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GROWTH KINETICS AND LIGNIN PEROXIDASE PRODUCTION BY *PHANEROCHAETE CHRYSOSPORIUM* IN A ROTATING BIOLOGICAL CONTACTOR

By

Susan Carol Jones

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ABSTRACT

GROWTH KINETICS AND LIGNIN PEROXIDASE PRODUCTION BY *PHANEROCHAETE CHRYSOSPORIUM* IN A ROTATING BIOLOGICAL CONTACTOR

By

Susan Carol Jones

The ligninolytic system of the white-rot fungus *Phanerochaete chrysosporium* offers potential applications for wastewater treatment and for the efficient conversion of lignocellulosic materials into useful products. To enable large-scale reactor design for high lignin peroxidase production, enzyme expression and cell adhesion of *P. chrysosporium* immobilized in a rotating biological contactor (RBC) were studied.

The fungus was cultured on roughened polymer discs in a bench-scale RBC. The reactor conditions necessary for biomass adhesion and expression of the ligninolytic system were identified. The growth kinetics of the fungus were modelled and used to determine the specific growth rate and the biomass carrying capacity of the discs. A comparison of film and pellet morphologies shows that film growth closely resembles pellet growth although some important differences exist.

To

Sharon, Randy, Richard
and, of course, most especially,
my parents

for the Love and Light they have imparted to my life.

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CHAPTER 1. INTRODUCTION

The white-rot fungus *Phanerochaete chrysosporium* produces a family of lignin peroxidase isoenzymes which efficiently degrade the complex, three-dimensional lignin polymer. This offers potential applications in the pulp and paper industry for biopulping and biobleaching, and in improving the conversion of lignocellulosic materials into animal feeds, alternative fuels, and chemicals. In addition to degrading lignin, the ligninolytic system of this fungus has been found to decolorize and detoxify waste water effluents from pulping mills. The fungus is able to degrade such pollutants as DDT, polychlorinated biphenyls (PCBs), and polychlorinated dioxins.

The progress made in recent years in elucidating the metabolism and physiology of *P. chrysosporium* has led to a better understanding of the lignin biodegradation system. Thus far, researchers have used primarily stationary and agitated shake flask cultures in studying the fungus. The use of alternative reactor systems such as stirred tank reactors, air-lift fermentors, and various types of immobilized film reactors has been explored to a small extent as a preparatory step toward the scale-up of the fungal system for industrial applications. The rotating biological contactor (RBC) is a film reactor which

offers advantages for bench-scale study of fungal growth as well as for large-scale applications.

Large-scale RBCs have proven to be reliable reactors for wastewater treatment applications. Bench-scale RBCs also offer advantages for the study of microorganisms. Successful modelling of growth kinetics in the RBC will be important in reactor design for commercial processes. Because the technology for operating industrial RBCs already exists, bench-scale results would have added relevance.

The work presented here involves the study of the adhesion and growth of *P. chrysosporium* in a bench-scale RBC. The primary objectives of this work are:

1.) To identify the fermentation conditions in the RBC necessary to obtain both adhesion of the fungus to the discs and expression of the ligninolytic system. This includes studying selected parameters related to RBC operating conditions, disc surface characteristics, and growth medium conditions.

2.) To model the growth kinetics of *P. chrysosporium* in the RBC and determine the biomass carrying capacity of the discs; to compare the behavior of the fungus in the RBC to growth in agitated shake flasks.

Although previous researchers have cultured *P. chrysosporium* in the RBC, a more complete study of the factors influencing fungal growth and enzyme production was necessary. Earlier work with the RBC has been limited to decolorization studies and the modelling of decolorization kinetics. The work presented here is the first attempt reported

at examining the growth kinetics of the fungal biofilm in a RBC and relating growth to culture parameters for the production of lignin peroxidase.

This thesis is presented primarily in five chapters. Chapter 2 provides a broad overview of lignin biodegradation research including a description of the lignin polymer, the factors which affect the growth and ligninolytic expression of *Phanerochaete chrysosporium*, the use of a rotating biological contactor, fungal growth models, and potential applications for this fungal system. Chapter 3, the materials and methods section, includes details relating to media and inocula preparation, analytical methods, and a characterization of the RBCs used for experiments. Chapter 4 describes the results and presents a discussion of the optimization of the RBC culture conditions. Results of growth modelling experiments are presented in Chapter 5. Finally, concluding remarks and considerations for future work are given in Chapter 6.

CHAPTER 2. LITERATURE REVIEW

An overview of the lignin biodegradation research involving the white-rot fungus *P, chrysosporium* is presented. This research has provided a more complete understanding of the ligninolytic system of the fungus, the factors which affect growth and enzyme expression, and the potential applications of the depolymerization reaction performed by this biosystem.

2.1 STRUCTURE OF LIGNIN

Lignin is the most abundant organic compound after cellulose.[50] The biodegradation of this biopolymer is critical to the carbon and oxygen cycle of the biosphere. Wood and other vascular plants are composed of 20-30% lignin[11] as well as cellulose, hemicellulose, and pectin. Lignin occupies the spaces between cellulose fibrils and is also found in the cell walls. It provides the plant cell with structural rigidity and defends the plant from microbial attack by shielding the polysaccharides from enzymatic degradation.

The term "lignin" refers to a family of related aromatic polymers. These polymers are heterogeneous in structure, three-dimensional, and cross-linked. The precursors of lignin are coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol which are shown in Figure 2.1. The relative amounts of each of these p-hydroxycinnamyl alcohols determines the type of lignin. For example, softwood lignins are primarily composed of coniferyl

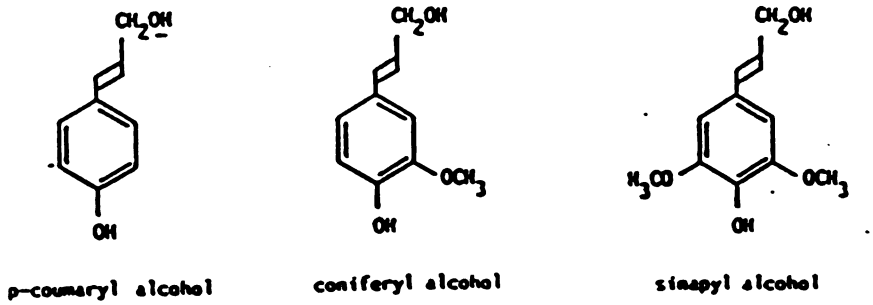


Figure 2.1 Lignin precursor molecules.[10]

alcohol polymers while hardwood lignins are polymers made of coniferyl and sinapyl alcohols.

Lignin is formed by a free radical copolymerization of the p-hydroxycinnamyl alcohols. In this process, peroxidase oxidizes the hydroxyl group of the alcohols to form free radicals. The free radicals non-enzymatically and randomly combine to form the lignin polymer. Figure 2.2 is a schematic representation of the structure of lignin. The polymer contains over ten different types of stable interunit linkages although over 50% of the linkages are β-o-4 bonds.[36] This type of bond exists between rings 1 and 2 in Figure 2.2. Unlike other natural polymers such as cellulose and proteins, lignin does not contain a bond that is easily hydrolyzable.[44]

The complex, heterogeneous structure of lignin makes it difficult to isolate in a natural state. Since it contains no bond that is easy to hydrolyze, the possibility of assaying for specific enzymatic reactions and accurately measuring the biodegradation of the polymer is eliminated. For these reasons synthetic lignins, low molecular weight lignin model compounds and polymeric dyes are used to study the ligninolytic system of the fungus.

One synthetic lignin polymer used for biodegradation studies is a dehydropolymerizate, or DHP, of coniferyl alcohol. The conversion of ^{14}C-labeled DHPs to ^{14}C-labeled water-soluble intermediates and to $^{14}CO_2$ is monitored to determine the extent of degradation. The drawback of using DHPs is that the preparation of this synthetic lignin has not been

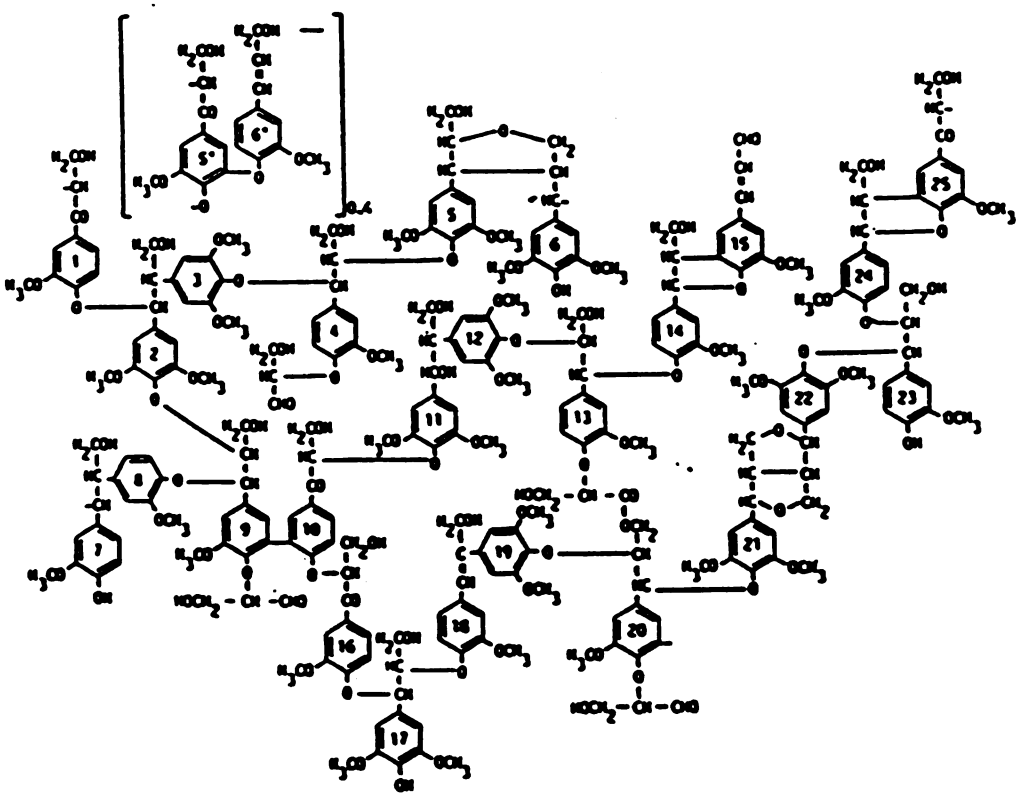


Figure 2.2 Schematic representation of lignin polymer.[10]

standardized.[10] Comparisons of results from these studies must be made carefully.

Low molecular weight lignin models are compounds with structural characteristics similar to lignin molecules. These compounds are used to study the chemical mechanisms of degradation. Some examples of lignin model compounds are the methoxylated aromatic acids veratric acid, vanillic acid, and syrignic acid as well as compounds which contain veratrylglycerol-β-(o-methoxy-phenyl)ether and veratrylglycerol-β-guaiacyl ether. It is assumed that what is learned from the biodegradation of model compounds will provide insight into the mechanisms of lignin biodegradation.

Kraft lignin and lignosulfonates, by-products of commercial pulping mills, are examples of chemically modified lignin. They are easily available and are often used in decolorization studies of waste water effluents. Researchers have shown that polymeric dyes such as Remazol brilliant blue, crystal violet, and phenol red are broken down by lignin-degrading fungi.[10,13] These dyes provide a rapid, inexpensive means of selecting for lignin-degrading mutants.[13]

2.2 FUNGAL DEGRADATION OF LIGNIN

Lignin is extremely resistant to attack from most microorganisms. The biodegradation of lignin is an aerobic process[36,39] and is thought to occur in nature through the cooperative efforts of bacteria and fungi. Some soil bacteria have been found to degrade lignin to CO_2 only to a limited

extent although they do intracellularly degrade lignin-derived soluble compounds.

Extensive degradation of lignin is achieved by wood-decaying fungi. There are three types of these fungi: soft-rot, brown-rot, and white-rot fungi. Both soft-rot and brown-rot fungi partially degrade lignin in order to obtain access to the preferred polysaccharides. White-rot fungi, most of which are basidiomycetes, are the most efficient microbes in degrading lignin. These fungi have the ability to totally degrade at equivalent rates all of the three major components of wood to carbon dioxide and water.[11] This is accomplished by the production of extracellular enzymes which are cellulolytic, hemicellulolytic, and lignolytic in nature.[44]

The most extensively studied white-rot fungus is the basidiomycete *Phanerochaete chrysosporium*. This fungus has previously been refered to as *Chrysosporum lignorum* or *Sporotrichum pulverlentum*.[19] Researchers have studied various strains of *P. chrysosporium*. The wild-type strains BKM-F-1767 (ATCC 24725) and ME-446 (ATCC 34541) are the most widely used. BKM-F produces the highest activity of lignin-degrading enzymes and exhibits the fastest rate of lignin degradation.[31]

Mutant strains have been developed with altered characteristics. Cellulase-negative strains have been obtained[20] which have potential uses in the pulping and bleaching industry. For this application, it would be desirable to minimize cellulolytic activity while enhancing ligninolytic activity. The mutant strain INA-12 has produced high

ligninolytic activity in carbon-limited cultures.[3] Another mutant strain SC26 was observed to adhere better to discs in a rotating biological contactor and produced higher lignin peroxidase activity than the BKM-F strain.[37]

2.3 THE LIGNINOLYTIC SYSTEM OF *P. CHRYSOSPORIUM*

P. chrysosporium produces several enzymes which are responsible for the degradation of lignin. Included in these enzymes are lignin peroxidases, manganese-dependent peroxidases, and H_2O_2-producing enzymes. Because this fungus is able to degrade various types of lignins, the enzymes involved must act in a non-specific manner on the lignin polymer.[70]

The first of the lignin-degrading enzymes to be isolated from extracellular fluid was lignin peroxidase, or ligninase.[28,68] Lignin peroxidase has since been shown to be a family of six heme protein isoenzymes which have molecular weights ranging between 38-46 kDa.[65] These enzymes require H_2O_2 for activity[26,68] and catalyze several reactions involved in the depolymerization of lignin: oxidative C_α-C_β cleavage, oxidation of benzyl alcohols to aldehydes to ketones, intradiol cleavage in phenylglycol structures, and hydroxylation of benzylic methylene groups.[44]

The isoenzymes are glycoproteins containing 13% carbohydrate by weight.[67] A high degree of homology exists between the primary and tertiary structure of the isoenzymes.[65] The isoelectric points range between 4.7 and 3.3.[23] Lignin peroxidase is now considered to function as a peroxidase and not

an oxygenase as previously thought.[30] The lignin peroxidase isoenzymes can be characterized by their ability to catalyze the oxidation of veratryl alcohol to veratryaldehyde in the presence of H_2O_2.[66] This reaction provides a method for the quantitative measurement of lignin peroxidase.

The second enzyme to be purified from the extracellular fluid of *P. chrysosporium* was manganese-dependent peroxidase.[42] This enzyme is also part of a family of lignin peroxidase isoenzymes and consists of four heme proteins which oxidize vanillylacetone in the presence of H_2O_2 and Mn^{2+}.[23] Unlike lignin peroxidase, these enzymes do not oxidize veratryl alcohol. The complete family of ten isoenzymes, six lignin peroxidases and four manganese-dependent peroxidases, are designated H1 through H10 according to their HPLC elution profile. A high degree of homology exists among these peroxidases.[45] They can be distinguished by their isoelectric points[23] as well as by their ability to decolorize polymeric dyes.[13]

In addition to the peroxidases, *P. chrysosporium* has been shown to produce another enzyme which may have importance in the ligninolytic system. This enzyme, glucose-2-oxidase, is produced during glucose starvation and oxidizes hexoses and xylose resulting in the formation of hydrogen peroxide.[21] The peroxidases involved in lignin depolymerization are dependent on H_2O_2 for activity. The reactions catalyzed by the peroxidase enzymes of *P. chrysosporium* during depolymerization of the lignin molecule are discussed in the following section.

2.4 THE MECHANISM OF LIGNIN DEGRADATION

The mechanism of lignin degradation by *P. chrysosporium* is a non-specific oxidative process. The enzymes involved catalyze a few types of reactions including demethylation, hydroxylations, and ring-cleavage reactions to produce lower molecular weight lignin-derived molecules and CO_2. The primary reaction of lignin peroxidase with lignin is a one-electron transfer to yield radical intermediates.[62] The intermediates then undergo various reactions to produce lower molecular weight lignin fragments.

The proposed mechanism of C_α-C_β cleavage in lignin model compounds is shown in Figure 2.3. In this mechanism, the methoxylated aromatic ring transfers a single electron to a high redox potential center. This transforms the substrate to a radical cation which then undergoes a C_α-C_β bond cleavage to yield veratrylaldehyde and a benzylic radical. Two possible reaction paths exist for the benzylic radical. The products of these reactions are phenyl ketol and phenylglycol. This mechanism accounts for the formation of products observed during lignin degradation studies.

The function of Mn^{2+}-dependent peroxidases in lignin degradation is unknown. These enzymes appear and reach a maximal activity earlier than lignin peroxidases indicating that they have a different role in the ligninolytic system.[45] Although lignin peroxidases are able to partially depolymerize lignin, they are unable to carry out complete degradation alone.

