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THE DEVELOPMENT OF A STIRRED TANK REACTOR SYSTEM
FOR THE PRODUCTION OF LIGNIN PEROXIDASE
BY PHANEROCHAETE CHRYSOSPORIUM

presented by

Frederick Carl Michel Jr.

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Major professor

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THE DEVELOPMENT OF A STIRRED TANK REACTOR SYSTEM FOR THE PRODUCTION OF LIGNIN PEROXIDASE BY *PHANEROCHAETE CHRYSOSPORIUM*

by

Frederick Carl Michel Jr.

A THESIS

Submitted to
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1988

ABSTRACT

THE DEVELOPMENT OF A STIRRED TANK REACTOR SYSTEM FOR THE PRODUCTION OF LIGNIN PEROXIDASE BY *PHANEROCHAETE CHRYSOSPORIUM*

by

Frederick C. Michel Jr.

Lignin Peroxidases (ligninases) produced by the white rot fungus *Phanerochaete chrysosporium* have several important potential industrial applications. A bench scale stirred tank reactor system for the production of lignin peroxidases was developed. Scale-up studies included the optimization of a low-cost media, examination of the mechanism of pellet formation, and the effects of agitation in shake flask cultures. Higher levels of lignin peroxidase activity were obtained with acetate buffer compared to 2,2-dimethyl succinate buffer and other buffers. Concentrations of 0.05% (w/v) Tween 80 and 0.4 mM veratryl alcohol gave optimal lignin peroxidase activities in this medium. The agitation rate affected pellet size, the number of pellets formed and enzyme activity. A model was proposed for the production of lignin peroxidase in shake flask cultures. In a baffled stirred tank reactor high lignin peroxidase titres were observed when the agitation rate, oxygen dispersion and foaming were closely controlled.

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CHAPTER I: INTRODUCTION

Lignin Peroxidases[19,46] are extracellular, hydrogen peroxide-dependent heme proteins which catalyze the oxidation of the C_α-C_β linkages in lignin, a major constituent of all vascular plants and the second most abundant renewable organic polymer in the biosphere[44]. These enzymes are produced during idiophase in nitrogen or carbon limited cultures of certain white rot fungi, as exemplified by the basidiomycete Phanerochaete chrysosporium[31]. Lignin peroxidases have several potential industrial applications including biopulping, detoxification of recalcitrant organo-pollutants such as DDT, dioxins and PCB's[6,7,13,22] degradation of pulp and paper mill effluents[17,28] and pretreatment of lignocellulosic biomass for efficient bioconversion to feeds, fuels and chemicals[42,24]. Unfortunately, the low levels of lignin peroxidases produced under current culture conditions limit the study of lignin peroxidases and their applications. Procedures for large scale production have not been adequately developed.

Lignin peroxidases were first produced in the laboratory in shallow stationary cultures of P. chrysosporium[46]. This filamentous fungi grows in the form of a mycelial mat or mycelial pellets and requires a pure oxygen environment for optimal lignin

peroxidase production. Enzyme production is induced during secondary metabolism by lignin or lignin metabolites such as veratryl alcohol. Initial attempts to produce lignin peroxidase in submerged cultures were unsuccessful due to the organism's sensitivity to agitation[32]. Recently it has been shown that the addition of surfactants, particularly Tween 80, enables P. chrysosporium to produce lignin peroxidase under mildly agitated conditions[26]. This made the production of lignin peroxidase using biocatalyst pellets feasible in a stirred tank reactor system. The development of a stirred tank reactor system for lignin peroxidase production however has not been described to date. This type of reactor is readily available at both the laboratory and commercial scale.

The hypothesis of this study was that lignin peroxidases could be produced in a stirred tank reactor system from mycelial biocatalyst pellets of P. chrysosporium grown in a low-cost media. A three part study was undertaken to test this hypothesis. First a low-cost culture medium was optimized for the production of lignin peroxidase in shake flask cultures. Next, the mechanism of biocatalyst pellet formation, growth and the effects of agitation were studied. Using the optimized media and an understanding of the characteristics of the pellet biocatalysts, a stirred

tank reactor system for the production of lignin peroxidases was developed. This system produced the lignin peroxidases at levels comparable to shake flask cultures.

This thesis is divided into five chapters, including the introduction (Chapter 1). The literature review (Chapter 2), describes work previously undertaken to understand the lignin degrading system in the white rot fungus P. chrysosporium and the factors that affect the production of the lignin peroxidase enzymes by this organism. Also described are reactor systems that have been developed for lignin peroxidase enzyme production. In Materials and Methods (Chapter 3), the techniques, and procedures used in this thesis are described. In the Results (Chapter 4), the findings of the three part study, as described above, are presented, primarily through the use of figures and tables. The major findings and future directions are explored in the Discussion (Chapter 5). Finally, in the appendix, an unsteady state model for the batch culture production of lignin peroxidases is described. This model used the data from optimization experiments and from other sources[26]. It accounted for the growth, substrate utilization, effects of media components and product formation in lignin peroxidase producing cultures of P. chrysosporium. Also presented in the appendix are the

description of a trickle bed reactor system which was developed but produced no detectable lignin peroxidase (Appendix B). The tabulated data used in this thesis are presented in Appendix C.

CHAPTER II: LITERATURE REVIEW

2.1 Lignin biodegradation

Lignocellulosics, 25% to 35% of which are lignin, represent a vast renewable resource for the production of fuels and chemicals. Lignin exists in the cell wall of vascular plants to provide structural rigidity and protection from pathogenic organisms[44]. It is a chemically and structurally complex aromatic biopolymer (Figure 2.1). Lignin is formed by the dehydrogenative polymerization of cinnamyl alcohol derivatives of which coumaryl alcohol, sinapyl alcohol and coniferyl alcohol are the primary monomers[44].

Certain micro-organisms, particularly the white-rot fungi, are known to metabolize lignin[11,14]. Lignin however, does not serve as a growth substrate for these cells[29]. The primary function of lignin metabolism by the micro-organisms appears to be to free plant polysaccharides protected by lignin which can serve as growth substrates. The filamentous fungus Phanerochaete chrysosporium has been shown to be particularly capable of degrading lignin based on the conversion of ^{14}C radio labeled lignin to CO_2[32], as well as the ability to produce the extracellular enzyme lignin peroxidase[46]. This organism displays rapid growth, metabolism of

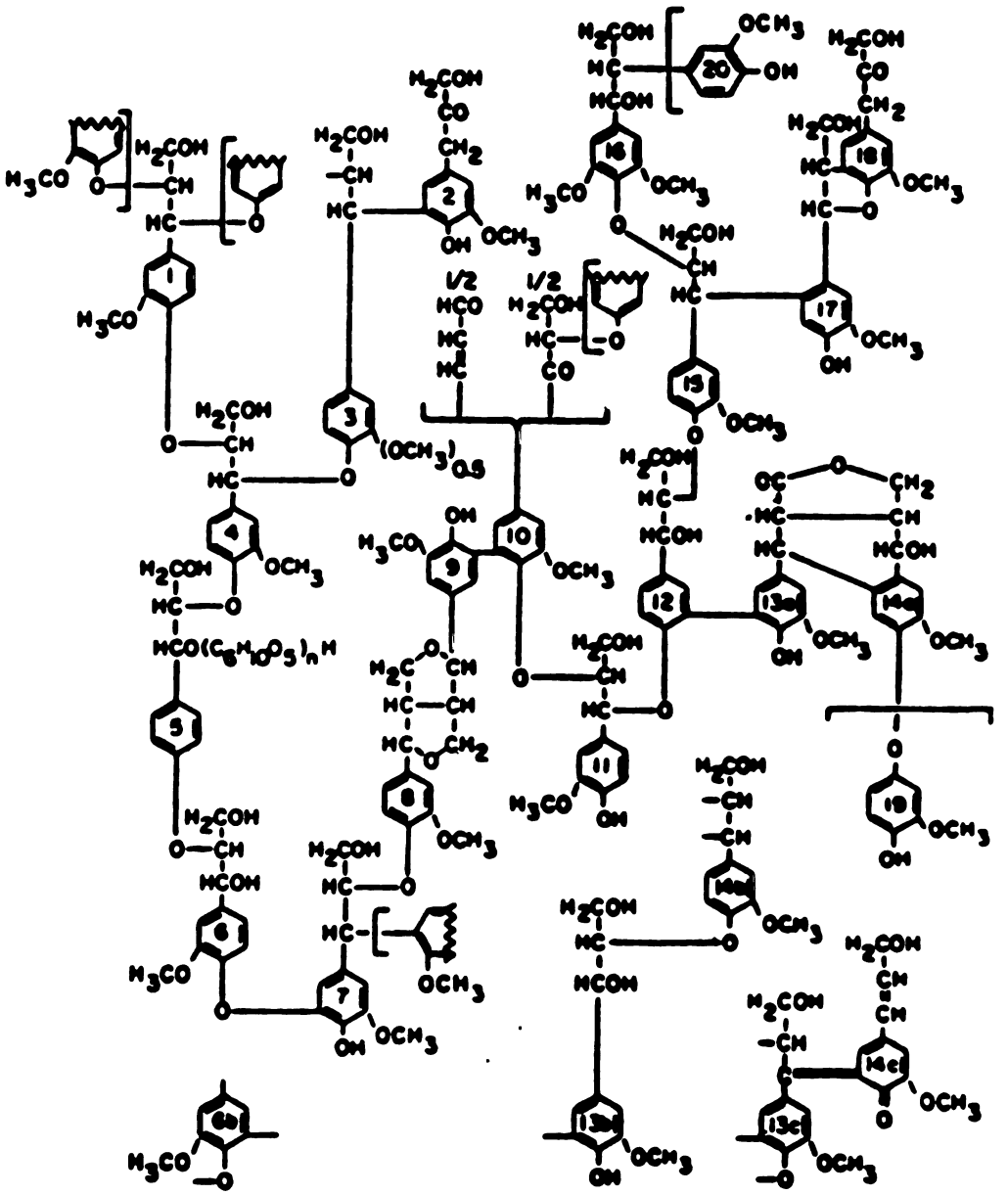


Figure 2.1 Typical structure of lignin [44].

lignin, prolific conidiation (spore production), a high temperature optimum of 40° C and the ability to grow on a range of media[32]. In addition to degrading lignin, P. chrysosporium has been shown to degrade environmental pollutants such as DDT, polychlorinated biphenyls and dioxins[6,7,8,13,21,22]. These pollutants have chemical structures similar to lignin metabolites and may be degraded by the same mechanism as lignin.

2.2 Lignin Peroxidases from Phanerochaete chrysosporium

Lignin peroxidases are produced during the stationary phase (idiophase) by P. chrysosporium when growth is limited by carbon or nitrogen[15]. Under shallow stationary conditions P. chrysosporium grows in the form of a mycelial mat[32]. Under the influence of agitation, P. chrysosporium grows in the form of mycelial pellets[26,30,50]. P. chrysosporium has been shown to produce optimal ligninolytic activity in a buffered media (pH = 4.5) consisting of a nitrogen source and a carbon source (one of which must be limiting), trace elements, basal elements and the vitamin thiamine[32].

Both carbon and nitrogen limited cultures of P. chrysosporium produce lignin peroxidases. Nitrogen limited conditions more closely approximate the natural environment encountered by white rot fungi (the nitrogen content of wood is low)[31]. Therefore, nitrogen

limitation is generally used in the laboratory to study lignin peroxidase production. Carbon limited cultures have been shown to only transiently degrade lignin and to autolyse[27]. However, despite this finding, many researchers have successfully used carbon limited cultures to produce high lignin peroxidase titres.[2,9,36,38]

The time course of lignin peroxidase production and the growth of P. chrysosporium in nitrogen limited cultures is presented in Figure 2.2. Cultures grow rapidly and reach the stationary phase during the second day. During this period, the nitrogen in the medium is depleted and becomes incorporated into the growing mycelium[26]. At the same time, mycelial dry weight increases and glucose is steadily depleted. The mycelial dry weight continues to increase after the second day due to the production of an extracellular polysaccharide. Glucose is consumed both in the growth and the stationary phase, declining linearly throughout the fermentation. Lignin peroxidase activity first appears 5 days after inoculation and reaches a maximum on the sixth or seventh day. After maximum activity is reached, lignin peroxidase activity declines. The decline in activity may be due to enzyme degradation by proteases.

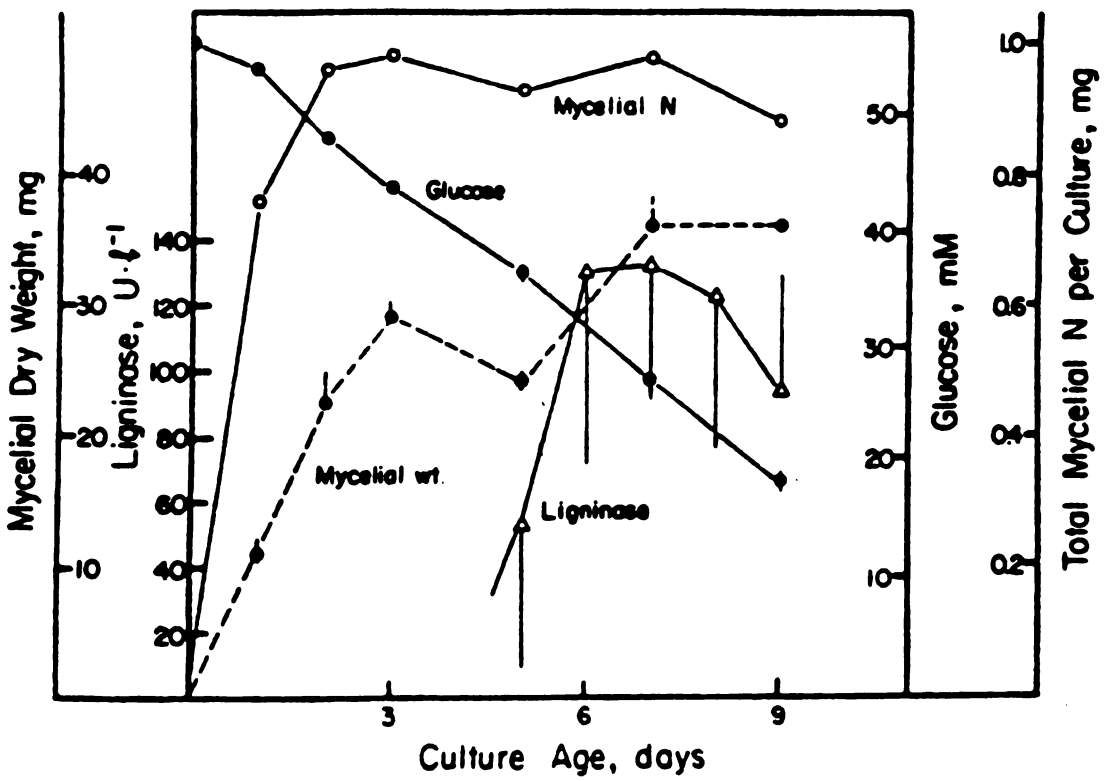


Figure 2.2 Time course of growth, substrate utilization and lignin peroxidase activity of agitated cultures of P. chrysosporium [26].

2.2.1 Strains of *P. chrysosporium*

Various strains of *P. chrysosporium* exist which have been used to study lignin peroxidase production. The wild type strains BKM-F-1767[26,46] (ATCC 24725) and ME-446[16,51] (ATCC 34541) have been predominantly used. Of the wild type strains, BKM-F-1767 has been shown to be the most rapid lignin degrader and produces the highest lignin peroxidase activities[26,33]. Two mutant strains have also been studied; SC26[30,33], derived from BKM-F-1767 and INA-12[2,9]. Strain SC26 was developed for the production of lignin peroxidase in a rotating biological contactor[30]. It produces higher lignin peroxidase activities than the wild type[33] and more easily adheres to surfaces. Strain INA-12 has produced the highest lignin peroxidase activities to date[2] in carbon limited cultures but is not available.

2.2.2 Characteristics of Lignin Peroxidase

Tien and Kirk[46,47] and Gold et al[19] first separated and characterized the extracellular lignin degrading enzyme from six day old cultures of *P. chrysosporium*. This enzyme non-specifically catalyzed several oxidations in the alkyl side chains of lignin related compounds. It was a heme containing glyco-protein[46] that required hydrogen peroxide for activity[19]. The enzyme belonged to a group of isozymes which catalyzed the oxidative depolymerization of lignin[30]. Collectively

these isozymes were called "ligninases". They share the ability to catalyze the oxidation of veratryl alcohol to veratryl aldehyde in the presence of H_2O_2, have a MW of between 39,000 and 43,000[31], and cross react with polyclonal antibodies raised against H8 one of the predominant lignin peroxidase isozymes[30]. Further studies[38,46] have shown that these extracellular enzymes have a pH optimum of 2.5 to 3.0 and a $pI_5o_{C.}$ between 3.5 and 4.65. The Michaelis-Menten constant, Km, for H_2O_2 and veratryl alcohol was found to be 80 μM and 60 μM respectively[46].

