was not assayed.

The microorganism immobilized in polyurethane foam gave good yields of lignin peroxidase. Moreover, clumping formation during growth was avoided and the variation in activity between flasks was reduced. Lignin peroxidase production by free and foam immobilized shaken culture in a medium containing 0.6% molasses plus 1% glucose as the carbon source is shown in figure 5.1. When free cells were used, pellet size was quite variable with pellets aggregating to form clumps. However, entrapment of the fungus within the cubes of foam, led to a control of pellet size by the physical properties of the foam itself. Restriction of growth and clump formation by the foam matrix might also promote the development of a culture that is physically less heterogeneous and where oxygen might have been more evenly distributed than in the free cells culture. These factors would explain the smaller discrepancy in lignin peroxidase activity among flasks when the microorganism was foam immobilized.
Figure 5.1 Production of Lignin Peroxidase by Free and Immobilized Cells of P. chrysosporium (Shaken Conditions)

A : Immobilized Cells
B : Free Cells
Reactivation of the foam immobilized microorganism was possible and allowed the repeated production of high activities of lignin peroxidase. Because the microorganism was restricted to the foam, handling was much simpler than in the case of free cells. The difficulty in separating cells and growth medium made successive reactivations of the free cells quite impossible. Free cells showed good yields of lignin peroxidase only when they were first reactivated. After that, reactivation of the free cells led to much lower levels of lignin peroxidase activity. Breakage and loss of some cells, and disturbance of the clumps formed, might be the reasons for the decline in activity. On the other hand, foam immobilized cells allowed reactivation to take place three or four times before lignin peroxidase activity started to decline. The production of lignin peroxidase by reactivated foam immobilized microorganism and by reactivated free cells is shown in figure 5.2 (first reactivation). The foam immobilized microorganism reactivated for the second time showed the same level of lignin peroxidase activity as the first time reactivated one. Second time reactivated free cells showed less than 50% of the activity of the first time reactivated cells. The stored (4°C) immobilized fungus also showed good levels of activity after being reutilized three times (levels of activity similar to those reactivated without previous storage). Reactivation of the immobilized microorganism offers the possibility of scaling-up the process further.
Figure 5.2 Production of Lignin Peroxidase by Reactivated Free and Immobilized Cells of *P. chrysosporium*

A : Free Cells
B : Immobilized Cells
The column containing the foam immobilized microorganism gave very low yields of lignin peroxidase. Compaction and consequently poor oxygenation might have been the main problems for low activity in this case. The use of a column where recirculation of the medium and equal distribution of oxygen is made possible might allow better production of the enzyme.

5.4 Summary

Lignin peroxidase has been immobilized in calcium alginate gel and in affigel. In both cases, successful immobilization has not been achieved. The low pH of the reaction and the presence of hydrogen peroxide might have provoked the breakage of the gel, liberating the enzyme, which was then lost through the washing of the gel beads after the first use. In the case of affigel 10, proper binding of the protein to the gel might not have occurred.

Immobilization of P. chrysosporium in calcium alginate led to leakage of the microorganism suspension resulting in a system that exhibited the same behaviour as a non-immobilized system.

Immobilization of P. chrysosporium in cubes of polyurethane foam had a number of advantages over the use of free cells. Uniformity of pellet size was controlled by the physical nature of the foam matrix and clumping was avoided. This resulted in a high reduction of the variation in lignin peroxidase among flasks which is observed when free cells are used. Reutilization of the foam immobilized microorganism was successfully accomplished.
Chapter 6
Lignocellulolytic Enzymes
LIGNOCELLULOLYTIC ENZYMES

6.1 Introduction

The potential for the use of lignocellulosic materials in bioconversion processes is well recognised (Lamptey, 1986). The fact that there are virtually no commercial processes based on the large scale use of lignocelluloses for the production of fuels and food, reflects the uneconomic state of such technologies. If, however, the production rate and yield of fermentable sugars from lignocellulosics could be increased economically, their use for the manufacture of various fermentation products would become more attractive.

The detection and characterization of the enzyme systems involved in lignocellulose degradation by basidiomycete fungi may allow the application of various techniques to improve the use of these fungi, and/or enzymes produced by them as bioconversion agents.

Lignocellulosic materials are composed of cellulose (a linear polymer of glucose linked in the B-1,4 configuration); hemicellulose or xylan (a linear polymer of xylose with side chains of glucose, mannose, glucuronic acid and arabinose) and lignin, which is a polymer of phenylpropane units (Kirk, 1983a and Brown, 1985). Once the lignin barrier is removed or broken down by ligninolytic enzymes or by the action of microorganisms, the cellulose and hemicellulose components are exposed and their utilization will depend on the action of various cellulases and hemicellulases (Lewis, 1983; Kirk, 1983a and Leisola & Fiechter, 1985).
In this chapter the investigation of the presence of cellulases and hemicellulases (xylanases) in the low nitrogen medium for lignin peroxidase production is described. Various carbon sources were used and the extracellular and intracellular appearance of lignin peroxidases, cellulases and xylanases was followed.

6.2 Methods
6.2.1 Determination of B-Glucosidase and Cellobiase Activities

O-Nitrophenyl-B-D-glucopyranoside and cellobiose are both substrates for the determination of B-glucosidase activity (Coughlan, 1985).

In order to make it simpler, in this chapter B-glucosidase will refer to the activity of the enzyme towards the substrate o-Nitrophenyl-B-D-glucopyranoside (ONPG). Cellobiase will refer to the activity of the enzyme towards the substrate cellobiose.

B-Glucosidase activity was determined according to the method of Evans (1985), by incubating 0.4 ml of enzyme solution (culture supernatant) and 1.0 ml of substrate (5 mM ONPG) in 50 mM sodium acetate buffer, pH 5.0 for 15 minutes at 50 °C. The reaction was stopped by the addition of 2 ml of 1 M sodium bicarbonate and the release of ONP (o-nitrophenol) was measured spectrophotometrically at 405 nm. Activity was expressed as umoles of ONP produced per minute per ml of enzyme solution for the extracellular enzyme. Intracellular B-glucosidase activity was expressed as umoles of ONP produced per minute per mg of dry weight of microorganism.

Cellobiase activity was determined according to Evans
(1985), by incubating 0.4 ml of enzyme solution and 1.0 ml of 1% cellobiose solution in 50 mM sodium acetate buffer at pH 5.0 for 15 minutes at °50 C. The reducing sugars produced were measured, after appropriate dilution, using the Glucose GOD-Perid test, as described in chapter 2. Activity was expressed as umoles of reducing sugar produced per ml per minute of culture supernatant for the extracellular enzyme. Intracellular activity was expressed as umoles of reducing sugar produced per minute per mg of dry weight of microorganism.

6.2.2 Determination of Filter Paper Hydrolysis Ability

Filter paper activity (Mandels et al, 1976), was measured by the release of reducing sugar from a mixture of 0.4 ml of enzyme solution (culture supernatant), 1 ml of 50 mM acetate buffer at pH 5.0 after incubation at 50°C for 60 minutes. Reducing sugar and activity were determined as described for cellobiase activity.

6.2.3 Determination of Xylanase Activity

Xylanase activity was determined according to the method of Yu et al (1987), by estimating the reducing sugar liberated from 1 ml of 1% oat spelts xylan solution in 50 mM acetate buffer at pH 5.0, incubated with 0.4 ml enzyme solution at 50°C for 30 minutes. The reducing sugar released and xylanase activity were determined and expressed as described above for cellobiase activity.
6.2.4 SDS-PAGE Electrophoresis

SDS-PAGE of the intracellular proteins was carried out as described in chapter 2, section 2.6.

6.2.5 Enzyme Source

Extracellular and intracellular enzymes were obtained as described in chapter 2, section 2.1.

6.3 Results

6.3.1 Cellulolytic Enzymes in Stationary Cultures of P. chrysosporium Using Various Carbon Sources

Table 1 and Table 2 show the cellulase enzymes produced in 1% glucose as the carbon source.

Extracellular xylanase was only detected on the 6th day of growth whereas the intracellular xylanase was present on the 3rd day. The intracellular activity was very low on the 5th day and an increase was observed on the 6th day, suggesting perhaps the appearance of a peak of activity later on. Extracellular and Intracellular cellobiase showed a similar behaviour to xylanase. There was a decrease of activity on the 5th day when compared to the activity on the 3rd day, followed by an increase on the 6th day, which was more accentuated for the extracellular cellobiase. Extracellular and Intracellular filter paper activity exhibited approximately the same behaviour. Activity peaked on the 3rd day and then decreased towards the 6th day. Extracellular B-glucosidase (ONPG) activity showed a maximum on the 3rd day which decreased towards the 5th and 6th day. Intracellular B-glucosidase showed a peak on the 3rd day and a peak on the 6th day. The
Table 1  *P. chrysosporium* grown in 1% glucose in tartrate buffer (stationary conditions)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>NONE</td>
<td>0.85</td>
<td>1.82</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>NONE</td>
<td>0.52</td>
<td>1.66</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>0.43</td>
<td>1.15</td>
<td>0.70</td>
<td>2.2</td>
</tr>
</tbody>
</table>

TABLE 2  *P. chrysosporium* grown 1% glucose in tartrate buffer (stationary conditions)

Intracellular Enzyme Activity (umoles/min/mg dry weight)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.062</td>
<td>0.090</td>
<td>0.053</td>
<td>0.930</td>
</tr>
<tr>
<td>5</td>
<td>0.003</td>
<td>0.015</td>
<td>0.045</td>
<td>0.490</td>
</tr>
<tr>
<td>6</td>
<td>0.050</td>
<td>0.074</td>
<td>0.052</td>
<td>0.920</td>
</tr>
</tbody>
</table>
Figure 6.1 Total Lignocellulases for \textit{P. chrysosporium} grown in 1% Glucose - Stationary Conditions

1 : Extracellular Xylanase Activity (umoles/min/100 ml flask)
2 : Intracellular Xylanase Activity (umoles/min/100 ml flask)
3 : Extracellular Cellobiase Activity (umoles/min/100 ml flask)
4 : Intracellular Cellobiase Activity (umoles/min/100 ml flask)
5 : Extracellular Filter Paper Activity (umoles/min/100 ml flask)
6 : Intracellular Filter Paper Activity (umoles/min/100 ml flask)
Figure 6.2 Total Lignocellulases for *P. chrysosporium* grown in 1% Glucose - Stationary Conditions

7 : Extracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

8 : Intracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

9 : Lignin Peroxidase Activity (umoles/min/100 ml flask)
intracellular activity on the 5th day was much smaller than the activity on the 3rd and 6th day.

Figures 6.1 and 6.2 show the total intracellular and extracellular lignocellulases present on days 5 and 6 of growth on 1% glucose. As can be seen, except for B-Glucosidase the levels of the other cellulases are low when compared to lignin peroxidase activity. B-Glucosidase (intracellular) showed the highest levels of activity. Except for extracellular B-glucosidase and extracellular xylanase (5th day), all extracellular cellulases showed higher levels of activity than the respective intracellular enzymes.

Table 3 and Table 4 show the cellulase enzymes produced in 0.5% xylan as carbon source.