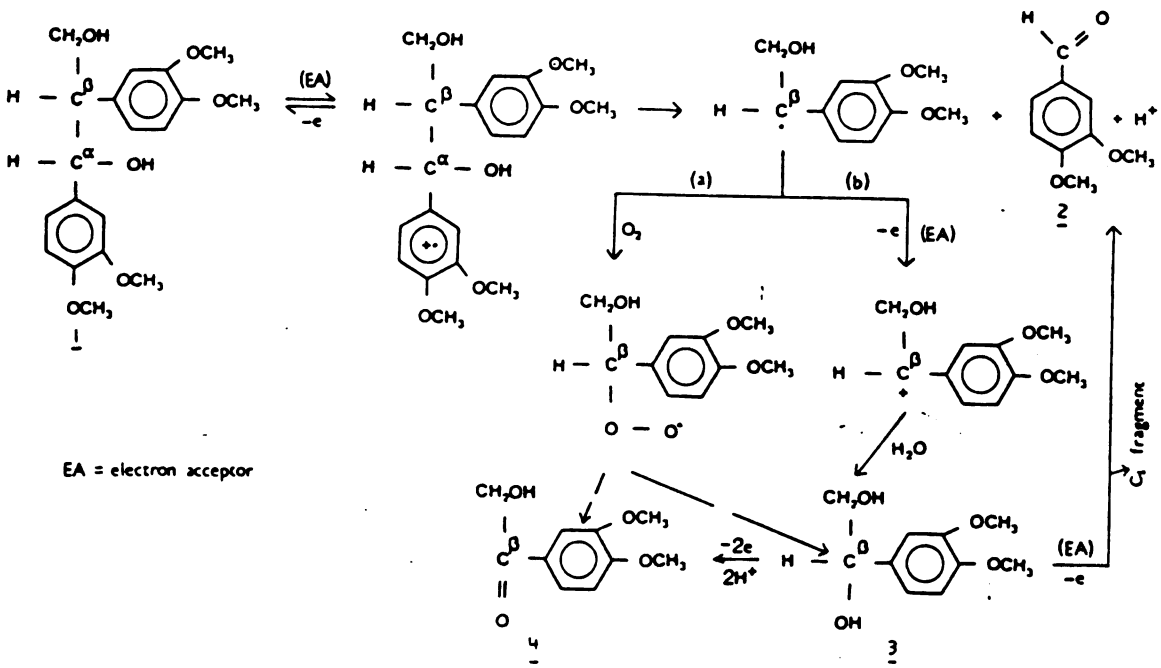


Figure 2.3 Mechanism of lignin degradation.[62]

2.5 GROWTH OF *PHANEROCHAETE CHRYSOSPORIUM*

Much of the research concerning the ligninolytic system of *P. chrysosporium* has been done using either stationary or agitated flasks. The fungus forms a thin mat of mycelia, or mass of hyphal filaments, on top of the medium when grown in shallow stationary cultures. Mycelial pellets are formed when cultured in agitated shake flasks. The cultures are generally inoculated with homogenized mycelium fragments grown from conidia (spores). The medium includes a carbon substrate, a nitrogen substrate, buffer, basal elements, trace elements, thiamine, and veratryl alcohol; in agitated cultures, a surfactant is added. Either the carbon or nitrogen source must be limiting for lignin peroxidase production to occur. The medium is buffered to pH 4.5 and cultures are maintained at 39^{o}C. Agitated cultures are periodically oxygenated.

The time-course of mycelial growth, glucose consumption, and lignin peroxidase production in agitated cultures using nitrogen-limited medium can be seen in Figure 2.4. Mycelium growth, measured as nitrogen uptake in the mycelium, occurs rapidly for two days until the nitrogen source is depleted. The fungus then enters secondary phase (idiophase), although mycelium dry weight continues to increase due to the formation of extracellular polysaccharides. Glucose is continously consumed at a linear rate until it is depleted at which time the fungus enters a death phase.

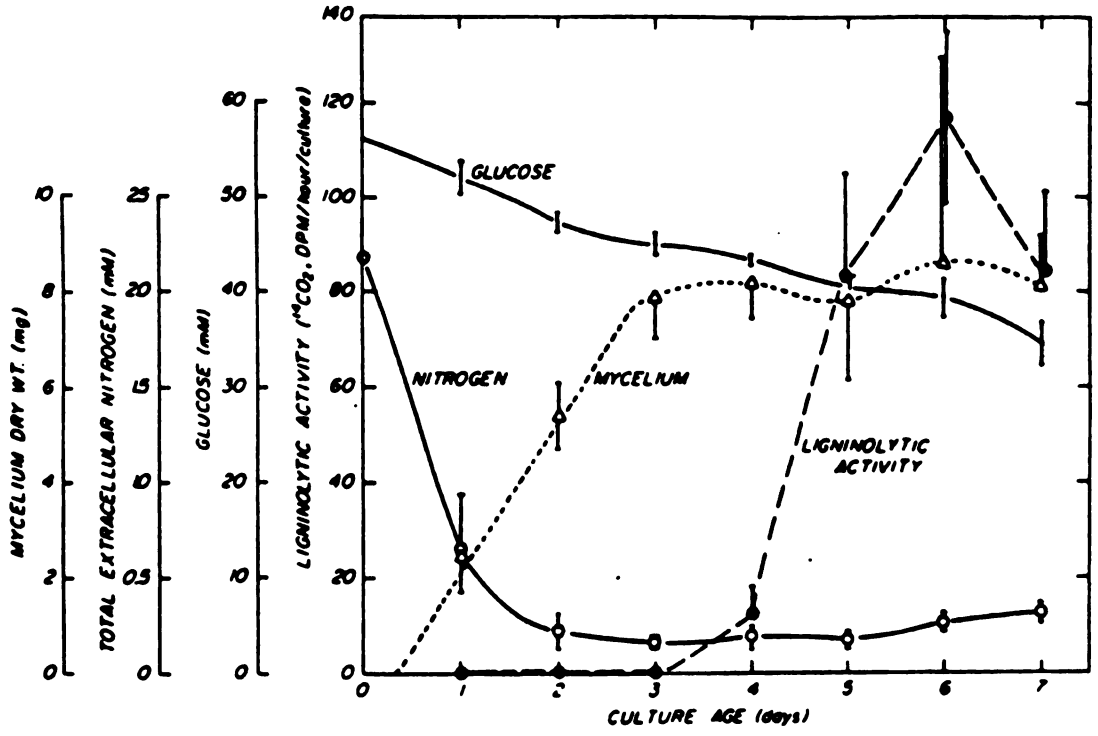


Figure 2.4 Relationship between several culture parameters for growth of *P. chrysosporium* in stationary flasks.[34]

The production of lignin peroxidase and Mn^{2+}-dependent peroxidase occurs during the idiophase.[24] Mn^{2+}-dependent peroxidase activity appears first and reaches a maximum on day 3. Lignin peroxidase activity begins on day 4, reaches a maximum on day 7, and then steadily declines. The next section describes some of the factors which influence the expression of these peroxidase enzymes in *P. chrysosporium*.

2.6 FACTORS AFFECTING THE LIGNINOLYTIC SYSTEM OF *PHANEROCHAETE CHRYSOSPORIUM*

Several nutritional, physiological, and environmental factors affect the ligninolytic system of *P. chrysosporium*. One of these factors is the requirement for a growth substrate. Lignin alone is not a sufficient carbon source for growth and must be supplemented by glucose, glycerol, or cellulose.[10,35] In addition to not being an essential substrate for growth of the fungus, the presence of lignin is also not required for the development of the ligninolytic system.[34] Lignin decomposition, a secondary metabolic activity, is triggered by the depletion of carbohydrate, sulfur, or nitrogen in the medium.[32] Most experiments use nitrogen-limiting conditions for growth which simulate the natural nitrogen-poor environment of wood encountered by the white-rot fungus.[36]

Lignin degradation is an oxidative process and is influenced by oxygen concentration. Researchers have found that the extent and rate of lignin decomposition was significantly higher in cultures grown in a 100% oxygen atmosphere compared to

cultures grown in air.[38,61,71] An optimum range of oxygen concentration appears to exist. Transient oxygenation in agitated shake flasks was found to result in higher lignin peroxidase activity and lower production of lignin-degrading proteolytic enzymes than continuous oxygenation.[15] Leisola showed that partial oxygen limitation was a major cause of poor lignin degradation.[46] In stationary cultures grown in a 100% oxygen environment, the oxygen concentration was negligible at depths greater than 1 mm from the surface of the mycelial mat.

The ligninolytic system of *P. chrysosporium* is sensitive to pH and requires a buffered medium in order to produce high titres of enzyme. The optimum pH range for lignin degradation is between pH 3.5 and pH 5.5.[10] The most widely used buffer is the relatively expensive 2,2 dimethylsuccinate (DMS) buffer. Researchers have studied the lignin peroxidase activity obtained with other buffers. In early work, Kirk compared tartrate, aconitate, and phthalate buffers in stationary flask cultures and found that the highest enzyme production occurred in phthalate-buffered cultures.[38] Acetate buffer was not tested since it was believed to be toxic to *P. chrysosporium*. Fenn reported a lignin degradation rate in stationary flasks two times higher with DMS buffer compared to cultures buffered with o-phthalate.[24] More recently, Michel compared several buffers in agitated shake flask cultures and found 0.02 M acetate buffer to give the highest enzyme activity.[56]

In early work with *P. chrysosporium*, researchers reported that the ligninolytic system was not expressed in agitated

cultures of mycelial pellets. Jager and coworkers found that the addition of such detergents as Tween 20 (polyoxyethylene-sorbitan monolaureate), Tween 80 (polyoxyethylene-sorbitan monooleate), or CHAPS (3-[(3-colamidopropyl)-dimethylamminio]1-propanesulfonate) enables the entire lignin-degrading system to be expressed in agitated cultures.[31] The mechanism of this effect has not been determined although it is known that detergents affects lipid metabolism.[3] It is believed that detergents have a physiological effect on the fungus and do more than simply aid in the release of enzyme from the mycelia.[31]

Extracellular protease activity has been measured during the growth phase and late stationary phase (day 8) of *P. chrysosporium* cultures.[14] These proteolytic enzymes promote the degradation of lignin peroxidase which accounts for the rapid decrease in lignin peroxidase activity after the maximum level is reached on about day 6.[15] The factors which have been found to decrease lignin peroxidase and manganese peroxidase activity have the reverse effect of increasing the production of extracellular proteases and polysaccharides.[16] Protease activity decreased in response to glucose addition beginning on day 6 and increased in response to veratryl alcohol addition.[14]

Veratryl alcohol is a secondary metabolite which is synthesized *de novo* by *P. chrysosporium* during ligninolytic activity.[25] Lignin peroxidase catalytically oxidizes veratryl alcohol to veratryl aldehyde. Veratryl alcohol acts as an inducer of lignin peroxidase production although the role of this aromatic compound in lignin degradation has not been

determined. It is possible that the veratryl alcohol cation radical formed by lignin peroxidase catalysis is involved in attacking particular lignin structures during decomposition.[69] Several researchers have found that the addition of veratryl alcohol to *P. chrysosporium* enhances lignin peroxidase production.[22,37,47] Tonon and Odier have concluded that veratryl alcohol protects lignin peroxidase from inactivation by hydrogen peroxide, another secondary metabolite.[69]

Another method that has been used to protect lignin peroxidase from damage by hydrogen peroxide is by the addition of 1 g/l solid manganese(IV)oxide to cultures at the onset of ligninolytic activity.[33] This has resulted in increased lignin peroxidase production and stability. It is hypothesized that MnO_2 associates with the hyphal surface and protects the proteins by catalytic decomposition of H_2O_2.

Conditions of high glucose levels and nitrogen limitation trigger the production of extracellular polysaccharides. The purpose of fungal polysaccharide formation is not clear although it has been suggested that polysaccharides serve as a carbon reserve.[43] Other researchers have proposed that polysaccharides are important in the regulation of glucose metabolism and hydrogen peroxide production.[7] Hydrogen peroxide, a necessary component for lignin breakdown, is synthesized from the oxidation of sugars. Glucose-oxidizing enzymes are catabolite repressed when the fungus is grown under conditions of glucose addition. Polysaccharide formation lowers the glucose concentration to a level where repression is no longer in

effect. This allows glucose oxidation to again proceed and hydrogen peroxide to be formed. A glucan-hydrolyzing enzyme converts polysaccharide back to glucose when the glucose levels become too low. Researchers have also suggested that polysaccharides are involved in maintaining optimum pH conditions for the activity of the lignin-degrading enzymes.[7]

In addition to shake flasks, *P. chrysosporium* has been cultured in other reactor systems including air-lift fermentors[16], continuous stirred tank reactors[56], and rotating biological contactors (RBC).[37] A description of fermentations carried out using a RBC is presented below.

2.7 USE OF A ROTATING BIOLOGICAL CONTACTOR

The rotating biological contactor (RBC) is a fixed film biological reactor used in wastewater treatment applications to remove organic materials. The reactor is composed of large-diameter plastic discs mounted on a horizontal shaft which is slowly rotated in a tank containing wastewater. The RBC offers the advantages of high surface area per unit volume, low energy consumption, low maintenance requirements, resistance to shock loadings, and simple operation.[2] Also, the RBC provides a low-shear environment which may be beneficial for culturing organisms sensitive to high rates of agitation.

Large-scale RBCs utilizing mixed organism populations have been used for the treatment of municipal wastes as well as industrial wastes from dairy, meat, poultry, food-processing, pulp and paper, and refinery processes. Figure 2.5 is a process

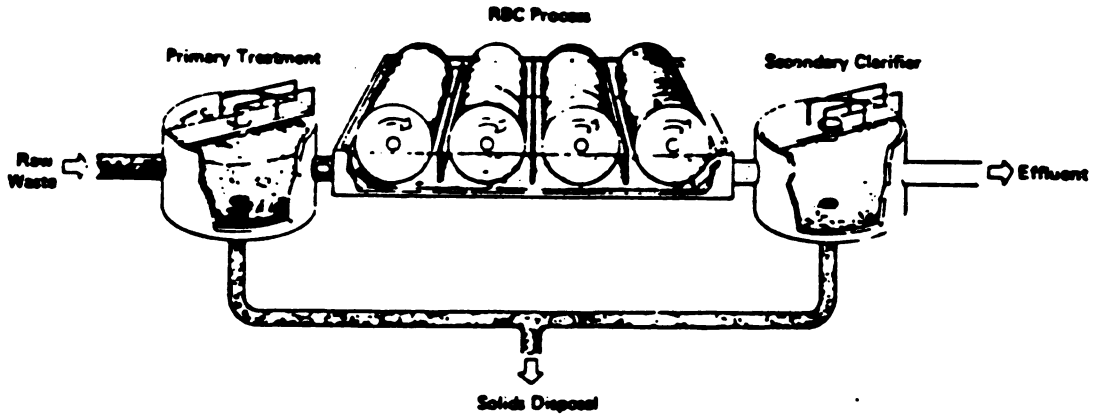


Figure 2.5 Process flow diagram for a waste treatment facility utilizing an RBC.[2]

flow diagram for a waste treatment facility. This process includes a primary clarifier where large objects, or materials which float or settle are separated from the influent. A multistage RBC system follows where a mixture of organisms removes organic matter. And finally, a secondary clarifier separates excess biomass from effluent.

Bench-scale rotating disk reactors have been used to immobilize whole cells and enzymes. *Saccharomyces cerevisiae* was immobilized on disks made of commercial sponge material and used for continuous ethanol production.[8] In other work, enzyme kinetics of lactate dehydrogenase were investigated by covalently attaching the enzyme to a nylon disk which was then rotated in a fluid.[12] Citric acid production by *Aspergillus niger* in a rotating disc fermentor has also been examined.[1]

P. chrysosporium wild-type strain BKM-F and the mutant strain SC26 have been grown in a bench-scale RBC containing 2.5 liters of medium buffered with 2,2-dimethylsuccinate.[37] BKM-F produced negligible amounts of lignin peroxidase while SC26 produced activity comparable to what is obtained in agitated shake flasks. This was attributed to the ability of SC26 to better adhere to the disc material which may be due to the development of a thicker polysaccharide hyphal sheath.

Decolorization and dechlorination of bleach plant effluents in the RBC have been studied for several years at North Carolina State University and the Forest Products Laboratory in Madison, WI. They have developed and patented a process termed the MyCoR (Mycelial Color Removal) process.[63,72] Color reduction of 60%

has been achieved in the RBC in one day with simultaneous BOD and COD reduction of 40%.[18] In these studies, decolorization was found to include three stages beginning with a period of rapid decolorization in the first hour. This was followed by a period described by zero-order kinetics and finally a period described by first-order kinetics.[73] Dechlorination of bleach plant effluents involves the degradation primarily of chlorinated phenols to veratryl alcohol and minor quanitites of other metabolites.[6]

Besides the RBC, other methods of immobilization have been used to culture *P. chrysosporium*. A brief summary of these techniques is given below.

2.8 OTHER IMMOBILIZATION TECHNIQUES

Alternate methods of immobilizing *P. chrysosporium* in order to enhance lignin peroxidase production have been explored. Immobilization of the fungus in cubes of polyurethane foam offers the potential of a semi-continuous system for enzyme production.[40] The fungus has been grown on silicon tubing wrapped around stainless steel rods in a 1-liter commercial bioreactor. Spore inocula were used to compare immobilization and enzyme activity using agar, agarose, and κ-carrageenan gel beads, nylon web, and polyurethane foam.[50,51,52] Carrageenan beads were not a successful immobilization medium whereas nylon web proved to be the best carrier. Further experiments using nylon web inside of a Biostat E bioreactor (6.7 liters of

medium) obtained high lignin peroxidase activity and may have application in large-scale enzyme production.[49]

Decolorization of effluents by *P. chrysosporium* has been studied using shake flask cultures and rotating biological contactors. In addition, decolorization has been observed using a 10-l aeration reactor.[60] This reactor is composed of an acrylic (Plexiglass) cylinder which is aerated and mixed using a diffuser located on the bottom of the reactor. The reactor was aerated with air instead of oxygen. Color removal rates of 1300 units per liter per day were obtained which is less than the rate observed in the RBC, although a longer fungal lifetime was achieved in the aeration reactor.