First thought to be oxygenases, ligninases are now known to be typical peroxidase enzymes[45] and are now referred to as lignin peroxidases. The key reaction is the one electron oxidation of aromatic nuclei to produce unstable cation radicals[31]. The evidence supporting this finding is that chemically catalyzed one electron oxidation of lignin model compounds gives the same reaction products as lignin peroxidases[23] and UV/Spectroscopic studies which have shown that lignin peroxidases change in the same way as other peroxidases during reaction[31]. The lignin model compounds have been shown to be degraded by lignin peroxidase via the following mechanism[23,29,45,46] (Figure 2.3). First, the lignin peroxidase heme forms an oxo-iron (IV) porphyrin cation radical with hydrogen peroxide. This removes an

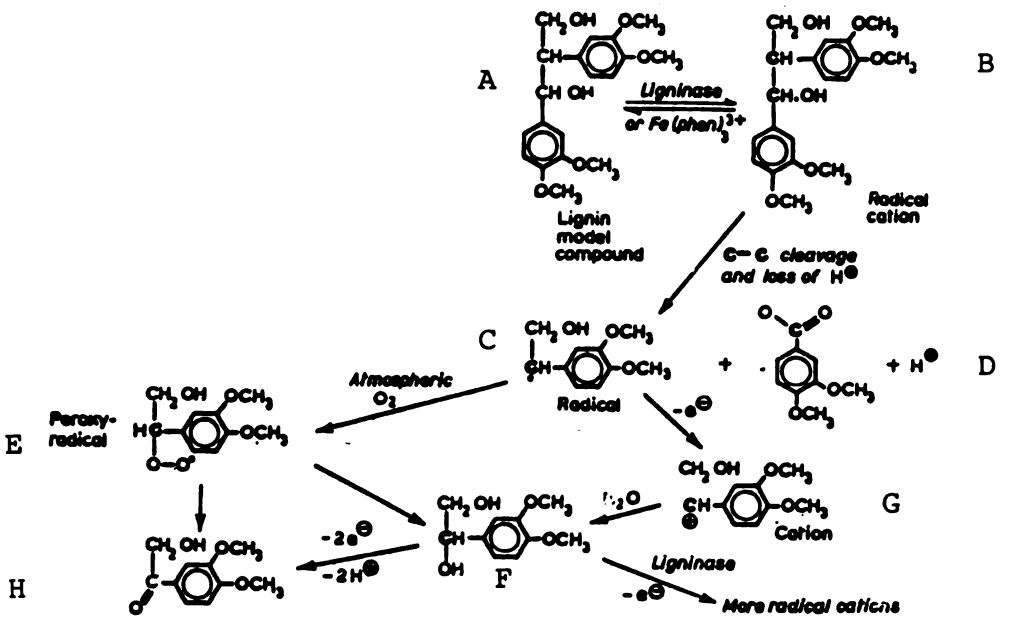


Figure 2.3 The mechanism of the biodegradation of a lignin model compound [46].

electron from an oxygenated aryl ring of a lignin model compound to form an aromatic cation radical (A to B). Cleavage of a lignin C_α-C_β bond follows resulting in two products (C and D). One of these products (C) may incorporate atmospheric oxygen forming a peroxy radical (E) which oxidizes to an aldehyde (H) or other products (G). The other cation product (F) may be oxidized by water forming an alcohol.

2.2.3 Factors Affecting Lignin Peroxidase Production

The production of lignin peroxidases by cultures of P. chrysosporium is influenced by many factors. These include oxygen tension, strain, buffer, the presence of enzyme inducers[16], agitation[16,28], trace element concentration, and the presence of surfactants.

Oxygen Partial Pressure. The partial pressure of oxygen in cultures of P. chrysosporium has a profound affect on the production of lignin peroxidase. Barlev and Kirk[4], found that in shallow stationary cultures of P. chrysosporium, lignin was degraded to CO_2 faster in a pure oxygen environment than in air. In these cultures growth was unaffected by the oxygen partial pressure. However, the oxygen partial pressure did affect both the amount of the ligninolytic system produced and the rate of lignin oxidation. Faison and Kirk[15] measured a twenty fold increase in lignin peroxidase activity in cultures grown in pure oxygen

compared to cultures grown in air. Leisola et al[36] found that in agitated cultures with a 100% oxygen atmosphere, lignin peroxidase activity increased ten fold compared to cultures with an air atmosphere. The mechanism by which oxygen stimulates lignin peroxidase production is not known.

Buffer and pH. A pH of 4.5 has been shown to be optimal for the expression of ligninolytic activity in P. chrysosporium[32]. The choice of buffer in the culture medium, however, can affect the activity of the ligninolytic system. Cultures buffered with polyacrylic acid have been shown to more rapidly degrade lignosulfones than cultures buffered with the widely used 2,2-dimethyl succinate buffer[31]. Acetate buffer has been reported to be toxic to P. chrysosporium[32] however other investigators have mentioned that acetate buffered cultures produce higher lignin peroxidase activities than 2,2-dimethyl succinate buffered cultures. Compared to phthalate buffered cultures, aconitate and tartrate buffered cultures mineralized lignin more slowly[32]. Some buffers (succinate and citrate) may be used as carbon sources by the growing organism. The buffer, 2,2-dimethyl succinate, is commonly used by investigators but is expensive. A more effective and less expensive buffer would greatly reduce media costs for lignin peroxidase production.

Enzyme Inducers. Lignin peroxidase activity has been shown to be inducible by lignin and by products of lignin degradation, particularly veratryl alcohol (3,4-dimethoxybenzyl alcohol). Faison and Kirk[15], found that birch, spruce and synthetic lignins, the lignin monomers syringyl and vanillyl alcohol as well as veratryl alcohol, veratryl aldehyde and veratrylglycerol increase the lignin peroxidase activity of stationary nitrogen limited cultures. Of these, veratryl alcohol gave the greatest increase in lignin peroxidase activity. Further study[16] also revealed increases in some extracellular lignin peroxidase proteins when veratryl alcohol was added. Leisola et al[35] detected no lignin peroxidase activity in agitated nitrogen limited cultures without veratryl alcohol. With the addition of veratryl alcohol lignin peroxidase activity appeared and reached a maximum 10 to 12 hours later. These results indicate that veratryl alcohol is an enzyme inducer. It may affect messenger RNA (mRNA) transcription.

Surfactant Addition. Agitated cultures of P. chrysosporium initially produced little or no lignin peroxidase[15,32]. Jager et al[26] demonstrated that agitated cultures could produce lignin peroxidase at levels greater than or equal to stationary cultures when the surfactants Tween 80, Tween 20 or 3-[(3-

colamidopropyl) dimethylammonio] 1-propanesulfonate (CHAPS) were added. Tween 80 and Tween 20 can supply fatty acids to the fungus however CHAPS cannot. Thus a mechanism other than fatty acid supplementation is thought to affect lignin peroxidase production. Asther et al[2] found that the addition of oleic acid emulsified in Tween 80 further increased lignin peroxidase production. They measured lipase activity in the extracellular fluid during the primary growth phase and found that fatty acids emulsified in Tween 80 were depleted by the organism. The mechanism by which surfactants enhance lignin peroxidase activity has not been established. Surfactants may modify the cell membrane affecting the uptake and release of compounds.

Effect of Agitation. It has been reported that increased agitation reduces lignin peroxidase production in agitated cultures of P. chrysosporium[15,32]. In shallow agitated cultures of strain INA-12 Asther et al[2] found that at agitation speeds greater than 50 rev min^{-1}, lignin peroxidase activity markedly decreased. Leisola et al[36] have found that pellet size and concentration affects lignin peroxidase activity although they did not quantify pellet size. Wase et al[48] have reported an analogous result in disc turbine agitated cultures of cellulase-producing mutants of Aspergillus niger. They found that increasing

the agitation rate from 100 to 300 rev min^{-1} caused a decrease in enzyme activity. They also found an increase in extracellular protein with increased agitation. They implied that, "Mycelium was being disrupted by shear forces generated by the impeller, liberating protein.", and measured low levels of protease activity in the liberated protein. The effect of agitation on the production of lignin peroxidase by P. chrysosporium has been overcome by the use of surfactants (see above)[26]. The mechanism by which agitation affects lignin peroxidase production by P. chrysosporium is not known.

2.3 Fungal biocatalysts

Fungal biocatalysts are used industrially for the production of organic acids[40], antibiotics, enzymes and the biotransformation of pharmaceuticals[3,10,41,50].

2.3.1 Fungal pellet reactor systems

Many filamentous fungi including, P. chrysosporium form pellets in submerged culture[12]. Pellets are formed by the aggregation of growing hyphae. Their formation is influenced by the rate of agitation, medium composition, strain, inoculum concentration and other factors[41,50]. Fungal pellets provide a convenient means of immobilizing the biomass within a reactor. Once formed pellets can serve as biocatalysts for the production of secondary metabolites.

Fungal pellets have been used in reactor systems

for the production of lignin peroxidase. A 1.5 liter air-lift reactor has been developed which produced lignin peroxidases from mycelial pellets. In this reactor pellets were formed by air circulation however the enzyme yields of this reactor were low (60 U l^{-1})[37]. A 78 ml column reactor using preformed mycelial pellets has been reported[38] which produced lignin peroxidases continuously (172 U l^{-1}). The development of a stirred tank reactor system for the production of lignin peroxidases has not been described.

2.3.2 Fungal film reactor systems

Filamentous fungi generally exhibit a strong affinity for surfaces. In fungal film bioreactors, mycelia attaches to immobilization support material submerged in the culture medium. Suitable support materials must not be toxic to the organism, encourage mycelial attachment and be steam sterilizable. Kirk et al developed a rotating biological contactor to produce lignin peroxidase from an adsorbed fungal film of strain SC26[30]. In this reactor disks of polyethylene were slowly rotated (1 rev min^{-1}) through a cylinder half filled with medium. This reactor produced lignin peroxidase activities comparable to stationary flask cultures (130 U l^{-1}). A 1-liter Biostat M fermenter (designed for tissue cultures) has also been used to produce lignin peroxidases from fungal films[49]. In this

reactor system the wild type strain BKM-F-1767 was immobilized on silicone tubing wrapped around stainless steel rods. Lignin peroxidase activities of 200 U l^{-1} were achieved. Kirkpatrick and Palmer[34] developed a method to produce lignin peroxidase semi-continuously using polyurethane foam immobilized mycelia in a 1 liter shake flask. Very recently, a 10 liter bioreactor system was developed in which the mycelia was immobilized on nylon web carrier[38]. This system produced over 700 U l^{-1} of lignin peroxidase activity in carbon limited medium.

The drawbacks to the scale up of fungal film reactors are that free mycelia foul sensors and clog lines and high culture viscosity hinders oxygen transport. In industrial scale fermentations the immobilization support material must be cleaned following each fermentation.

2.4 Research Objectives

1. The currently used media for lignin peroxidase production are costly. This is due primarily to the use of the expensive buffer 2,2-dimethyl succinate. The first objective of this study is to optimize a low cost defined medium for the production of lignin peroxidase using a less costly buffer.

2. Pellet formation, growth and the effects of agitation on lignin peroxidase production by *P. chrysosporium* are not well understood. Experiments will be conducted to gain an understanding of the mechanism of pellet formation and to determine the consequences of varying the agitation rate.

3. Existing reactor systems which have been developed for the production of lignin peroxidases can not be readily scaled up. The primary objective of this study is to develop a stirred tank reactor system to produce lignin peroxidases from biocatalyst pellets of *P. chrysosporium*.

CHAPTER III: MATERIALS AND METHODS

3.1 Culture Characteristics

All experiments were conducted in nitrogen limited cultures of *Phanerochaete chrysosporium*. Strains BKM-F-1767 (ATCC # 24725) and SC26 (obtained from Dr. T.K.Kirk, U.S.Forest Products Laboratory, Madison, WI) were used. The culture medium used was a defined low nitrogen (nitrogen limited) medium[26] buffered at pH 4.5, with veratryl alcohol (an enzyme inducer), and Tween 80. Cultures were initiated by a conidial inoculum. After 2 days growth in stationary Fernbach flasks, the mycelia were blended and then inoculated into Erlenmeyer flasks (125ml or 2l) containing low nitrogen medium. The cultures were incubated at 39^{o} C. Lignin peroxidase activity as measured by the oxidation of veratryl alcohol to veratryl aldehyde, in the presence of hydrogen peroxide, was generally detected on day 5. Maximum lignin peroxidase enzyme activity occured on day 6 or 7.

3.2 Solution and media preparation

3.2.1 Stock solutions

The following stock solutions were prepared and sterilized as aliquots and stored until use at 4^{o} C.

Deionized water (DI) was used in the preparation of all stock solutions.

Basal medium was prepared by adding 20 gm KH_2PO_4, 5 gm $MgSO_4$ and 1 gm $CaCl_2$ to a final volume of one liter. The solution was sterilized by passing it through a 115 ml 0.45u Nalgene filter sterilizer unit, (Nalge Co., Rochester N.Y., type S CN #245-0045).

Trace element solution was prepared by first dissolving 1.5 g nitrilotriacetate in 800 ml H2O and adjusting the pH to 6.5 using 1.0 M KOH. Then 3.0 gm $MgSO_4 \cdot 7H_2O$, 0.5 gm $MnSO_4$, 1.0 gm NaCl, 0.1 gm $FeSO_4 \cdot 7H_2O$, 0.1 gm $CoCl_2$, 0.1 gm $ZnSO_4 \cdot 7H_2O$, 0.1 gm $CuSO_4$, 10 mg $AlK(SO_4)_2 \cdot 12H_2O$, 10 mg H_3BO_3 and 10 mg $Na_2MoO_4 \cdot 2H_2O$ were added to make a final volume of one liter. The solution had a faint yellow color. The solution was sterilized by passing it through a 115 ml 0.45u Nalgene filter sterilizer unit.

10% Glucose (560 mM) was prepared by dissolving 100 gm glucose in a final volume of one liter. The solution was sterilized by autoclaving at 115 C for 15 min.

Buffer Solutions. Acetate Buffer (0.2 M, pH 4.5) was prepared by adding 5.98 g Na-acetate$\cdot 3H_2O$ and 3.23 ml acetic acid to 800 ml DI water. The pH was checked (should be 4.5) and the solution was brought to 1 liter using DI water. Malate (0.2 M), citrate (0.1 M),

tartarate (0.1 M), succinate (0.1 M) and oxalate (0.1 M) buffers were prepared similarly according to the procedure of Gomori[20]. 2,2-Dimethyl succinate buffer (0.1 M) was prepared using 2,2-dimethyl succinic acid and 1.0 M NaOH to adjust the pH. All buffer solutions were sterilized by autoclaving at 115° C for 20 minutes.

Ammonium Tartrate (8 g/l, 43.4 mM) was prepared by dissolving 8.0 gm ammonium tartrate in 800 ml DI water and then bringing the volume to one liter. The solution was sterilized by autoclaving at 115° C for 20 minutes.

Thiamine (1.0 g/l, 2.96 mM) was prepared by dissolving 0.10 g thiamine (Nutritional Biochemical Corp., Cleveland OH) in a final volume of 100 ml. The solution was sterilized by passing it through a 115 ml Nalgene 0.45μ filter sterilizer unit.

Veratryl alcohol (400 mM) was prepared by adding 6.73 gm of veratryl alcohol (Aldrich Chem. Co. Inc., Milwaukee, WI. #D13,300-0) to DI water to a final volume of 100 ml. The solution was sterilized by passing it through a 115 ml Nalgene 0.45μ filter sterilizer unit.

Tween 80 (1.0 %) was prepared by adding 1.0 gm Tween 80 (Sigma Chem. Co., PO Box 14500, St.Louis, MO, #P-1754) to 80 ml of DI water and bringing the solution to 100ml. The solution was sterilized by autoclaving at 115° C for 20 min.

3.2.2 Media preparation

Media components were combined asceptically using autoclaved graduated cylinders, pipets and Erlenmeyer flasks. Mixtures were brought to the final volume using autoclaved DI water. Once prepared, the media were used immediately. The compositions of the three culture media used in this study are presented in Table 3.1.

Culture medium #1 is a malt extract medium which was used to maintain the fungus and to produce conidia for inoculation of starter cultures. It was prepared by combining the ingredients into 1 liter of deionized water (final volume), adjusting the pH to 4.5 using 0.1 M hydrochloric acid, and autoclaving. The medium was cooled to approximately 50^{o} C and asceptically poured into 20 ml Petri dishes to a depth of approximately 1 cm (15 ml per plate) and allowed to solidify at room temperature for 2 hours before use.

Culture medium #2 was used for producing lignin peroxidase in stationary cultures and for preparing inocula for agitated cultures. Culture medium #3 was used for producing lignin peroxidase in agitated cultures. Stock solutions prepared as described above were used for making media 2 and 3 (Table 3.1).these two media. The concentrations of some media components were varied in optimization experiments.

Table 3.1 Culture Media Compositions

CULTURE MEDIUM #1: Spore Production and Culture Maintenance

Glucose	20 g
Malt Extract	20 g
Peptone	1 g
Agar Bacto	15 g
0.1 M HCl	to pH 4.5
DI water	to make one liter

CULTURE MEDIUM #2: Stationary Starter Cultures

10% Glucose (autoclaved)	100 ml
Ammonium Tartrate (8 g/l stock, autoclaved)	25 ml
Trace elements (filter sterilized)	70 ml
Thiamine (1 g/l stock, filter sterilized)	1 ml
Buffer solution, pH 4.5 (autoclaved)	100 ml*
Veratryl Alcohol (400 mM stock; filter sterilized)	1 ml*
Basal medium (filter sterilized)	100 ml*
Conidia	to 0.1 OD*
DI water (autoclaved)	~700 ml
TOTAL VOLUME	1 l

CULTURE MEDIUM #3: Agitated and STR Cultures

10% Glucose (autoclaved)	100 ml
Ammonium Tartrate (8 g/l stock, autoclaved)	25 ml
Trace elements (filter sterilized)	70 ml
Thiamine (1 g/l stock, filter sterilized)	1 ml
Buffer solution, pH 4.5 (autoclaved)	100 ml*
Veratryl Alcohol (400 mM stock; filter sterilized)	1 ml*
Basal medium (filter sterilized)	100 ml*
Tween 80 (1% stock, autoclaved)	50 ml*
DI water (autoclaved)	~700 ml
TOTAL VOLUME	900 ml

(* Media components whose concentrations were optimized.)