Extracellular xylanase was only detected on the 6th day of growth, whereas intracellular xylanase was present on the 3rd, 5th and 6th days. The extracellular xylanase produced on the 6th day had an higher activity than the intracellular xylanase, independent of the time of growth. Extracellular cellobiase was present on the 3rd day and an increase in activity was observed up to the 6th day. On the other hand, intracellular cellobiase activity was also present on the 3rd day, but a decrease in activity was observed up to the 6th day. Extracellular filter paper activity was not present on the 3rd day. Activity was observed on the 5th day which showed an increase on the 6th day. The intracellular filter paper activity showed higher activity on the 3rd and 6th day when compared to the 5th day. Extracellular B-glucosidase was present in the three days
Table 3  *P. chrysosporium* grown in 0.5% xylan (stationary conditions)

**Extracellular Enzyme Activity (umoles/min/ml of culture medium)**

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
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<td>3</td>
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<td>0.36</td>
<td>NONE</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>NONE</td>
<td>1.17</td>
<td>0.11</td>
<td>5.5</td>
</tr>
<tr>
<td>6</td>
<td>0.094</td>
<td>1.5</td>
<td>0.25</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Table 4  *P. chrysosporium* grown in 0.5% xylan (stationary conditions)

**Intracellular Enzyme Activity (umoles/min/mg dry weight)**

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.023</td>
<td>0.092</td>
<td>0.023</td>
<td>2.66</td>
</tr>
<tr>
<td>5</td>
<td>0.011</td>
<td>0.078</td>
<td>0.010</td>
<td>1.90</td>
</tr>
<tr>
<td>6</td>
<td>0.028</td>
<td>0.069</td>
<td>0.028</td>
<td>1.82</td>
</tr>
</tbody>
</table>
Figure 6.3 Total Lignocellulases for *P. chrysosporium* grown in 0.5% Xylan - Stationary Conditions

1: Extracellular Xylanase Activity (umoles/min/100 ml flask)
2: Intracellular Xylanase Activity (umoles/min/100 ml flask)
3: Extracellular Cellobiase Activity (umoles/min/100 ml flask)
4: Intracellular Cellobiase Activity (umoles/min/100 ml flask)
5: Extracellular Filter Paper Activity (umoles/min/100ml flask)
6: Intracellular Filter Paper Activity (umoles/min/100ml flask)
Figure 6.4 Total Lignocellulases for *P. chrysosporium* grown in 0.5% Xylan - Stationary Conditions

7: Extracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

8: Intracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

9: Lignin Peroxidase Activity (umoles/min/100 ml flask)
assayed and showed a peak of activity on the 5th day. Intracellular B-glucosidase showed maximum activity on the 3rd day with less towards the 5th and 6th day.

Figures 6.3 and 6.4 show the total intracellular plus extracellular lignocellulases present on days 5 and 6 of growth on 0.5% xylan. As can be seen extracellular and intracellular B-glucosidase showed the highest levels of activity, which were higher than lignin peroxidase itself. Extracellular cellobiase also showed a high level of activity when compared to lignin peroxidase, but this was less than 50% of the B-glucosidase activity.

Table 5 and Table 6 show the cellulase enzymes produced in 0.5% xylan plus 0.5% glucose as carbon source.

Extracellular xylanase was present on the 3rd day, showed a peak of activity on the 5th day and a decrease of activity on the 6th day. Intracellular xylanase activity was higher on the 3rd day and decreased towards the 5th and 6th day. Extracellular cellobiase activity was observed on the 3rd, 5th and 6th days of growth. Maximum activity was observed on the 5th day. Intracellular cellobiase showed higher activity on the 3rd day. The same levels of activity were observed on the 5th and 6th days, which were lower than the activity on the 3rd day. Extracellular filter paper activity was observed on the 3rd day and there was a slight increase in activity towards the 5th and 6th days. Intracellular filter paper activity was fairly constant throughout the 3rd, 5th and 6th days. Extracellular B-glucosidase showed a peak of activity on the
Table 5  *P. chrysosporium* grown in 0.5% xylan plus 0.5% glucose (stationary conditions)

Extracellular Enzyme Activity (umoles/min/ml of culture medium)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.42</td>
<td>0.30</td>
<td>0.053</td>
<td>2.75</td>
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<tr>
<td>5</td>
<td>0.13</td>
<td>0.41</td>
<td>0.058</td>
<td>1.81</td>
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<tr>
<td>6</td>
<td>0.07</td>
<td>0.32</td>
<td>0.066</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Table 6  *P. chrysosporium* grown in 0.5% xylan plus 0.5% glucose (stationary culture)

Intracellular Enzyme Activity (umoles/min/mg dry weight)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
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</thead>
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<tr>
<td>3</td>
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<td>0.051</td>
<td>0.015</td>
<td>1.12</td>
</tr>
<tr>
<td>5</td>
<td>0.013</td>
<td>0.036</td>
<td>0.011</td>
<td>0.91</td>
</tr>
<tr>
<td>6</td>
<td>0.010</td>
<td>0.036</td>
<td>0.014</td>
<td>1.13</td>
</tr>
</tbody>
</table>
Figure 6.5 Total Lignocellulases for *P. chrysosporium* grown in 0.5% Xylan + 0.5% Glucose - Stationary Conditions

1 : Extracellular Xylanase Activity (umoles/min/100 ml flask)
2 : Intracellular Xylanase Activity (umoles/min/100 ml flask)
3 : Extracellular Cellobiase Activity (umoles/min/100 ml flask)
4 : Intracellular Cellobiase Activity (umoles/min/100 ml flask)
5 : Extracellular Filter Paper Activity (umoles/min/100 ml flask)
6 : Intracellular Filter Paper Activity (umoles/min/100 ml flask)
Figure 6.6 Total Lignocellulases for *P. chrysosporium* grown in 0.5% Xylan + 0.5% Glucose - Stationary Conditions

7 : Extracellular β-glucosidase Activity (umoles ONP/min/100 ml flask)

8 : Intracellular β-glucosidase Activity (umoles ONP/min/100 ml flask)

9 : Lignin Peroxidase Activity (umoles/min/100 ml flask)
3rd day, which decreased towards the 5th and 6th days. Intracellular B-glucosidase was similar on the 3rd and 5th day but slightly lower on the 6th day.

Figures 6.5 and 6.6 show the total intracellular plus extracellular lignocellulases present on days 5 and 6 of growth in 0.5% xylan plus 0.5% glucose. Intracellular B-glucosidase showed the highest levels of activity, followed by lignin peroxidase and extracellular B-glucosidase and extracellular cellobiase.

When compared to the cellulases produced in 0.5% xylan only, xylanases (intracellular and extracellular) showed higher activity in 0.5% xylan plus 0.5% glucose medium. On the other hand, all others activities were better expressed in 0.5% xylan only medium. However, lignin peroxidase showed higher yields in 0.5% xylan plus 0.5% glucose medium.

Table 7 and Table 8 show the cellulase enzymes produced in 1% cellobiose as carbon source.

Extracellular xylanase activity was observed on the 3rd day of growth, which increased towards the 5th and 6th days. Intracellular xylanase activity was constant during the 3rd, 5th and 6th days and was lower than the extracellular xylanase, regardless of the day. Extracellular cellobiase activity was present on the 3rd day, and showed a peak of activity on the 5th day which remained constant towards the 6th day. Intracellular cellobiase activity was maximum on the 3rd day, and showed a slight decrease towards the 5th and 6th days. Extracellular filter paper activity was observed on the 3rd
Table 7  *P. chrysosporium* grown in 1% cellobiose (stationary conditions)

Extracellular Enzyme Activity (umoles/min/ml of culture medium)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
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<td>0.048</td>
<td>0.19</td>
<td>0.86</td>
</tr>
<tr>
<td>5</td>
<td>0.17</td>
<td>0.11</td>
<td>0.29</td>
<td>0.80</td>
</tr>
<tr>
<td>6</td>
<td>0.34</td>
<td>0.11</td>
<td>0.041</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 8  *P. chrysosporium* grown in 1% cellobiose (stationary conditions)

Intracellular Enzyme Activity (umoles/min/mg dry weight)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.100</td>
<td>0.023</td>
<td>2.520</td>
</tr>
<tr>
<td>5</td>
<td>0.010</td>
<td>0.088</td>
<td>0.025</td>
<td>2.300</td>
</tr>
<tr>
<td>6</td>
<td>0.011</td>
<td>0.082</td>
<td>0.012</td>
<td>2.134</td>
</tr>
</tbody>
</table>
Figure 6.7 Total Lignocellulases for *P. chrysosporium* grown in 1% Cellobiose - Stationary Conditions

1 : Extracellular Xylanase Activity (umoles/min/100 ml flask)
2 : Intracellular Xylanase Activity (umoles/min/100 ml flask)
3 : Extracellular Cellobiase Activity (umoles/min/100 ml flask)
4 : Intracellular Cellobiase Activity (umoles/min/100 ml flask)
5 : Extracellular Filter Paper Activity (umoles/min/100 ml flask)
6 : Intracellular Filter Paper Activity (umoles/min/100 ml flask)
Figure 6.8 Total Lignocellulases for *P. chrysosporium* grown in 1% Cellobiose - Stationary Conditions

7 : Extracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

8 : Intracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

9 : Lignin Peroxidase Activity (umoles/min/100 ml flask)
day, and showed a peak of activity on the 5th day, that which decreased towards the 6th day. Intracellular activity was similar on the 3rd and 5th day and showed a decrease on the 6th day. Extracellular B-glucosidase activity was higher on the 3rd day, and showed a slight decrease towards the 5th and 6th days. Intracellular activity showed similar behaviour but the levels of activity were much higher than the extracellular activity.

Figures 6.7 and 6.8 show the total extracellular and intracellular lignocellulases present on days 5 and 6 of growth in 1% cellobiose. Intracellular B-glucosidase showed the highest levels of activity. Compared to 1% glucose medium, the lignocellulases produced in 1% cellobiose showed lower activity, except for intracellular B-glucosidase.

Table 9 and Table 10 show the cellulase enzymes produced in 1% lactose as the carbon source.

Extracellular xylanase activity was similar on the 3rd and 5th day. Intracellular xylanase activity was not observed on the 3rd day but it was detected on the 5th day. Extracellular cellobiase activity was higher on the 3rd day than on the 5th day. Intracellular cellobiase activity showed similar behaviour. Extracellular filter paper activity was observed on the 3rd day, which showed an increase towards the 5th day. Intracellular activity showed similar behaviour. Extracellular B-glucosidase activity was observed on the third day and showed a slight decrease towards the 5th day. Intracellular B-glucosidase activity was high and showed a sharp decrease towards the 5th day.
Table 9  *P. chrysosporium* grown in 1% lactose (stationary conditions)

Extracellular Enzyme Activity (umoles/min/ml of culture medium)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.091</td>
<td>0.078</td>
<td>0.019</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>0.10</td>
<td>0.033</td>
<td>0.041</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 10  *P. chrysosporium* grown in 1% lactose (stationary conditions)

Intracellular Enzyme Activity (umoles/min/mg dry weight)

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
<th>B-GLUCOSIDASE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
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<td>0.290</td>
<td>0.006</td>
<td>7.36</td>
</tr>
<tr>
<td>5</td>
<td>0.010</td>
<td>0.010</td>
<td>0.018</td>
<td>3.14</td>
</tr>
</tbody>
</table>
Figure 6.9 Total Lignocellulases for *P. chrysosporium* grown in 1% Lactose - Stationary Conditions

![Graph showing total lignocellulases for *P. chrysosporium* grown in 1% lactose under stationary conditions. The graph displays the activity of various enzymes (extracellular and intracellular) over time. The y-axis represents activity (umoles/min/100 ml flask) and the x-axis represents time of culture (DAYS).]

1 : Extracellular Xylanase Activity (umoles/min/100 ml flask)
2 : Intracellular Xylanase Activity (umoles/min/100 ml flask)
3 : Extracellular Cellobiase Activity (umoles/min/100 ml flask)
4 : Intracellular Cellobiase Activity (umoles/min/100 ml flask)
5 : Extracellular Filter Paper Activity (umoles/min/100 ml flask)
6 : Intracellular Filter Paper Activity (umoles/min/100 ml flask)
Figure 6.10 Total Lignocellulases for *P. chrysosporium* grown in 1% Lactose - Stationary Conditions

7: Extracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

8: Intracellular B-glucosidase Activity (umoles ONP/min/100 ml flask)

9: Lignin Peroxidase Activity (umoles/min/100 ml flask)
Figures 6.9 and 6.10 shows the total intracellular and extracellular lignocellulases present on day 5 of growth in 1% lactose. B-glucosidase intracellular showed the highest levels of activity. No lignin peroxidase was observed under these conditions. Perhaps if some glucose had been added to the medium, to serve as a growth substrate, completely different enzyme levels would have been observed.

Overall, except for intracellular B-glucosidase and intracellular cellobiase (produced in 1% cellobiase), all extracellular lignocellulases showed higher levels of activity than the respective intracellular enzymes.

Figures 6.11 and 6.12 show the kinetic constant Km for extracellular and intracellular B-glucosidase derived from Lineweaver-Burk plots of 1/V against 1/[S], using ONPG as substrate.