2.9 FUNGAL GROWTH MODELS

Several types of models have been used to describe fungal growth. These include biochemical models which consider changes in macromolecular components such as RNA, DNA, or protein within the cell. Morphological models are another type of model which describe changes in hyphal structure, colony formation, or pellet growth. Logistic growth models consider the numbers of individuals in a population. Some models are empirical in nature and offer the advantages of simplicity and ease of use. Mechanistic models, which are based on a proposed mechanism of a cellular event are more complicated to formulate but may prove useful for a specific process. A brief overview of fungal growth characteristics and some of the models used to describe

fungal growth on solid media and in submerged culture are discussed.

The mechanism for growth used by mycelial fungi resembles growth in plant cells. Growth occurs at the hyphal tips. The filaments of biomass, called hyphae, are not discrete cells but instead are separated by diaphragms which allow the passage of cytoplasmic material toward growth regions. The cytoplasm is pushed into new regions by the formation of vacuoles in older regions. Differentiation exists in hyphae since hyphal tips have been found to contain slightly higher levels of proteins, protein-bound sulfhydryl groups, and ribonucleic acids than other portions of the hyphae.[74]

These observations have led to the development of mechanistic models which describe hyphal tip growth. These models relate parameters such as tip shape or transport of cytoplasmic materials into growth regions to the rate of hyphal extension. Other models examine branching phenomena and relate this to hyphal growth.

The growth of mycelial fungi exhibits some differences from the growth of other organisms. Bacteria and yeast maintain a state of balanced growth where the exponential growth rates of all extensive properties of a culture increase at the same rate. For instance, parameters such as biomass dry weight, concentration of subcellular components, and total cell length of the population should all increase at the same rate for a particular doubling time. Researchers have observed that filamentous fungi do not maintain a state of balanced growth.

In particular, the physical size of a colony growing on a solid surface increases linearly with exponential growth.[41] The colony size may be characterized by measuring the radius, surface area, or volume of the colony.

Fungal growth on solid media has been examined by several researchers. The model proposed by Pirt assumes initial colony growth to increase exponentially until nutrient starvation restricts growth in the center of the colony.[59] From that point, exponential growth occurs in an annular region surrounding the center. A model presented by Koch[41] based on population considerations predicts a decrease in growth rate due to overcrowding on a solid surface. The model has the form:

$$\frac{dN}{dt} = \lambda N \left(\left(K - N / N \right) \right)^{n}$$

where N = mycelial mass
λ = maximum specific growth rate constant
K = carrying capacity of the solid media.
n = parameter based on the geometry of the growth space

The growth of fungi in submerged culture is similiar to the growth of bacteria in some respects. For instance, the classic phases of lag, exponential, deceleration, and stationary growth phases are observed. A major difference between bacterial and fungal growth is that pellet formation occurs with fungi in submerged culture. This requires the development of different models to describe fungal growth.

Pirt[55] showed that the exponential phase of pellet growth follows cube-root kinetics according to the equation:

$$M_t^{1/3} = (\text{constant})(M_o^{1/3})(t)$$

where M_t is the biomass at time t and M_o is the initial biomass. Several workers have studied the diffusion of nutrients into fungal pellets. Yano modelled the pellets as spheres and assumed oxygen diffusion into the pellets to be the limiting substrate.[55] His solution is presented as a plot of effectiveness factor versus Thiele modulus where the effectiveness factor is related to oxygen consumption and the Thiele modulus is a ratio of the radius and the critical radius of the pellet. Aynsley[5] and coworkers have modelled fungal growth in submerged culture by modelling a hypha as a self-extending tubular reactor. Nutrients absorbed along the length of the reactor are transformed into cell precursors and transported to the growth tip region. This model can predict changes in the rate of hyphal extension, branching, and pellet fragmentation.

Although it is known that many parameters have an influence on biomass growth, most models present biomass growth as a function of only time. A kinetic model developed by Michel[63] relates biomass growth to glucose and ammonia concentration in the media. In addition, the model treats each of the phases of the fungal lifetime (lag, primary, stationary, and death) with separate mathematical equations. Biomass growth predicted by the model closely fits experimental data taken over a range of fermentation conditions. This type of biomass growth model

coupled with model equations for enzyme production will be useful in reactor design for large-scale applications.

2.10 POTENTIAL APPLICATIONS FOR THE LIGNIN-DEGRADING SYSTEM OF *PHANEROCHAETE CHRYSOSPORIUM*

Several areas of potential applications for the ligninolytic system of *P. chrysosporium* have been discussed in the literature. These applications include treatment of bleach plant effluents, biopulping and biobleaching, and the conversion of lignocellulosic materials into feeds, fuels, and chemicals. Decolorization and detoxification of waste effluents hold the greatest promise of being the first industrial application of the lignin biodegradation system of *P. chrysosporium*.

2.10.1 Decolorization of Bleach Plant Effluents

Bleach plant effluents from the first alkali extraction stage of the paper pulping process contain, in addition to other pollutants, colored high-molecular weight chlorinated lignins. Current biological, chemical, and physical methods available for treating wastewater effluents do not satisfactorily remove these substances. The removal of colored chlorolignins is desirable for aesthetic reasons as well as for compliance to regulatory standards which presently exist or are expected to be passed in the future. Chlorolignins are formed during chemical bleaching of pulp. They are not highly toxic in themselves but may be broken down in the environment to low molecular weight

chlorinated aromatics which are toxic, mutagenic, and carcinogenic.

The primary structures believed to result in the color of lignin are quinoid structures, double bond and carbonyl groups conjugated with aromatic rings, and metal complexes.[53] Ferric complexes of phenolic hydroxyl groups and catecholic structures form chromophores which absorb visible light. It is believed that the brown color of effluents may increase water temperature and decrease photosynthesis resulting in lower levels of dissolved oxygen.[58] This may have detrimental effects on the aquatic ecosystem.

P. chrysosporium has the ability to degrade chlorinated lignins and decolorize bleach plant effluents.[54] This is accomplished either by breakdown of the lignin polymer to colorless or volatile products or by attack on the chromophores of the polymer or both.[64] Evidence indicates that decolorization initially involves physical adsorption of compounds to the fungal biomass. Modification by the ligninolytic system of the fungus then occurs. A color reduction of 60% was achieved in 2-4 days using mycelial pellets in shake flasks.[17]

Decolorization experiments have shown that the rate of decolorization is independent of initial glucose concentration. In one set of experiments, the initial glucose concentration was reduced from the widely-used 10 g/l level to 2 g/l without affecting the decolorization rate.[58] Reducing the glucose concentration would have the additional benefit of decreasing the final COD level in wastewater treatment processes.

Besides decolorization and dechlorination of lignin molecules, *P. chrysosporium* is able to degrade several other undesirable molecules. These include DDT (1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane), polychlorinated biphenyls (PCBs), polychlorinated dibenzo(p)dioxins, as well as other polycyclic aromatic hydrocarbons.[10,48] Biosystems have been proposed which would use *P. chrysosporium* to carry out chemical detoxification of wastes.[10] This fungus has also been found to absorb polymeric dyes such as Poly R, Poly B, phenol red, Remazol blue, and crystal violet dye. These dyes are environmental pollutants which suggests potential applications for this organism in wastewater treatment of dye-related industries.[27]

2.10.2 Biopulping and Biobleaching

A second promising application of the ligninolytic system of *P. chrysosporium* is for pretreatment of lignocellulosic materials. Although the white-rot fungus degrades all components of wood, lignin is removed more rapidly than other components. This delignification of wood, referred to as biopulping, can reduce the need for more expensive mechanical pulping methods. Removal of even a small percentage of lignin from wood results in substantial energy cost reductions of up to 20% in the pulping process.[44] Pulping increases enzymatic hydrolysis of cellulose to products such as acetate, ethanol, and methane by anaerobic organisms. Cellulase-free mutants have

been developed which leave the desired cellulosic units of wood intact.

A third use for the ligninolytic system of *P. chrysosporium* in the pulp and paper process besides biopulping and treatment of waste effluents is in biobleaching of wood pulp. After the pulping step, chlorinated chemical bleaching agents are used to remove lignin residues. The use of bleaching agents, which are harmful pollutants, could be eliminated by using fungal biobleaching. The primary disadvantage associated with biobleaching is that long exposure times are needed which prevent the use of a continuous process in the paper industry. This problem could possibly be avoided by using only the lignin-degrading enzymes for bleaching instead of the entire fungal biosystem.

2.10.3 Conversion of Lignocellulosics into Feeds, Fuels, and Chemicals

Lignocellulosic material could potentially be converted to animal feeds. Lignin shields polysaccharide components of wood from hydrolytic enzymes. Treatment of lignocellulosics with white-rot fungi would reduce the lignin content and increase the digestibility and nutritional value for animal consumption.

The eventual depletion of oil supplies has prompted research on the bioconversion of lignocellulosics to liquid fuels. The saccharification process is rate-limited by the lignocellulosic pretreatment step.[10] This suggests the possible use of lignin-degrading organisms to efficiently reduce the

lignin content of lignocellulosic materials and eliminate chemical or physical methods currently used.

The bioconversion of lignin into chemicals presently made from petroleum is an inviting alternative due to the chemical substructures of lignin and the abundance of lignin as a renewable resource and waste material. Waste lignins such as lignin sulfonates and kraft lignins are currently used in many products as additives or to enhance the properties of the product. Examples of these products include emulsifiers, stabilizers, grinding aids, dispersants for oil recovery, drilling muds, and surfactants.[10,11] Vanillin and dimethyl sulfoxide are examples of low-molecular weight chemicals made from lignin sulfonates and kraft lignins.[10] Microbial conversion of lignocellulosic materials would be less energy intensive than conventional methods but would require a longer production time.

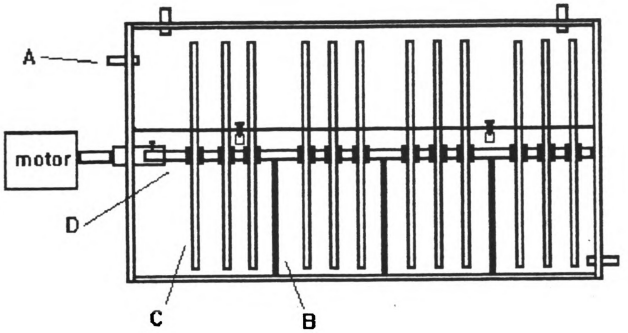
Previous work using *P. chrysosporium* in the RBC has been limited to decolorization and dechlorination studies of bleach plant effluent. Further studies to optimize the medium and RBC operating conditions are necessary to enhance biomass growth and increase enzyme expression. Although the kinetics of decolorization have been modelled for the RBC, no modelling has been reported for biomass growth. This thesis presents the first attempt to examine and model fungal growth in the RBC. These efforts are necessary in order to evaluate and design a large-scale process using the ligninolytic system of *P. chrysosporium*.

CHAPTER 3. MATERIALS AND METHODS

A rotating biological contactor was used for lignin peroxidase production from *Phanerochaete chrysosporium* immobilized on polyethylene discs. The discs were slowly rotated at 1.5 rpm alternately through a nitrogen-limited medium and a 100% oxygen atmosphere. A continuous oxygen flow rate of 0.35 l/min entered the reactor. Fermentations were conducted at 37°C. The reactor was inoculated with homogenized mycelia grown in Fernbach flasks. Biomass growth data were obtained by removing discs from the reactor during the course of the experiment and determining the biomass dry weight per disc. Lignin peroxidase activity began on day 4 and reached a maximum on day 6 or 7.

3.1 DESCRIPTION OF THE ROTATING BIOLOGICAL CONTACTOR

Experiments were run using two rotating biological contactors (RBCs) constructed by the Physics and Astronomy Machine Shop of Michigan State University. The electrical wiring of the motor and speed controller was connected by the Electronics and Computing Service of MSU. As shown in Figure 3.1, the RBC is a cylindrical reactor constructed from a 3/8 inch Plexiglass tube. It measures 13 inches in length and has a diameter of 9 inches. The top of the cylinder opens and closes as a lid. The lid closes upon a layer of silicone rubber in order to provide an air-tight seal.



A = sample ports
B = baffles
C = shaft
D = discs
E = hinge on reactor lid

Figure 3.1. Schematic diagram of the rotating biological contactor (front view).

The reactor was constructed so as to divide the main chamber into four compartments with three 1/4-inch thick Plexiglass baffles. The first compartment is larger than the others due to design constraints. Each baffle contains a removable Teflon threaded plug 3/8-inch in diameter placed toward the bottom of the baffle on alternating sides. This allows the reactor to be operated either as four isolated compartments when the plugs are in place or as a single batch reactor when the plugs are removed.

A 3/8-inch diameter stainless steel shaft is seated axially in the cylindrical reactor on top of the baffles. The shaft is removable which provides a convenient means of mounting and removing discs. The shaft is rotated by a Dayton AC/DC right angle gear motor mounted outside of the reactor. One RBC is fitted with a 2.8 full load rpm motor (Grainger, stock #2Z797) and the other reactor has a 6.7 full load rpm motor (Grainger, stock #2Z798). The motor is connected to a speed controller (Grainger, stock #4X796) which can vary the shaft rotation speed from 20-100% of the maximum motor speed. The reactor and motor are mounted on a stainless steel platform for support.

Discs made from various materials are mounted on the shaft and rotated through liquid medium contained in the reactor. The discs placed on the shaft between stainless steel washers which are held in place with screws. The discs have a diameter of 8 inches and are 1/8-inch thick. Discs were constructed from polyethylene, polypropylene, nylon, polytetrafluoroethylene (Teflon), and polymethamethacrylate (PMM). Polymer sheets for

disc construction were obtained from Almac Plastics, Inc., Grand Rapids, MI. The discs were scored in a cross-hatch design using a scribe. Most experiments were run using the polyethylene and polypropylene discs. One to three discs may be placed on the shaft in each compartment. The space between discs when three discs are used in a compartment is 3/8-inch. The total disc surface area covered by the mycelia for twelve discs in the RBC and 2.8 liters of medium is 7310 cm^2. The surface area to volume of media ratio for these conditions is 2610 cm^2/l. A summary of the major design characteristics of the RBC is presented in Table 3.1.

Table 3.1 Design characteristics of the RBC.

Reactor	
Material	Plexiglass
Length x Diameter	1 ft x 8.5 in
Volume of Medium	25% of RBC volume
Baffles	Four separate compartments
Mode of Operation	Continuous, batch, recycle
Discs	
Material	Polypropylene, polyethylene
Diameter	8 inches
Number	Up to twelve
Shaft	
Material	Stainless steel
Shaft rpm	1 - 10 rpm

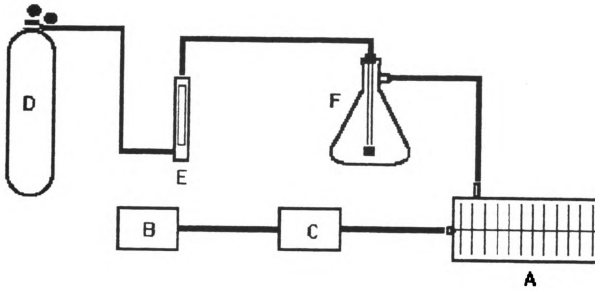
Four ports have been designed into the reactor to enable materials to be added or removed. During operation, water-saturated oxygen flows into one of the ports of the reactor

through Tygon tubing connected to a syringe. When not in use, the ports are closed with a rubber stopper. The ports provide the option of operating the reactor in a continuous flow or recycle mode. The reactor was placed in an environmental growth chamber during fermentations to maintain a constant temperature of 37°C. A schematic diagram of the experimental setup is presented in Figure 3.2.

Prior to experiments, the RBC was washed with Alconox detergent and rinsed with distilled water. The discs were soaked in an Alconox/Tergazyme solution, scrubbed with a handbrush to remove mycelial fragments, and rinsed with distilled water. The discs were then mounted on the shaft and placed into the RBC. The reactor and discs were rinsed with 95% ethanol and rinsed four times with sterile distilled water. At this point, the reactor was ready for inoculation. No problems of contamination were observed with this procedure of sterilization. Autoclaving the reactor was not a feasible sterilization method since high temperature warps the RBC materials.

3.2 PREPARATION OF STOCK SOLUTIONS

The following stock solutions were prepared as described by Michel[56] and used for media preparation. These solutions were sterilized either by autoclaving at 120°C for 15 minutes or by filtration using a 115 ml-45μm Nalgene disposable filter unit. The solutions were stored at 4°C until needed.



A ROTATING BIOLOGICAL CONTACTOR
B VARIABLE SPEED CONTROLLER
C MOTOR
D OXYGEN TANK
E FLOWMETER
F WATER-SATURATOR

Figure 3.2. Schematic diagram of experimental setup.

STOCK SOLUTIONS

Basal Medium

Add the following ingredients to 800 ml DI water, mix, and then bring the solution to 1 liter using DI water. Sterilize solution by passing it through a 115 ml-0.45µm Nalgene filter sterilizer unit (Nalge Co. #245-0045).

KH_2PO_4	20 g
$MgSO_4$	5 g
$CaCl_2$	1 g

10% Glucose

Dissolve 100 g glucose in 800 ml DI water and make up the volume to one liter using DI water. Sterilize by autoclaving at 120°C for 15 minutes.

0.1 M Dimethylsuccinate Buffer (DMS)

Dissolve 14.6 of 2,2-dimethylsuccinic acid (Sigma Chemical Co., #D-3394) in 800 ml. Adjust pH to 4.5 using 2 N NaOH. Bring volume to 1000 ml. Sterilize using a 0.45µm Nalgene filter.

Trace Element Solution

Dissolve 1.5 g nitrilotriacetic acid in 800 ml water. Adjust pH to 6.5 with 1 M KOH. Dissolve the following ingredients into the solution. Add DI water to make 1 liter. Solution will be faint yellow in color. Sterilize using a 0.45µm Nalgene filter.

$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.1 g
$AlK(SO_4)2 \cdot H_20$	10 mg
H_3BO_3	10 mg
$Na_2NMoO_4 \cdot 2H_2O$	10 mg

Ammonium Tartrate (8 g/l)

Dissolve 8 g ammonium tartrate in 800 ml DI water. Bring volume to 1 liter. Sterilize by autoclaving at 120°C for 15 minutes.