3.3 Culture Inoculation Methods

3.3.1 Spore Inoculum Preparation

Phanerochaete chrysosporium BKM-F-1767 and SC26 were maintained through periodic transfers on malt extract agar plates as previously described[32]. A loop full of conidia from stock cultures maintained on malt extract agar slants (Medium #1) was added to 5 ml of sterilized DI water. Approximately 0.5 ml of this conidial susupension was added to a Petri dish containing 10 ml of solidified culture medium #1. The conidial suspension was spread over the surface of the plate and the plate was incubated for 4 or 5 days at 39^{o} C until white conidia covered the plate. The conidia were collected asceptically by adding 5 ml of sterilized DI water onto a plate and then stirring the surface of the plate using a bent glass rod. The conidial suspension was vacuum filtered, to remove mycelia, through sterilized glass wool into a sterile Erlenmeyer flask to remove mycelia.

The concentration of the conidia in the suspension was determined by preparing a 1:10 dilution of the conidial suspension, placing it on a haemocytometer and counting the conidia using phase contrast microscopy. The optical density of a 1:100 dilution of the conidial suspension was also determined at a wavelength of 650

nm. An optical density of 1.0 cm^{-1} corresponds to approximately 5 X 10^6 conidia ml^{-1}.

This conidial suspension was used to inoculate Fernbach flask starter cultures, used to inoculate additional malt extract plates or stored in 2.0 ml cryogenic vials at -15° C for up to 2 months for future use.

3.3.2 Starter culture preparation

Starter cultures were shallow stationary cultures of P. chrysosporium (culture medium #2) used to create biomass for inoculating agitated cultures (Table 3.2 type 1). Fernbach flasks (3.0 liter volume), plugged with cotton wool wrapped in cheese cloth, were autoclaved along with flasks for media preparation. After autoclaving for 20 minutes at 15 psig and 121° C, 75 ml of culture medium #2 was added to each Fernbach flask using a sterile graduated cylinder. The Fernbach flask was inoculated to an optical density 0.1 at 650 nm using fresh or frozen conidia prepared as described above. The starter cultures were incubated at 39° C and not disturbed for 48 hr (any disturbance invariably caused the mycelia to clump and affected lignin peroxidase activity). After 48 hr, the contents of the Fernbach flasks were blended for five minutes at the highest setting in a Sorval omni-mixer blender, using an

TABLE 3.2 Culture Inoculation and Conditions

Type*	Flask vol.	Medium type	Volume of culture medium	Inoculum size and type	Agitation rate
1	3 l	#2	75.0 ml	0.1 OD spores	stationary
2	125 ml	#3	40.5 ml	4.5 ml mycelia	200 rev min^{-1}
3	2 l	#3	675.0 ml	75.0 ml mycelia	125 rev min^{-1}
4	1 l	#3	500.0 ml	pellets	100 rev min^{-1}

* 1- Fernbach flask starter cultures.

2- Optimization, pellet formation and growth study cultures.

3- Stirred tank reactor pellet production cultures.

4- Stirred tank reactor (STR) cultures.

autoclaved blender cup (500 ml volume). Incomplete blending resulted in clumps and agglomerates which gave large irregular mycelial growths during the pellet formation process. The blender cup was immersed in a mixture of ice and water to reduce heating during the preparation of the blended mycelial suspension. After blending, the contents of the blender cup were checked to be sure that all of the mycelia had been homogenized and that no strings or clumps existed. This suspension consisted of approximately 1.1 g of dry mycelia per liter of mycelial suspension. It was used as a 10 % (v/v) mycelial inoculum for agitated cultures.

3.3.3 Agitated culture inoculation

Agitated shake flask cultures were used for media optimization, and pellet formation and agitation studies (Table 3.2 type 2). About 40.5 ml of culture medium #3 was inoculted with 4.5 ml of the mycelial suspension prepared as described above. The suspension was transferred from the autoclaved blender cup to the flask using a sterilized, cotton plugged 5 ml pipet.

3.3.4 Stirred tank reactor inoculation

The stirred tank reactor was inoculated with pellets which were prepared in a 2 liter shake flask culture (Table 3.2 type 3). Culture medium #3 (675 ml) was inoculated with 75 ml of blended mycelial inoculum

prepared as described above. After incubation at 39° C at an agitation rate of 125 rev min^{-1} for 48 hrs, the formed pellets and medium were transferred asceptically into a 1 liter stirred tank reactor (Table 3.2 type 4).

3.4 Culture conditions

3.4.1 Flask culture conditions

All cultures were incubated at 39° C. Agitated cultures were incubated in a New Brunswick Shaker bath with a 1.25 cm diameter of rotation. The 45 ml cultures were agitated at an agitation rate of 200 rev min^{-1} (and other agitation rates as indicated) and the 750 ml cultures were agitated at a rate of 125 rev min^{-1}. The rate of rotation was measured both by counting the number of cycles during a timed interval and using a phototachometer. The 750 ml cultures were also sometimes incubated in the Labline incubator shaker which has a diameter of rotation of 2.5 cm. Stationary cultures were incubated in a Lunaire environmental chamber, and were not disturbed.

Shake flask cultures were oxygenated by flushing the head space of the culture flask with at least 10 flask volumes of 99.5 % oxygen. Agitated cultures were oxygenated at the time of inoculation and daily thereafter (usually for 1.0 minute at a flowrate between

6 and 10 liters min^{-1}). Stationary Fernbach flask cultures were not oxygenated. A sterile Pasteur pipet plugged with cotton was used to convey the oxygen from the oxygen tank to the flask. The pipet tip was flamed repeatedly to maintain sterility.

3.4.2 STR culture conditions

After 48 hrs growth, pellets grown in a 2 liter Erlenmeyer flask culture were used to inoculate a stirred tank reactor (New Brunswick Sci. Co. model C30, operating volume 500 ml). The stirred tank reactor had a temperature controller and a heating element which were used to control the culture temperature at 39^{o} C Agitation was provided by a stainless steel agitator with four impeller blades mounted on a thick disk, at the bottom of the reactor vessel at a rate of 100 rev min^{-1}. The agitator was powered by a magnetic stirrer beneath the reactor vessel. Humidified, sterile oxygen was metered continuously through a rotameter and added via a gas dispersion tube with a fine glass frit located directly above the agitator. Antifoam (Dow Corning, Silicone Emulsion 10%) was used to reduce culture foaming.

3.5 Assays

Samples for assays were collected asceptically using Pasteur pipettes. Generally a 0.5 ml sample was collected. The samples were centrifuged for 10 minutes in a bench top centrifuge at 8000 rev min^{-1} to remove suspended mycelial and other culture debris. The cultures were reoxygenated after samples were taken. Lignin peroxidase activity samples were generally collected on days 3,4,5,6,7 and 8.

3.5.1 Lignin Peroxidase

The lignin peroxidase activity of a culture filtrate or enzyme preparation was assayed by monitoring the increase in absorbance at 310 nm due to the oxidation of veratryl alcohol (3,4-dimethoxy benzyl alcohol) to veratryl aldehyde (3,4-dimethoxy benzylaldehyde) at room temperature[46]. Veratryl aldehyde absorbs strongly at a wavelength of 310 nm whereas veratryl alcohol does not.

A 500 μl volume of the supernatant was transferred to a test tube along with 400 μl of 125 mM tartarate buffer, adjusted to the enzyme's pH optimum of 2.5, and 50 μl of 40 mM veratryl alcohol. The sample was poured into a 2.0 ml quartz cuvette with a path length of 1.0 cm and the baseline was measured at a wavelength of 310 nm using a UV spectrophotometer (Varian Cary Model #

219). A chart speed of 10 sec cm^{-1} and an optical density (OD) range of 1.0 was used. To begin the enzyme reaction, 50 μl of freshly prepared 5.4 mM hydrogen peroxide was added to the sample (this saturates the available enzyme since the Km for hydrogen peroxide is 80 μM). The increase in absorbance over time at 310 nm was measured for a period of at least one minute. This value was used to quantify the units of enzyme activity.

A unit of lignin peroxidase enzyme activity is defined as the oxidation of one μmole of veratryl alcohol to veratryl aldehyde per minute. Using the molar extinction coefficient of veratryl aldehyde (9300 M^{-1} cm^{-1}) and the absorbance change of the sample, the units of lignin peroxidase activity per liter of sample can be calculated according to the following formula.

$$\text{Lignin Peroxidase (U } l^{-1}\text{) Activity} = \frac{\text{Absorbance change (min}^{-1}\text{) * 1 X 10}^{6}\text{ (umol/mol)}}{9300\ (M^{-1}\ cm^{-1})\ *\ 1.0\ (cm)\ *\ 0.5\ (ml\ sample/ml\ assay)}$$

3.5.2 Protein

The protein concentration of culture filtrates was determined with Coomassie blue according to the method of Bradford[5].

Samples were collected and centrifuged at 8000 rev min^{-1} for 10 minutes in a tabletop centrifuge to remove mycelia. A Biorad protein staining solution (100 μl) was added to 400 μl of sample in a test tube and mixed by shaking. The reaction mixture was then added to a 2.0 ml cuvette and the absorbance at a wavelength of 595 nm was measured using a Varian spectrophotometer. This absorbance value was recorded and compared to a standard calibration curve to estimate the protein concentration of the sample.

A calibration curve was constructed using bovine serum albumin as the standard protein. A BSA solution with a concentration of 1.0 mg ml^{-1} was made and stored at 0^{o} C This solution was diluted ten fold with uninoculated media (or 1 mM Acetate pH 6.0 for FPLC samples) giving a solution with a concentration of 100 μg ml^{-1}. This solution, in turn, was diluted to give solutions with protein concentrations of 0.0, 2.0, 5.0, 10.0, 15.0 and 20.0 μg ml^{-1}. Biorad protein staining solution (200 μl) was added to 800 μl of the standard solutions. The absorbance of the standard solutions was measured at a wavelength of 595 nm and the values were recorded. A least squares linear regression was used to determine the slope and intercept of the calibration curve.

3.5.3 pH measurement

The pH of culture samples was measured using a Corning digital expanded scale pH meter with a Corning general purpose pH electrode (Cat #476531). The pH meter was calibrated before each measurement with standard buffers at pH 4.01 and pH 7.00. Samples were measured at room temperature.

3.5.4 Dry weight measurement

The cell dry weight was measured using an O'haus balance. The mycelia was vacuum filtered through tared 9.0 cm diameter Whatmann GF/C grade filter paper and rinsed with deionized water. The filter cake was dried to a constant weight at 39^{o} C and weighed.

3.5.5 Pellet size measurement

Pellet sizes and size distributions were measured using U.S.A. standard screen sieves with 2.0, 1.4, 1.18, 1.0, 0.85, 0.5 and 0.212 mm opening sizes. Samples were added directly to stacked sieve trays and rinsed with deionized water to move the pellets over the screen openings. The number of pellets in each tray from a sample of known volume was counted. During the first 24 hours pellets were too small to be counted using sieve trays so a microscope and a haemacytometer was used to count pellets.

3.6 Protein characterization by FPLC

The culture filtrate was collected and generally concentrated. An Amicon ultra concentrator cell (10,000 MW cut-off) was used at a temperature of 4.0° C The filter was prepared by stirring DI water over it for 30 minutes. The extracellular fluid was concentrated 100 fold using Nitrogen at 60 psi. After concentration, the fluid was dialysed against 5 mM potassium phosphate buffer (pH 6.5) overnight. Usually 4 liters of potassium phosphate solution was used to dialyze 40 ml of concentrated extracellular fluid. The concentrated fluid was stored at 4° C until used. Samples generally lost approximately half of their activity per month when stored in this manner.

The concentrated and dialyzed extracellular fluid was applied to a Mono Q column (Pharmacia, Uppsala, Sweden) using a gradient of acetate buffer (pH 6.0) from 10 mM to 1.0 M°. Thirty-eight ml of dialyzed extracellular fluid was purified using injections of 2 ml. The sample was loaded with the acetate buffer gradient and eluted for 40 minutes.

CHAPTER IV: RESULTS

4.1 Media optimization

The culture media were optimized with regard to the strain and buffer used, the inoculum size, and the concentrations of various media components. Low cost acetate buffered media was found to give improved enzyme activity. All experiments were conducted in 125 ml Erlenmeyer flasks using culture medium #3 as described in Materials and Methods.

4.1.1 Strain selection

Phanerochaete chrysosporium strains SC26 and BKM-F-1767 were compared based on lignin peroxidase activity. Strain BKM-F-1767 was found to give greater maximum lignin peroxidase activity (Figure 4.1). Furthermore, strain BKM-F-1767 formed pellets more readily, was easier to culture and more consistently produced the enzyme than strain SC26. Hence, strain BKM-F-1767 was used in all subsequent experiments.

4.1.2 Acetate buffer selection

A comparison was made between the widely used 2,2-dimethylsuccinate (DMS) buffer and a number of less costly buffers, based on lignin peroxidase activity in shake flask cultures. The data are from two separate experimental runs. Acetate buffer (20 mM, pH 4.5)

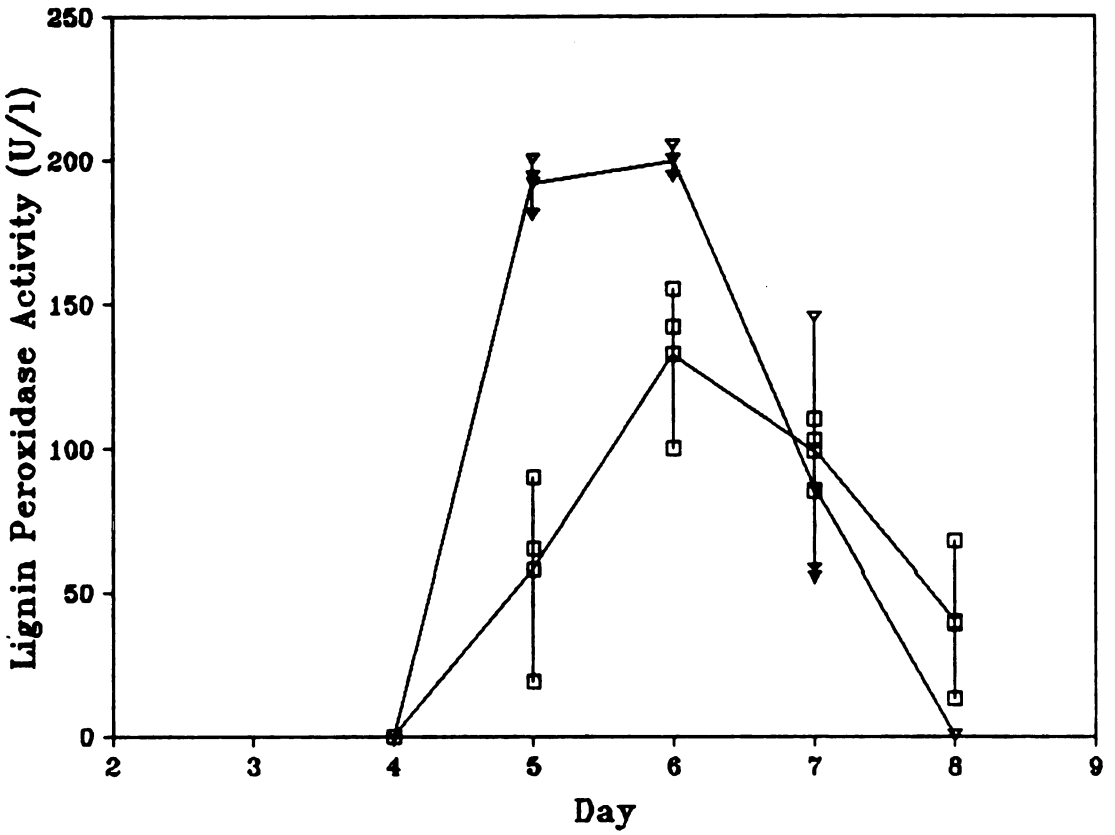


Figure 4.1 Comparison of P. chrysosporium strains SC26 (□) and BKM-F-1767 (▽) for the production of lignin peroxidase.

produced greater maximum lignin peroxidase activity compared to DMS buffer (10 mM) on both occasions (Figure 4.2). The maximum activity ocurred later in acetate buffered cultures than in DMS buffered cultures. Tartrate (10 mM) and oxalate (10 mM) buffered cultures produced no detectable lignin peroxidase activity. Citrate (10 mM) and succinate (10 mM) buffered cultures gave slightly less average activities than DMS buffered cultures. The data of Jager et al[26] is presented as a comparison.

In DMS buffered cultures the culture pH was more stable than in other cultures. In DMS buffered cultures pH rose only slightly after 8 days whereas the culture pH rose approximately 1.0 pH unit after 8 days in all other cultures. This may be due to the metabolism of the acid component of some of the buffers causing the media pH to rise.

4.1.3 Optimization of spore inoculum concentration

The amount of conidia added to Fernbach flasks appeared to affect lignin peroxidase activity (Figure 4.3). Maximal lignin peroxidase activities were obtained when conidial inocula of 2.5×10^5 to 5.0×10^5 conidia ml^{-1} were used. These conidial (spore) concentrations correspond to optical density values of 0.05 and 0.1 OD respectively. The concentration of conidia used in the following experiments was 5.0×10^5 spores ml^{-1}.