Intracellular B-glucosidase : Km = 0.25 mM
Extracellular B-glucosidase : Km = 10 mM

Figure 6.13 shows the behaviour of extracellular B-glucosidase activity produced in 1% glucose as carbon source, incubated without agitation and under both, oxygenation and non oxygenation conditions. Up to the 3rd day of growth the behaviour was the same since there was no difference in culture conditions. On the 3rd day, oxygenation was carried out in some flasks; whereas others were kept without oxygenation. Under oxygenated conditions a peak of activity was observed on the 3rd day, after which a decrease in activity was observed up to the 9th day, when a very low increase was observed. Under non
Figure 6.11 Lineweaver-Burk Plot for Extracellular β-Glucosidase from *P. chrysosporium*

\[ V = \text{umoles/min/ml of culture medium} \]

\[ [S] = \text{mM} \]

\[ S = \text{ONPG} \]
Figure 6.12 Lineweaver-Burk plot for Intracellular \( \beta \)-Glucosidase from \( P. \) chrysosporium

\[ \frac{1}{V} (\text{umoles/ml/min}) \]

\[ \frac{1}{S} \text{ mM}^{-1} \]

\( V = \text{umoles/min/ml of intracellular extract} \)

\( [S] = \text{mM} \)

\( S = \text{ONPG} \)

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Figure 6.13 Extracellular B-Glucosidase in Culture Medium of
P. chrysosporium (Stationary Conditions)

A : Culture With Oxygenation on the 3rd Day
B : Culture Without Oxygenation on the 3rd Day
oxygenation conditions, after the peak of activity on the 3rd day a decrease in activity was observed up to the 5th day, followed by a lower peak of activity on the 6th day. The subsequent decrease in activity was followed by a sharp increase on the 10th day of growth.

Figure 6.14 shows the behaviour of intracellular B-glucosidase produced in 1% glucose as carbon source under the same conditions as described for figure 6.13. After oxygenation on the 3rd day, the peak of activity observed was followed by a decrease up to the 6th day when a lower peak of activity appeared, followed by a slight decrease up to the 10th day. When no oxygenation was carried out a sharper decrease was observed after the peak of activity on the 3rd day. From the 4th to the 7th day there was very little increase in activity. A lower peak was observed on the 8th day followed by slight decrease in activity up to the 10th day of growth.

Figure 6.15 shows the total extracellular lignin peroxidase and B-glucosidase and intracellular B-glucosidase produced in 10 ml medium /100 ml flask using 1% glucose as carbon source and under oxygenation conditions. As can be seen the peak of lignin peroxidase appears from the 7th to the 8th day of growth when a lower peak of intracellular B-glucosidase was also present. Maximum activity peaks for intracellular and extracellular B-glucosidase however, were observed on the 3rd day of growth.

Figure 6.16 shows the total extracellular lignin
Figure 6.14 Intracellular β-Glucosidase in *P. chrysosporium* (Stationary Conditions)

A : Culture With Oxygenation on the 3rd Day

B : Culture Without Oxygenation on the 3rd Day
Figure 6.15 Total Lignin Peroxidase and B-glucosidase of *E. chrysosporium* - With oxygenation (Stationary Conditions)

**A**: Total Lignin Peroxidase Activity  
(umoles/min/10 ml of culture medium)

**B**: Total Extracellular B-Glucosidase  
(umoles ONP/min/10 ml of culture medium)

**C**: Total Intracellular B-Glucosidase  
(umoles ONP/min/mg of dry weight in 10 ml culture medium)
Figure 6.16 Total Lignin Peroxidase and B-glucosidase of *P. chrysosporium* - Without oxygenation (Stationary Conditions)

A : Total Lignin Peroxidase Activity
(umoles/min/10 ml of culture medium)

B : Total Extracellular B-Glucosidase
(umoles ONP/min/10 ml of culture medium)

C : Total Intracellular B-Glucosidase
(umoles ONP/min/mg of dry weight in 10 ml culture medium)
peroxidase and B-glucosidase and intracellular B-glucosidase produced in 10 ml medium / 100 ml flask using 1% glucose as carbon source without carrying out oxygenation on the 3rd day. A similar behaviour as described for figure 6.15 was observed in this case, except for the much lower level of lignin peroxidase observed under non oxygenation conditions.

6.3.2 Lignocellulases in Cultures of *P. chrysosporium* Grown Under Shaken Conditions

Table 11 shows the extracellular cellulases produced in 1% glucose as the carbon source under shaken conditions. No extracellular xylanase activity was found on the 2nd day of growth. Activity was observed on the 6th day, which decreased towards the 7th day and disappeared towards the 8th day. Extracellular cellobiase activity was low on the 2nd day but an increase was observed on the 6th and 7th day. Filter paper activity was observed on the 2nd day, was higher on the 6th day and decreased towards the 7th day.

Table 12 shows the extracellular cellulases produced in 0.6% molasses plus 1% glucose as the carbon source under shaken condition. Extracellular xylanase was observed on the 6th day and decreased towards the 7th day. No activity was observed on the 8th day. Extracellular cellobiase was observed on the 2nd day, was higher on the 6th day and decreased towards the 7th day. The same behaviour was observed for extracellular filter paper activity.
Table 11  Extracellular cellulases produced by *P. chrysosporium* grown in 1% glucose under shaken conditions.

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NONE</td>
<td>0.043</td>
<td>2.21</td>
</tr>
<tr>
<td>6</td>
<td>4.66</td>
<td>3.58</td>
<td>3.96</td>
</tr>
<tr>
<td>7</td>
<td>2.85</td>
<td>3.65</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Table 12  Extracellular cellulases produced by *P. chrysosporium* in 0.6% molasses plus 1.0% glucose under shaken condition.

<table>
<thead>
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<th>TIME (DAYS)</th>
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<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
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<tbody>
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<td>1.11</td>
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<tr>
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<td>3.96</td>
<td>6.53</td>
<td>4.67</td>
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<tr>
<td>7</td>
<td>2.89</td>
<td>3.40</td>
<td>0.73</td>
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</tbody>
</table>
Table 13 Extracellular cellulases produced by *P. chrysosporium* in 0.5% xylan plus 0.5% glucose under shaken condition.

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>XYLANASE ACTIVITY</th>
<th>CELLOBIASE ACTIVITY</th>
<th>FILTER PAPER ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>18.72</td>
<td>0.23</td>
<td>0.063</td>
</tr>
<tr>
<td>6</td>
<td>14.55</td>
<td>0.12</td>
<td>0.090</td>
</tr>
</tbody>
</table>
Table 13 shows the extracellular cellulases produced in 0.5% xylan plus 0.5% glucose under shaken condition. Extracellular xylanase was high on the 5th day and suffered a small decrease towards the 6th day. Extracellular filter paper and cellobiase activities showed similar behaviour as xylanase activity but they were both present in much lower levels.

The appearance of lignocellulases under shaken conditions was similar to those under stationary conditions, but higher levels of activity were observed under shaken conditions.

The activities of intracellular cellulases were not determined, but in order to find out if there was any similarity in the appearance of the enzymes, a sodium dodecyl sulphate-polyacrylamide gel electrophoresis, of an intracellular extract of some samples from cultures of P. chrysosporium incubated under both shaken and non shaken conditions, was carried out. The protein banding patterns are shown in figure 6.17, and similarity in the protein banding can be observed, indicating the presence of intracellular cellulases.

Figure 6.18 shows the extracellular B-glucosidase and lignin peroxidase produced in 1% glucose as the carbon source in 1L flasks under shaken conditions. B-glucosidase showed a peak of activity on the 1st day of growth, which decreased sharply towards the 2nd day and then decreased steadily up to the 7th day. Lignin peroxidase activity was seen from the 4th to the 5th day, showed a peak of activity on the 7th day followed by a sharp decrease towards the 8th day.
Figure 6.17 SDS-PAGE of Intracellular Extract from *P. chrysosporium*

1 & 4: Molecular Weight Markers
2: 3rd day; Stationary Conditions; 0.5% Xylan + 0.5% Glucose
3: 3rd day; Stationary Conditions; 1% Glucose
5: Same as 2 but grown under shaken conditions
6 & 7: 3rd day; Shaken conditions; 0.6% molasses + 1% glucose
8: 6th day; Stationary Conditions; 0.5% Xylan + 0.5% Glucose
9: 6th day; Shaken Conditions; 0.6% molasses + 1% glucose
10: 6th day; Shaken Conditions; 0.5% Xylan + 0.5% glucose
11: 3rd day; Stationary Conditions; 1% Cellobiose
Figure 6.18 Extracellular B-Glucosidase & Lignin Peroxidase from P. chrysosporium Grown in 1% Glucose - Shaken Conditions

A : Extracellular B-Glucosidase Activity (umoles ONP/min/ml of culture medium)

B : Lignin peroxidase Activity (umoles/min/ml culture medium)
Figure 6.19 shows the extracellular B-glucosidase and lignin peroxidase produced under shaken condition using 0.6% molasses plus 1% glucose as the carbon source. B-glucosidase showed a peak of activity on the 2nd day followed by a decrease up to the 6th day of growth. Lignin peroxidase activity started from the 3rd to the 4th day. A peak of activity was observed on the 6th day followed by a decrease towards the 7th and 8th days.

Figure 6.20 shows the extracellular B-glucosidase and lignin peroxidase produced under shaken condition by foam immobilized cells of *P. chrysosporium* in 0.6% molasses plus 1% glucose as the carbon source. A peak of activity on the 1st day was observed for B-glucosidase which decreased steadily towards the 3rd day and sharply to the 5th day. Low activity was observed from the 5th to the 7th day. Lignin peroxidase activity started from the 3rd to the 4th day, and showed a peak on the 6th day followed by a decrease.

Figure 6.21 shows the extracellular B-glucosidase and lignin peroxidase produced under shaken condition using 0.8% molasses plus 1% glucose as the carbon source. B-glucosidase showed a peak of activity on the 3rd day which decreased towards the 4th and 5th day showing a second lower peak of activity on the 6th day. Lignin peroxidase showed a peak of activity from the 5th to the 6th day.

Figure 6.22 shows the extracellular B-glucosidase and lignin peroxidase produced under shaken condition using 1%
Figure 6.19 Extracellular B-Glucosidase & Lignin Peroxidase from *P. chrysosporium* Grown in 1% Glucose + 0.6% Molasses – Shaken Conditions

A : Extracellular B-Glucosidase Activity (umoles ONP/min/ml of culture medium)

B : Lignin peroxidase Activity (umoles/min/ml culture medium)
Figure 6.20 Extracellular B-Glucosidase & Lignin Peroxidase from Foam Immobilized *P. chrysosporium* Grown in 1% Glucose + 0.6% Molasses - Shaken Conditions

A : Extracellular B-Glucosidase Activity (umoles ONP/min/ml of culture medium)

B : Lignin peroxidase Activity (umoles/min/ml culture medium)
Figure 6.21 Extracellular β-Glucosidase & Lignin Peroxidase from *P. chrysosporium* Grown in 1% Glucose + 0.8% Molasses – Shaken Conditions

A : Extracellular β-Glucosidase Activity (umoles ONP/min/ml of culture medium)

B : Lignin peroxidase Activity (umoles/min/ml culture medium)
Figure 6.22 Extracellular B-Glucosidase & Lignin Peroxidase from *P. chrysosporium* Grown in 1% Glucose + 1% Molasses - Shaken Conditions

A : Extracellular B-Glucosidase Activity (umoles ONPG/min/ml of culture medium)

B : Lignin peroxidase Activity (umoles/min/ml culture medium)
Figure 6.23 Extracellular B-Glucosidase & Lignin Peroxidase from *P. chrysosporium* Grown in 0.5% Glucose + 0.5% Xylan - Shaken Conditions

A : Extracellular B-Glucosidase Activity (umoles ONP/min/ml of culture medium)

B : Lignin peroxidase Activity (umoles/min/ml culture medium)
molasses plus 1% glucose as the carbon source. B-glucosidase activity showed a similar behaviour to that described in figure 6.19. Lignin peroxidase had a peak of activity from the 6th to the 7th day.