Thiamine (1 g/l)

Dissolve 0.10 g thiamine (Sigma Chemical Co., #T-4625) in 80 ml DI water. Bring volume to 100 ml. Sterilize using a 0.45µm Nalgene filter.

400 mM Veratryl Alcohol

Add 6.73 g veratryl alcohol (Aldrich Chemical Co., #D13,300-0) to 80 ml DI water. Bring volume to 100 ml. Sterilize using a 0.45µm Nalgene filter.

1% Tween 80

Add 1 ml Tween 80 (polyoxyethlene sorbitan monooleate)(Sigma Chemical Co., #P-1754) to 80 ml DI water. Bring volume to 100 ml. Sterilize using a 0.45µm Nalgene filter.

3.3 PREPARATION OF BUFFERS

Various buffers were used in the RBC for comparison studies. These buffers were prepared according to Gomori[29] at a concentration of 0.1 M and pH 4.5 as shown below. The buffers were diluted to a final concentration of 0.01 M in the RBC.

0.1 M Acetate Buffer

A. 0.1 M sodium acetate: 0.82 g into 100 ml water
B. 0.1 M acetic acid: 0.5776 ml into 100 ml water
Buffer: 22 ml of A + 28 ml of B + water to 100 ml

0.1 M Citrate Buffer

A. 0.1 M citric acid: 2.101 g into 100 ml water
B. 0.1 M sodium citrate: 2.941 g into 100 ml water
Buffer: 26.75 ml of A + 23.25 ml of B + water to 100 ml

0.1 M Succinate Buffer

A. 0.1 M succinic acid: 1.181 g into 100 ml water
B. 0.1 M NaOH: 4 g into 100 ml water
Buffer: 25 ml of A + 18.35 ml of B + water to 100 ml

0.1 M Phthalate Buffer
A. 0.1 M potassium hydrogen phthalate: 2.042 g into 100 ml water
B. 0.1 M NaOH
Buffer: 25 ml of A + 4.93 ml of B + water to 100 ml

0.1 M Tartrate Buffer
A. 0.1 M tartaric acid: 1.51 g into 100 ml water
B. 0.1 M sodium tartrate: 2.31 g into 100 ml water
Buffer: 25 ml A + 2.73 ml B + water to 100 ml

0.1 M Oxalate Buffer
0.1 M oxalic acid: 1.261 g into 100 ml water

0.1 M Dimethylsuccinate (DMS) Buffer
0.1 M DMS: 1.46 g DMS into 100 ml water

3.4 MEDIA PREPARATION

Media were prepared using autoclaved graduated cylinders, disposable pipet tips, and Erlenmeyer flasks plugged with foam stoppers and capped with aluminum foil.[15] Distilled water was sterilized by autoclaving at 120°C for 15 minutes.

Petri Plate Medium was used to make 2% malt agar plates for conidia (spore) production. This medium was prepared as described in Table 3.2 and then autoclaved at 120°C for 15 minutes. The medium was removed from the autoclave and poured immediately into 100 x 15 mm polystyrene petri plates. The plates were filled to about one half of their capacity. The plates were allowed to cool at room temperature for several hours until solidified and then stored at 4°C until used. The plates were generally used within 24 hours of being poured. One liter of Petri Plate Medium makes approximately 30 plates.

Table 3.2. Petri Plate Medium for maintenance of conidia.

Petri Plate Medium	
Glucose	20 g
Malt Extract	20 g
Peptone	1 g
Agar Bacto	20 g
0.1 M HCl	to pH 4.5
DI Water	to 1 liter

Culture Medium was used for growth of *P. chrysosporium* in the RBC. This medium was prepared from stock solutions as described in Table 3.3. In some experiments, the medium used was identical to Culture Medium with the exception that 50 ml of 1% Tween 80 was substituted for an equal volume of distilled water.

Table 3.3 Culture Medium used in the RBC.

Culture Medium	
Basal Medium	100 ml
10% Glucose	100 ml
0.2 M DMS Buffer	100 ml
Trace elements	70 ml
Ammonium Tartrate (8 g/l)	25 ml
Thiamine (1g/l)	1 ml
400 mM Veratryl Alcohol	1 ml
Adjust to pH 4.5 with 2 N NaOH or 1 M HCl	
DI Water	to 1 liter

3.5 MAINTENANCE OF *PHANEROCHAETE CHRYSOSPORIUM*

Phanerochaete chrysosporium Burds BKM-F 1767 (ATCC 24725) and the mutant strain SC26 were used in these experiments. The fungus was maintained by transferring 0.5-1.0 ml of thawed conidia onto 2% malt agar plates. The conidia were spread

evenly over the agar surface using a sterile bent glass rod. The plates were incubated at 37°C for 4-5 days or until the plates were covered with white conidia. Five ml of sterilized distilled water was added to each plate, and the plates were scraped with a sterile bent glass rod to remove the conidia. The suspension was vacuum filtered through glass wool to remove any mycelia, and the harvested conidia were collected in sterile 12 x 75 mm polystyrene tubes with caps. The conidia were stored at 4°C for up to three months. The frozen conidia were thawed to room temperature when used for inoculation.

The optical density (OD) of the conidia was measured in a Varian Series 634 spectrophotometer at 650 nm in 1.5 ml polystyrene cuvettes. Appropriate dilutions were made. An absorbance of 1.0 is approximately equal to 5 x 10^6 conidia per ml.[65]

3.6 INOCULUM PREPARATION

Fernbach flasks capped with aluminum foil were autoclaved at 120°C for 15 minutes. 75 ml of Culture Medium were added to each flask. The flasks were inoculated with thawed conidia to either 0.05, 0.1, or 0.2 OD. The following formula was used to determine the volume of conidia needed for inoculation:

(75 ml medium)(OD of conidia) =

(OD of flask)(x ml of conidia)

where OD of flask = 0.05, 0.1, or 0.2 OD
x = volume of thawed conidia needed for inoculation.

The flasks were incubated at 37°C for 48 hours to form thin mycelial mats. Care was taken not disturb the flasks during incubation as the ligninolytic system of the fungus is easily affected by disturbances. The contents of the flasks were then added to an autoclaved blender container and homogenized using a Waring blender at high speed for seven minutes. The blender container was placed in an ice-water bath several times during homogenization to prevent heat damage to the mycelia. The homogenized mycelial suspension was used as inoculum for the RBC.

3.7 GROWTH EXPERIMENTS IN THE RBC

Experiments were conducted using the RBCs to gather biomass growth data. Fermentations were run for 10 to 16 days. Each reactor contained a total of 2.8 liters of Culture Medium and were inoculated with the volume of homogenized mycelia indicated in Table 3.4. The shaft was rotated at 1.5 rpm. Oxygen of medical grade purity was used at a continuous flowrate of 0.35 l/min into the reactor. The baffle plugs were in place for most experiments. Samples of extracellular fluid were withdrawn daily and assayed for glucose, extracellular protein, and pH level. Discs were periodically removed from the shaft and the dry weight of biomass per disc was recorded.

Table 3.4 Volume of inoculum and medium used in the RBC.

Compartment of RBC	Volume Mycelia (ml)	Volume Medium (ml)
1	50	1000
2	25	600
3	25	600
4	25	600
Total	125	2800

3.8 ANALYTICAL METHODS

Samples of culture fluid were periodically withdrawn in 3 ml aliquots from compartments of the RBC using a Gilson Pipetman pipet. The samples were assayed using the following methods.

3.8.1 Glucose Measurement

Glucose was measured using DNS reagent which was prepared as described in Table 3.5. DNS reagent was stored up to three weeks at 4°C. Samples were prepared by adding 0.9 ml of distilled water to 0.1 ml of extracellular fluid in a test tube. Three ml of DNS reagent was added to each sample. The test tubes were vortexed and placed in a boiling water bath. After five minutes, the test tubes were removed and immediately placed in a water bath at room temperature to quench the reaction. The test tubes were cooled to room temperature. The contents of the test tubes were then transferred to 1.5 ml polystyrene cuvettes and the absorbance at 640 nm was recorded using a Varian Series

634 spectrophometer. Care was taken to cool the samples thoroughly as temperature affected absorbance measurements.

Table 3.5. DNS Reagent used for Glucose Assay

DNS Reagent	
Distilled water	1416 ml
3.5 Dinitrosalicylic acid	10.6 g
Sodium hydroxide	19.8 g
Dissolve above chemicals, then add:	
Potassium sodium tartrate	306 g
Phenol (melted)	7.6 ml
Sodium meta bisulfite	8.3 g

A calibration curve was prepared using a glucose standard. A 1 g/l glucose stock solution was made and stored at room temperature. A tenfold dilution of the stock solution was used to make standards in concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 g/l. Three ml of DNS reagent was then added to each standard. The standards were vortexed and placed in the boiling water bath with the culture fluid samples. After cooling, the absorbance of the standards was recorded and used to determine a calibration curve using linear regression analysis. The concentration of glucose in the samples was determined from the calibration curve.

3.8.2 Lignin Peroxidase Assay

The lignin peroxidase assay described by Tien and Kirk[65] was used. This assay is based on the oxidation of veratryl alcohol to veratryl aldehyde in the presence of hydrogen

peroxide. The absorbance of veratryl aldehyde was monitored at 310 nm using a Varian Cary 219 spectrophotometer. Veratryl alcohol does not absorb at this wavelength.

The tartaric acid and veratryl alcohol solutions used in this assay were prepared and stored at room temperature for a maximum time of three months. The hydrogen peroxide solution was made fresh daily. A sample with a total volume of 1 ml was prepared in a test tube by combining the solutions in Table 3.6 according to the order in which they are listed. The contents of the test tube were gently mixed by inverting the test tube several times. The contents were poured into a 2 ml quartz cuvette with a path length of 1 cm and placed into the spectrophotometer. The increase in absorbance as a function of time was measured using a chart recorder.

Table 3.6. Preparation of Sample for Lignin Peroxidase Assay

Solution	Volume (ml)
culture fluid	500
125 mM tartaric acid pH 2.5	400
40 mM veratryl alcohol	50
8 mM hydrogen peroxide	50

For most experiments, a chart speed of 0.1 cm/s was used and 1.0 OD was equivalent to 100 squares. A baseline was established on the chart paper before the sample was inserted

into the spectrophotomer. The change in OD of the sample was measured as the vertical deviation from the baseline in squares over a horizontal distance of 4 cm on the chart paper. The ligninase activity was calculated as units per liter according to the formula below using a molar extinction coefficient of 9300 $M^{-1}cm^{-1}$.

ligninase activity (U/l) =

$$\frac{(\text{\# of squares})(1.0\ OD)(60s/min)(1000ml/500ml)(1x10^{6}mmol/mol)}{(4\ cm)(10\ s/cm)(100\ squares)(9300\ M^{-1}cm^{-1})(1\ cm)}$$

One unit of ligninase activity is defined as one micromole of veratryl alcohol oxidized to veratryl aldehyde per minute.

3.8.3 Biomass Dry Weight Measurement

The weight of each disc was recorded before being mounted in the RBC. At selected time intervals during fermentation, a disc was removed from the RBC and placed on a piece of preweighed aluminum foil. The disc and foil were dried to a constant weight at 37°C and the dry weight of biomass was determined.

3.8.4 Protein Assay

Protein was measured by the method of Bradford[9] using the BIO-RAD Laboratory protein microassay procedure using bovine serum albumin (BSA) standards. A 5 mg/ml stock solution of bovine serum albumin was prepared and could be stored at 4°C for six months. A tenfold dilution was made from the stock

solution for each experiment and stored at 4°C when not in use. Standards were prepared using the diluted BSA solution in concentrations of 2.5, 6.25, 12.5, 18.75, and 25 mg/ml.

Samples for the protein assay were prepared by pipeting 800 ml of culture fluid into a test tube. 200 ml of BIO-RAD Dye Reagent Concentrate (BIO-RAD Laboratories, catalog #500-0006) was added to both standards and samples and the test tubes were vortexed. After five minutes, the absorbance at 595 nm was measured using 1.5 ml polystyrene cuvettes and a Varian Series 634 spectrophotometer. A calibration curve was determined using linear regression analysis on the absorbance measurements of the standards. Protein concentration in the samples was calculated from the calibration curve.

3.8.5 Measurement of pH

The pH of culture fluid samples was measured using a Corning General Purpose Combination electrode (catalog #76530) and a Corning 240 pH meter. The electrode was calibrated with standard buffers of pH 4 and 7.

3.8.6 Preparation of Samples for SEM Micrographs

Scanning electron micrographs were taken of mycelia from the RBC at the Center of Electron Optics of Michigan State University. A sample was prepared for SEM by first fixing it in a 4% glutaraldehyde/0.2 M phosphate solution for one hour. The sample was transferred to 0.2 M phosphate buffer for 10 minutes and then dehydrated using a series of ethanol solutions (25, 50,

75, 95, and two solutions of 100%) for 10 minutes each. The samples were critical-point dried using CO_2, mounted on stubs, gold-sputtered to 20 nm thickness, and then inspected using the scanning electron microscope.

CHAPTER 4. EFFECT OF VARIOUS PARAMETERS ON LIGNIN PEROXIDASE PRODUCTION BY *P. CHRYSOSPORIUM* IN THE RBC

Fungal attachment to the discs of the RBC and expression of the ligninolytic system of *P. chrysosporium* were obtained in the RBC. Many factors affect fungal growth and enzyme production including reactor operating conditions and fermentation conditions. During the process of obtaining measurable quantities of enzyme activity, certain parameters were selected as having an important influence on the fungus. The results found from studying the effects of these parameters on fungal behavior are presented below.

4.1 OXYGEN FLOW RATE

Fermentations in the RBC were run using two different continuous oxygen flow rates of 1.4 l/min and 0.35 l/min. This corresponds to 0.5 l/(l·min) and 0.125 l/(l·min) for a medium volume of 2.8 liters. Lignin peroxidase activity was either very low or non-existent in experiments oxygenated at the higher flow rate. When the flow rate was reduced by a factor of four to 0.35 l/min, higher enzyme activity was consistently obtained in the RBC. Results presented in this work are from experiments which were continuously oxygenated at 0.35 l/min of pure oxygen.

4.2 STRAIN COMPARISON

The behavior of two strains of *P. chrysosporium* was observed in the RBC. The wild-type strain BKM-F and the mutant

strain SC26 were compared on the basis of lignin peroxidase production. The results of this experiment are shown in Figure 4.1. Enzyme activity began about one day earlier (day 4) with SC26 compared to BKM-F. The maximum activity measured for both strains was roughly equivalent, but enzyme activity decreased more rapidly with the SC26 strain. No enzyme activity was measured for either strain after day 11. Because BKM-F gave higher overall lignin peroxidase activity, this strain was used in subsequent experiments with the exception of the growth modelling experiments for which SC26 was used.

Researchers have grown various strains of *P. chrysosporium* in the RBC including BKM-F, SC26, and ME-446.[37,57,72] Kirk and his coworkers[37] reported higher lignin peroxidase production with SC26 than with BKM-F. They suggest that SC26 is able to better adhere to the discs and therefore produce higher lignin peroxidase activity because of its thicker polysaccharide hyphal sheath compared to BKM-F.

In experiments done in this work, good adhesion and enzyme production were observed with both strains. Scanning electron micrographs of BKM-F and SC26 mycelia taken from a disc in the RBC on day 5 of a fermentation are presented in Figures 4.2 and 4.3. Also shown for comparison purposes is an SEM micrograph of a BKM-F mycelial pellet cultured in a shake flask. (Figure 4.4) These pictures show that the fungus looks similar whether it is cultured as an immobilized biofilm or as a pellet. The hyphal diameters of the BKM-F and SC26 strains are equivalent and

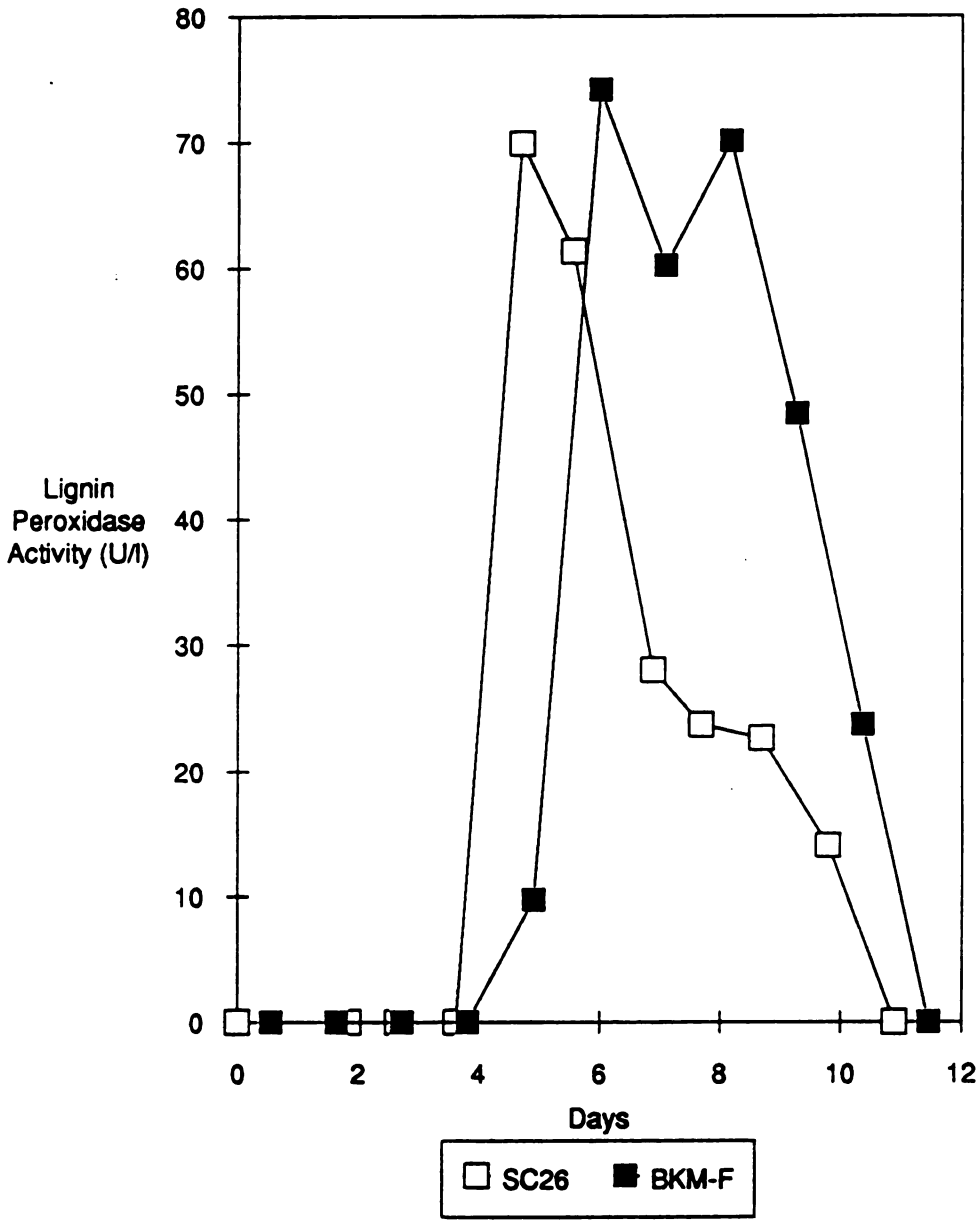
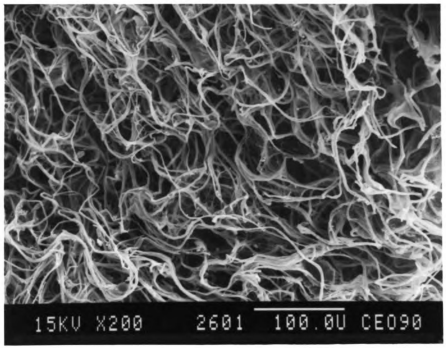


Figure 4.1 Lignin peroxidase activity for BKM-F and SC26 strains.