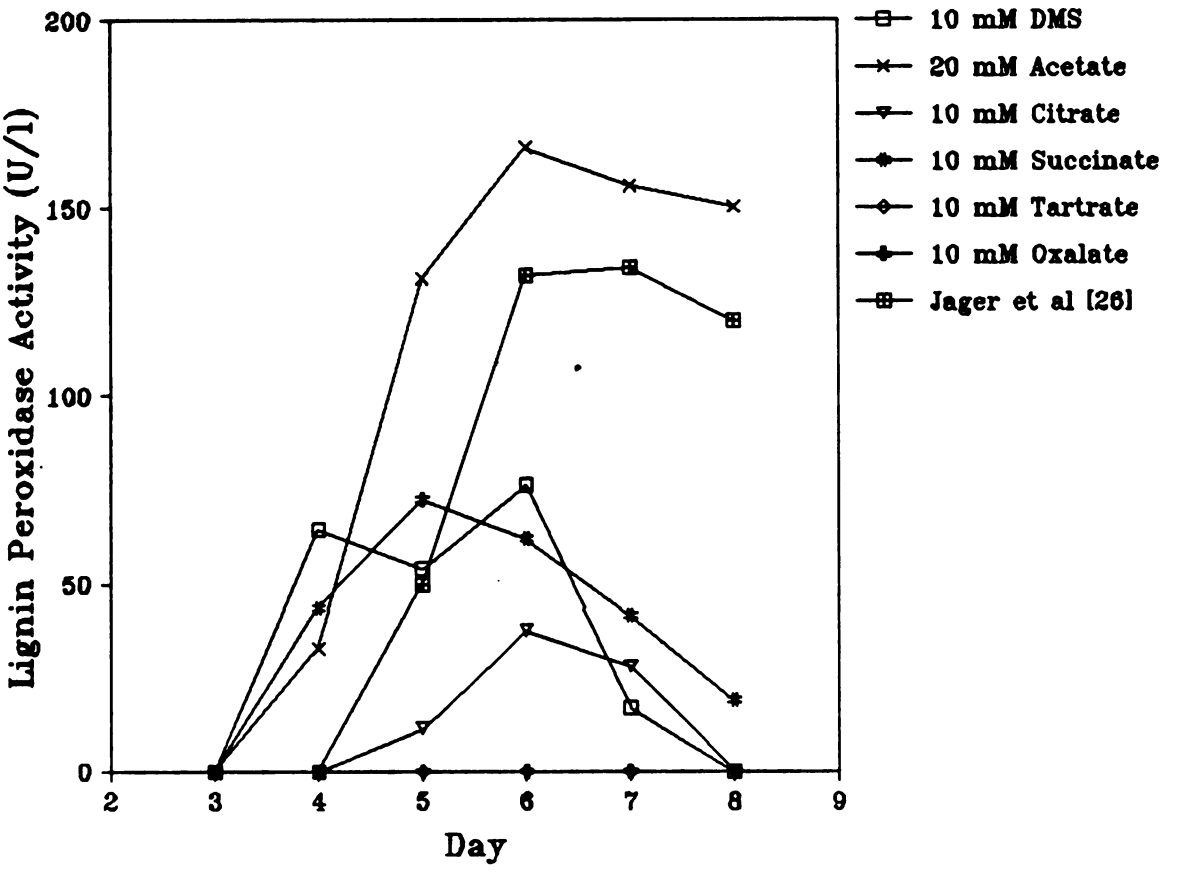


Figure 4.2 Comparison of various buffers for the production of lignin peroxidase.

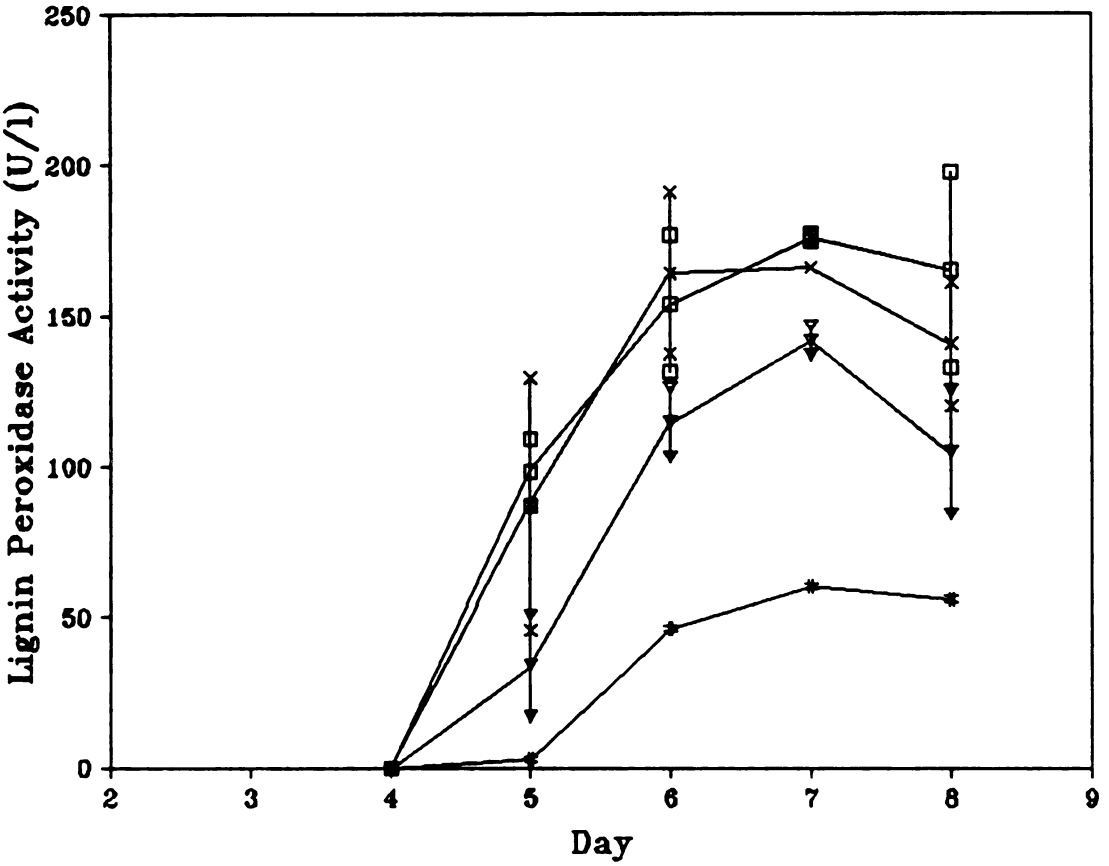


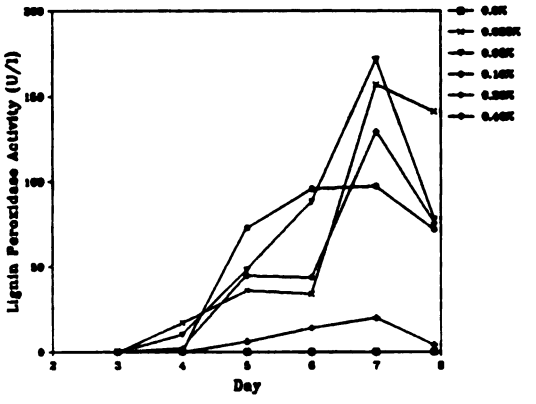
Figure 4.3 Time course of lignin peroxidase activity for four different spore inoculum concentrations.
(□) 2.5×10^5 spores ml^{-1}
(x) 5.0×10^5 spores ml^{-1}
(▽) 1.5×10^6 spores ml^{-1}
(#) 3.0×10^6 spores ml^{-1}

4.1.4 Optimization of Tween 80, veratryl alcohol and basal medium concentrations

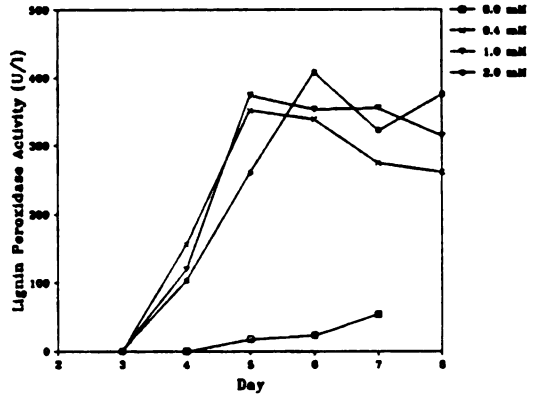
The concentrations of the media components Tween 80, veratryl alcohol and basal medium which support optimal lignin peroxidase activity in acetate buffered cultures were determined. The culture conditions of Jager et al[26] were used as a starting point for media optimization experiments except that acetate buffer (20 mM, pH 4.5) was substituted for DMS buffer.

Tween 80, which allows lignin peroxidase production in agitated cultures, gives maximal lignin peroxidase activity at a concentration of 0.05 wt% in DMS buffered cultures.[26] In acetate buffered cultures, as in DMS buffered cultures, no activity was detected in the absence of Tween 80 (Figure 4.4a) and optimal enzyme levels were observed at a Tween 80 concentration of 0.05 wt%. Lignin peroxidase activity decreased when the Tween 80 concentration was increased to 0.4 wt%, but the decreases in enzyme activity observed with 0.2% and 0.025% concentrations of Tween 80 were not significant.

Veratryl alcohol is known to be an inducer of lignin peroxidase activity[16,35]. In the absence of veratryl alcohol, little or no activity was observed whereas optimal activity was seen at 0.4 mM veratryl alcohol concentration. No significant increase in lignin peroxidase activity was observed for veratryl alcohol



A



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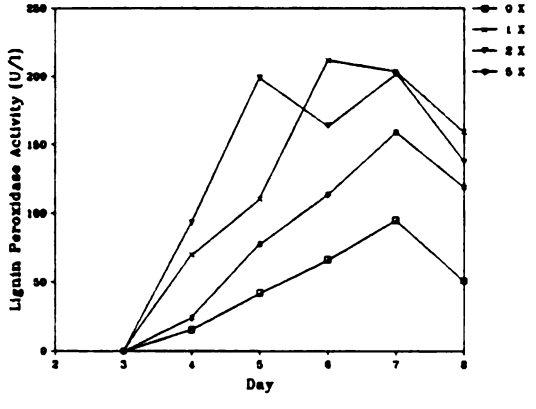


C

Figure 4.4 Time course of lignin peroxidase activity as affected by: Tween 80 concentration (A), veratryl alcohol concentration (B) and basal medium concentration (C).

concentrations above 0.4 mM (Figure 4.4b).

The effect of basal medium concentration on lignin peroxidase production is presented in Figure 4.4c. Lignin peroxidase activity was obtained even in cultures to which no basal medium was added. Maximal activity occured in cultures to which 1 or 2 times the normal amount of basal medium was added.

The optimized media used in all subsequent experiments had the composition indicated in Table 4.1.

4.1.5 Culture characteristics using optimized media

The time course of pH, dry cell weight and lignin peroxidase activity were determined for cultures grown in the optimized media described above (Figure 4.5).

The cell dry weight increased dramatically during the first 24 hours after incubation. After culture nitrogen was depleted (24 to 48 hrs after inoculation), cell dry weight continued to increase through the sixth day. This was probably due to the formation of polysaccharides from the excess glucose available in the medium (see Kirk et al[30]).

The culture pH rose steadily throughout the fermentation. After eight days, culture pH had risen from 4.5 to more than 5.6. This rise was not observed in DMS buffered cultures.

The lignin peroxidase activity of cultures grown in the optimized media varied tremendously from run to run.

Table 4.1 Optimized culture media for lignin peroxidase production.

Optimized Culture Medium #2

10% Glucose (autoclaved)	100 ml
Ammonium Tartrate (8 g/l stock, autoclaved)	25 ml
Trace elements (filter sterilized)	70 ml
Thiamine (1 g/l stock, filter sterilized)	1 ml
Acetate Buffer solution, (0.2 M, pH 4.5)	100 ml
Veratryl Alcohol (400 mM stock; filter sterilized)	1 ml
Basal medium (filter sterilized)	100 ml
Conidia	5.0×10^5 spores/ml
DI water (autoclaved)	~700 ml
TOTAL VOLUME	**1 l**

Optimized Culture Medium #3

10% Glucose (autoclaved)	100 ml
Ammonium Tartrate (8 g/l stock, autoclaved)	25 ml
Trace elements (filter sterilized)	70 ml
Thiamine (1 g/l stock, filter sterilized)	1 ml
Acetate Buffer solution, (0.2 M, pH 4.5)	100 ml
Veratryl Alcohol (400 mM stock; filter sterilized)	1 ml
Basal medium (filter sterilized)	100 ml
Tween 80 (1% stock, autoclaved)	50 ml
DI water (autoclaved)	~700 ml
TOTAL VOLUME	**900 ml**

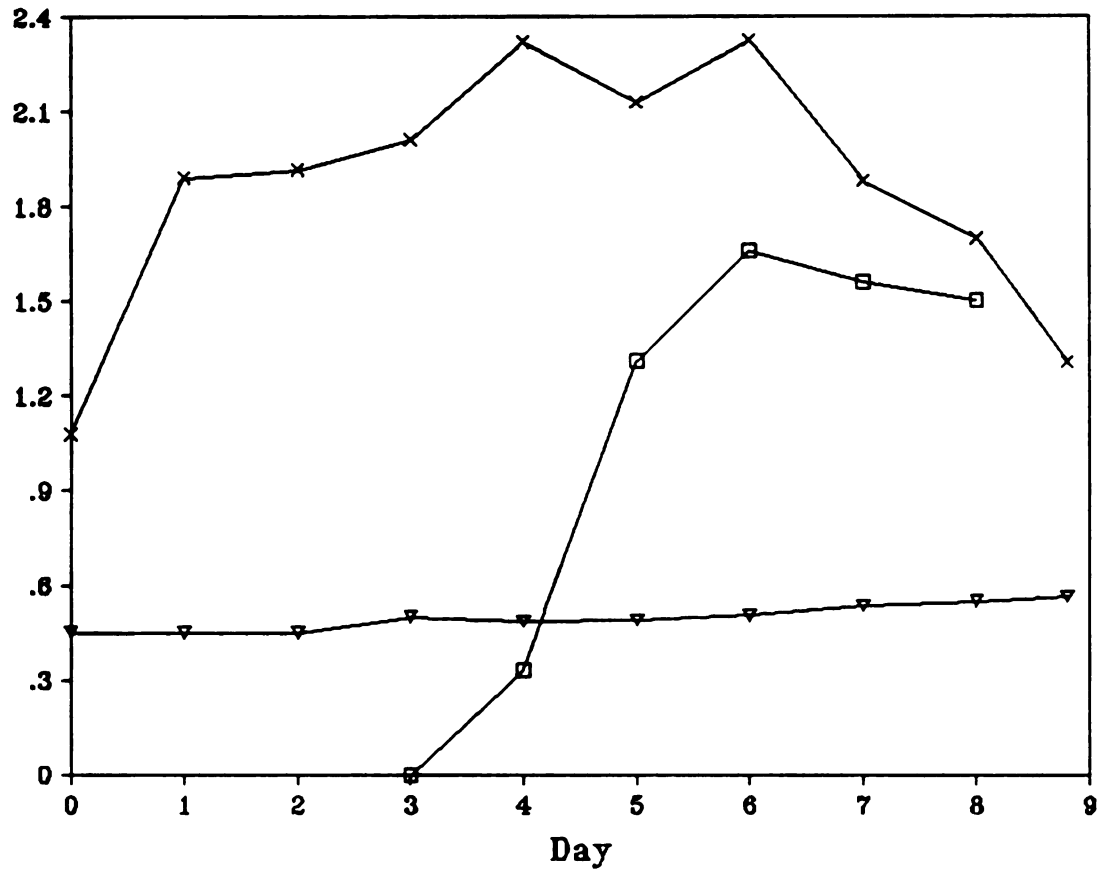


Figure 4.5 Characteristics of agitated cultures grown in optimized media.
(□) Lignin peroxidase activity (U l^{-1} * 10^{-2})
(x) Dry cell weight (g l^{-1})
(▽) pH * 10^{-1}

In some experimental runs no activity at all was detected. Variation within the same experimental run however, was less significant. The average lignin peroxidase activity from over twenty cultures grown in the optimized media is plotted in Figure 4.5. For each point the standard deviation of the average lignin peroxidase activity generally exceeded half the value of the mean. Cultures in which lignin peroxidase activity was not detected were not included. A model was proposed for the growth, substrate utilization and lignin peroxidase enzyme production. It is presented in Appendix A.

4.2 Biocatalyst Pellets

Agitation of submerged cultures of P. chrysosporium leads to pellet formation. Agitation has also been reported to inhibit ligninolytic activity in these cultures. The pellet formation mechanism and the effects of agitation on lignin peroxidase producing cultures of P. chrysosporium have not previously been studied.

4.2.1 Pellet formation mechanism

To study the mechanism of pellet formation, the number of mycelial fragments in the inoculated Erlenmeyer flasks was determined as a function of time (see methods). A semi-log plot of the number of hyphal fragments vs. time is presented for cultures in both 125 ml and 2 l Erlenmeyer flasks in Figure 4.6. The hyphal fragments which numbered approximately 1×10^7 per liter initially, aggregated to between 2.0 and 4.0×10^4 pellets per liter in less than 8 hours. This number remained constant throughout the rest of the fermentation. The number of hyphal fragments per pellet was between 2×10^2 and 6×10^3, indicating a coagulative mechanism for pellet formation. This conclusion was further supported by observations of pellet formation using a light microscope (Figure 4.7). Once formed the pellets remained intact throughout the fermentation and did not break apart even at high agitation rates.