Figure 6.23 shows the extracellular B-glucosidase and lignin peroxidase produced under shaken condition using 0.5% xylan plus 0.5% glucose as the carbon source. B-glucosidase showed a peak of activity on the 3rd day and a decrease towards the 6th day. Lignin peroxidase showed a peak of activity from the 5th to the 6th day followed by a decrease from the 6th day on.
6.4 **Discussion**

In virtually all microorganisms examined to date, the synthesis of cellulases is induced by the presence of celluloses and repressed by the presence of glucose or other readily metabolized sugar in the growth medium (Tsao, 1988; Morrison et al, 1987; McHale & Morrison, 1986 and Coughlan, 1985). *Sporotrichum pulverulentum* (*P. chrysosporium*) has been reported to have five endoglucanases (Ericksson & Pettersson, 1975a), one exoglucanase (Ericksson & Pettersson, 1975b) and two B-glucosidases (Deshpande et al, 1978), in a medium containing cellulose as the carbon source. Similarly, Smith & Gold (1979), have reported that glucose represses the formation of extracellular B-glucosidase in *P. chrysosporium*. B-glucosidases have a vital role in the saccharification of cellulose because they hydrolyse the products of cellulases action and because of their glycosyl transfer abilities (Coughlan, 1985). Jafelice et al (1988), have reported the appearance of B-glucosidase in a medium containing 1% glucose as the carbon source and suitable for the production of lignin peroxidase by *P. chrysosporium*. Here, it has been shown that extracellular and intracellular B-glucosidase exhibited good levels of activity, under both stationary and shaken conditions, independently of the carbon source used, when *P. chrysosporium* was grown under the conditions required for establishing the ligninolytic system. Maximum extracellular and intracellular B-glucosidase was observed during the first period of biosynthesis, before growth ceased. This contradicts the earlier findings of Deshpande et al (1978), who found that
there was a lack of B-glucosidase when P. chrysosporium was grown on glucose and that this enzyme was not produced constitutively by this fungus. However, Kubiceck (1982), has shown that during cultivation of Trichoderma pseudokoningii (a filamentous fungi), on various carbon sources, two different patterns of B-glucosidase were observed. On carbon sources that allowed fast growth, a relative high percentage of the total activity was found in the culture fluid, which decreased as the culture became older, but which increased again during the phase of cell lysis. On carbon sources which allowed slow growth, excretion was initially low, but increased at later culture stages. A similar behaviour was observed here (see figures 6.13, 6.14 and 6.18 to 6.23), where the carbon source (glucose), allowed fast growth to take place. When 0.5% xylan was used as the only carbon source, promoting slower growth than in 1% glucose, the highest level of activity was observed during the secondary phase of growth (see table 3). However, when a combination of 0.5% xylan plus 0.5% glucose was used as the carbon source, growth rate was similar to the growth in 1% glucose, and higher levels of B-glucosidase activity were observed in the first phase of growth (see table 5). Growth in 1% cellobiose only allowed low levels of extracellular B-glucosidase activity (towards both substrates, ONPG and cellobiose), whereas intracellular levels were the highest observed, with the exception of intracellular B-glucosidase in 1% lactose. This agrees with the findings of Smith & Gold (1979), who found that cellobiose was a very poor inducer for extracellular B-glucosidase, but induced maximal intracellular
activity levels of the enzyme. Lactose was not used as a source of carbon by the workers mentioned above. Umile & Kubicek (1986), observed that when *Trichoderma reesei* was grown in 0.5% glucose as the sole carbon source, whole cells exhibited significant B-glucosidase activity. The extracellular glucose and cellobiose levels might be important in the regulation of cellulolytic enzymes and control of these levels could be relevant to the overall control of lignocellulose degradation. It has been observed that intracellular, as well as extracellular B-glucosidase activity is related to the morphogenesis and development of fungal organisms associated with intra or extra cellular B-glucoside hydrolysis (Lusis & Becker, 1973; Canevascini & Meyer, 1979; Meyer & Canevascini, 1981; Woodward & Wiseman, 1982). This could also be one of the reasons for the presence of extra and intra cellular B-glucosidase in the exponential phase of growth. When 1% lactose was used as the carbon source, B-glucosidase might also have been of inducible forms of the enzyme. Lactose has been reported to induce B-glucosidase production in the thermophilic fungus *Talaromyces emersonii* (McHale & Morrison, 1986 and Morrison et al, 1987).

It has also been proposed that B-glucosidase may be important for glycoprotein synthesis (Woodward & Wiseman, 1982). This could explain the appearance of an intracellular peak of B-glucosidase activity in the later stages of growth as shown in figures 6.14 to 6.16 inclusive, when lignin peroxidase, a glycoprotein, is present in the extracellular medium.

The intracellular B-glucosidase showed higher affinity
(Km=0.25 mM, figure 6.12), towards the substrate ONPG, than the extracellular enzyme (Km=10 mM, figure 6.11). It is possible that these B-glucosidases are two different enzymes. According to Smith & Gold (1979), several forms of B-glucosidases are produced by *P. chrysosporium* and it has been suggested that the intracellular and the extracellular B-glucosidase could be products of different structural genes. Meyer and Canevascini (1981), have reported the presence of two distinct B-glucosidases in *Sporotrichum (Chrysosporium) thermophile*, a white-rot fungus. One possesses only an aryl B-glucosidase activity and a large molecular weight, 440000. The other shows cellobiase activity, with little activity towards aryl B-glucosides, and has a molecular weight of 40000.

The presence of other cellulases might have been of constitutive forms. However, induction of these enzymes by polysaccharides formed in the medium during growth of *P. chrysosporium* (Leisola et al, 1982b), should also be considered. Production of xylanase on simple sugar might also have been induced by hyphal wall constituents following substrate depletion. The higher extracellular xylanase activity observed under shaken conditions, in 0.5% xylan plus 0.5% glucose as the carbon source (see table 13), might have been a result of better oxygenation and homogenisation of the medium due to agitation. The high lignin peroxidase activity observed under this condition could be caused by the presence of the hemicellulose (oat-spelts xylan). In woody materials, lignin is usually associated with hemicelluloses. It has been suggested that xylanases may play an important role in the mechanism of
lignin removal, through the disruption of hemicellulose-lignin matrices (Jurasek & Kirk, 1987).

6.5 Summary

In this chapter, cellulases and xylanases produced in the low nitrogen medium suitable for lignin peroxidase production were investigated. Under the stationary condition of culture, the extracellular and intracellular enzymes were assayed for cellulases and xylanases activity, whereas only the extracellular enzymes produced under the shaken conditions of culture were investigated.

For both conditions of culture, extracellular B-glucosidase (ONPG activity), showed a peak of activity during the first three days of growth, which might be related to fungal development. Intracellular B-glucosidase showed a peak of activity on the 3rd day and another lower peak of activity during the stationary phase of fungal culture. These activities might be associated with fungal growth and production of glycoproteins respectively. SDS-PAGE for the intracellular enzymes produced under both conditions of culture was carried out and a similarity in the protein banding was observed, suggesting the presence of the same enzymes during growth under stationary and shaken conditions. The intracellular B-glucosidase produced showed a higher affinity to the aryl glucoside substrate (ONPG) than the extracellular enzyme, indicating that they might be different forms of the enzyme.

The other cellulases and xylanases investigated showed lower levels of activity than B-glucosidase (ONPG) activity and
a different sequence of appearance and maximum activity with time, depending on the carbon source used. These activities might have been of constitutive or inducible forms of the enzymes.

Lignin peroxidase produced under shaken conditions using xylan plus glucose as the carbon source showed good levels of activity, suggesting that the presence of the xylan had a positive effect on lignin peroxidase production.
Chapter 7

Isolation and Some Studies on the RNA of \textit{P. chrysosporium}
7 ISOLATION AND SOME STUDIES ON THE RNA OF *P. chrysosporium*

7.1 Introduction

Bioprocessing of lignocellulosic materials depend on a balance of a number of different enzymes that can promote the breakdown of the substrates. The efficiency of the process is determined by the balance of these enzyme activities which could be improved by manipulating the levels at which the encoding genes are expressed.

In order to develop efficient processes for the utilization of lignocellulosics it is necessary to obtain more information about the biochemistry and molecular biology of the organisms able to utilize these materials. The precision of recombinant-DNA technology, which allows the isolation of specific genes, is then required for further significant progress in the study of the extremely complex lignocellulose breakdown system.

Lignin degradation in *P. chrysosporium* is a secondary metabolic event (Kirk & Farrel, 1987). Identification of the genes regulating the onset of secondary metabolism and secretion of extracellular enzymes is of fundamental interest to the understanding of the mechanism involved in the regulation of lignin biodegradation. By using the mRNA isolated from cultures in primary and secondary metabolism, the genes coding for specifically secondary metabolic activities can be identified and used for *in vitro* translation to produce cDNA or to identify cDNA clones in a cDNA library. Isolation of cDNA species coding for ligninolytic enzymes opens up the possibility of producing enzymes in large quantities by cloning.
in bacteria and/or yeasts (Farrell et al., 1988). The information obtained from the cloned genes will make possible the construction of new lignocellulolytic organisms and the production of improved enzymes.

In this chapter the isolation of total RNA from P. chrysosporium in different stages of growth and characterisation by gel electrophoresis was carried out. Northern blotting of the electrophoretically resolved RNA fragments was undertaken followed by hybridisation to a radiolabelled oligonucleotide lignin peroxidase probe. RNA dot blots was also carried out and the amount of lignin peroxidase specific mRNA sequences was determined by hybridisation with the same radiolabelled oligonucleotide probe as used for Northern blotting. This oligonucleotide probe was as described by Tien & Tu (1987), which was first synthesised according to a sequence of amino acid residues near the N-terminus of purified ligninase H8 (Tien & Tu, 1987).

7.2 Methods

7.2.1 Extraction of RNA by the TNS Buffer Method

This was carried out as described by Haylock et al (1985), where the TNS extraction buffer was 1% tri-isopropyl-naphthalenesulphonic acid sodium salt (TNS), 6% p-aminosalicylic acid (PAS), 200 mM Tris HCl plus 25 mM EDTA adjusted to pH 7.8 and 250 mM NaCl.

P. chrysosporium was grown under stationary condition as described in section 3.2.2. Samples that received oxygenation on the 3rd day and controls, without oxygenation, were collected at different days of growth. Mycelium was washed with cold
sterile distilled water, filtered through two layers of muslim to remove the water and stored at -70°C.

The frozen mycelium was ground in a mortar and pestle with the addition of small amount of TNS extraction buffer. 3 volumes of TNS extraction buffer was added and this mixture was transferred to a 50 ml centrifuge tube and homogenised with the use of a polytron homogeniser. Debris were removed by centrifugation at 4000 rpm for 20 minutes. The debris pellet was washed with 1 volume of ice cold TNS extraction buffer and the supernatants pooled. 1 ml of phenol (water saturated) was added to each ml of the supernatants pooled and mixed by gentle agitation. One half volume of chloroform was then added and the flask shaken in order to form a homogeneous mixture. The phases were separated by allowing the mixture to settle. The upper, aqueous phase was collected and the lower, organic phase discarded. Care was taken throughout the procedure to maintain the temperature below 15°C. 2 volumes of ethanol plus 0.1 volume of 3M sodium acetate solution was added to the upper phase containing the RNA. This mixture was left overnight at -20°C in order to precipitate the RNA. Centrifugation to pellet RNA was then carried out at 10000 rpm for 15 minutes and the supernatant discarded. The RNA was then vacuum dried for 15 minutes. The RNA was dissolved in 0.5% SDS solution for 10 minutes at 65°C. The concentration of the RNA was determined by reading the absorbance of the diluted solution at 260 nm. Protein contamination was determined by the absorbance at 280nm. The ratio (Abs 260nm/Abs 280nm) for a pure RNA solution should be around 1.8.
The RNA samples were stored at -70°C prior to use.

7.2.2 Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction

This was carried out as described by Chomczynski & Sacchi (1987).

Preparation of stock solution D:
100 g of guanidine thiocyanate was dissolved with 117.2 ml of sterile distilled water in the manufacturer's bottle. 7.04 ml of 0.75 M sodium citrate pH 7.0 and 10.56 ml of 10% sarcosyl was then added.

Working solution D: 0.36 ml of 2-mercaptoethanol added to 50 ml stock solution D.