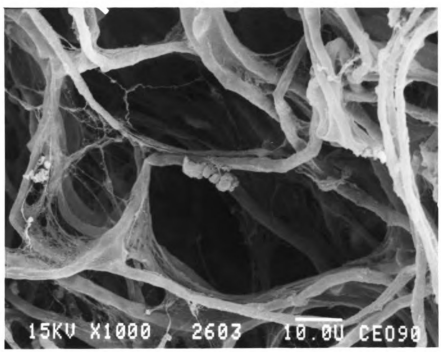
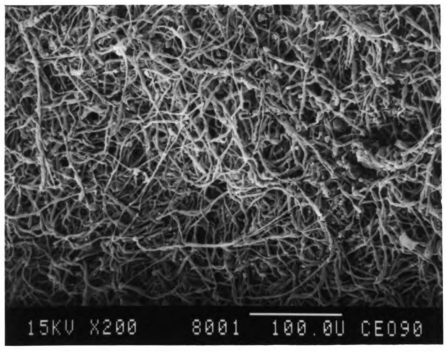


Figure 4.2. SEM micrographs of 8-day old SC26 mycelia from RBC disc.



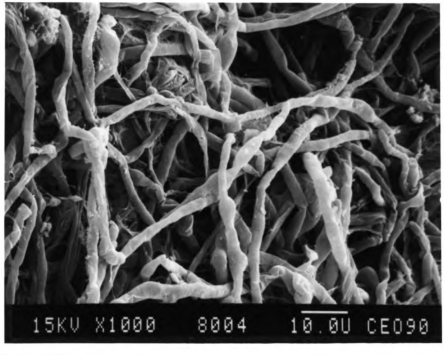
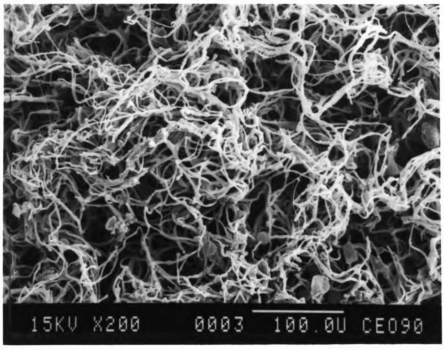


Figure 4.3. SEM micrographs of 8-day old BKM-F mycelia from RBC disc.



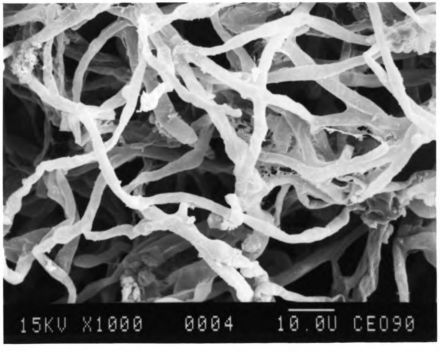


Figure 4.4. SEM micrographs of 8-day old BKM-F mycelial pellet cultured in a shake flask.

are about 2.5 microns. The major difference between the two strains is the density of the mycelial mats. The BKM-F developed a denser biofilm. Whether a denser biofilm necessarily improves lignin peroxidase production or not is unknown. Other factors such as oxygen diffusion into the film may limit enzyme production.

4.3 DISC TEXTURE AND MATERIAL

It was necessary to select an appropriate material from which to fabricate the RBC discs. Selection was based on which material would promote sufficient biomass adhesion to the discs. The effect of disc texture and disc material on fungal immobilization was studied using smooth and roughened discs made of five different materials. The materials used were polyethylene, polypropylene, nylon, polytetrafluoroethylene (Teflon), and polymethamethacrylate (PMM). Adhesion was determined by measuring the dry weight of mycelia on each disc after four days of fermentation.

No biomass growth was measured on smooth discs of any of the carrier materials. Figure 4.5 shows the biomass growth obtained on roughened discs. The values shown are averaged biomass dry weights measured from two to four discs. Adhesion was observed on all of the roughened disc materials except Teflon. The greatest amount of biomass growth occurred on nylon discs followed by polypropylene and polyethylene. Biomass growth on these discs consisted of a thin mycelial mat (1-3 mm) which evenly covered the entire disc surface. Poor adhesion was

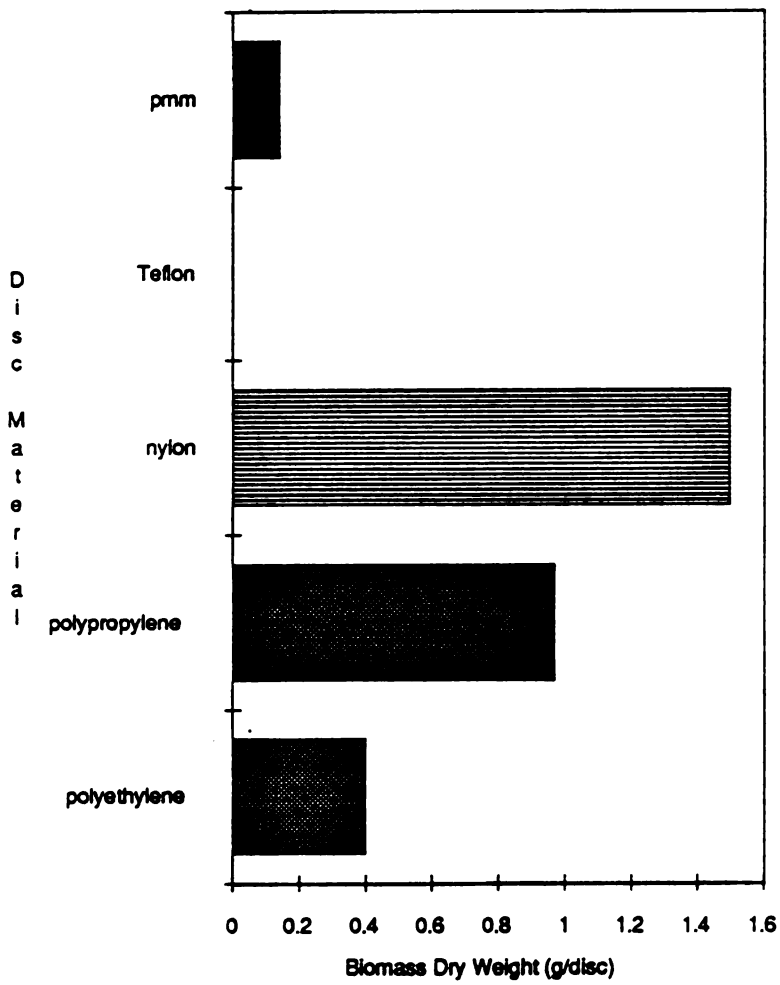


Figure 4.5 Biomass growth on roughened discs constructed from various materials.

observed on the polymethamethacrylate discs. Biomass growth on these discs was spotty and could be easily removed from the discs. Figure 4.6 is a photograph showing mycelial adhesion on a roughened polypropylene disc.

For the remaining experiments in this work, roughened discs constructed from polypropylene and polyethylene were used. Biomass adhesion was even and covered the entire surface of these discs, and the ligninolytic system of *P. chrysosporium* was expressed using these materials. Although the best adhesion was obtained using nylon, this material was not selected for disc construction due to its significantly higher cost.

The surface energy of the disc polymer has an effect on adhesion. The disc materials tested span a range of low (Teflon), medium (polypropylene, polyethylene), and high surface energy (nylon). Biomass adhesion is correlated to the surface energy of the discs with greater adhesion observed on materials of higher surface energy.

The results show that the texture of the immobilization surface and the surface energy of the disc materials are important factors in biomass adhesion. Roughened surfaces and materials of high surface energy greatly enhance adhesion. Other carrier surface characteristics which influence adhesion are hydrophobic-hydrophilic interactions, rugosity (wrinkling), overall morphology, and porosity.[4] These factors would also need to be studied in order to select the optimum disc material for fungal adhesion in the RBC.

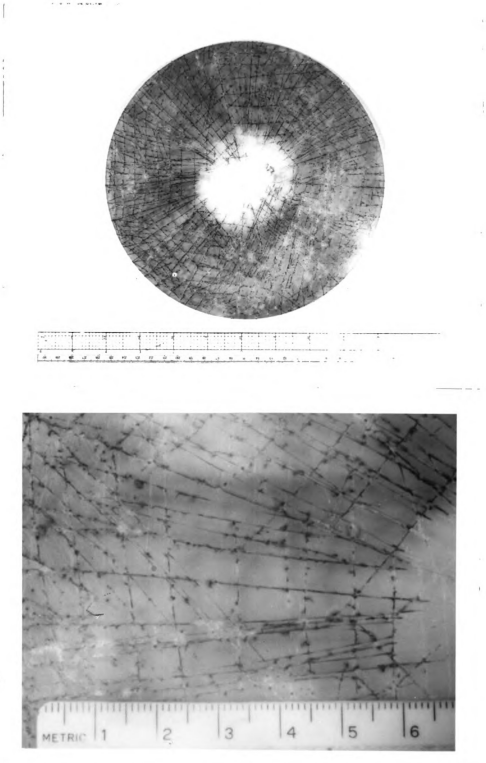


Figure 4.6 Photographs of roughened polypropylene discs.

4.4 MYCELIAL AND CONIDIAL INOCULA

Two types of inocula were used in the RBC. Thawed conidia and homogenized mycelia prepared from Fernbach flask cultures both resulted in biomass growth in the RBC. Fungal growth was delayed by 2-3 days when using a conidial inoculum compared to the mycelial inoculum. Because experiments are time-consuming and only two reactors were available, all experiments in the RBC were run using an inoculum of homogenized mycelia.

4.5 EFFECT OF INOCULUM CONCENTRATION

The effect of the concentration of conidial inoculum in the Fernbach flasks on biomass growth and lignin peroxidase production in the RBC was studied. Fermentations were run using spore inocula in the Fernbach flasks of 0.05 OD (2.5×10^5 conidia/ml) and 0.2 OD (1×10^6 conidia/ml). Biomass growth for 0.05 OD and 0.2 OD using 5% initial glucose concentration is shown in Figure 4.7. Lignin peroxidase activity is shown in Figure 4.8. These plots show that higher biomass growth on the discs did not result in greater enzyme production.

4.6 VOLUME OF MYCELIAL INOCULUM

The effect of inoculum volume on biomass growth and lignin peroxidase activity was examined. The inocula used in the RBC were obtained by homogenizing mycelial mats grown for 48 hours in Fernbach flasks which had been inoculated to 0.1 OD with thawed conidia. The three inoculum volumes tested in the RBC

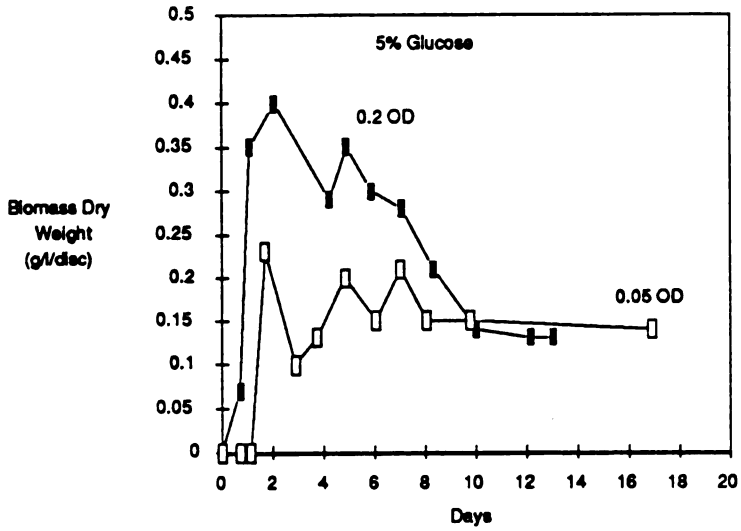


Figure 4.7 Effect of inoculum concentration on biomass growth using 5 g/l initial glucose concentration.

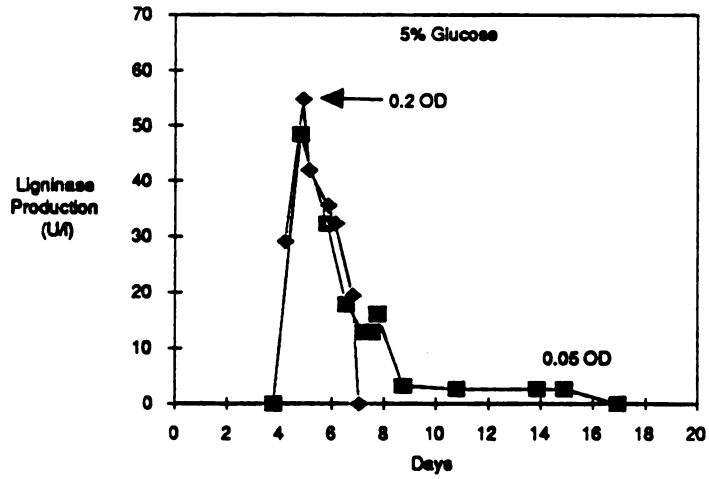


Figure 4.8 Effect of inoculum concentration on lignin peroxidase production using 5 g/l initial glucose concentration.

were 25,50 and 75 ml of inoculum per 600 ml of medium. Biomass growth was measured as mycelial dry weight per disc on day 10 of the fermentation. Lignin peroxidase activity was measured daily throughout the experiment.

The biomass dry weight measured for each inoculum volume is presented in Figure 4.9. The values shown are average weights for three discs. The standard deviation, s, is also listed. As seen in the figure, doubling or tripling the inoculum volume does not double or triple the amount of biomass which adheres to the disc. This is not unexpected since all the mycelia which are inoculated into the RBC compartment do not adhere to the discs. Some of the inoculum grows as free mycelia in the medium. The free mycelia are removed from the RBC after two days in order to prevent mycelial strands in the medium from attaching to the discs. The results indicate that 25 ml of inoculum contains sufficient mycelia to give full, even coverage of the disc surface.

These results indicate that a maximum disc carrying capacity exists. Excess mycelia from the inoculum, which do not adhere to the discs, consume nutrients in the medium until it is removed from the RBC. This most likely has an affect on the ligninolytic system of the immobilized fungus since it is known that nitrogen depletion stimulates the onset of secondary metabolism and excess glucose has an inhibitory affect on the production of lignin peroxidase.

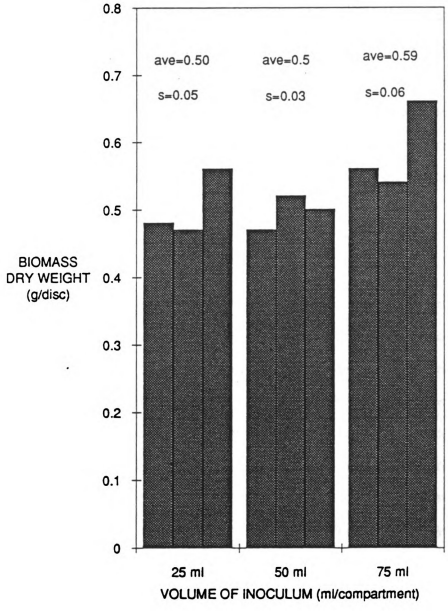


Figure 4.9 Effect of inoculum volume on biomass growth.

The lignin peroxidase production obtained for each volume of inoculum is shown in Figure 4.10. This plot shows that the inoculum volume does affect the amount of lignin peroxidase activity obtained in the RBC. The highest enzyme activity was observed using a 75-ml inoculum although a rapid decrease in activity occurred between days 6 and 7. Although the 50-ml inoculum had a lower maximum level of activity, the activity decreased less rapidly resulting in higher overall production of enzyme during the fermentation. For this reason, an inoculum volume of 50-ml was used in subsequent experiments.