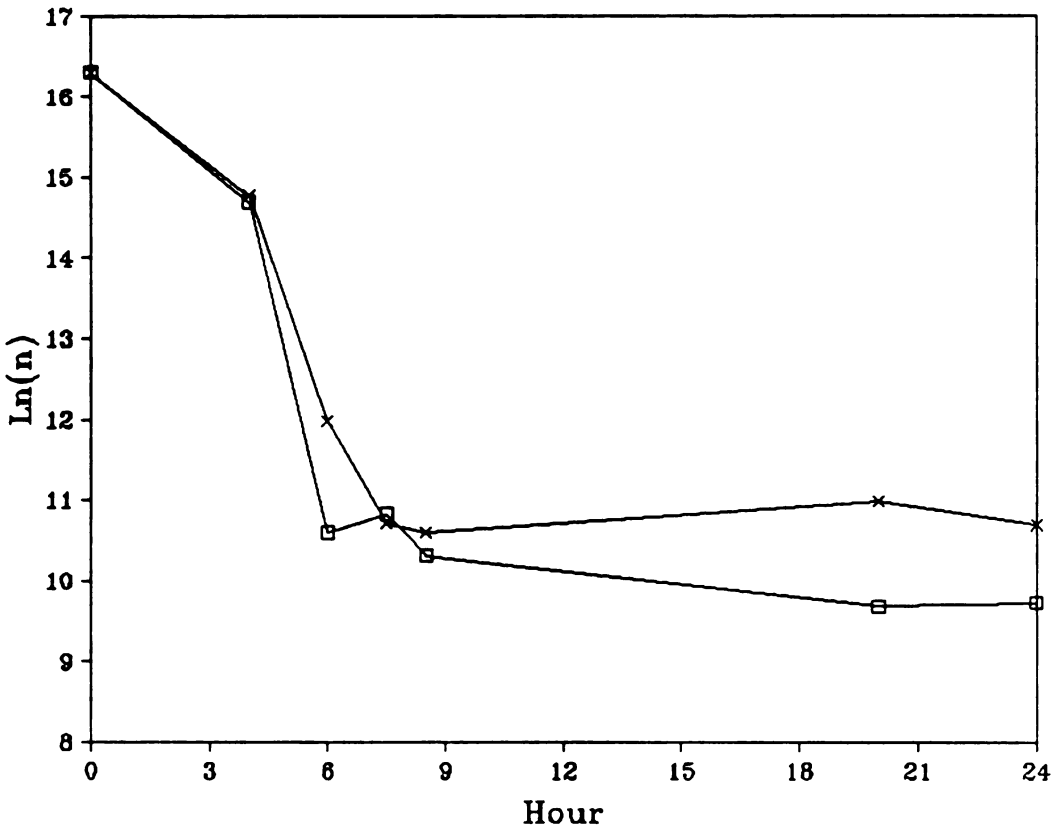


Figure 4.6 Semi-log plot of the number of hyphal agglomerations (n) as a function of time;
(x) 2 liter flask culture,
(□) 125 ml flask culture.

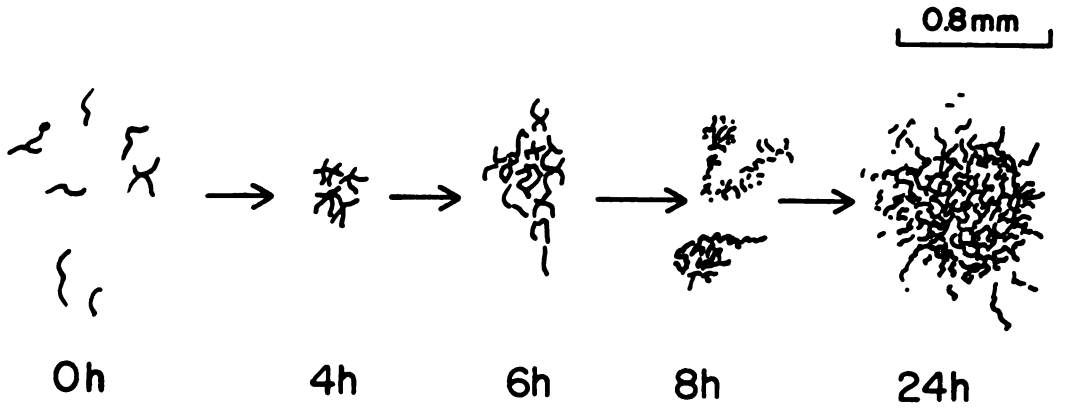


Figure 4.7 Schematic representation of the mechanism of pellet formation by P. chrysosporium observed using a light microscope (not to scale).

4.2.2 Pellet growth

After aggregation ceased, the pellet size increased due to cellular growth. The Sauter mean diameter[25] of the pellets was calculated according to the formula:

$$\text{Sauter Mean Diameter} = D_{32} = (\sum_{1}^{n} d^3/d^2)/n \qquad (1)$$

where D_{32} is the Sauter mean diameter, d is the pellet diameter as measured by screen sieves and n is the number of pellets (Figure 4.8). The Sauter mean diameter averages particle size based on the ratio of volume to surface area, which is appropriate for catalyst applications. The average pellet size was 1.29 ± .21 mm after eight days of growth. The standard deviation, which indicates the range of pellet sizes, indicates that pellet size is fairly uniform.

A photo-micrograph of an 8 day old mycelial pellet (Figure 4.9) reveals that pellets consisted of two regions; a dense inner region consisting of round structures (arthrospores) and branched hyphae packed closely together, and a less dense outer region consisting primarily of long hyphal filaments.

4.2.3 Agitation Effects

It has been reported previously that increased agitation has a negative effect on lignin peroxidase enzyme production in agitated cultures of P.

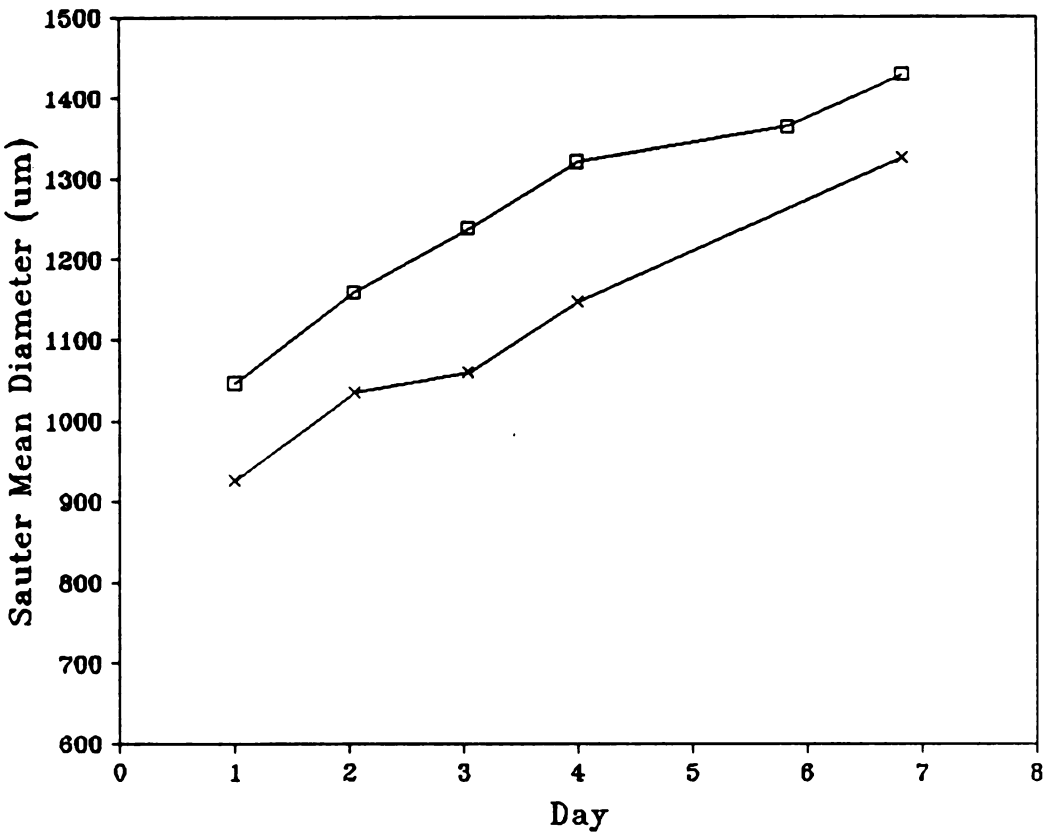


Figure 4.8 P. chrysosporium pellet size as a function of culture age. Pellet size is expressed as the Sauter mean diameter.
(x) STR culture,
(□) 125 ml flask culture.

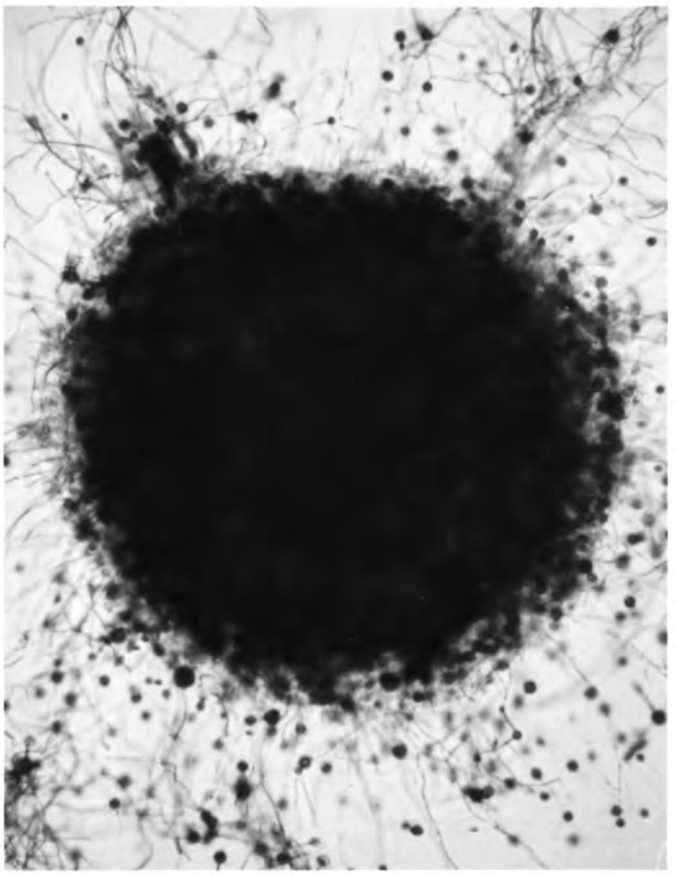


Figure 4.9 Photo micrograph of an eight day old mycelial pellet of P. chrysosporium.

chrysosporium[45]. In this study the size of pellets, the number of pellets produced, and the expression of lignin peroxidase were all affected by the rate of agitation.

The time course of lignin peroxidase activity for three agitation rates is presented in Figure 4.10. Cultures agitated at a rate of 260 rev min^{-1} had significantly less lignin peroxidase activity compared to flasks agitated at 200 and 150 rev min^{-1}. Some of the properties of the biocatalyst pellets produced at three different agitation rates were determined (Table 4.2). These data (Table 4.2) show that higher agitation rates result in an increase in extracellular protein, a decrease in pellet size and an increase in the pellet concentration. Pellet size was significantly affected by the agitation rate. Photographs of pellets formed in shake flask cultures agitated at three different agitation rates are presented in Figure 4.11. At a low agitation rate (<75 rev min^{-1}), a single mycelial pellet is formed. At higher agitation rates pellet size decreases and the number of pellets increases as the agitation rate increases.

Shear rate often relates to particle size in liquid-liquid and solid-liquid dispersions. A characteristic shear rate experienced by the liquid phase in a rotating flask is given by rotation rate (RPM) times the diameter

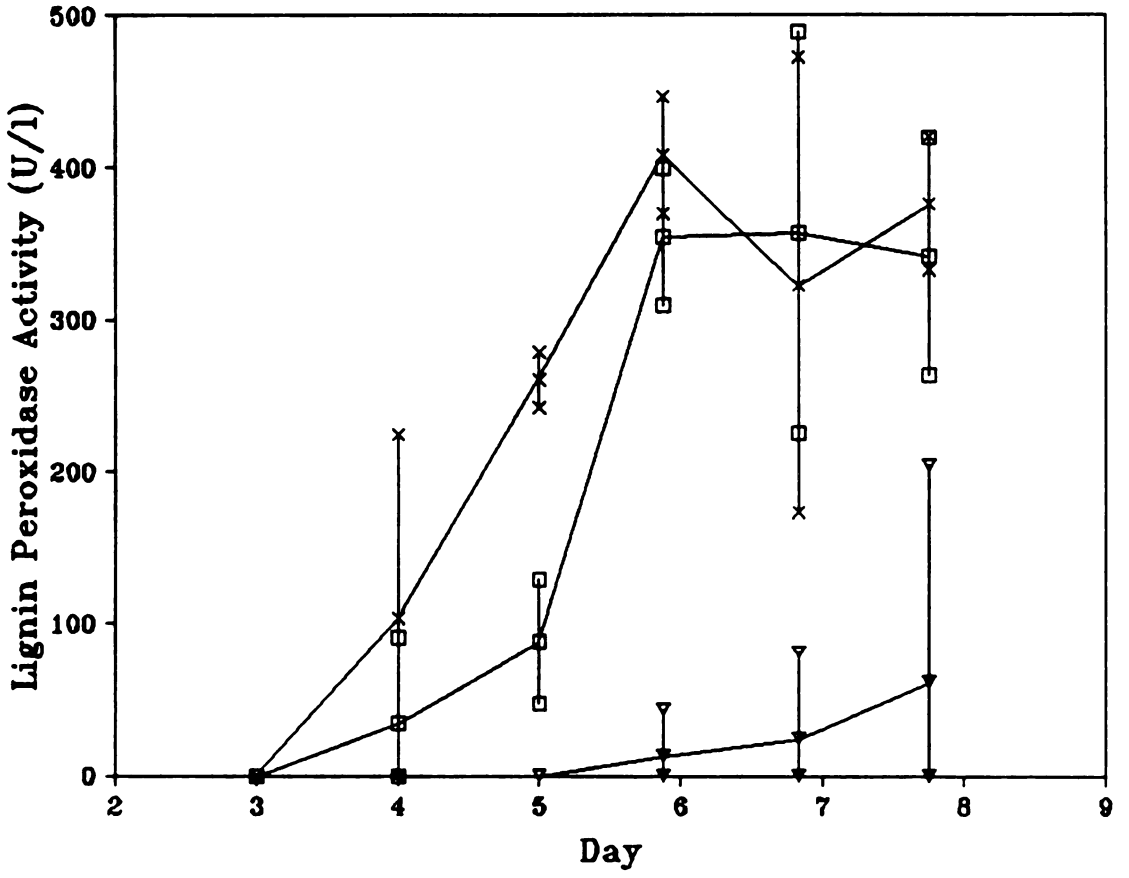
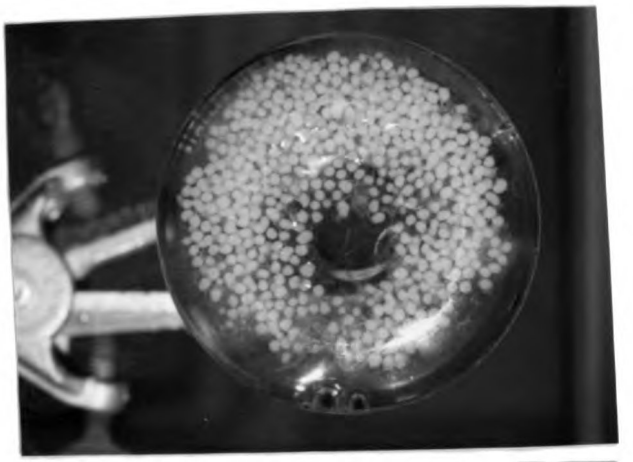


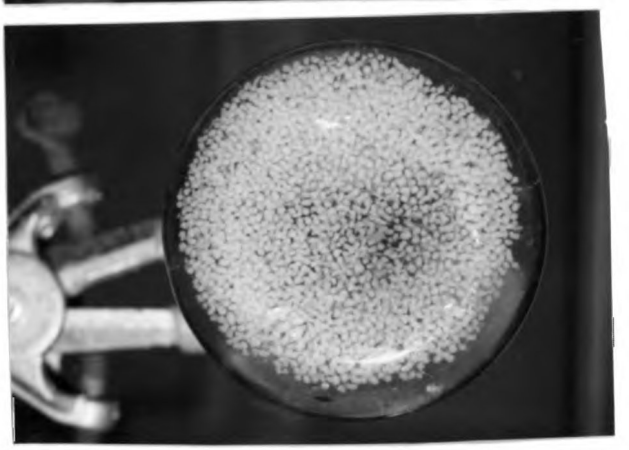
Figure 4.10 Effect of the agitation rate on lignin peroxidase activity.
(□) 150 rev min^{-1}
(x) 200 rev min^{-1}
(▽) 260 rev min^{-1}

TABLE 4.2 Culture parameters for 8 day old cultures of *P. chrysosporium* at three agitation rates.