Frozen mycelium from 1 flask was ground in mortar with a pestle and then transferred to a 50 ml centrifuge tube. 10 ml of working solution D was added and the mixture homogenised with a polytron homogeniser. Sequentially, 1.0 ml of 2 M sodium acetate solution pH 4.0, 10 ml of phenol (water saturated) and 2 ml of chloroform:isoamyl alcohol (49:1) were added to the homogenate, with thorough mixing after the addition of each reagent. The final mixture was shaken vigorously for 10 seconds and cooled on ice for 15 minutes. Samples were then centrifuged at 10000 rpm for 20 minutes at 4°C. After centrifugation RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a clean tube, mixed with equal volume of isopropanol and kept at -20°C for 2 hours. Centrifugation at 10000 rpm for 20 minutes at 4°C was carried out and the resulting RNA pellet was dissolved in 0.3 ml of solution D,
transferred into a 1.5 ml Eppendorf tube and precipitated with 1 volume of isopropanol at -20 °C for 1 hour. After centrifugation in a Eppendorf centrifuge for 10 minutes at 4 °C the RNA pellet was resuspended in 75% ethanol, sedimented and vacuum dried for 15 minutes, and dissolved in 50 ul of 0.5% SDS solution at 65 °C for 10 minutes. The concentration of RNA was determined as described in section 7.2.1 and samples stored at -70 °C prior to use.

7.2.3 **Agarose Electrophoresis of the RNA** (Maniatis et al, 1982)

Agarose (1.05 g) was dissolved in RNAse-free water (50.4 ml), in an 80 ml Duran bottle, in a microwave oven. Rapidly, 12.6 ml of formaldehyde and 7 ml of 10 x gel running buffer were added and the mixture agitated. The resulting mixture was poured into a rectangular mould whose base and two parallel sides consisted of perspex and whose other two sides were sealed by autoclave tape. A simple perspex comb was assembled at one end of the mould so that there was about 1 mm space between the base of the teeth and the plate.

The RNA samples were prepared in RNAse-free eppendorf tubes. The material listed below was vortexed and centrifuged: 20 ug RNA; 2.0 ul of 10 x buffer; 3.5 ul of formaldehyde; 10 ul of formamide (deionised). Next, the samples were warmed to 65 °C for 15 minutes, then cooled quickly on ice to room temperature. Loading buffer (2 ul) was added and the samples re-centrifuged and vortexed.

The final sample solution was loaded onto a gel and run at about 40V (100 mA), in 1 x running buffer, until bromophenol
blue had run approximately 3/4 of the length of the plate. The RNA in the gel was stained with 25 μl of ethidium bromide (20 mg/ml) in 250 ml 1 x running buffer for 30 minutes. Destained of the gel was carried out by washing it in 1 x running buffer for 1 hour. Rna was then visualised under ultraviolet light.

7.2.4 The Northern Blot Technique

This procedure was first described by Alwine et al (1977) and is an adaptation of the method for blotting DNA described by Southern (1975). It involves the transfer of the electrophoretically resolved RNA fragments from the agarose gel (as described in section 7.2.3) onto a filter. In this case the filter used was Hybond-N nylon membrane. The experiment was as follows:

The blot was set up as shown in figure 7.1.

4 large rubber bungs were placed on the bottom of a plastic tray and 600 ml of 20 x SSC solution was added to the tray. A glass plate (14 cm x 19 cm) was placed on the top of the rubber bungs. 3 layers of wet (in 20 x SSC solution) Whatman paper were placed on the top of the plate and all air bubbles were removed with the help of a glass rod. The paper was long enough to be in contact with the solution in the tray. The agarose gel containing the RNA was then placed on the top of the paper and all air bubbles removed again. All the edges of the gel were covered with clingfilm, tucking excess under the tray but avoiding covering any area of the gel. This was done to ensure that all the 20 x SSC solution passed evenly through the gel. The Hybond nylon membrane was then laid on the surface of the gel and all air bubbles were removed. 3 sheets of pre-soaked
(in 20 x SSC solution) filter paper were laid over the membrane and air bubbles were removed. 10 cm of dry Kleenex paper towels was placed over the filter paper and a glass plate on top of these towels. A kilogram weight was put on the top of the glass plate and left to blot for a minimum of 24 hours, after which the blot was disassembled. The membrane containing the RNA was washed in 5 x SSC solution, dried in between two layers of filter paper, baked for 10 minutes at 80 °C and exposed for 3 minutes under ultraviolet lamp. This membrane was then used for hybridisation with a radiolabelled oligonucleotide probe as described later in this chapter.

**Figure 1 Northern Blot Apparatus**
7.2.5 The Dot Blot Assay for RNA

RNA samples were diluted to give concentrations of 50 ug to 0.1 ug in 100 ul of sterile distilled water. 300 ul of a solution containing equal parts of formaldehyde and 20 x SSC was added to each RNA sample. This mixture was incubated at 65 °C for 15 minutes and cooled down in ice to room temperature.

The Dot Blot (BRL) apparatus was washed with absolute ethanol and left to dry. The Hybond-N nylon membrane was wetted in water (5 minutes), then in 10 x SSC for 5 minutes and apparatus assembled.

Vacuum was adjusted and the complete volume of RNA mixture (400 ul) was applied to the bottom of the wells. Each well containing sample was then washed with 400 ul of 10 x SSC.

The apparatus was disassembled and the membrane containing RNA was dried in between two pieces of Whatman 3M paper. The membrane was then exposed to ultraviolet light for 3 seconds and used for hybridisation experiments.

7.2.6 Labelling the Oligonucleotide Probe Used

The probe used was synthesised by AFRC (Institute of Animal Physiology and Genetics Research), Cambridge.

The nucleotide sequence was as follows:

5' -GCG-TCG-CCG-ACA-GTC-TTG-CCG-TT 3'

The DNA 5' - End labelling was carried out according to the supplier's catalogue (Boehringer Mannheim), as follow: 200 ng of the oligonucleotide probe was dissolved in a final volume of 15.5 ul of sterile distilled water and incubated at 70 °C for 1 minute. 2.5 ul of 10 x kinase buffer, 5 ul of (γ-32P)ATP (Amersham) and 2 ul of T4 Polynucleotide Kinase (BRL) was added
to the oligonucleotide solution and the final mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 2 ul of 0.5 M EDTA and cooled in a ice bath. Radioactivity was measured by a Geiger counter. The product of the above reaction was passed through a NACS column (BRL), that had been previously equilibrated in 1M NaCl in TE buffer and in 0.1 M NaCl in TE buffer. The column containing the radiolabelled probe was washed with 10 ml of 0.1 M NaCl in TE buffer and the probe was eluted with 3 x 200 ul 1M NaCl in TE buffer. The eluate was boiled boiled for 2 minutes and then used for hybridisation experiments.

7.2.7 Hybridization of the Oligonucleotide Probe to The Northern Blots and Dot blots

Hybridization Mixture (Clontech Lab.), where the final concentration of the solution was as follows:

- 6 x SSC
- 2 x Denhardt's solution
- 50 ug/ml denaturated and sheared fish sperm DNA
- 50 ug/ml E. coli tRNA
- 0.1% SDS

The radiolabelled probe obtained as described in section 7.2.6 was added to 50 ml of the hybridization mixture and added to the hybond membrane that had been previously prehybridized in the above hybridization mixture for a period of at least 3 hours at 37°C. The hybridization with the probe was conducted for 18 to 22 hours at 37°C. The membranes were then washed, first with 2 x SSC plus 0.05% SDS for one hour at room temperature, and then for 20 minutes with 6 x SSC plus 0.1% SDS at 55 C (20°C below the Td). The blots were scanned with a Geiger counter and allowed to air dry. They were then placed in a appropriate tray, covered with plastic to prevent X-ray film
sticking to the blot, exposed to the X-ray film and stored at -70 °C for 5 days, after which the films were developed.

All the recipes for the reagents used in this section are given in appendix 2.

7.3 Results and Discussion

7.3.1 Extraction of RNA

The RNA sample used in the experiments described in this chapter were obtained by extraction with the TNS extraction buffer as outlined in section 7.2.1. This method was found to be time consuming and high volume of reagents were necessary for extraction. The RNA extracted had protein contamination, which was observed by A260:A280 ratio of around 1.6. The high amount of polysaccharides formed during growth of the microorganism might have been in part responsible for the yields of extraction obtained. Extraction of RNA using the single step method described in section 7.2.2 showed low contamination with protein. A ratio A260:A280 higher than 1.7 was obtained for samples extracted by this procedure. The method was found to be less time consuming and a higher number of samples could be handled simultaneously. However, no characteristic rRNA bands were observed after agarose gel electrophoresis for any of the samples used. This method has been successfully used for the extraction of RNA from mammalian cells and tissues (Chomczynski & Sacchi, 1987). It could be that the characteristics of the fungal cells and/or the amounts of reagents used did not allow a satisfactory extraction of fungal RNA. Non inactivation of the RNases by the reagents used
7.3.2 **Variation of the amount of cellular RNA with growth of *P. chrysosporium***

Table 1 and Table 2 show the amount of the cellular RNA extracted from *P. chrysosporium* at different time of growth. As can be seen, the amount of the cellular RNA decreased with growth. A sharp decrease was observed from the third day to the fourth day of growth, which coincides with the beginning of the stationary phase of growth. The decrease in RNA might be due to the transition from the growing state (active cell cycle) to the stationary or resting state. Growing cells have more RNA than resting cells.

7.3.3 **Total RNA and Lignin Peroxidase**

Table 3 and Table 4 show the amount of RNA and the lignin peroxidase activity in culture of *P. chrysosporium*, in various days of growth, for 1% glucose and 1% glucose plus 0.6% molasses as carbon sources respectively. As can be seen, lignin peroxidase appearance and decrease in amount of RNA occurs simultaneously. Wallace et al (1983) have reported an increase in the intracellular level of cyclic AMP, after nutrient nitrogen depletion in cultures of *P. chrysosporium*, which coincides with the initiation of ligninolytic activity. It is also known that reduction in the replicatory growth cause increased levels of cyclic AMP. Bu'lock (1975) has reported that cAMP-activated protein promotes transcription of some genes directly and that this cAMP-mediated process may cause different σ-factors to be produced for the control of messenger RNA initiation so that a different range of genes becomes
Table 1 Amount of RNA present in different days of growth of *P. chrysosporium* in 1% glucose as the carbon source

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>mg of RNA/g of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.39</td>
</tr>
<tr>
<td>4</td>
<td>0.89</td>
</tr>
<tr>
<td>5</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 2 Amount of RNA present in different days of growth of *P. chrysosporium* in 0.6% molasses plus 1% glucose as the carbon source

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>mg of RNA/ g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.40</td>
</tr>
<tr>
<td>3</td>
<td>1.87</td>
</tr>
<tr>
<td>4</td>
<td>0.94</td>
</tr>
<tr>
<td>5</td>
<td>0.61</td>
</tr>
</tbody>
</table>
Table 3  Lignin peroxidase activity and RNA concentration in cultures of *P. chrysosporium* grown in 1% glucose as the carbon source

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>mg RNA/10 ml Medium</th>
<th>LIGNIN PEROXIDASE ACTIVITY (umol/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>51.01</td>
<td>NONE</td>
</tr>
<tr>
<td>4</td>
<td>36.49</td>
<td>0.78</td>
</tr>
<tr>
<td>5</td>
<td>47.45</td>
<td>1.41</td>
</tr>
<tr>
<td>5</td>
<td>40.61</td>
<td>5.50</td>
</tr>
</tbody>
</table>

Table 4  Lignin peroxidase activity and RNA concentration in cultures of *P. chrysosporium* grown in 0.6% molasses plus 1% glucose as the carbon source

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>mg RNA/10 ml MEDIUM</th>
<th>LIGNIN PEROXIDASE ACTIVITY (umol/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>69.12</td>
<td>NONE</td>
</tr>
<tr>
<td>3</td>
<td>68.63</td>
<td>NONE</td>
</tr>
<tr>
<td>4</td>
<td>38.54</td>
<td>1.57</td>
</tr>
<tr>
<td>5</td>
<td>26.50</td>
<td>NONE</td>
</tr>
<tr>
<td>5</td>
<td>26.10</td>
<td>9.4</td>
</tr>
</tbody>
</table>
accessible for transcription. This cAMP-mediated process could be one of the factors responsible for the late appearance of lignin peroxidase activity when growth rate and total RNA content are low, implying that mRNA encoded by genes of the ligninolytic system is regulated by cAMP-activated proteins.