4.7 USE OF SURFACTANT

Other researchers have shown that the use of surfactant in the medium promotes lignin peroxidase production from mycelial pellets grown in shake flasks. Earlier work involving the growth of *P. chrysosporium* in the RBC for lignin peroxidase production or in decolorization experiments did not include a surfactant in the medium.[37,73] The effect of the addition of the surfactant Tween 80 to the RBC medium on lignin peroxidase activity was observed in this work. A comparison of enzyme activity was made between a culture with 0.05% w/v Tween 80 and a fermentation using no Tween 80.

As shown in Figure 4.11, the presence of Tween 80 in the medium significantly increased lignin peroxidase activity. Overall enzyme production was approximately double the amount measured in the case of no Tween 80. Tween 80 was included in the RBC medium in the remaining experiments of this work.

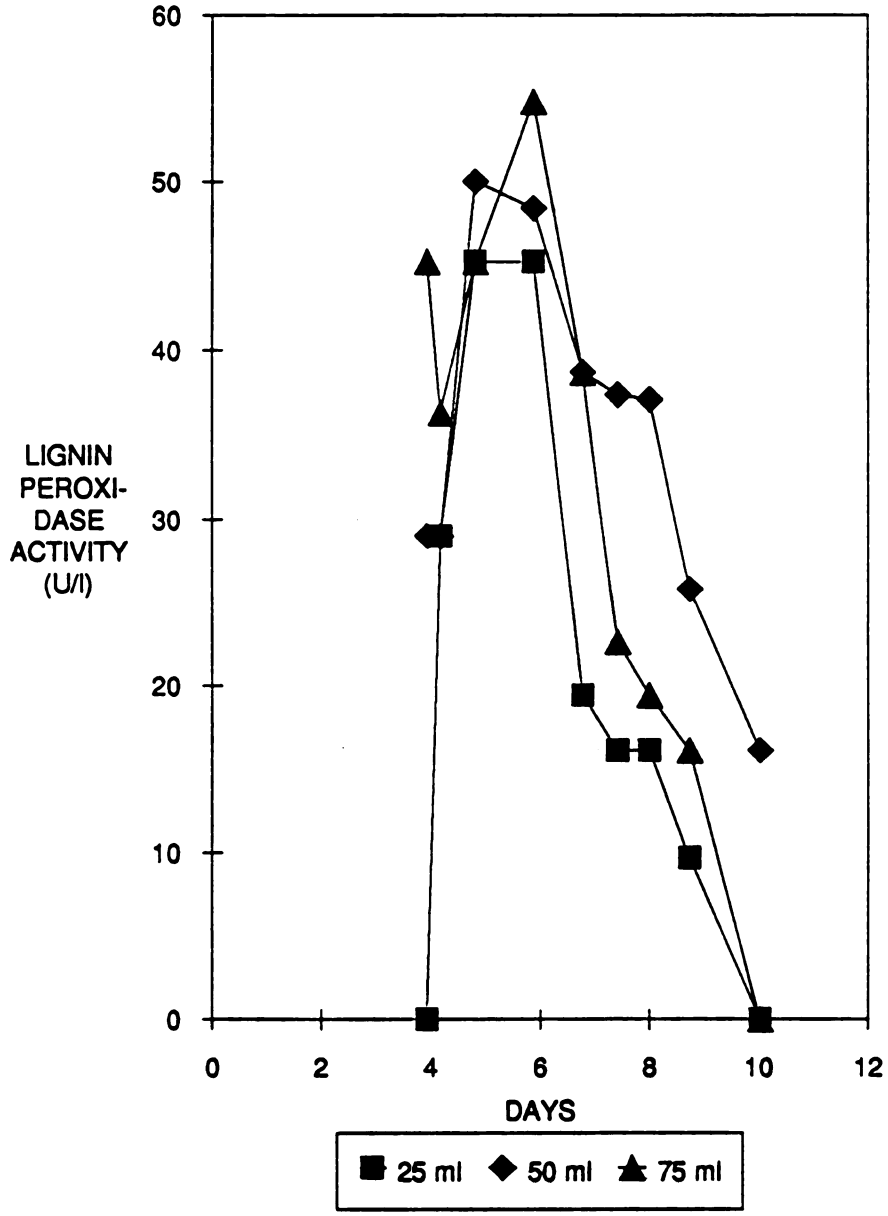


Figure 4.10 Lignin peroxidase activity for various inoculum volumes.

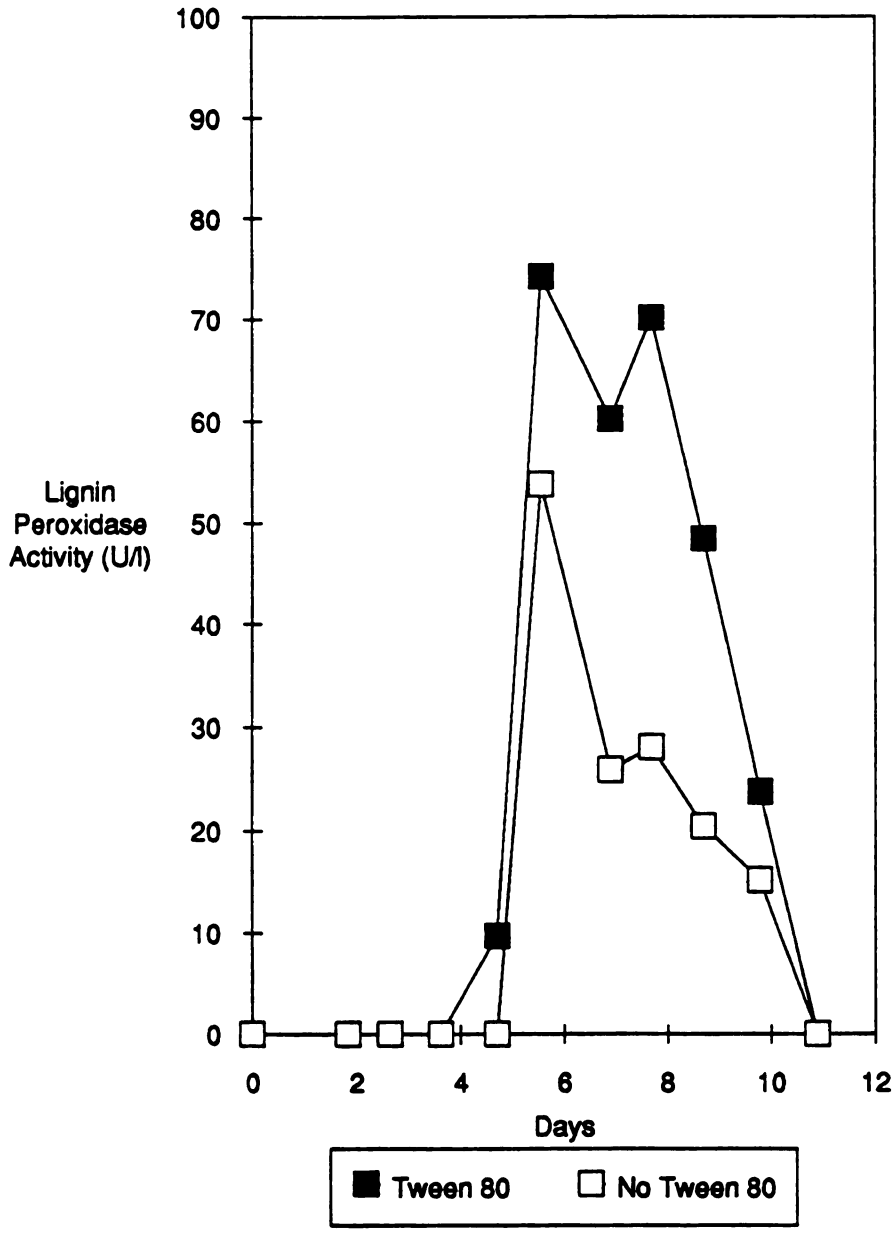


Figure 4.11 Effect of surfactant on lignin peroxidase production.

Increasing the concentration of Tween 80 in shake flask cultures to 0.2% w/v was not found to enhance lignin peroxidase activity.[56] In addition, 0.4% w/v Tween 80 concentration resulted in decreased enzyme production. Tween 80 most likely serves as a protective agent, rather than as an activator, during fermentation by preventing extracellular proteases from degrading the lignin peroxidase enzymes.

4.8 BUFFER SELECTION

The effect of various buffers on lignin peroxidase production in the RBC and on pH control of the medium was studied. The seven buffers compared were: acetate, citrate, succinate, phthalate, tartrate, oxalate, and 2,2-dimethylsuccinate (DMS) buffer. Each of these buffers was used in the RBC at a concentration of 0.01 M.

The amount of lignin peroxidase measured using each of the buffers is presented in Figure 4.12. The highest lignin peroxidase activity was observed for succinate buffer. Phthalate buffer gave the second highest lignin peroxidase activity followed by DMS, acetate, tartrate, and citrate buffer. No activity was measured for oxalate buffer although biomass growth did occur.

The pH level maintained by each of the buffers during fermentation is presented in Figure 4.13. In general, lower lignin peroxidase activity was measured in compartments where the pH of the medium was 4.8 or higher. The highest enzyme

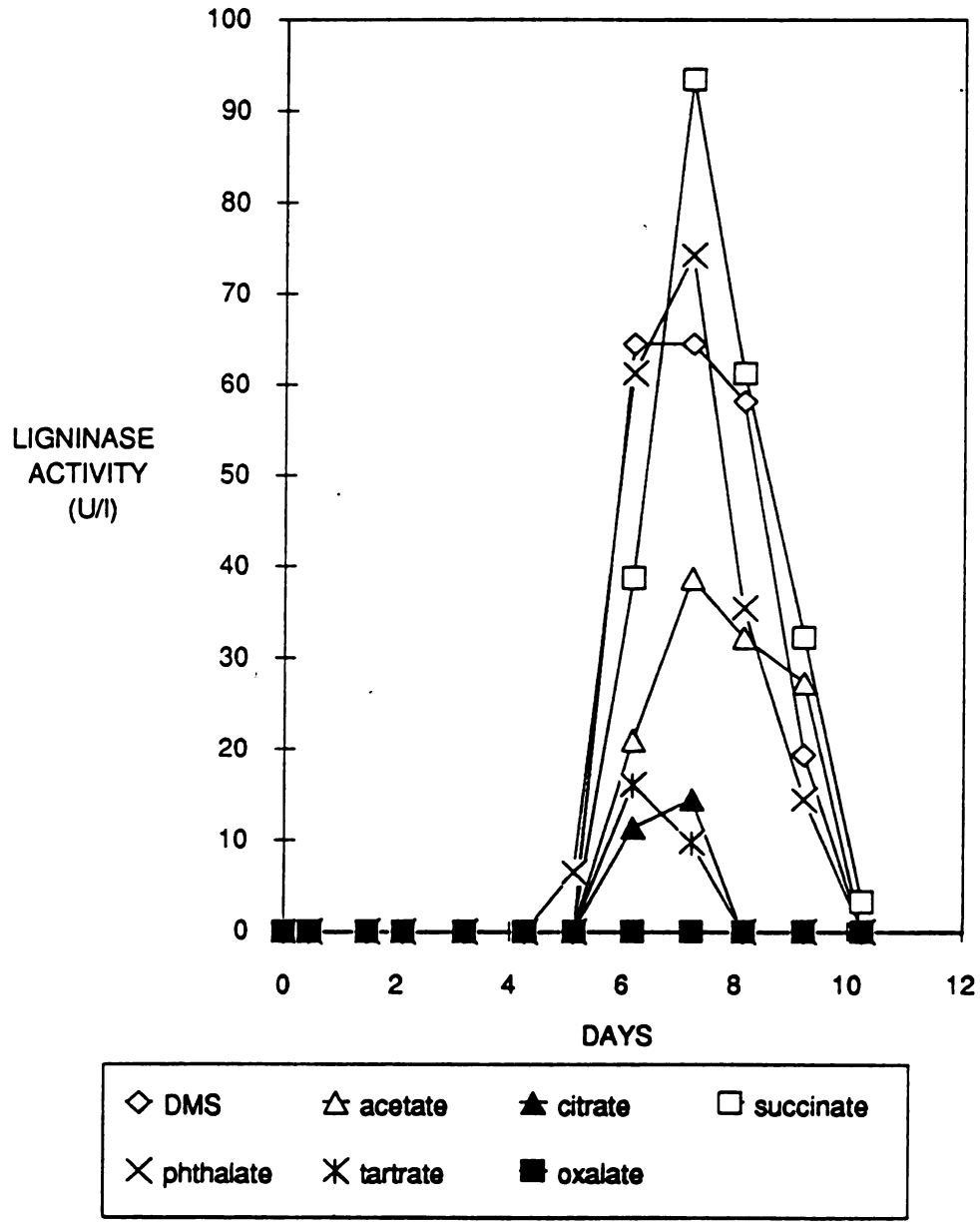


Figure 4.12 Lignin peroxidase activity for various buffers.

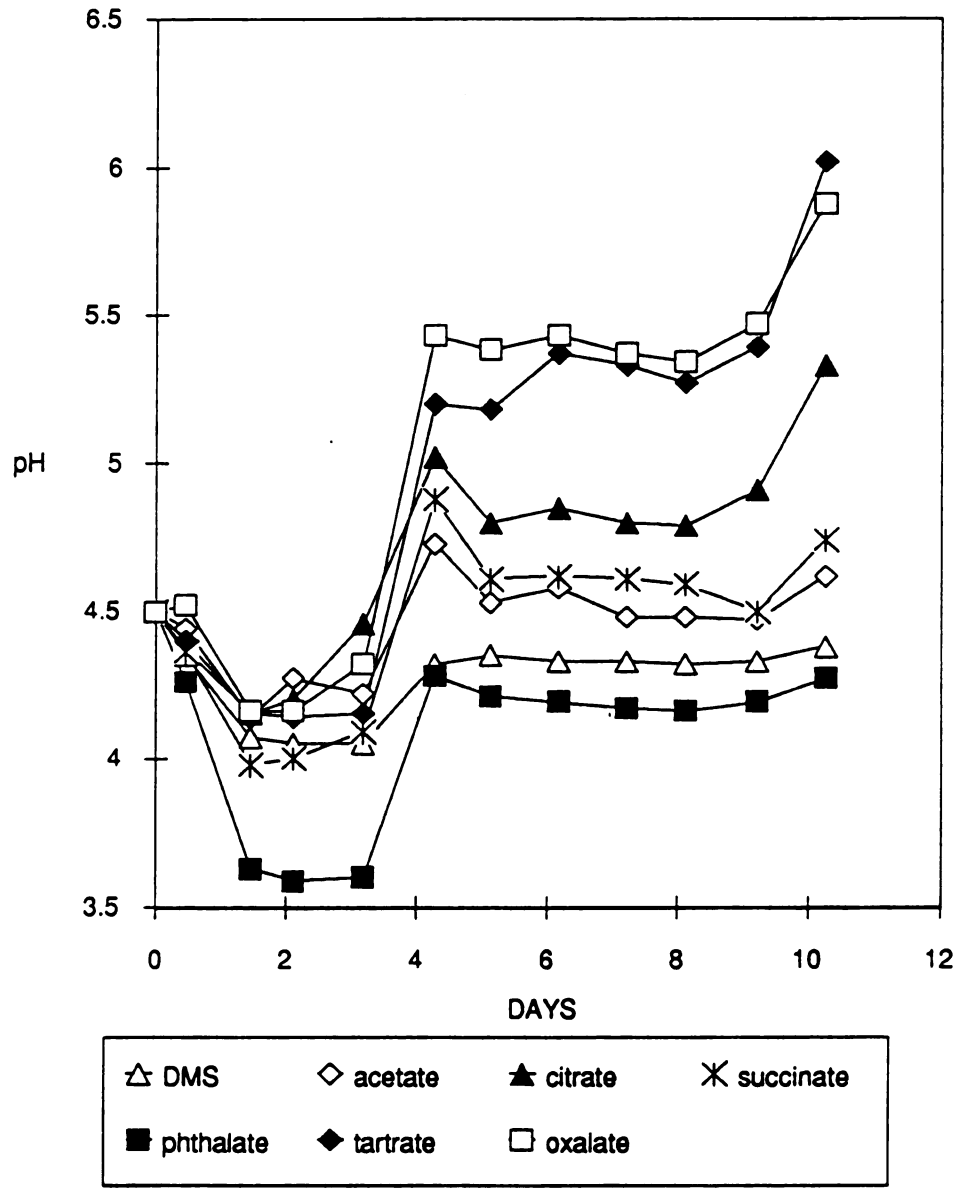


Figure 4.13 Measurement of culture fluid pH for various buffers.

activity occurred using buffers which controlled the pH between 4.2 and 4.6 during secondary metabolism.

In previous work involving the growth of *P. chrysosporium* in a RBC, researchers have used only 0.01 M DMS buffer.[37] Results of the work presented here indicate that higher lignin peroxidase activity is obtained with succinate and phthalate buffers. In addition, these buffers are less expensive than DMS buffer. With the exception of DMS buffer, the remaining buffers used in this experiment are involved in fungal metabolism. For lignin peroxidase production from mycelial pellets grown in agitated shake flasks, Michel[56] found 0.02 M acetate buffer to give the best results of the buffers tested. In this work, relatively low amounts of enzyme activity were obtained in the RBC using acetate buffer. It appears that some differences exist in the behavior of the fungus when cultured in shake flasks compared to the RBC.

4.9 SHAFT ROTATION SPEED

The rotation of the discs alternately through the medium and oxygen atmosphere provides essential nutrients to the biomass. The disc rotation through the medium also serves to remove excess biomass from the discs. The RBC provides a relatively low shear environment compared to other reactor systems for organisms which are sensitive to agitation effects. In addition, lower energy requirements are associated with the slow rotation speeds of the RBC.