RPM	Lignin peroxidase activity(U/l)	Extra-Cellular Protein(ug/ml)	Mean Pellet Diameter(mm)	Total Number of Pellets per l
150	341 ± 73	9.2 ± 1.0	1.83 ± .32	10000 ± 7000
200	376 ± 33	10.9 ± 1.4	1.29 ± .21	32600 ± 3700
260	62 ±100	11.9 ± 2.8	1.27 ± .23	36500 ± 8500



150 rev min^{-1}



200 rev min^{-1}

260 rev min^{-1}

Figure 4.11 Photographs of mycelial pellets of P. chrysosporium formed at three different agitation rates.

of the flask at the liquid level (D), (D)*(RPM). The shear rate in a shake flask can be altered by varying either of these parameters. The Sauter mean pellet diameter for cultures agitated at seven different agitation rates from 125 to 300 rev min^{-1} was measured. The data was plotted using a log-log scale (Figure 4.12), and a straight line (equation 2) with a correlation coefficient of 0.70 was obtained.

$$D_{32} = 2.1 \times 10^{4} RPM^{-0.52} \qquad (2)$$

This equation, relating pellet size to the agitation rate in shake flasks, indicates that pellet size may be inversely proportional to the square root of the agitation rate.

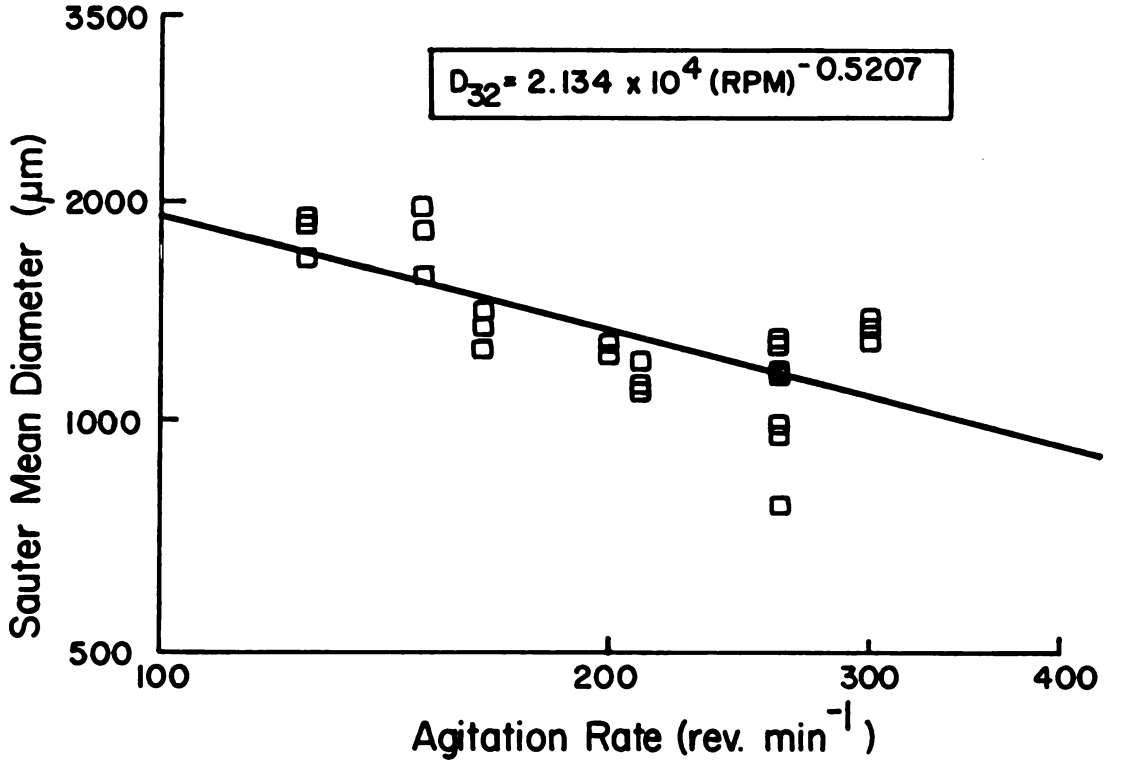


Figure 4.12 Correlation of the Sauter mean pellet diameter (D_{32}) and the agitation rate (RPM).

4.3 Lignin peroxidase production in a stirred tank reactor (STR)

Two reactor systems were examined for the production of lignin peroxidase by P. chrysosporium; A trickle bed reactor with an immobilized mycelia (see Appendix C) and a stirred tank reactor with mycelial pellets.

4.3.1 Development of operating conditions for a stirred tank reactor system

A bench scale stirred tank reactor system was developed for the production of lignin peroxidase (Figure 4.13). The reactor system produced lignin peroxidase activities comparable to shake flask cultures. Several factors were critical to obtaining lignin peroxidase activity in the stirred tank reactor (STR); biocatalyst pellet formation, reactor mixing, oxygen dispersion, mycelial adsorption to reactor surfaces and foaming.

Two methods for making pellet biocatalysts were used: inoculation of blended mycelia directly into the STR and transfer of pellets to the STR from 2 l shake flask cultures. Inoculation of blended mycelia to the STR did not give detectable lignin peroxidase activities. Some of the mycelia formed pellets and some adhered to reactor surfaces, forming large, irregular agglomerates.

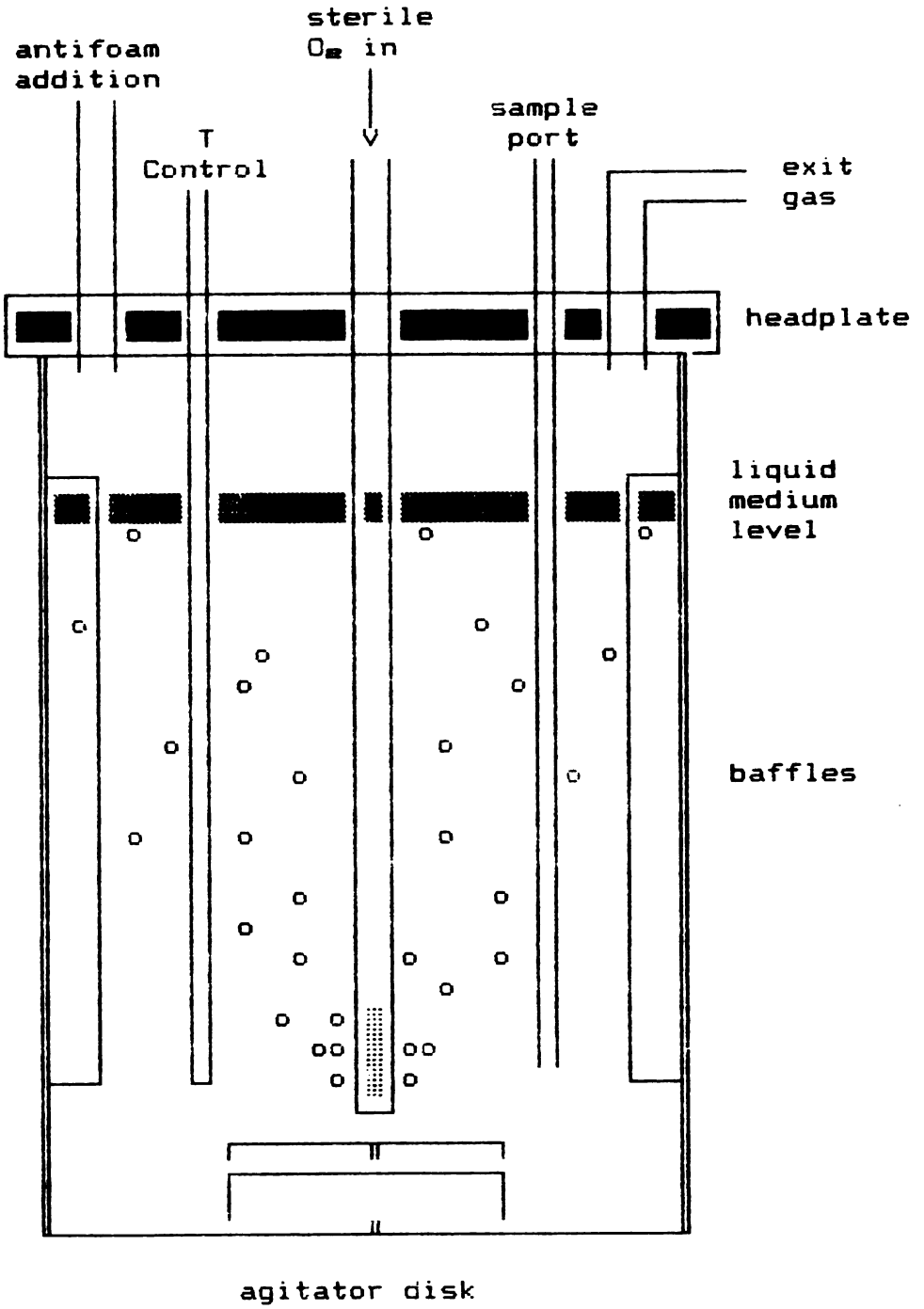


Figure 4.13 Stirred tank reactor system for the production of lignin peroxidase enzymes.

The pellets formed under these conditions failed to produce detectable amounts of lignin peroxidase. Removing the reactor baffles reduced the adsorption of mycelia and the amount of irregular agglomerates, but gave large pellets which did not produce lignin peroxidase in detectable amounts. When 2 day-old pellets formed in a 2 l shake flask culture were used to inoculate the STR lignin peroxidases at times and levels similar to shake flask cultures were produced. The agitation rate was adjusted to 100 rev min^{-1} to provide adequate suspension of the pellet biocatalysts . High agitation rates in the STR (200 rev min^{-1}) resulted in no measurable lignin peroxidase production.

Oxygen transfer affected lignin peroxidase activity in the STR. In shake flask cultures, oxygen is transferred readily into the culture medium due to agitation and a high surface to volume ratio. In stirred tank reactors oxygen transfer must be accomplished by sparging. In the bench scale STR, oxygen was introduced above the agitator using a fine glass frit at a rate of 0.5 liter min^{-1} (1.0 v/v/min). When a glass tube was used as a sparger, no lignin peroxidase activity was detected.

The small bubbles produced by the frit tube created foam which did not disperse easily. At an oxygen flowrate of 0.2 liter min^{-1}, foam overflowed the reactor

while at 0.05 liter min^{-1} foaming was roughly static. below 0.05 liter min^{-1} little foam was created. To enable STR operation without foaming silicone antifoam (Dow Corning FG-10 10% emulsion) was added and an oxygen flowrate of 0.5 liter min^{-1} (1.0 v/v/min) was maintained. At these levels, antifoam did not inhibit culture growth or enzyme activity.

4.3.2 Lignin peroxidase production by the STR system

A typical plot of the time course of lignin peroxidase activity and extracellular protein produced in an STR (agitated at 100 rev min^{-1}, oxygenated at 0.5 liter min^{-1} and with 5 ml of silicone antifoam emulsion added) is presented in Figure 4.14. One day after pellet biocatalyst transfer, the extracellular fluid became brownish yellow in color. On the third day the pellets turned a dark brown color (which is generally associated with lignin peroxidase production) and lignin peroxidase activity was detected. Lignin peroxidase activity peaked on the fourth day. Six days after transfer, the pellets began to collect around the reactor components and coagulate in the top third of the operating volume. This coagulation of pellets corresponded with the appearance of long filaments growing from the pellets.

4.3.3 Product recovery and characterization

An FPLC profile of the heme proteins in 38 ml of extracellular fluid from an STR culture four days

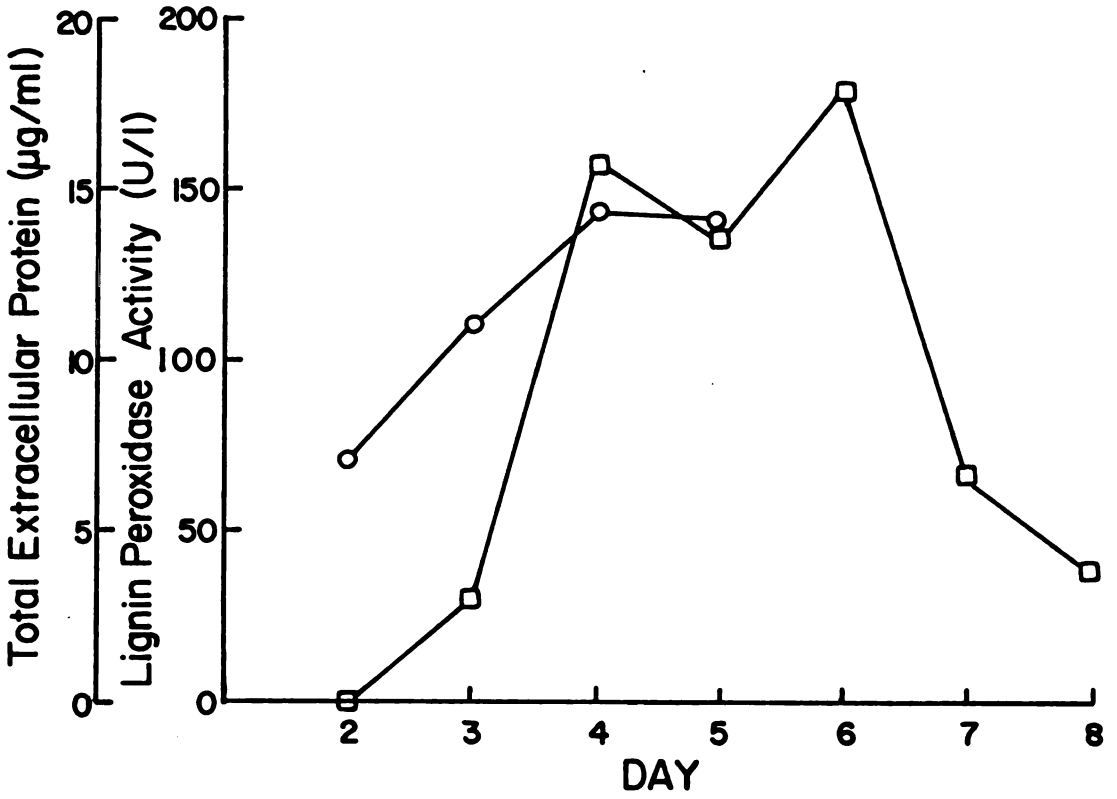


Figure 4.14 Lignin peroxidase activity (□) and total extracellular protein concentration (O) in a stirred tank reactor system.

after inoculation, was somewhat similar to the protein profile previously described by Kirk et al[17] (Figure 4.15). Peaks at an elution volume of 9, 15, 17, 21 and 26.5 ml all had lignin peroxidase activity. The peaks at elution volume 12 to 14 ml (probably manganese peroxidases which correspond to the H3 and H4 peaks in Kirk's profile)[17] were somewhat larger in comparison. Also the peak at an elution volume of 26 ml, which corresponds to the H8 protein peak in Kirk's profile, was reduced in size.

The total protein content, lignin peroxidase activity and specific activity versus the elution volume is presented in Appendix C. The protein peaks corresponding to H2 and H8 peaks described by Kirk et al had highest specific lignin peroxidase activities.

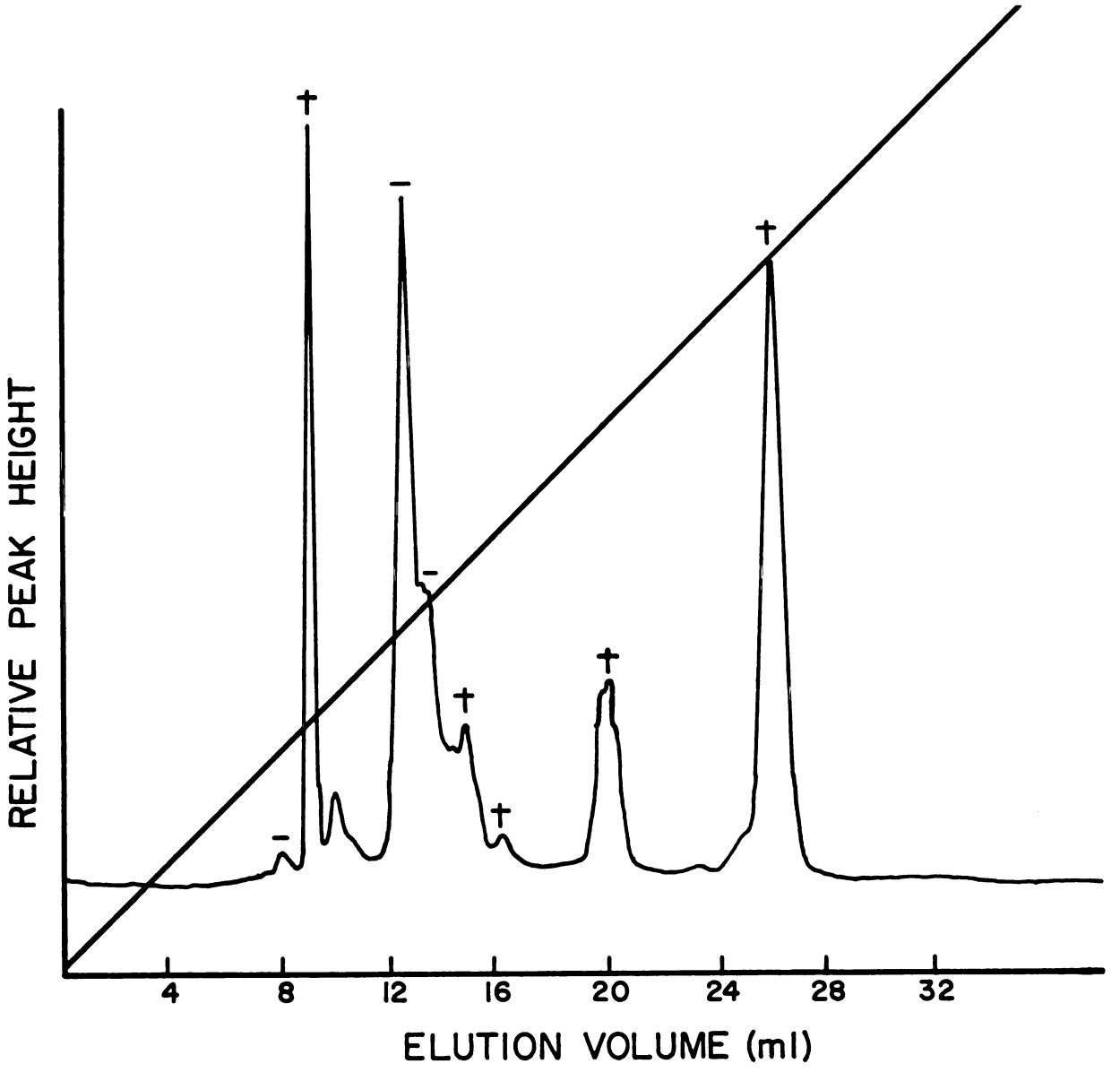


Figure 4.15 FPLC profile of heme-proteins in six day old stirred tank reactor system extracellular fluid, four days after inoculation.