7.3.4 Agarose Gel Electrophoresis of RNA Samples

The three main types of RNA, according to function, are ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). The rRNA and tRNA are of defined size and can be banded by gel electrophoresis. mRNA are of smaller and very heterogeneous size and can not be banded. Each eukaryotic ribosome contains three different molecules of rRNA classified by size as 28S, 18S, and 5S. Figures 7.2 and 7.3 show the electrophoretically-resolved RNA fragments from various RNA samples extracted from P. chrysosporium. The two high molecular weight rRNA bands should be well defined and the mRNA should be seen as a smear, mainly above and between the two ribosomal bands. As can be seen, only samples 2, 8 and 3 are presenting well defined bands. The other samples are showing poorly defined bands and degradation of RNA might have occurred.

7.3.5 RNA Dot Blot

Figures 7.4 and 7.5 show the dot blot hybridization of RNA samples with the oligonucleotide probe described in section 7.2.5. Samples from the 2nd to the fourth day of growth for both carbon sources showed the same pattern of hybridization, i.e., the hybridization signal intensity varied in the same way for these samples, being stronger for more concentrated samples and decreasing proportionally with the concentration of the
Key for figure 7.2

Track 1: RNA standard (size markers)

Track 2: RNA from a 4-days old culture of *P. chrysosporium*, grown in 1% glucose as the carbon source.

Track 3: Empty

Track 4: RNA from a 5-days old culture of *P. chrysosporium*, grown in 1% glucose as the carbon source. Lignin peroxidase activity = 5.50 umol/ml/min.

Track 5: RNA from a 4-days old culture of *P. chrysosporium*, grown in 0.6% molasses plus 1% glucose as the carbon source.

Key for figure 7.3

Track 1: RNA standard (size markers)

Track 2: RNA from a 5-days old culture of *P. chrysosporium*, grown in 1% glucose as the carbon source. Lignin peroxidase activity = 1.41 umol/ml/min

Track 3: RNA from a 5-days old culture of *P. chrysosporium*, grown in 1% glucose plus molasses as the carbon source. Lignin peroxidase activity = none

Track 4: RNA from a 5-days old culture of *P. chrysosporium*, grown in 1% glucose plus 0.6% molasses as the carbon source. Lignin peroxidase activity = 9.5 umol/ml/min.
Figure 7.2 Agarose Gel Electrophoresis of RNA Samples
Figure 7.3 Agarose Gel Electrophoresis of RNA Samples
Key for figures 7.4 and 7.5

Carbon Source = 1% glucose

Row 1: 4-days old culture, RNA concentrations (ug): Fig. 7.4 = 50; 30; 10; 5; 1 and 0.1; Fig. 7.5 = 10; 8; 5; 3; 1; 0.5; 0.1.

Row 2: 5-days old culture; Lignin peroxidase activity = 1.41 umol/ml/min; RNA concentrations: same as for row 1.

Row 3: Same as for row 2 but Lignin peroxidase activity = 5.50 umol/ml/min.

Row 9: 3-days old culture. RNA concentrations (ug): Fig. 7.4 = same as for row 1; Fig. 7.5 = not to be considered.

Row 10: 2-days old culture. RNA extracted by the single step method (see section 7.2.2). Not shown in fig. 7.5.

Row 11: 5-days old culture, no oxygenation on the 3rd day, no lignin peroxidase activity. RNA concentrations: same as for 1.

Carbon Source = 1% glucose plus 0.6% molasses

Row 4: 2-days old culture. RNA concentrations (ug): Fig. 7.4 = 50; 30; 10; 5; 0.1; empty; and 1. Fig. 7.5 = same as for row 1.

Row 5: 3-days old culture. RNA concentrations (ug): Fig. 7.4 = same as for row 1. Fig. 7.5 = 8; 10; 5; and 3.

Row 6: 4-days old culture. RNA concentrations (ug): Fig. 7.4 = same as for row 1; Fig. 7.5 = 8; 5; 3; and 1.

Row 7: 5-days old culture. RNA concentrations (ug): Fig. 7.4 and Fig. 7.5 = same as for row 1. No lignin peroxidase activity.

Row 8: 5-days old culture; Lignin peroxidase activity = 9.4 umol/ml/min. RNA concentrations (ug): Fig. 7.4 = 30; 50; 10; 5; 1; and 0.1. Fig. 7.5 = 10-1.
Dot Blot Hybridization of RNA

Figure 7.4

Figure 7.5
RNA. A RNA sample from the 5th day of growth and glucose plus molasses as the carbon source showed the same behaviour as for samples from the 2nd to the 4th day. However, a less intense hybridization signal was obtained from the sample grown with glucose as the only carbon source. Samples from the 5th day exhibiting poor lignin peroxidase activity showed an associated decrease in hybridization with the probe. Those samples giving no lignin peroxidase activity showed even lower hybridization. A sample from the 5th day which received no oxygenation and showed no lignin peroxidase activity, displayed greater hybridization than oxygenated samples from the same day of growth, that showed poor or no lignin peroxidase activity. It could be that the sample from the 5th day, which had no lignin peroxidase activity, had higher amount of lignin peroxidase specific mRNA, but environmental conditions did not allow transcription to take place. On the other hand the sample which showed poor lignin peroxidase activity, might have had smaller amount of lignin peroxidase specific mRNA.

7.3.6 Northern Blot of RNA

Figures 7.6 and 7.7 illustrate the Northern blot run for samples from the 2nd to 5th day of growth, where the carbon source used was 0.6% molasses plus 1% glucose. The blot was hybridized with the radiolabelled oligonucleotide probe as described in section 7.2.5 and 7.2.6. The bands seen in the figures represent specific RNA fragments which hybridized to the probe.

Lignin peroxidase has been shown to be produced in 6 to 7 days old cultures of P. chrysosporium grown in low nitrogen
Northern Blot of RNA

Figure 7.6

Track 1 & 2 = 2-days old culture.

Truck 3 = 3-days old culture.
Northern Blot of RNA

Figure 7.7

Truck 1 = 4-days old culture.
Truck 2 & 3 = 5-days old culture. No lignin peroxidase activity.
Truck 4 & 5 = 5-days old culture. Lignin peroxidase activity = 9.4 umol/ml/min.
medium. The enzyme is not detectable in 1 to 3 days old cultures, which are in primary growth. Therefore, one would have expected not to find RNA extracted from cultures in primary stage of growth or from older cultures (limited growth), which had no lignin peroxidase activity, to exhibit hybridization to a probe for lignin peroxidase.

The results obtained from the Dot blot and Northern blot hybridization suggest that lignin peroxidases might either be the product of the same genes that code for primary metabolites, but that have undergone post-transcriptional/post-translational modifications, or are products of different genes sharing sequence homology. Environmental factors may also play a role in the expression of lignin peroxidases, since cultures in secondary stage of growth, which had no lignin peroxidase activity, also showed hybridization to the probe used.

7.4 **Summary**

RNA has been extracted from 2-5 days old cultures of *P. chrysosporium* grown either in glucose or glucose plus molasses as carbon sources. A method using guanidinium isothiocyanate was found inadequate for the extraction of the extracellular RNA from *P. chrysosporium*. The method used contained the two anionic detergents TNS and PAS, which rapidly inactivate RNases and dissociate the nucleoprotein from the RNA.

Northern blotting and Dot blot hybridization was carried out using a probe which was first synthesized according to a sequence of amino acid residues near the N-terminus of purified ligninase H8. All RNA samples used were found to hybridize to
the probe, to some extent. The weaker hybridization signal was obtained from a RNA sample extracted from a 5 days old culture, which had no lignin peroxidase activity. A 5 days old control, which received no oxygenation on the 3rd day of growth and never exhibited lignin peroxidase activity, showed stronger hybridization signal than the latter.

Results obtained suggest that lignin peroxidase expression might either be regulated by environmental stresses or by post-transcriptional/post-translational modifications of the encoding gene(s).

It should be mentioned that these were only preliminary experiments which were not repeated due to shortage of time.
Chapter 8

Final Discussion and Conclusions
8 FINAL DISCUSSION AND CONCLUSIONS

As already described in chapter 1 (Introduction), research carried out during the last ten years has demonstrated that several nutritional and cultural parameters are important for establishing the ligninolytic system in *P. chrysosporium*:

1- Presence of a cosubstrate: *P. chrysosporium* metabolized various lignin preparations only when a simpler carbon source was present (Ulmer et al, 1983; Blanchette, 1984; Leisola et al, 1984; Otjen & Blanchette, 1985 and Leatham, 1986)

2- High oxygen tension: the presence of oxygen is crucial in determining the rate of lignin degradation (Bar-Levi & Kirk, 1981; Reid & Seifert, 1982 and Yu & Erickson, 1985)

3- Growth as mycelial mats rather than as submerged pellets in agitated cultures (Faison & Kirk, 1985)

4- Suitable choice of buffer (Kirk & Farrell, 1987)

5- Appropriate levels of certain minerals and trace elements

6- Growth limiting amount of nutrient nitrogen

Preliminary studies carried out here, in medium containing Indulin AT (a commercial form of lignin), as the carbon source, have demonstrated no ligninolytic activity, as determined by the veratryl alcohol oxidation assay, neither under stationary nor shaken conditions. Lignin peroxidase activity was later obtained using glucose as the carbon source, with limiting amounts of nutrient nitrogen and lignin itself was not necessary for the establishment of the ligninolytic system.

Cultures grown under stationary conditions showed much lower levels of lignin peroxidase activity if no oxygenation was carried out on the 3rd day of growth. Two kinds of stoppers
were used for non-oxygenated cultures, cotton wool wads and rubber bungs. In both cases, flasks were stoppered immediately after inoculation and incubated in an aerated incubator. The rubber bung sealed flasks never showed any lignin peroxidase activity. The cotton stoppered flasks showed 20-30% of the activity of an oxygenated culture and evaporation of the growth medium was observed to occur. This suggests that exchanges in the air due to evaporation might be the factor responsible for the presence of this lower level of activity. These results are in agreement with those obtained by Faison and Kirk (1985), where around 10% of lignin peroxidase was obtained with non-oxygenated cultures, as compared to oxygenated cultures.

The buffer DHS used as described in section 3.2.4, was not found to be the best choice for the production of lignin peroxidase by *P. chrysosporium*. A wide variation in lignin peroxidase activity was observed among the flasks and although a few flasks showed high levels, many others showed little or no lignin peroxidase activity. In experiments carried out using DMS as the only carbon source, it was found that *P. chrysosporium* could metabolize it and produce some lignin peroxidase activity. The fact that *P. chrysosporium* was able to grow in DMS as the only carbon source could be one of the reasons for the lower rate of commercial lignin mineralized to CO2 in DMS buffered medium compared to other buffers (Kirk & Farrell, 1987). When tartrate buffer was used, the variation in lignin peroxidase activity among flasks was smaller and a lower number of flasks did not show any activity at all. Very poor growth and no lignin peroxidase activity were observed when
tartrate buffer was the only carbon source.

The balance of minerals was also found to affect lignin peroxidase production. When the mineral solution used was prepared in a way that would lead to the precipitation of some of the components, forming a cloudy solution, no lignin peroxidase activity was obtained. Kirk et al (1986), have reported that the balance of trace metals is important for enzyme production as are some minerals, such as Cu²⁺ and Mn²⁺ which promote an increase in the total lignin peroxidase activity.

Cultures grown under stationary conditions were found to be very sensitive to any disturbance of the mycelium mat formed. If the mycelium was disturbed (for example, at the time of oxygenation), no lignin peroxidase activity was subsequently produced. This might indicate that the breakage of cells liberates metabolites that interfere with the production of the enzyme (e.g. catalase). *P. chrysosporium* was observed to have intracellular catalase activity as indicated by the liberation of oxygen on the addition of hydrogen peroxide. Kersten & Kirk (1987), have reported that the microorganism exhibits a strong intracellular catalase activity which might be related to the production of hydrogen peroxide during lignin peroxidase appearance. The disturbance of the mycelium might also lead to an uneven distribution of oxygen which in turn may disturb the production of lignin peroxidase.