The effect of shaft rotation speed on biomass growth and lignin peroxidase activity in the RBC was studied. Comparisons were made for the shaft rotation speeds of 1.5 rpm and 10 rpm. Biomass growth on the discs was about the same for both speeds. The lignin peroxidase activity obtained for each case is presented in Figure 4.14. Although slightly higher activity was observed for 10 rpm, enzyme production decreased much faster at this speed than for 1.5 rpm. This could be due to shearing effects on the enzyme. Subsequent experiments were run using a shaft rotation speed of 1.5 rpm.

4.10 LIGNIN PEROXIDASE PRODUCTION IN THE RBC

The behavior of several parameters for a typical run in the RBC is presented in Figure 4.15. A lag time of 12-18 hours exists before measurable biomass adhesion occurs on the discs. A rapid growth phase follows as the nitrogen in the medium is consumed. Secondary metabolism begins on about day 3 or 4 with the production of manganese peroxidase. The amount of biomass remains fairly constant during the rest of the fermentation until eventually a gradual decrease in biomass is observed. The decrease coincides with the depletion of glucose in the medium. The thickness of the biofilm on the discs was generally 1-3 mm. The film remained attached to the disc during the entire fermentation.

Lignin peroxidase production generally begins on day 4 or 5 and continues through day 9 or 10. Maximum activity occurs around day 7. The highest activity obtained in the RBC was

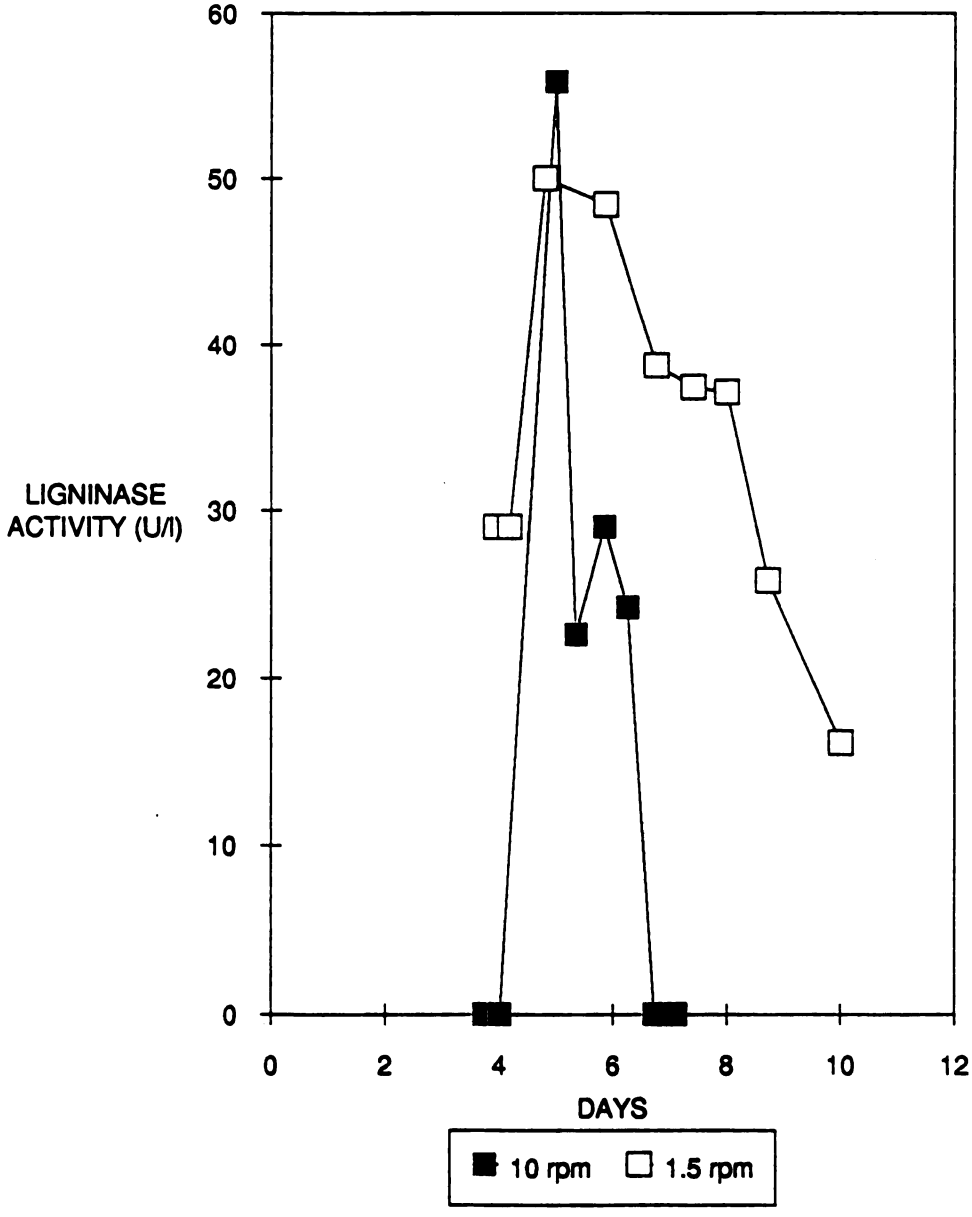


Figure 4.14 Effect of shaft rotation speed on lignin peroxidase activity.

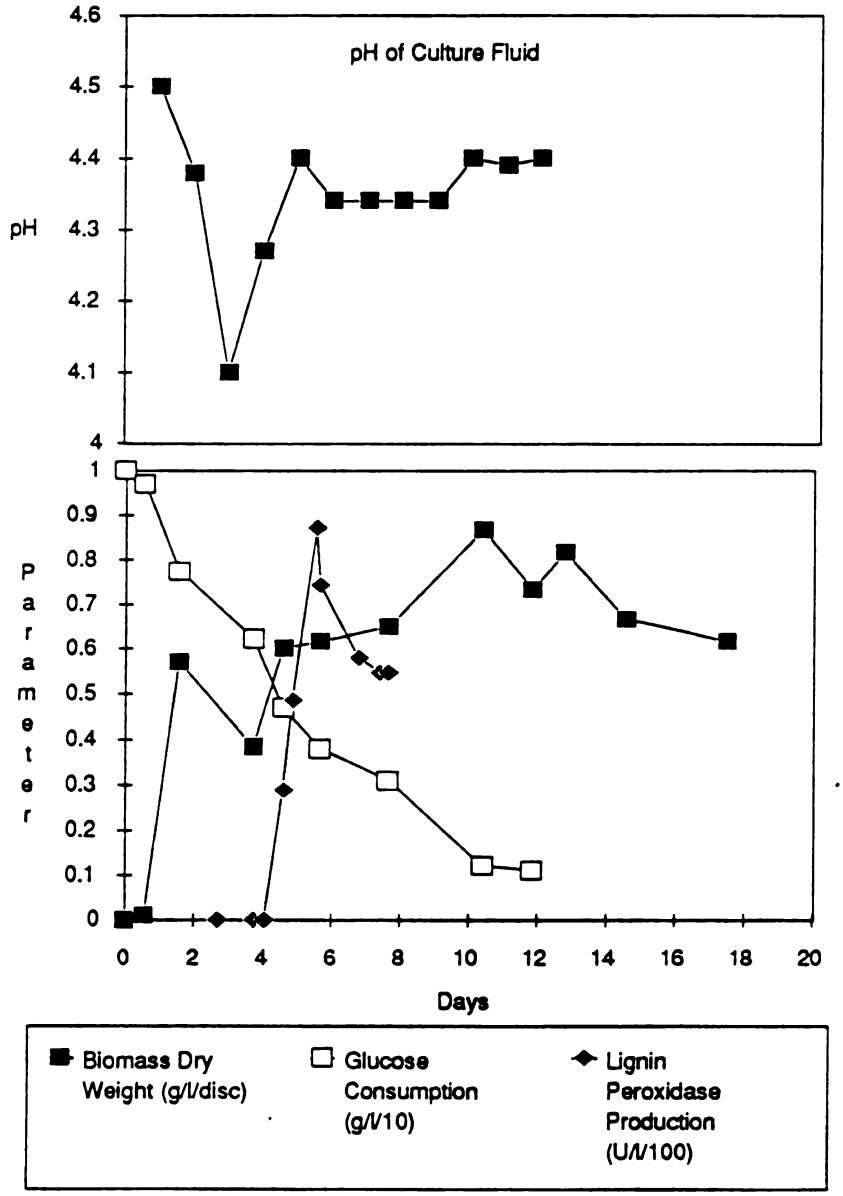


Figure 4.15 Relationship between several parameters for a typical RBC fermentation.

about 95 U/liter of sample although the average range was 50-70 U/l. This is comparable to the enzyme activity reported in other work for the RBC[37] but is much less than activity obtained in shake flask experiments (300-400 U/l).[14,56] Glucose in the medium is consumed at a rate of about 1 g/(liter·day) over a
10-day period. The pH of the medium, which is initially 4.5, drops to about pH 4.0 and then increases to about pH 4.2-4.3 by day 4. The pH remains constant until the death phase begins at which time an increase in pH is observed.

Biomass dry weight data for the RBC were found to contain more scatter than data collected for shake flask pellet experiments. One factor to account for this is the existence of disc-to-disc surface variations. Although an attempt was made to construct the discs as identically as possible, differences in the surface roughness exist which would affect fungal adhesion. In general, fermentations run under identical conditions in the RBC showed lower reproducibility than shake flask cultures.

4.11 TEMPERATURE OF FERMENTATION

In commercial applications using *P. chrysosporium*, it may be expensive to maintain a controlled temperature environment for optimum growth and secondary metabolism activity. Variations in temperature may be encountered which would affect the fungus. In order to study the affect of temperature on *P. chrysosporium*, fermentations were conducted at 37, 20, and 5°C

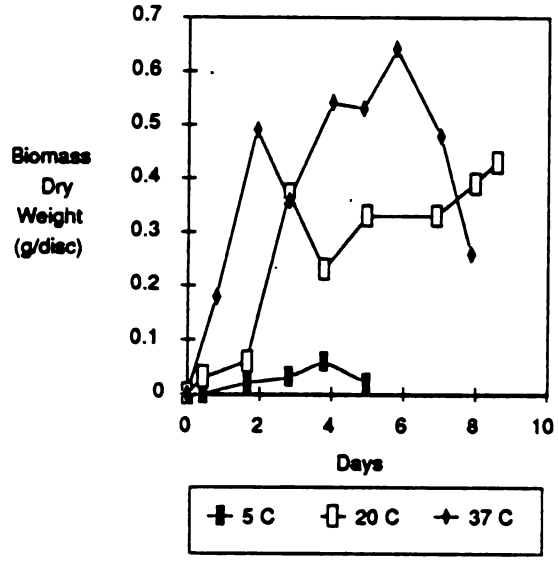


Figure 4.16 Biomass growth for various fermentation temperatures.

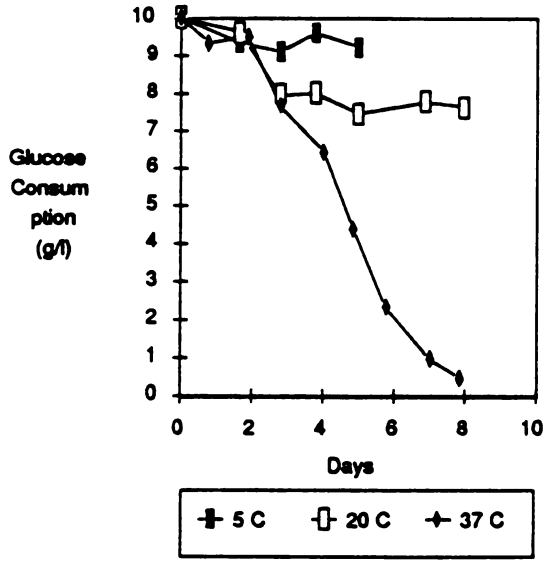


Figure 4.17 Glucose consumption for various fermentation temperatures.

and were compared on the basis of biomass growth, lignin peroxidase production, and glucose consumption. The preparation of the reactor inocula was done as described in Section 3.6. The reactors were placed in their respective temperature environment and then inoculated.

The results of these experiments are presented in Figures 4.16 and 4.17. No biomass adhesion occurred on the discs in the RBC operating at 5°C. Very little glucose was consumed from the medium indicating that the free mycelia in the medium experienced virtually no growth. The fermentation run at room temperature resulted in biofilm growth on the discs as well as lignin peroxidase activity, although both were significantly less than the growth and enzyme activity obtained in the RBC held at 37°C. These results indicate that fermentation temperature is a significant factor which must be considered when designing a large-scale reactor system or in attempting to obtain mutant strains of *P. chrysosporium* which are mesophilic or psychrophilic in nature.

CHAPTER 5. GROWTH KINETICS OF *P. CHRYSOSPORIUM* IN THE RBC

The ability to predict the behavior of a microorganism for different fermentation conditions is an important aspect in evaluating a biosystem for commercial applications and in making process design decisions. The ligninolytic system of *P. chrysosporium* offers promising potential for wastewater treatment of pulp and paper effluents, the degradation of certain hazardous substances such as dioxins and DDT, and in the treatment of lignocellulosic materials. For this reason, an understanding of the growth behavior of the fungus is desirable.

5.1 Growth Model

The linear increase in physical dimension during the exponential growth phase of fungi growing in various geometries has been reported.[41] It has also been shown that the biomass increases in quantitatively different ways depending on whether fungus is growing as a hyphal extension, on a surface, or in submerged culture. These observations were considered by Koch[41] in the development of a mechanistic model to describe fungal growth in one, two, or three dimensions.

The model used in this work is a modification of Koch's model. The model has the following form:

$$\frac{dN}{dt} = \lambda N \left(K - N \right)^n$$

where N = biomass dry weight ($g \cdot l^{-1} disc^{-1}$)
λ = growth rate constant for initial growth ($disc \cdot l \cdot g^{-1} day^{-1}$)
K = maximum carrying capacity of growth region ($g \cdot l^{-1} disc^{-1}$)
n = a parameter based on the geometry of the growth space
(ie. for one-dimensional growth, n=0,
for two or three dimensional growth,
n varies from 1-2)

The equation is integrated as follows for the case of growth on a surface where n=1:

$$N = N_o e^{k\lambda t}(K/(K - N_o + N_o e^{k\lambda t}))$$

This equation was applied to experimental data obtained from the RBC in order to determine the specific growth rate of the fungus and the carrying capacity of the discs. Unlike cell count or cell weight quantifications used for bacterial cultures, dry weight of the mycelia is used to quantify the fungal biomass.

5.2 Experimental Conditions

Fermentations for biomass growth experiments were run in the RBC according to the method described earlier in Chapter 3. Fermentations were inoculated with a homogenized mycelia solution cultured from Fernbach flasks. Initial glucose concentrations of 5, 10, and 15 g/l were used. Biomass dry weight, glucose consumption, pH, and lignin peroxidase production were measured. One disc was sacrificed for each biomass data point. The time-course of glucose concentration and pH of the culture fluid during the fermentation are presented in Figures 5.1 and 5.2.

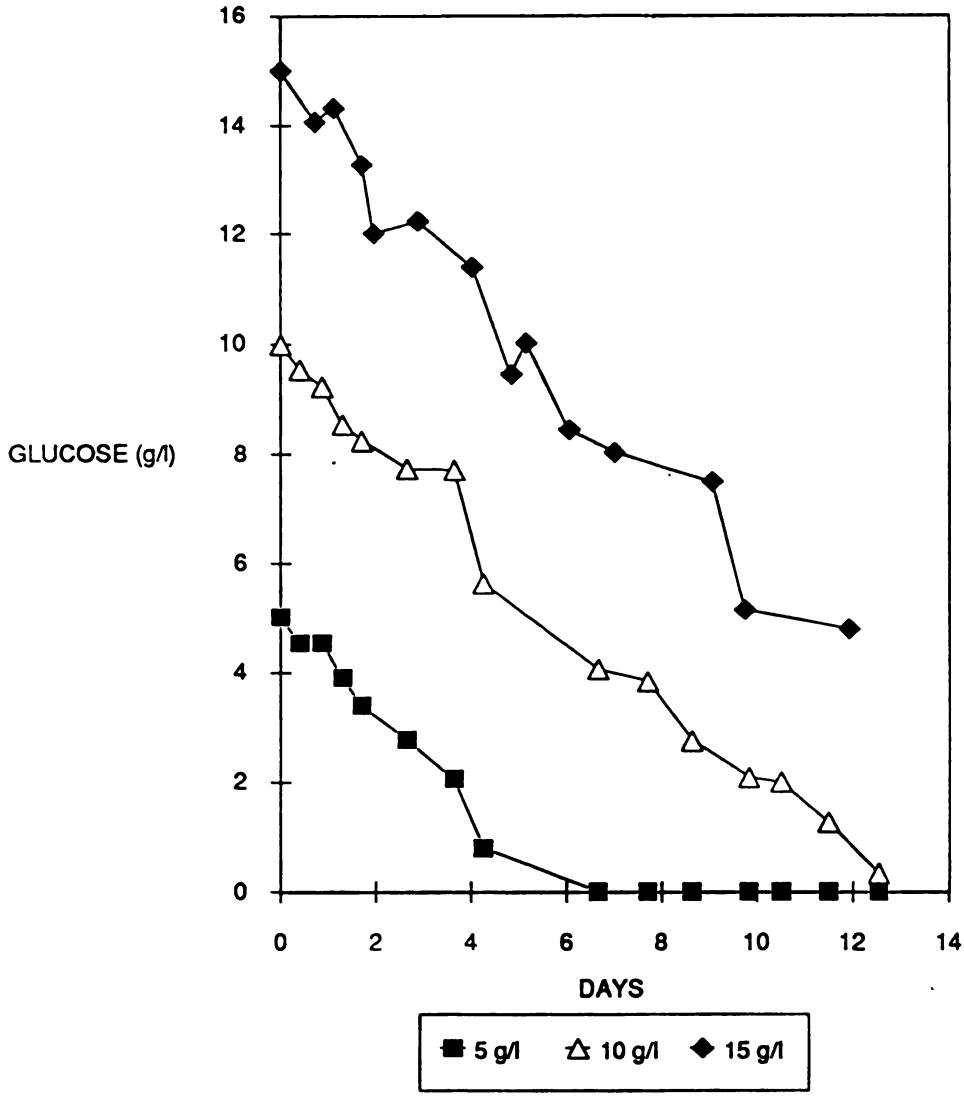


Figure 5.1 Glucose concentration as a function of time in the RBC for three initial glucose concentrations.