CHAPTER V: DISCUSSION

5.1 Significance

In this study the mechanism and kinetics of pellet formation in agitated submerged cultures of P. chrysosporium has been determined. Agitation has been found to have a pronounced effect on lignin peroxidase producing cultures of P. chrysosporium. A low cost acetate buffered media has been optimized for lignin peroxidase production. For the first time a STR system for the production of lignin peroxidases has been described. This reactor system utilized optimized media and biocatalyst pellets formed in a shaker flask. Lignin peroxidase was produced at concentrations comparable to that in shake flask cultures.

5.2 Comparison with other Systems

A comparison of this stirred tank reactor system with fungal film and pellet biocatalyst systems previously developed is presented in Table 5.1. During the preparation of this thesis two articles appeared in the literature in which immobilized fungal films were used to obtain high lignin peroxidase activities (see Table 5.1)[38,48]. Another fungal film system previously developed by Kirk et al[30] is presented. In these systems, nylon, silicone or polyethylene immobilization support materials were used to which the growing mycelia

TABLE 5.1 Comparison of the characteristics of various reactor systems for the production of lignin peroxidases by *P. chrysosporium*

	Volume (l)	Maximum Activity (U/l)	Strain used	Nutrient limitation
Fungal Film Bioreactors				
RBC Polyethylene disc immobilized Kirk et al[30] 1986	2.5	130	SC26	Nitrogen
Biostat E Silicone tube immobilized Willer-shausen et al[49] 1987	1.0	232	BKM	Nitrogen
Biostat M Nylon-web immobilized S.Linko[38] 1988	7.6	730	BKM	Carbon
Pellet Biocatalyst Reactors				
Airlift Mycelial pellets Leisola et al[37] 1986	1.5	60	BKM	Nitrogen
Column reactor Y.Y.Linko et al[39] Mycelial pellets 1987	0.078	172	BKM	Carbon
STR Mycelial pellets (this work) 1988	0.5	180	BKM	Nitrogen

adsorbed. In fungal film reactors the mycelia present in the culture fluid makes product separation difficult and frequently clogs pumps lines and filters. Cleaning of mycelia from immobilization support materials and sensor fouling also pose problems. Pellet biocatalyst reactors should offer an advantage over fungal film reactors for the large scale production of lignin peroxidase.

The pellet biocatalyst reactor system developed in this work compares favorably to other small scale pellet biocatalyst reactors. The airlift reactor and developed by Leisola et al[37] used a spore inoculum and produced low enzyme activities (60 U l^{-1}). The column reactor developed by Linko et al[39] was a very small volume reactor (78 ml) in which continuous production of lignin peroxidases was demonstrated. Lignin peroxidase activities above 150 U l^{-1} were observed in this reactor.

5.3 Future Directions

Many obstacles remain for further scale-up of the stirred tank reactor system for lignin peroxidase production. It is especially important to devise methods for producing pellets in the stirred tank itself and for improving the consistency of enzyme production consistency. Methods for efficient product recovery and separation must also be developed.

The inoculum scale-up procedure in this study

consisted of inoculating a stationary culture with conidia, blending the mycelial mat which forms after 48 hours, and using the blended mycelia as a ten percent inoculum for 750 ml of medium in a 2 liter Erlenmeyer flask. This procedure gives approximately 75 ml of blended mycelial inoculum per each 3 liter (flask volume) stationary culture. Using this same procedure to create an inoculum for a ten liter fermenter would require over 15 stationary cultures or 45 for a 30 liter fermentor. This is impractical for large scale production. An improved method of producing biomass for inoculation must be developed. This might involve using an agitated starter culture. Using agitated starter cultures a 15 fold increase in biomass could be achieved in the same total flask volume. Other investigators have used blended mycelial pellets as inocula for scale-up[12]. Thus it may be possible to blend growing pellets (<48 hr old) to give adequate biomass for large scale inoculations.

Pellet formation in the 500 ml stirred tank reactor system was accomplished using large 2 liter shake flask cultures to produce pellets for inoculation into the STR. At a larger scale this procedure would also be impractical. If this procedure were used, the culture volume in the shake flask cultures used to produce pellets would equal the volume of the pilot scale

reactor. Therefore for a large scale fermentor methods of producing pellets in the reactor itself must be developed. The primary problem with producing pellets in the small STR was that the growing mycelia adsorbed to reactor components and to the agitator. It may be that moving to a larger volume, free of protrusions and uneven surfaces to which mycelia can adsorb, would minimize mycelial adsorption and allow pellet formation in the reactor itself. Intrestingly, in the bench scale STR, removing the baffles greatly increased the amount of mycelia which formed pellets when blended mycelia was used as the inoculum.

Finally, the consistency with which reactor systems produce lignin peroxidase must be improved. Although the bench scale STR did produce lignin peroxidase on three separate occasions, on approximately 15 attempts, no detectable enzyme was produced. These failures were in some cases attributable to operational errors however generally they were not. More understanding of the process of enzyme production and excretion is necessary. By more closely monitoring and controlling culture parameters such as dissolved oxygen, glucose and nitrogen depletion, pH, agitation rate and biomass production the consistency of enzyme production may be improved.

APPENDIX

Appendix A

Proposed model for the production of lignin peroxidases by P. chrysosporium

Model for Enzyme Production

A kinetic model to describe the growth, substrate utilization and lignin peroxidase production by cultures of P. chrysosporium has not previously been developed. Detailed structured models have been developed for the production of anti-biotics by filamentous fungi during secondary metabolism[3,43]. A model was developed to account for substrate utilization, biomass growth and lignin peroxidase production in submerged cultures of P. chrysosporium. In this model the data for biomass growth, lignin peroxidase activity, nutrient depletion and the effects of media components were used. This model accurately predicted the effects of the media components Tween 80, veratryl alcohol and basal medium on lignin peroxidase production.

Biomass growth

Changes in the cell dry weight in cultures of P. chrysosporium occur due to growth on the limiting nutrient nitrogen, growth on glucose and cell death. The Monod equation can be used to describe microbial

growth due to the uptake of the limiting nutrient. In lignin peroxidase producing cultures of P. chrysosporium, nitrogen is limiting (equation 1). In this equation N is

$$\left.\frac{dX}{dt}\right|_{nitrogen} = \frac{Umn * N}{Kn + N} * X \qquad (1)$$

the nitrogen source (g l^{-1}), X is the biomass (g l^{-1}) and Umn (hr^{-1}) and Kn (g l^{-1}) are the Monod constants for nitrogen. Cell growth also occurs due to the formation of polysaccharides from glucose (G)[31]. An additional term is used to account for this growth (equation 2).

$$\left.\frac{dX}{dt}\right|_{glucose} = B * G * X \qquad (2)$$

The cell death rate is represented by a first order death constant (Kd).

$$\left.\frac{dX}{dt}\right|_{death} = - Kd * X \qquad (3)$$

Combining equations 1 to 3 gives the total rate of cell growth due to nitrogen uptake, glucose uptake and cell death (equation 4).

$$\frac{dX}{dt} = \frac{Umn * N}{Kn + N} * X + (B * G * X) - (Kd * X) \qquad (4)$$

Substrate utilization

As cell growth occurs, nitrogen and glucose substrates are depleted in the medium. The rate of substrate utilization is equal to the rate of growth due to the particular substrate (equations 2 & 3) multiplied by

$$\frac{dN}{dt} = -\frac{Umn * N}{Kn + N} * X * Yxn \qquad (5)$$

$$\frac{dG}{dt} = - B * G * X * Yxg \qquad (6)$$

proportionality constants (Yxn, Yxg) equivalent to the mass of substrate utilized per mass dry weight created.

Product formation

The production of lignin peroxidase occurs during idiophase, generally two to three days after nitrogen has been depleted. Time dependent genetically structured models have been used to describe the production of secondary metabolites[3,43]. In an individual cell, messenger RNA for the lignin peroxidase enzyme is transcribed only after a lag of some time (ti) (equation 7). If it is assumed that only cells which were growing at time t-ti will produce mRNA, then the production of mRNA is equal to a constant (Kr) times the growth rate

$$\frac{dmRNA}{dt} = Kr * \left(\frac{dX}{dt}\right)_{t-ti} - (Krd * mRNA) \qquad (7)$$

at time t-ti. Within the cell mRNA is deactivated at some rate which is modelled using a first order death

rate expression (Krd).

The rate of production of lignin peroxidase is proportional to the rate of mRNA production in the individual cell times the total cell weght. The production of lignin peroxidase enzymes as affected by media composition is accounted for using an empirical constant; M (equation 8). Kld (hr^{-1}) is a first

$$\frac{dL}{dt} = (M * mRNA * X) - (Kld * L) \qquad (8)$$

M = media effects = f(tween, veratryl alc, other) order enzyme deactivation constant.

Since the mechanism of action of tween 80, veratryl alcohol and basal medium are not well understood, an empirically determined variable (M) was used to account for the effect of these media components on the production of lignin peroxidase. The effect of veratryl alcohol concentration on the production of lignin peroxidase was modeled assuming Michaelis-Menten kinetics. The effect of Tween 80 and basal medium concentration on lignin peroxidase production required the use of second order models since maxima were apparent. The media effects variable represents the collective effect of media components on lignin peroxidase production.

$$M = \frac{T}{kt + T^2} * \frac{B}{kb1 + B^2} + kb2 * \frac{V}{kv + V} \qquad (9)$$

To evaluate the model, data for glucose and nitrogen depletion, cell growth, lignin peroxidase production and mRNA production were needed. Empirical data was readily available for the depletion of nitrogen and glucose by nitrogen limited cultures of P. chrysosporium[26]. The growth rate and the production of lignin peroxidase are easily measured. The rate of mRNA production is not known nor is it readily measurable, however this is not essential to the model. By beginning with reasonable estimates for constant values and modifying the parameter values, the model can be made to fit the data.

The data used to evaluate the model parameters were from two sources; Jager et.al.[26], and this work. Since the depletion of glucose and nitrogen was not measured in this work, the data for substrate utilization was obtained from the work of Jager et.al.[26] (see Figure 2.2), in which agitated cultures identical (except for the use of DMS buffer) to the optimized cultures used in this work were used. The data for cell dry weight, lignin peroxidase activity and the effects of media components are presented in section 4.1.

Euler's method was used with a time interval of 4 hours to simultaneously solve the differential equations describing growth, substrate utilization and lignin

peroxidase formation. Data curves were fitted by programming the equations into a PC computer using SC4 software, and modifying the model parameters until the model curves fit the data. A summary of the model equations (Table A1.1) and the model parameter values are presented (Table A1.2). The time course of nutrient depletion, lignin peroxidase activity, and cellular growth as predicted by the model for optimal conditions is presented in Figure A1.1. Compared to Figure 4.5, the model curves accurately reflect the trends of the data.

The model predicted media effects are presented in Figure A1.2. These model curves closely reflect the trends in the data observed in Figure 4.4 a,b,c.

TABLE A1.1 Model equations for *P. chrysosporium* growth and enzyme expression

Biomass growth

$$\frac{dX}{dt} = \frac{Umx*N}{Kn + N} * X + (B * G * X) - (Kd * X)$$

Substrate utilization

$$-\frac{dN}{dt} = \frac{Umx * N}{(Kn + N)} * X * Yxn$$

$$-\frac{dG}{dt} = B * G * X * Yxg$$

Gene expression

$$\frac{dmRNA}{dt} = Kr * \left(\frac{dX}{dt}\right)_{t-ti} - Krd * mRNA$$

Lignin peroxidase expression

$$\frac{dL}{dt} = M * mRNA * X - Kld * L$$

Media effects variable

$$M = \frac{T}{kt + T^2} * \frac{B}{kb1 + B^2} + kb2 * \frac{V}{kv + V}$$

TABLE A1.2 Model Equation Symbols and Parameter Values

Symbols

X	Biomass (g l^{-1})	G	Glucose (g l^{-1})
N	Nitrogen source (g l^{-1})	L (U l^{-1})	Lignin peroxidase activity
B	Basal medium (-)	mRNA	- Messenger RNA
V	Veratryl alcohol (mM)		
M	Media constant (U g^{-1} hr^{-1})		
T	Tween 80 concentration (g 100 ml^{-1})		

Parameter values

Umn	Monod constant for N	0.009	hr^{-1}
Kn	Monod constant for N	0.020	g l^{-1}
Kd	Biomass death constant	0.011	hr^{-1}
Yxn	N Proportionality coefficient	0.40	-
Yxg	G Proportionality coefficient	1.50	-
B	Glucose constant	0.0018	hr^{-1}
ti	time lag constant	84.0	hr
Kr	mRNA production constant	0.30	l g^{-1}
Krd	mRNA deactivation constant	0.04	hr^{-1}
Kld	Lignin peroxidase degradation	0.01	hr^{-1} l
ktw	tween effects constant	0.0015	g^{2} $100ml^{-2}$
kb1	basal medium effects constant	2.00	-
kb2	basal medium effects constant	0.35	-
kv	veratryl alcohol constant	0.01	mM

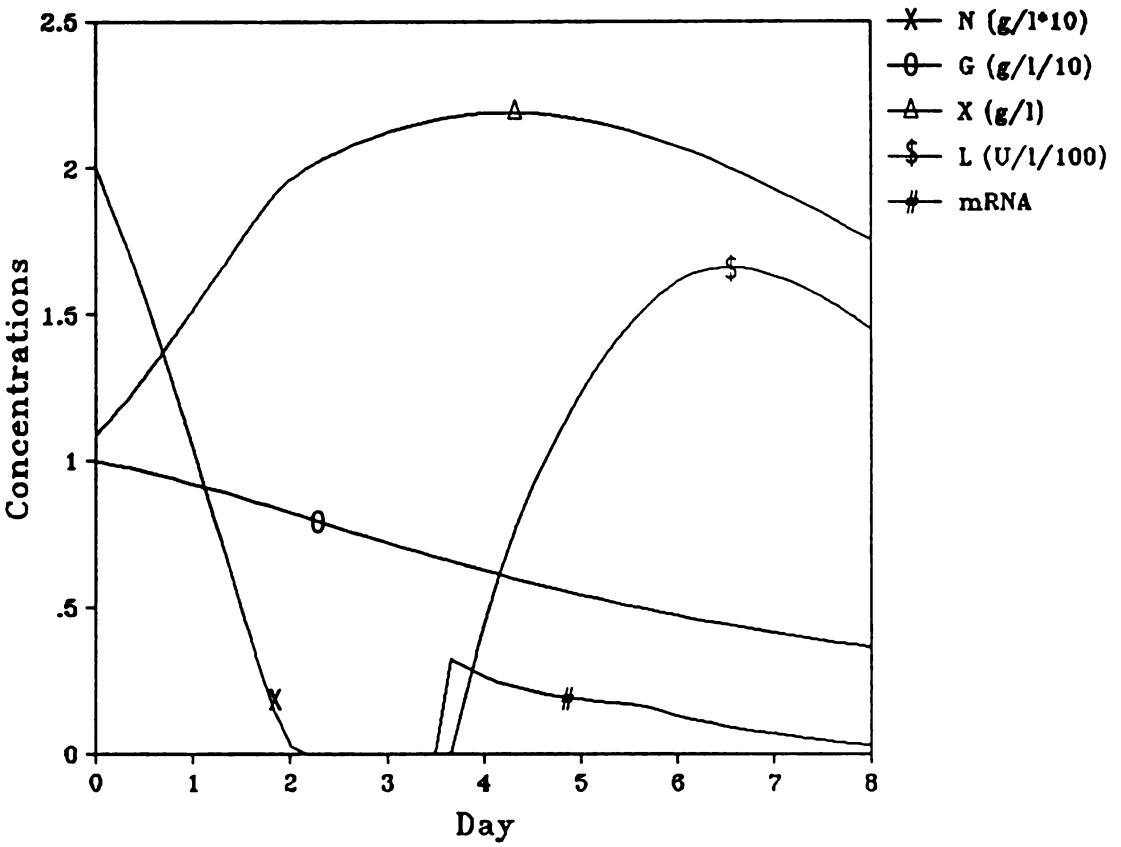


Figure A1.1 Model for growth, substrate utilization and lignin peroxidase production in batch cultures.