Culture agitation, which is usually used to increase oxygenation, strongly suppressed lignin peroxidase production (Faison & Kirk, 1985). Here, lignin peroxidase has been
produced under agitated conditions and the presence of molasses was shown to stimulate its production. In medium containing 1% glucose as the carbon source, maximum activity under shaken condition was observed from the 7th to the 8th day of incubation. However, when molasses was present, maximum activity appeared 24 hours earlier and it was about 50% higher than the activity produced in glucose-only medium. Molasses have been reported to contain products originating from microbial and chemical degradation of lignins during processing of cane and cane juice. These compounds have been identified as phenolic and phenylpropanetriolic glucosides (Paella, 1983). The aglycon part of some of these compounds are, 3,4-dimethoxyphenol; 3-methoxy-4-hydroxyphenol, 3,5-dimethoxyphenol-4-hydroxyphenol, vanillic acid, veratric acid and syringic acid. It could be suggested that the presence of these compounds might be responsible for the stimulation of lignin peroxidase activity. This finding may be compared to recent works where veratryl alcohol (Palmier, 1987 (personal communication); Tien & Kirk, 1988; Linko, 1988) or benzyl alcohol (Kirk & Farrell, 1987) were used to stimulate production of lignin peroxidase under shaken conditions. Dialysis of the molasses resulted in a delay in the peak of activity, which was lower than the activity obtained in non-dialysed molasses (about 60%), perhaps due to the loss of some low molecular weight compounds responsible for the stimulation of the enzyme. It should be mentioned here that the dialysed molasses retained most of its colour. Among the molasses' fraction identified by Palla (1983), there was a brown portion which, after enzymatic and
acid hydrolysis, released UV absorbing products with similar characteristics to the phenylglycosides and phenylpropanetriols mentioned above. This brown fraction was 68% retained in dialysis experiments by membranes with retention limits of 10000 (Palla, 1983). The same brown fraction and its hydrolysis products might also explain the later appearance and lower activity of lignin peroxidase when the dialysis of molasses was carried out.

The fact that molasses promote the enhancement of lignin peroxidase activity under shaken conditions, opens up the possibility of scaling up its production through the use of a natural compound that is cheap and readily available. Also, it could be possible that the cane juice itself would produce similar results, since the compounds present in the molasses that are derived from lignin, would also be present in the juice. It would be interesting to know whether P. chrysosporium would grow on sucrose as the only carbon source. This would make the use of sugar cane juice attractive, since it would contain most of the requirements necessary for lignin peroxidase production, such as the carbon source, minerals, vitamins, the enzyme "inducers" and most probably a good buffering capacity. If P. chrysosporium is not able to grow on glucose as the sole carbon source, a process where some of the glucose necessary for growth could be obtained from the sucrose itself (e.g. use of immobilized invertase) would be desirable. The fact that lignin peroxidase was able to decolorize molasses' pigment might also make possible its utilization as an anti-pollution agent for waste water from sugar cane
refineries and distilleries. Bumpus & Aust (1987) and Hammel et al, (1987), have reported that *P. chrysosporium* has the ability to degrade a wide variety of structurally diverse organic compounds, including a number of environmentally persistent organopollutants such as DDT, polychlorinated biphenyls and benzo(a)pyrene. This biodegradative ability was found to be dependent on the lignin degrading system of the fungus and its non-specificity and partially extracellular nature suggests that it might be useful as a supplementary means to treat organochemical wastes (Bumpus & Aust, 1987). Brazil has the biggest alcohol programme in the world but one of its drawbacks is the huge amount of liquid waste or stillage that is produced during the process (Rosillo-Calle & Hall, 1988). Stillage is a serious polluter because it is rich in organic matter and consumes oxygen when discharged into rivers and lakes, killing plants and animals. The possibility of using *P. chrysosporium* and its non-specific lignin degrading system, as a biological agent to degrade the organic matter in this stillage is something worth trying.

During growth under agitation conditions lignin peroxidase was found to be produced by *P. chrysosporium* only when pellets with a hairy surface were formed. Compact smooth pellets did not exhibit any lignin peroxidase activity. This same phenomenon was observed by Haemmeli (1988), but no explanation was given. It is interesting to mention here some facts about the fungal growth on solid surfaces. When filamentous fungi, such as *P. chrysosporium*, grow on solid surfaces, it resembles roots in the soil (interpenetrating and ramificated). The
tubular body of the fungal filament grows alongside the solid particle using it as a source of nutrients. In the case of lignin-degrading fungi, the growing tips of the filament produce powerful extracellular lignin degrading enzyme, which act as a "chemical drill" (Weiland, 1988), penetrating the substrate and converting lignin to metabolic products. An illustration of this process is presented in figure 1. Perhaps by analogy, the formation of the hairy surface could be compared to the growing filament tips of the fungus on solid substrates (see figure 1). This would explain the importance and role of this hairy surface on the production of lignin peroxidase by *P. chrysosporium*. It was also observed that an agitation rate higher than 90 rpm suppressed the formation of lignin peroxidase. It could be that this rate of agitation was enough to disturb or even disrupt the hairy surface and thus prevent the appearance of lignin peroxidase in a similar way to the disturbance of the mycelial mat under stationary condition described above.

Figure 1  Fungal growth on solid substrate
The establishment of fungal cultures, specially continuous flow types presents a number of technical problems and sometimes requires the construction of specialised equipment for laboratory and large scale fermentations. Accumulated growth on surfaces inside the fermenters and within pipes and valves constitute serious problems in the continuous culture of filamentous fungi. Surface growth of this type decreases the culture volume, creates a physiologically heterogeneous culture and may also cause premature depletion of nutrients (Smith & Berry, 1976). The possibility of producing lignin peroxidase by immobilized P. chrysosporium under shaken conditions opens up the way to large scale enzyme production in a fermenter. Herein, lignin peroxidase has been produced in repeated batch shake cultures by foam immobilized P. chrysosporium. In the control method (free cells), pellet size was variable and clump formation was unavoidable, leading to a large variation in lignin peroxidase activity among flasks. However, in the presence of foam, growth was restricted to the pores of the foam (polyurethane) matrix. Thus pellet size was controlled by the physical properties of the foam itself. Uniformity of pellet size and prevention of clumping could be the possible explanation for the much lower variation in lignin peroxidase activity among flasks. Also, the support used does not seem to interfere with the formation of the hairy surface, since high activity of the enzyme was obtained. Perhaps, it might even offer some kind of protection against shear forces developed in the medium due to agitation, which might promote the breakage of the hairy filaments. This kind of protection is very useful.
for the production of the enzyme in large fermenters. In fact, lignin peroxidase has been produced in a 10 l Biostatic E bioreactor which was modified to function as a packed-bed aerated fermenter (Linko, 1988). A maximum activity of 700U/l was reached. Here, an attempt to produce lignin peroxidase in continuous operation through the use of a glass column packed with foam immobilized P. chrysosporium, produced very poor amounts of enzyme, but it was clear that some improvement in the process, such as better oxygenation and feeding of substrate, would have promoted better yields of the enzyme.

The normal pattern of wood decay by white-rot fungi involves a simultaneous attack on both the polysaccharides and the lignin. To date no organism is known that can grow on lignin as the only carbon source. When white-rot fungi grow in wood, enzymes diffusing from the fungal cell wall first degrade low molecular weight polysaccharides through the action of cellulases, which provide nutrients that allow the fungi to grow. When exposed polysaccharides are depleted by these enzymes, the fungus starts to starve and shifts over to secondary metabolism and only then does degradation of lignin begin (Ericksson, 1988). To be able to use white-rot fungi in biotechnological processes it is necessary to learn how their enzymes are secreted and under what conditions. This is the reason why P. chrysosporium was grown on various carbon sources and the lignocellulolytic enzymes were investigated. P. chrysosporium is known to produce extracellular cellulases in various growth media and in solid substrates, but the production of cellulases produced under nitrogen
starvation in media for lignin peroxidase production had not been investigated. The first finding was the appearance of an earlier extracellular β-glucosidase peak of activity, well before the lignin peroxidase peak in a medium containing 1% glucose as the carbon source (Jafelice et al, 1988). Intracellular β-glucosidase was then also found to be present, and both an earlier peak of activity, before lignin peroxidase appearance, and a later peak, during lignin peroxidase appearance, were observed in 1% glucose medium. This β-glucosidase might be associated with development of fungal cell walls, formation of glycoproteins and possibly with the cellulases formed during the initial attack of wood, as mentioned above. Intracellular and extracellular lignocellulases were also found when other carbon sources, such as cellobiose and xylan were used. They might be of both constitutive and inducible form and might also reflect the utilization of lignocelluloses in wood. Good levels of lignin peroxidase and xylanase activities were produced under shaken conditions in a medium containing xylan plus glucose as the carbon source. The simultaneous appearance of xylanase and lignin peroxidase is desirable for several reasons: xylan is closely associated with lignin in wood, the monomer xylose, resulting from xylan degradation is a readily metabolizable sugar providing energy for lignin degradation. Recent findings also indicated that the presence of xylan speeds up both delignification and bleaching in wood pulp processes (Ericksson, 1988).
The understanding of the biodegradation of lignocelluloses represents a major challenge because of the complexity of the substrate and also because many organisms and enzymes are involved in its degradation. As described in the Introduction (chapter 1), genetic approaches have been applied to the study of the bioligninolytic system of *P. chrysosporium*, in order to elucidate fundamental principles, such as its primary and secondary metabolism, and regulation of ligninolytic activity, and also manipulate the system for industrial applications. Here, some preliminary studies on the RNA of *P. chrysosporium* were carried out. RNA extracted from samples from the 2nd and 3rd days of growth were also found to hybridize to the oligonucleotide probe used. This might suggest that some mRNA species specific for secondary metabolism occur earlier, during primary growth. Gene expression then might play a role in the establishment of secondary metabolism. Raeder et al (1987), have suggested that the shift from primary to secondary metabolism might be controlled not only at the transcriptional level, but also at the post-transcriptional level, e.g. through differential rates of processing of constantly present pre-mRNA pools, with coding capacity for both growth and ligninolytic functions.

The genetic approach, including strain improvement, manipulation of the lignocellulolytic system, selection for desired metabolic characteristics and development of new recombinant strains will very possibly help to elucidate the very complex biolignocellulolytic system. The potential applications of these systems include pulping wood, bleaching
pulps, converting by-products lignins to useful chemicals, upgrading lignocellulosics for animal feed, freeing the carbohydrates in lignocellulosics for further bioconversions, and treating various lignin derived wastes and also some organopollutants. As has been mentioned in chapter 1 (Introduction), the results obtained from the experiments carried out here have provided further information about the production of lignocellulases. The pH of the medium, and the choice of a buffer which has good buffering capacity and is not assimilated by the fungus, have been demonstrated to be relevant in the production of lignin peroxidase enzymes. The development of growth conditions suitable for the production high levels of lignin peroxidase activity has been achieved. Good yields of lignin peroxidase activity have been obtained under shaken conditions when molasses or xylan were added to the growth medium containing glucose. The results obtained when various carbon sources were used under both, stationary and shaken conditions, but more efficiently under shaken conditions, have indicated that there are many possibilities of manipulating culture parameters and growth media in order to obtain higher yields of lignocellulases. The investigation of the use of these various additives and/or carbon sources might lead to the formulation of growth media and culture parameters suitable for the production of very high yields of lignocellulases. In addition, it might also provide more information about the lignocellulolytic system of the fungus. The foam immobilization of the microorganism has been successfully applied to successive production of the enzyme.
The use of a widely-available and inexpensive support, such as polyurethane foam, opens the possibility of the use of fermenters for the production of lignocellulases. The optimization of a continuous system for the production of lignocellulases will eventually allow the best possible the utilization of lignocellulosic materials.

Recommendations For Future Research

More information about the lignin peroxidases from \textit{P. chrysosporium}, such as regulation and maximization of their production; secretory mechanisms and controls; properties of the various isoenzymes and their interaction; the importance of glycosylation on the activity of the enzymes, is necessary. Also, the possible role of Mn peroxidase system in lignin degradation and phenol-oxidizing enzymes should be clarified.

Research is needed to identify the some of the physiological hydrogen peroxide producing system or systems.

The possible roles of veratryl alcohol, which has been suggested to be a mediator of lignin peroxidase oxidation, in addition to its apparent roles as an inducer and protector of the enzyme need to be clarified.

Further research is needed to determine whether bacteria are able to produce extracellular enzymes capable of degrading the lignin polymer and also for a better understanding of the structure and function of the cellulases and ligninolytic activities of others microorganisms such as brown-rot fungi.

Major emphasis should be placed on the molecular biology of lignocellulose degradation, in order to understand how the
various systems interact so that in future it will be possible to construct new microorganisms producing a limited set of enzymes for the specific modification of lignocellulose for particular purposes.

Improvements in the bioprocesses, including the use of immobilized cells and enzyme systems and improved downstream processing are necessary for the establishment of economically viable processes. The development of an efficient biological pretreatment system or systems for lignocellulose materials is also required.
Appendices
### Appendix 1

**Mineral Solution:** *(g/litre)*

- Ammonium tartrate - 0.22
- Manganese sulphate - 0.66
- Iron III sulphate - 0.144
- Cobalt II sulphate - 0.10
- Calcium chloride - 0.082
- Zinc sulphate - 0.10
- Copper sulphate - 0.0064
- Aluminium potassium sulphate - 0.01
- Boric acid - 0.01
- Sodium molybdate - 0.01
- Sodium chloride - 1.0

**Vitamin Solution:** *(mg/ l)*

- Biotin - 2.0
- Folic acid - 2.0
- Thiamine-HCl - 5.0
- Riboflavin - 5.0
- Pyridoxine-HCl - 10.0
- Cyanocobalamin - 0.10
- Nicotinic acid - 5.0
- DL-Calcium pantothenate - 5.0
- Thiotic acid - 5.0
Appendix 2

10 x Gel Running Buffer: 0.4 M morpholinopropanesulphonic acid (MOPS) pH = 7.0
   100 mM sodiumacetate
   10 mM EDTA (pH = 8.0)

20 x SSC : 175.30 g sodium chloride
   88.20 g sodium citrate
   Final Volume - 1l
   pH = 8.0

TE Buffer pH = 7.4 : 10 mM Tris HCl (pH 7.4)
   1 mM EDTA (pH 8.0)

Denhardt's solution (50 x) : ficol - 5.0 g
   polyvinylpyrrolidone - 5.0 g
   BSA (Pentax Fraction V) - 5.0g
   Final Volume - 500 ml
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Ulmer, D.C.; Leisola, M.S.A.; Schmidt, B.H. & Fiechter, A.
Publications and Communications
Sequential appearance of \(\beta\)-glucosidase and lignin peroxidase in the exocellular fluid of a stationary phase culture of \textit{Phanerochaete chrysosporium}

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Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

\(\beta\)-d-Glucosidase (\(\beta\)-d-glucohydrolase EC 3.2.1.21) is an enzyme which can catalyse the hydrolysis of the \(\beta\)-glucosidic linkage of alkyl and aryl \(\beta\)-d-glucosides, in addition to glucosides containing only carbohydrate residues. This enzyme is of practical importance as a component of the cellulase system, which promotes the saccharification of cellulose. We have investigated the appearance of cellulolytic enzymes in the same culture fluid of a \textit{Phanerochaete chrysosporium} strain grown under nitrogen limitation suitable for lignin peroxidase production. In addition, we have determined lignin peroxidase activity, one of the most important components of the ligninase system. Lignin peroxidase, an extracellular enzyme involved in the degradation of lignin and lignin model compounds, has been isolated from cultures of the white-rot fungi \textit{P. chrysosporium} (Tien & Kirk, 1983). Many isoenzyme forms of this lignin peroxidase have been recently isolated and separated by SDS/polyacrylamide gel electrophoresis and isoelectric focusing techniques (Glenn et al., 1985; Kirk et al., 1986; LaRossa et al., 1987). In addition to lignin peroxidase, and depending upon culture conditions, \textit{P. chrysosporium} synthesizes a number of other biodegradative enzymes, included among them \(\beta\)-glucosidase (Deshpande et al., 1978; Erickson & Pettersson, 1975).

White-rot fungi, \textit{P. chrysosporium} ATCC 24725 was maintained in 2\% (w/v) malt agar slope at room temperature. A uniform portion, containing mycelium and spores was cultivated in a medium composed of (w/v): glucose 2\%, peptone 2\%, and yeast extract 1\% for 30 h at 39\(^\circ\)C without agitation. The mycelium was then filtered off, washed free of culture medium and used to inoculate the nitrogen-limited medium as described by Kirk et al. (1978). This medium contains glucose, 1\% (w/v) basal medium, minerals and vitamins. To ensure equal amounts of inoculated microorganism, the mycelium from one glucose-peptone-yeast extract flask (50 ml in 250 ml Erlenmeyer flask) was divided equally and placed into 100 ml Erlenmeyer flasks containing 10 ml of the nitrogen-limited medium described above. These flasks were incubated at 39\(^\circ\)C without agitation.

Correct oxygenation is known to be an essential for lignin peroxidase production (Kirk et al., 1978; Kirk, 1983; Matti & Fiechter, 1985). This was carried out on the third day when oxygen was flushed in for 1 min (to saturation), to a 100 ml flask scaled with a rubber bung, using a wide needle connected via rubber tubing to an oxygen cylinder.

Samples were collected every 24 h and checked for \(\beta\)-glucosidase and lignin peroxidase activities. \(\beta\)-Glucosidase activity was measured using \(o\)-nitrophenol-\(\beta\)-d-glucopyranoside (ONPG) as substrate (Evans, 1985). Activity was measured by incubating 0.4 ml of enzyme solution and 1.0 ml of substrate (10 mg/ml, pH 4.5) for 15 min at 40\(^\circ\)C. The reaction was stopped by the addition of 2 ml of 1 m-sodium bicarbonate and the release of \(o\)-nitrophenol was measured spectrophotometrically at 410 nm. Lignin peroxidase activity was assayed using veratryl alcohol, a simple lignin model substrate (Faison & Kirk, 1985). This is oxidized to the corresponding aldehyde in the presence of hydrogen peroxide. The formation of veratryl aldehyde is measured by the associated increase in absorbance at 310 nm.

Fig. 1 shows that the level of exocellular \(\beta\)-glucosidase peaks on day four well before the peak of lignin peroxidase on day seven. No intracellular ligninase could be detected, but disrupted (by grinding) mycelia released extra \(\beta\)-glucosidase in amounts that varied considerably with time of culture. It is of interest that levels of secreted enzymes fall after peaking, which is presumably due to cessation of biosynthesis rather than changes in the biodegradative ability of the culture fluid. The results are of considerable interest because of the clearly sequential appearance in the medium, even in the uninduced cells, firstly of constitutive enzyme (\(\beta\)-glucosidase) associated with the attack on the cellulose component of lignocellulose, followed by a constitutive lignin-attacking enzyme. This finding could be rationalized if the nutritional preference of these fungi is directed towards cellulose degradation in the first instance.

It may be surprising that we also find (data not shown) that the constitutive \(\beta\)-glucosidase produced later, in the stationary phase of culture does not appear to be secreted, whereas about 50\% is secreted during the first period of biosynthesis before growth ceases at about day four. This \(\beta\)-glucosidase is not apparently associated with nutrient supply considerations even if cellulose substrates were to be present. In that case, however, inducible \(\beta\)-glucosidases may be produced and secreted to attack such cellulose substrates. Removal of lignin from lignocellulose substrates would be a prerequisite for the availability of the cellulose moiety and a second secreted appearance of \(\beta\)-glucosidase may have been expected therefore. If lignin were present, secreted ligninase activity might be of inducible forms of these enzymes.

**Fig. 1.** Sequential constitutive biosynthesis and secretion of \(\beta\)-glucosidase and lignin peroxidase in \textit{P. chrysosporium} grown under N limitation

<table>
<thead>
<tr>
<th>Time of culture (days)</th>
<th>(\beta)-glucosidase activity (nmol ONPG/mg enzyme/2 h)</th>
<th>Lignin peroxidase activity (nmol veratryl aldehyde/mg enzyme/2 h)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
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</tr>
<tr>
<td>1</td>
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<tr>
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<tr>
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<td>0.6</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Growth of the micro-organism (g dry weight/100 ml of culture). (A—A); \(\beta\)-glucosidase activity (nmol ONPG/mg of micro-organism dry weight per min), (O—O); lignin peroxide activity (nmol veratryl aldehyde/mg of micro-organism dry weight per min). Values are the means of three determinations, bars indicate s.d.
It has been observed that intracellular as well as exocellular \(\beta\)-glucosidase activity is related to the morphogenesis and development of fungal organisms associated with intra- or exocellular \(\beta\)-glucoside hydrolysis (Lusis & Becker, 1973; Canevascini & Meyer, 1979; Meyer & Canevascini, 1981; Woodward & Wiseman, 1982), rather than with the production of breakdown products of cellulose for metabolic energy purposes.

We gratefully acknowledge Professor Howard Dalton, Department of Biological Sciences, University of Warwick, for providing the culture of \(P.\) chrysoporium ATCC 24725. One of us (L.R.S.J.) would like to thank The British Council for financial support for this research.


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INTRODUCTION

In most sources of lignocellulosic materials, the presence of lignin restricts access to the carbohydrate and hence prevents degradation. In the last few years, biological pretreatment of lignocellulose has become a subject of growing commercial interest because of the possibility of converting it to a food or energy source at a lower cost than present extraction processes. The white-rot basidiomycetes appear to be the major natural degraders of lignin, and of these Phanerochaete chrysosporium is the most widely studied species. Lignolytic activity in P. chrysosporium develops in the culture after the primary phase is completed, as a response to nitrogen, carbon or sulphur starvation. Here, we describe the production of ligninase by both free and immobilised cells of P. chrysosporium ATCC 24725 under shaken conditions, in medium containing 1% glucose, or 1% glucose plus different concentrations of molasses. In glucose only cultures, maximum activity was observed from the 7th to the 8th day of incubation in response to nitrogen limitation in the medium, apparently as part of secondary metabolism. However, when molasses were present, maximum activity appeared 24 hours earlier. It is concluded that under starvation conditions ligninase production can be manipulated by medium additives present in a cheap, readily-available, waste product.

METHODS

INOCULUM PREPARATION

P. chrysosporium ATCC 24725 was maintained at 30°C on 2% malt agar conical flasks. The inoculum was prepared by scraping the surface of the agar with a wire loop and washing off the spores with distilled water, and filtering the solution through two layers of glass wool.

FOAM PREPARATION

Foam cubes of length 0.5cm were washed with distilled water, autoclaved for 20 minutes at 15 psi, pressed to remove the water and were autoclaved again.

LIGNINASE PRODUCTION

The nitrogen-limited medium described by Kirk et al. (1978) was used throughout the experiment. This medium contains 1% glucose, basal medium, minerals and vitamins.

When molasses was used, it was added to the described medium to give the final concentration required for the experiment. 1 liter conical flask containing 250ml of medium were inoculated with spore suspension and foam cubes (5-60) were added, when immobilization studies were carried out. The flasks were incubated at 40°C and 140 rpm for 70 hours. After this period, 150 ml of the medium were poured off, the flasks sealed and flushed with oxygen. Incubation was then continued at 40°C and 60 rpm.

Ligninase activity was assayed using veratryl alcohol, which is oxidized to the corresponding aldehyde in the presence of the enzyme and hydrogen peroxide. The formation of veratryl aldehyde is measured by the increase in absorbance at 310nm.

RESULTS

Figure 1 shows ligninase activity (u/mglain) when different concentrations of molasses were used.

Figure 2 shows ligninase activity for molasses 0.6% where the foam immobilized microorganism had been kept at 4°C for a period of a month.

Table 1 shows ligninase activity for glucose-only cultures, glucose plus 0.6% molasses inoculated with free form of microorganism and glucose plus 0.6% molasses inoculated with microorganism foam immobilized.

REFERENCES

Table I  LARGE SCALE PRODUCTION OF LIGNINASE ACTIVITY (umol/ml/min)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TIME (DAYS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 1/2</td>
</tr>
<tr>
<td>MICROORG. IN FOAM</td>
<td>7.29</td>
</tr>
<tr>
<td>MOLASSES 0.6% + 1% GLUCOSE</td>
<td>5.20</td>
</tr>
<tr>
<td>1% GLUCOSE</td>
<td>2.42</td>
</tr>
</tbody>
</table>

LIGNINASE ACTIVITY FOLLOWING FLUSHING WITH OXYGEN vs TIME

LIGNINASE ACTIVITY vs MOLASSE CONCENTRATION