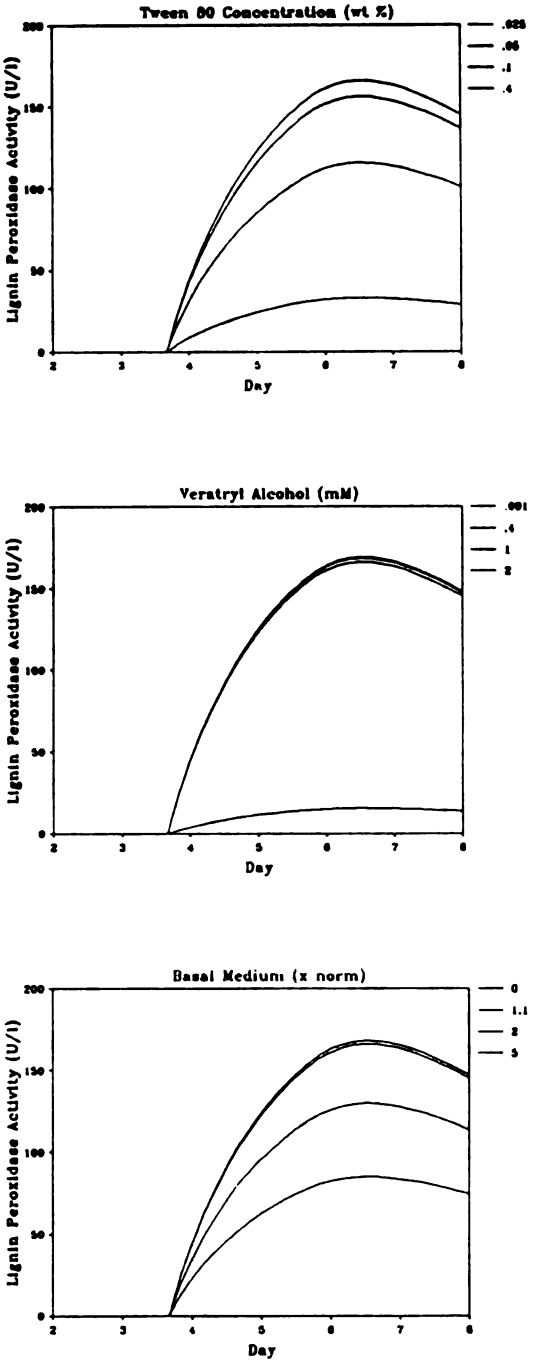


Figure A1.2 Model predicted media effects on the production of lignin peroxidase in batch cultures.

Appendix B

Operation of a trickle bed reactor

A bench scale trickle bed reactor system for the production of lignin peroxidases was developed (Figure A2.1). Experiments showed that both strains SC26 and BKM-F1767 would readily and completely adsorb to telleret rings composed of polyethylene and would produce small titres of lignin peroxidase in agitated shake flask cultures. Therefore, the plastic rings were used as a mycelial support material in a 1.5 l trickle bed reactor. Optimized culture media #3 was added to the lower third of the reactor volume and inoculated with blended mycelia. The inoculated media was circulated to the top of the reactor using a centrifugal pump. The temperature was controlled by a heating jacket surrounding the reactor and 99.5% oxygen was added continuously via a sparger located at the base of the reactor.

Unfortunately, this reactor system was plagued by problems which could not be overcome. Initially a medium was pumped directly to the top of the reactor column through a 1/2 " diameter steel pipe. With this sparger however, growth was limited to a thin stream of mycelium

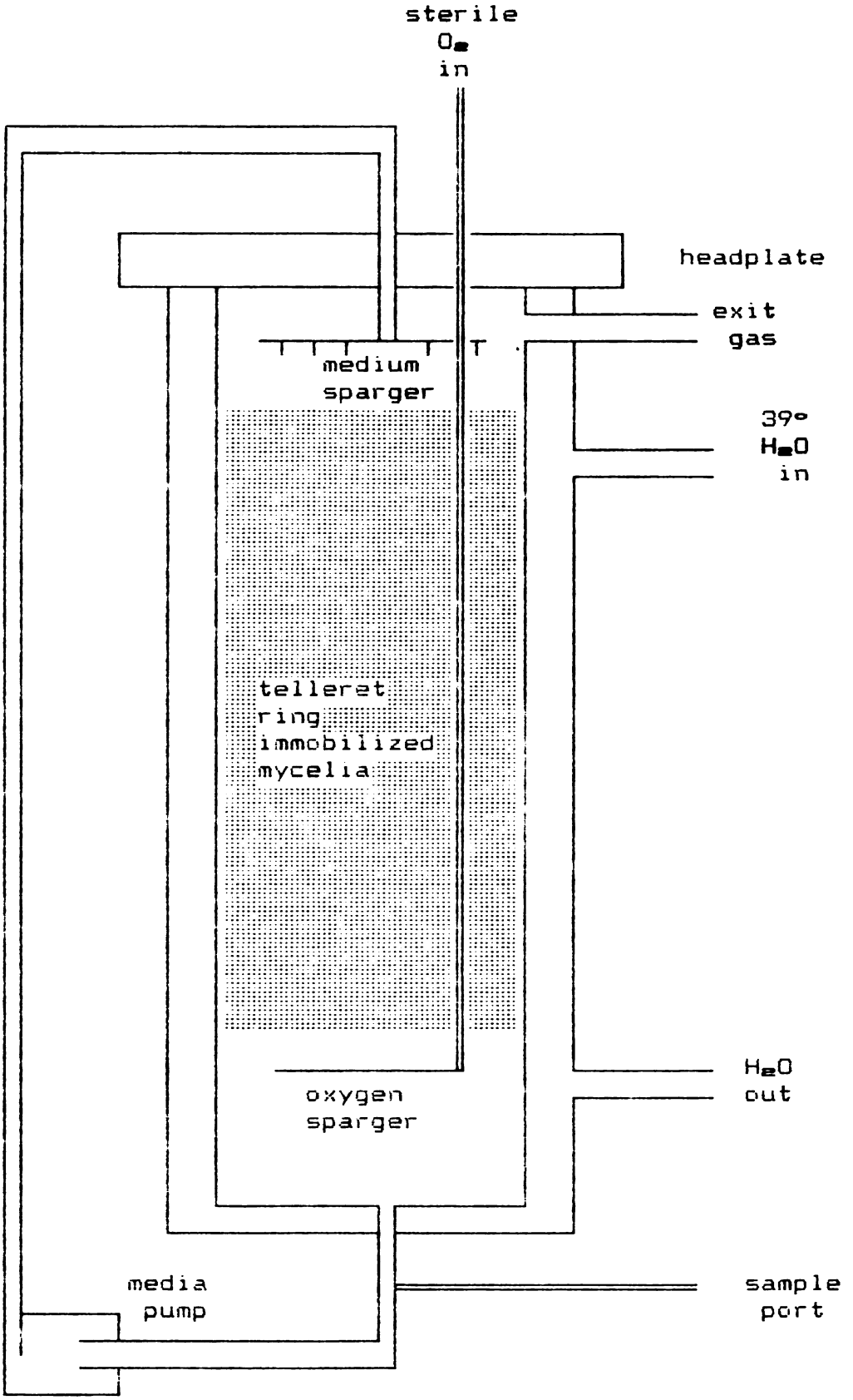


Figure A2.1 Design of a trickle bed reactor for the production of lignin peroxidase.

and no lignin peroxidase activity was detected. To improve medium sparging, a metal sparger was designed with a cross configuration and small diamaeter medium ports. With this sparger, growth was more disperse however bits of mycelia from the liquid phase of the reactor or from the supports, would invariably break loose and clog the sparger and pump. No activity was detected when the improved sparger was used. Clogging by mycelia is a typical problem encountered in fungal film reactor configurations. This reactor configuration was abandoned in favor of a stirred tank reactor system.

Appendix C

Tabulated data

Table A3.1 Tabulated data for strain comparison experiment. (refer to Figure 4.1)

Run 1: 9/14/87

	Lignin Peroxidase Activity (U/l)				
Day	**4**	**5**	**6**	**7**	**8**
Strain					
BKM-F-1767		181	200	55	0
	0	204	205	58	0
	0	194	194	145	0
SC26	0	19	100	110	13
	0	65	142	85	39
	0	90	155	103	68

Table A3.2 Comparison of various buffers for the production of lignin peroxidase. Tabulated data for Figure 4.2. Values are averages of triplicate cultures.

		Lignin Peroxidase Activity (U/l)			
Buffer	Day 3	4	5	6	7
10 mM DMS	0	64.25	54	76.25	17
20 mM Acetate	0	32.75	131.17	166.2	155.85
10 mM Citrate	0	0	11.5	37.5	28
10 mM Succinate	0	43.5	72.5	62	41.5
10 mM Tartrate	0	0	0	0	0
Jager et al	0	0	50	132	134
10 mM Oxalate	0	0	0	0	0

Table A3.3 Tabulated data for spore inoculum size optimization (refer to Figure 4.3).

Run 1: 8/25/87

		Ligninase Activity (U/l)			
Day	4	5	6	7	8
Inoculum OD					
.05 OD	0	106	138	177	142
		90.3	170	175	188
0.1 OD	0	58	145	166	126
		117	183	166	155
0.3 OD	0	21.9	106.4	145	119
		45.7	122.5	138	90
0.6 OD	0	23	0	0	20
	0	0	0	0	0

Table A3.4 Tabulated data for tween 80 optimization experiments (refer to Figure 4.4 A).

Run 1: 8/25/87

	Lignin Peroxidase Activity (U/l)				
Day	4	5	6	7	8
Tween 80% (w/v)					
0.05%	0	21.9	106.4	145	119
	0	45.7	122.5	138	90
0.1%	0	96	97	142	68
	0	50	95	53	76
0.2%	0	45	51.6	71	45
	0	54.8	90	94	56

Run 2: 3/12/88

	Lignin Peroxidase Activity (U/l)				
Day	4	5	6	7	8
Tween 80 % (w/v)					
0.025%	21.5	53	41	142	137
	26.9	0	19	142	135
	3	55	42	187	151
0.05%	22	48	65	169	71
	28	72	71	218	26
	0	53	77	187	84
0.2%	0	34	39	168	103
	10	38	18	152	93
	0	52	19	161	81
0.0%	0	0	0	0	0

Table A3.5 Tabulated data for veratryl alcohol concentration optimization experiments (refer to Figure 4.4 B).

Run 1: 2/20/88

	Lignin Peroxidase Activity (U/l)				
Day	4	5	6	7	8
Veratryl Alcohol					
0.4 mM	181	400	403	331	330
	129	354	306	274	242
	158	300	306	218	210
1.0 mM	123	400	452	387	290
	110	371	235	347	338
	126	350	371	331	314
2.0 mM	155	269	434	371	395
	155	242	371	403	395
	0	270	419	193	338
0.0 mM	0	17	23	54	

Table A3.6 Tabulated data for the optimization of basal medium concentration (refer to Figure 4.4 C).

Run 1: 1/22/88

	Ligninase Activity (U/l)				
Day	**4**	**5**	**6**	**7**	**8**
Basal Medium					
0X	20	39	70	89	0
	11	45	62	101	101
1X	76	32	264	222	169
	63	180	160	185	149
2X	109	201	177	222	149
	77	97	150	181	125
5X	48	142	124	185	121
	0	13	103	133	117

Table A3.7 Tabulated data for the characteristics of agitated cultures grown in optimized medium. (refer to Figure 4.5).

Day	Dry Weight (g/l)	pH
0	1.18	4.5
0	.98	4.5
1	1.92	4.5
1	1.86	4.5
2	1.71	4.5
2	2.12	4.5
3	2.04	5.0
3	1.98	5.0
4	2.19	4.8
4	2.45	4.9
5	2.15	4.9
5	2.11	4.9
6	2.38	5.0
6	2.27	5.1
7	1.89	5.4
7	1.87	5.3
8	1.59	5.5
8	1.81	5.5
9	1.24	5.6
9	1.37	5.7

Table A3.8 Tabulated data for the number of hyphal agglomerations as a function of time (refer to Figure 4.6)

Hour	125 ml flask n= number of pellets/l	2 liter flask n= number of pellets/l
0	12000000	12000000
4	2400000	2600000
6	40000	160000
7.5	50000	45000
8.5	30000	40000
20	16000	59000
24	16700	43700
48	23000	41000

Table A3.9 Pellet size as a function of culture age. Tabulated data for Figure 4.8.

125 ml Shake flask culture

Sieve size (mm)	Number of pellets per tray				
	24 hr	49 hr	73 hr	96 hr	140 hr
2	0	1	0	7	7
1.4	0	23	61	115	107
1.18	47	86	69	70	56
1	84	66	38	51	27
.85	18	15	12	14	1
.5	17	17	7	10	4
Sauter Mean Diam.(um)	1046.215	1159.398	1238.367	1320.730	1363.847

STR culture

	Number of pellets per tray				
	24 hr	49 hr	73 hr	96 hr	140 hr
2	0	0	0	0	
1.4	0	14	20	114	
1.18	70	55	116	150	
1	223	104	172	210	
.85	134	52	65	86	
.5	320	79	90	85	
Sauter Mean Diam.(um)	925.6399	1034.862	1059.837	1147.482	

Table A3.10 Effect of the agitation rate on lignin peroxidase activity. Tabulated data for Figure 4.10.

Run 1: 12/1/87

	Lignin Peroxidase Activity (U/l)				
	Day 4	5	6	7	8
Agitation Rate					
150 RPM	13	65	387	419	395
	90	129	354	225	258
	0	71	323	427	371
200 RPM	155	269	434	371	395
	155	242	371	403	395
	0	270	419	193	338
260 RPM	0	0	39	73	185
	0	0	0	0	0
	0	0	0	0	0

Run 2: 3/28/88

	Lignin Peroxidase Activity (U/l)				
	Day 4	5	6	7	8
Agitation Rate					
125 RPM	0	0	0	0	0
200 RPM	27	68	32	44	
	62	100	34	24	
260 RPM	34	54	8	15	
	38	32	8	5	

Table A3.11 Sauter mean pellet diameter as a function of the agitation rate. Tabulated data for Figure 4.12.

RPM	Sauter mean pellet diam. D32 (mm)	Ln (RPM)	Ln (D32)
150	2	5.010635	7.600902
150	1.620529	5.010635	7.390508
150	1.860691	5.010635	7.528703
200	1.257833	5.298317	7.137146
200	1.302042	5.298317	7.171689
200	1.301004	5.298317	7.170892
260	1.191148	5.560682	7.082673
260	1.319476	5.560682	7.184990
260	1.296639	5.560682	7.167531
125	1.708593	4.828314	7.443425
125	1.711006	4.828314	7.444837
125	1.911837	4.828314	7.555820
125	1.944835	4.828314	7.572933
165	1.361629	5.105945	7.216437
165	1.287960	5.105945	7.160815
165	1.409698	5.105945	7.251131
165	1.456255	5.105945	7.283623
210	1.146687	5.347108	7.044632
210	1.232677	5.347108	7.116944
210	1.137920	5.347108	7.036957
260	1.012458	5.560682	6.920136
260	.9753344	5.560682	6.882780
260	.9800289	5.560682	6.887582
260	.7886938	5.560682	6.670378
300	1.410078	5.703782	7.251400
300	1.384071	5.703782	7.232784
300	1.373099	5.703782	7.224825
300	1.314032	5.703782	7.180855

Table A3.12 Lignin peroxidase activity and total extracellular protein in a Stirred tank reactor system. Tabulated data for Figure 4.13.

Day	Lignin peroxidase activity (U/l)	Total extracellular protein (ug/ml)
2	0	7
3	29	11
4	156	14
5	141	13
6	180	
7	63	
8	43	

Table A3.13 Protein concentration and lignin peroxidase activity of STR FPLC heme-protein elution volumes.

elution volume (ml)	OD 595 nm	Total Protein (ug/ml)	Lignin Peroxidase Activity (U/l)
5	0.446	0.775	0
6	0.501	4.936	0
7	0.521	6.449	0
8	0.522	6.524	0
9	0.535	7.508	0
10	0.715	21.126	88.69
11	0.637	15.225	0
12	0.544	8.189	0
13	0.743	23.244	0
14	0.726	21.958	0
15	0.745	23.396	0
16	0.605	12.804	24.2
17	0.68	18.478	0
18	0.665	17.343	0
19	0.605	12.804	0
20	0.725	21.883	0
21	0.78	26.044	44.34
22	0.71	20.748	0
23	0.683	18.705	0
24	0.83	29.826	0
25	0.659	16.889	0
26	0.865	32.474	24.18
27	0.815	28.692	84.7
28	0.89	34.366	0
29	0.741	23.093	0
30	0.901	35.198	0
31	1.124	52.069	0
32	0.833	30.053	0

Table A3.14 Lignin peroxidase activity of all control flasks.

	Lignin Peroxidase Activity (U/l)				
	Day 3	4	5	6	7
Date					
8/25/87	0	0	106	138	177
	0	0	90.3	170	175
9/14/87	0	0	181	200	55
	0	0	200	205	58
	0	0	194	194	145
11/3/87	0	0	136	116	116
	0	0	149	171	206
12/3/87	0	155	269	434	371
	0	0	242	371	403
	0	155	270	419	193
1/23/88	0	76	32	264	222
	0	63	180	160	185
2/17/88	0	30	87	100	80
	0	0	90	87	86
3/6/88	0	22	48	65	169
	0	28	72	71	219
	0	0	53	77	187
3/30/88	0	27	68	32	44
	0	62	100	34	24
	0	37	56	16	2
Average	0	32.75	131.165	166.2	155.85
SD	0	48.37015	75.17486	123.5244	104.6514
+ one SD	0	81.12015	206.3399	289.7244	260.5014
- one SD	0	0	55.99014	42.67562	51.19856

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