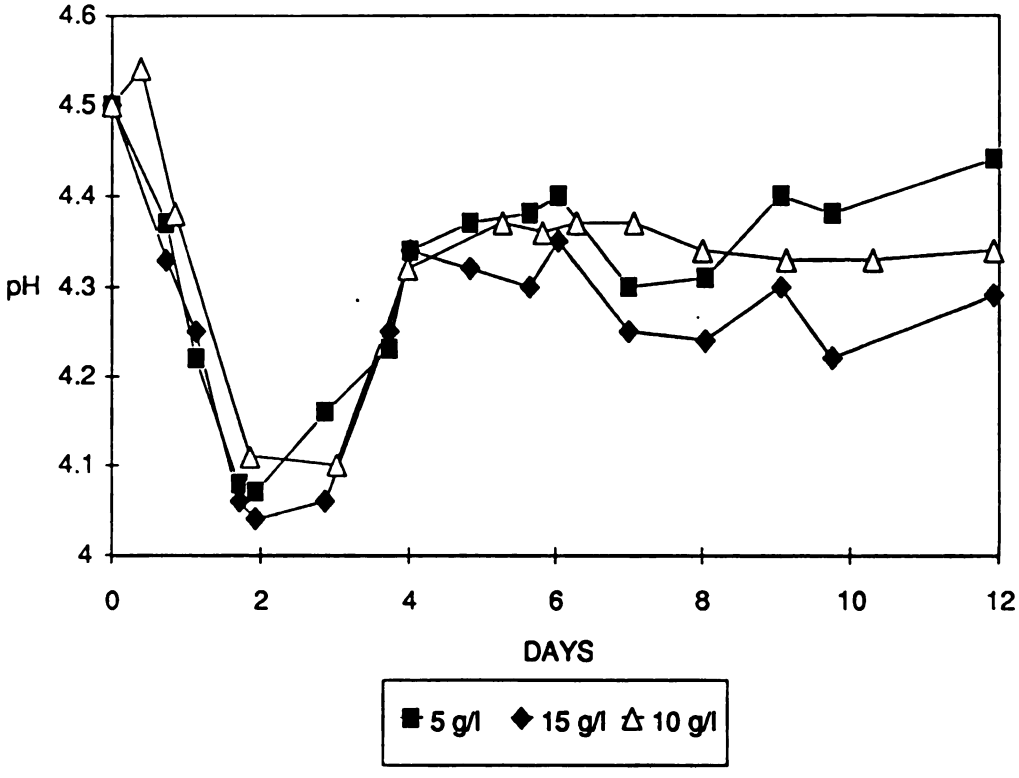


Figure 5.2 Culture fluid pH as a function of time in the RBC for three initial glucose concentrations.

5.3 Determination of Model Parameters

The two parameters of the model, K and λ, were obtained from the data. The disc carrying capacity, K, was found by inspection from the biomass growth curves. The value of this parameter was taken as the average maximum biomass dry weight for the experiment. The value of K from two experiments was averaged to give the value of K for the model.

The specific growth rate, λ, was then found using a trial and error approach. The difference between the sum of the squared values of the experimental data points and the corresponding predicted values for biomass dry weight during the time-course of the experiment was minimized. This is described by the equation below.

$$\text{difference} = \sum_{t=1,t} (\text{biomass data point at time t})^2 - \sum_{t=1,t} (\text{biomass prediction from model at time t})^2$$

The value of λ was found when the "difference" was minimized.The value of λ determined from two experiments was averaged to give the value of λ for the model. After the parameters of the model were found, a comparison of the biomass dry weight predicted by the model to experimental data was made.

5.4 Model Fit to Data

Predicted biomass dry weight values calculated using the model are shown in Figure 5.3 for the case of 10 g/l initial glucose concentration. Actual data normalized to medium volume

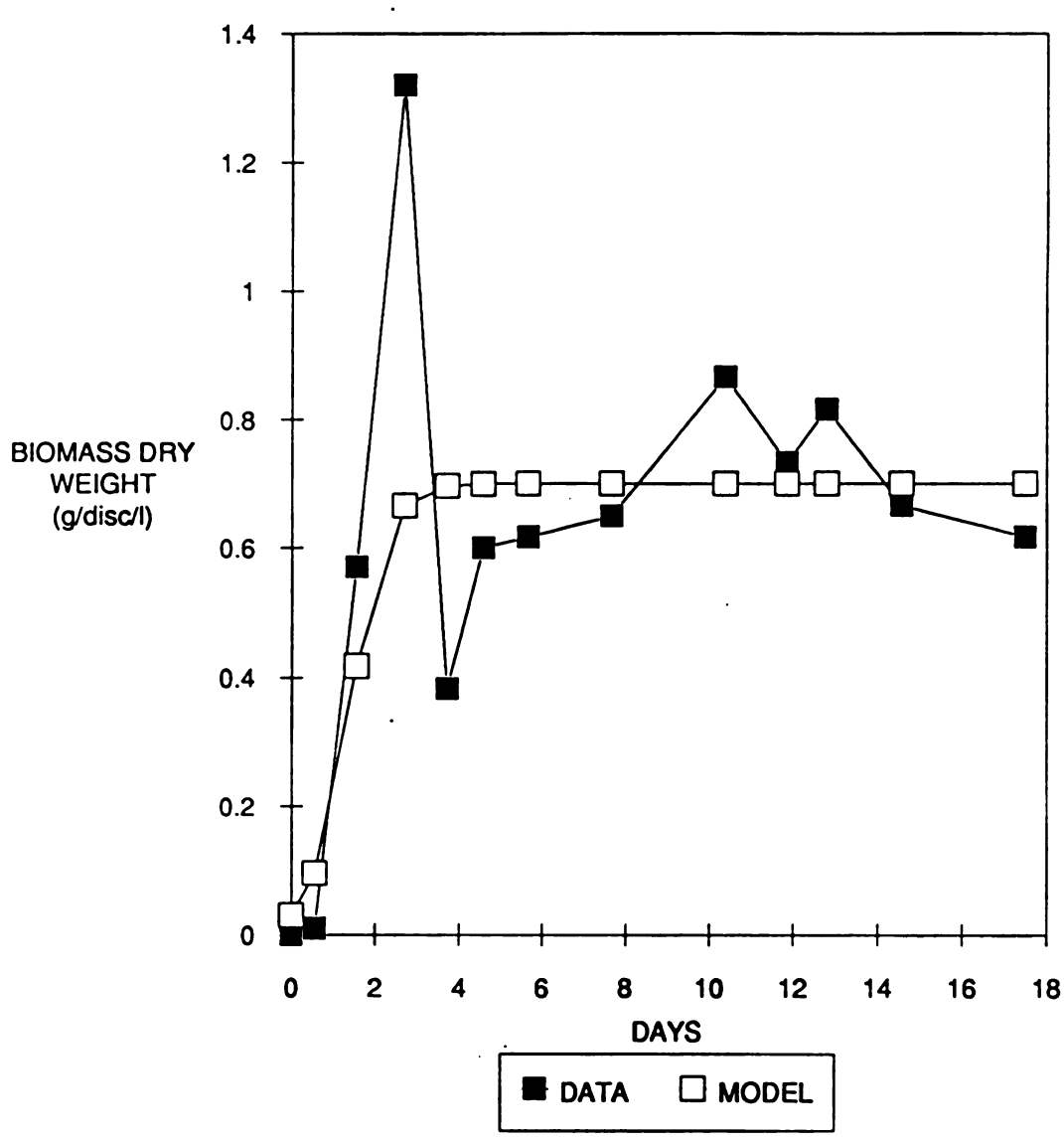


Figure 5.3 Comparison of biomass growth predicted by model to experimental data for 10 g/l initial glucose concentration. λ=3.2 disc·l·g^{-1}·day^{-1} and K=0.7 g·l^{-1}·disc^{-1}.

are also shown. (The data was normalized to a 1-liter volume of medium. This was done because compartment 1 in the RBC is larger than compartments 2, 3, and 4. See Table 3.4.) The single data point corresponding to day 2.5 is most likely due to mycelia from the medium having attached to the disc. The figure shows that the model provides a rough approximation of fungal growth in the RBC. The values of the model constants are listed in Table 5.1.

Table 5.1 Values of model constants for 0.1 OD inoculum and 10 g/l initial glucose concentration.

K	=	0.7 g/(l·disc)
λ	=	3.2 (l·disc)/(g·day)
lag time	=	13 hours

The disc carrying capacity, K, refers to the maximum quantity of biomass adhering to the disc. The value of K is dependent on initial glucose concentration since a different quantity of biomass grows on the discs for different conditions. An attempt was made to relate the initial glucose concentration to K and therefore to the biomass growth. Due to the variation in the data, a good correlation could not be determined; however the value of K increases with increasing initial glucose concentration.

The value of K is also dependent on the concentration of nitrogen in the media. This is another factor which is not considered in the first model. The fungus exhibits a rapid growth phase until the nitrogen is depleted in the medium after about 2 days. The characteristics of the disc surface also affect the value of K. Surface roughness and surface free energy influence the amount of bioadhesion.

5.5 Comparison of Film Growth to Pellet Growth

A physiological model as described earlier in Section 2.9 has been developed by Michel[56] to describe fungal growth and lignin peroxidase production for mycelial pellets in submerged culture. Michel's model relates biomass growth to initial glucose and nitrogen concentration. The equations and parameter values are presented in Tables 5.2 and 5.3. The equations are solved on a computer spreadsheet using Euler's method for integration. The specific growth rate for pellet culture determined from this model for 10 g/l initial glucose concentration and 0.01 OD inoculum concentration is 3.166 day^{-1}. The lag time reported for pellet growth was 8 hours. The lag time observed before the exponential growth phase began was longer for the film system than for the pellets.

The total surface area available for mycelial growth on the discs in the RBC was calculated and compared on a normalized basis of medium volume to the surface area of fungal pellets.

Table 5.2 Equations of Michel Growth Model

Phase	Symbol	Equation	Integral	Final value
LAG				
Time	t (dy)	0 <= t < tlag		t = tlag
Biomass	X (g/l)	dX/dt = 0	X = Xo	X = Xo
Glucose	G (g/l)	dG/dt = 0	G = Go	G = Go
Ammonia	N (g/l)	dN/dt = 0	N = No	N = No
PRIMARY				
Time	t (dy)	tlag <= t < t1		t1 = tlag + ln[1 + umax*No/Kn/Xo]/umax
Biomass	X (g/l)	dX/dt = umax*X	X = Xo*exp(umax*(t-tlag))	X1 = Xo + umax*No/Kn
Glucose	G (g/l)	dG/dt = -Kg*X	G = Go + Kg*Xo/umax*[1-exp(umax*(t-tlag))]	G1 = Go - Kg*No/Kn
Ammonia	N (g/l)	dN/dt = -Kn*X	N = No + Kn*Xo/umax*[1-exp(umax*(t-tlag))]	N1 = 0
STATIONARY				
Time	t (dy)	t1 <= t < t2		t2 = t1 + ln[1+ Yx/g*G1/X1]/Kg/Yx/g
Biomass	X (g/l)	dX/dt = Yx/g*Kg*X	X = X1*exp(Kg*Yx/g*(t-t1))	X2 = X1 + Yx/g*G1
Glucose	G (g/l)	dG/dt = -Kg*X	G = G1 + X1/Yx/g*[1-exp(Kg*Yx/g*(t-t1))]	G2 = 0
Ammonia	N (g/l)	dN/dt = 0	N = 0	N2 = 0
DEATH				
Time	t (dy)	t >= t2		t3 = infinity
Biomass	X (g/l)	dX/dt = -Kd*(X - F*X1)	X = F*X1 + (X2 - F*X1)* exp[-Kd*(t-t2)]	X3 = F*X1
Glucose	G (g/l)	dG/dt = 0	G = 0	G3 = 0
Ammonia	N (g/l)	dN/dt = 0	N = 0	N3 = 0

Table 5.3 Parameter definition for Michel growth model.

Parameter	Definition
μmax	maximum growth rate constant
K_g	glucose uptake rate constant
$Y_{x/g}$	biomass yield coefficient for glucose
K_n	ammonia uptake rate constant
K_d	death rate constant
tlag	lag time for growth
X_o	initial biomass concentration
N_o	initial ammonia concentration

Data for determining pellet surface area in agitated shake flasks at 200 rpm were reported by Michel.[63] The values are shown in Table 5.4. The similarity in normalized surface area for the two reactor systems indicates that the surface area of mycelia exposed to oxygen and nutrients in each reactor is roughly equivalent.

Table 5.4 Comparison of surface area for mycelial growth on RBC discs and of pellets from agitated shake flasks.

Mode of Growth	Surface Area (cm^2/l)
RBC - 8 discs	1740
RBC - 12 discs	2600
Pellets	1700

The Michel model was applied to biomass growth data obtained from the RBC. A comparison of the model prediction to experimental data is presented in Figure 5.4 for the case of 5 g/l initial glucose concentration. The parameters used in the model are listed in Table 5.5. As seen in the figure, the model gives a close approximation to the actual data for this case.

A comparison between the model and experimental data for the case of 15 g/l initial glucose concentration is shown in Figure 5.5. For this condition, the model prediction does not fit the data as closely as for the 5 g/l case. The model underpredicts biomass growth on the discs during the time period

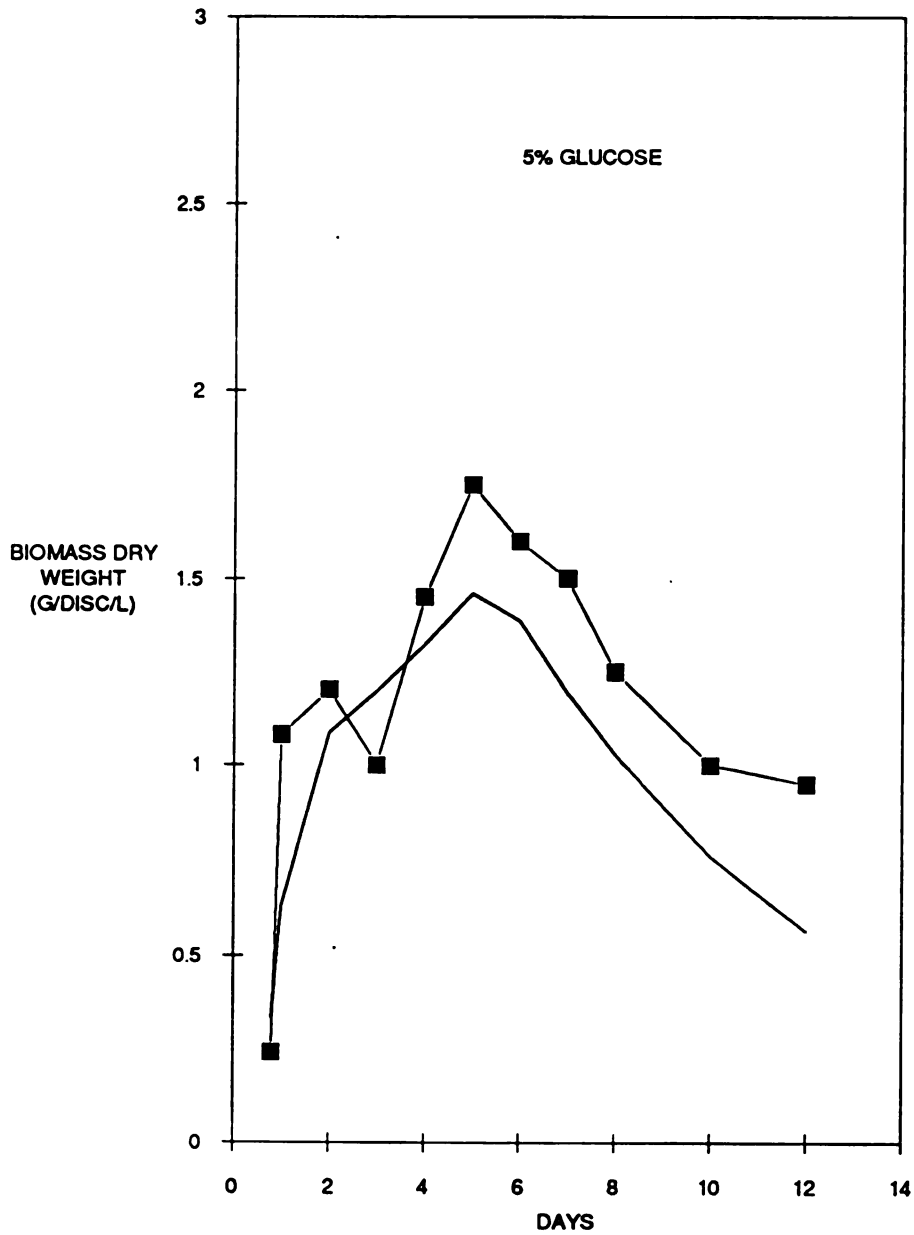


Figure 5.4 Comparison of biomass growth predicted by Michel model to experimental RBC data for 5 g/l initial glucose concentration.

Table 5.5 Values of parameters used in model.

Parameter	Value
μmax	3.17 g/(l·dy)
K_g	0.9 dy^{-1}
$Y_{x/g}$	0.11
K_n	0.138 g/(l·dy)
K_d	0.15 dy^{-1}
tlag	0.42
X_o	0.1
N_o	0.039 g/l

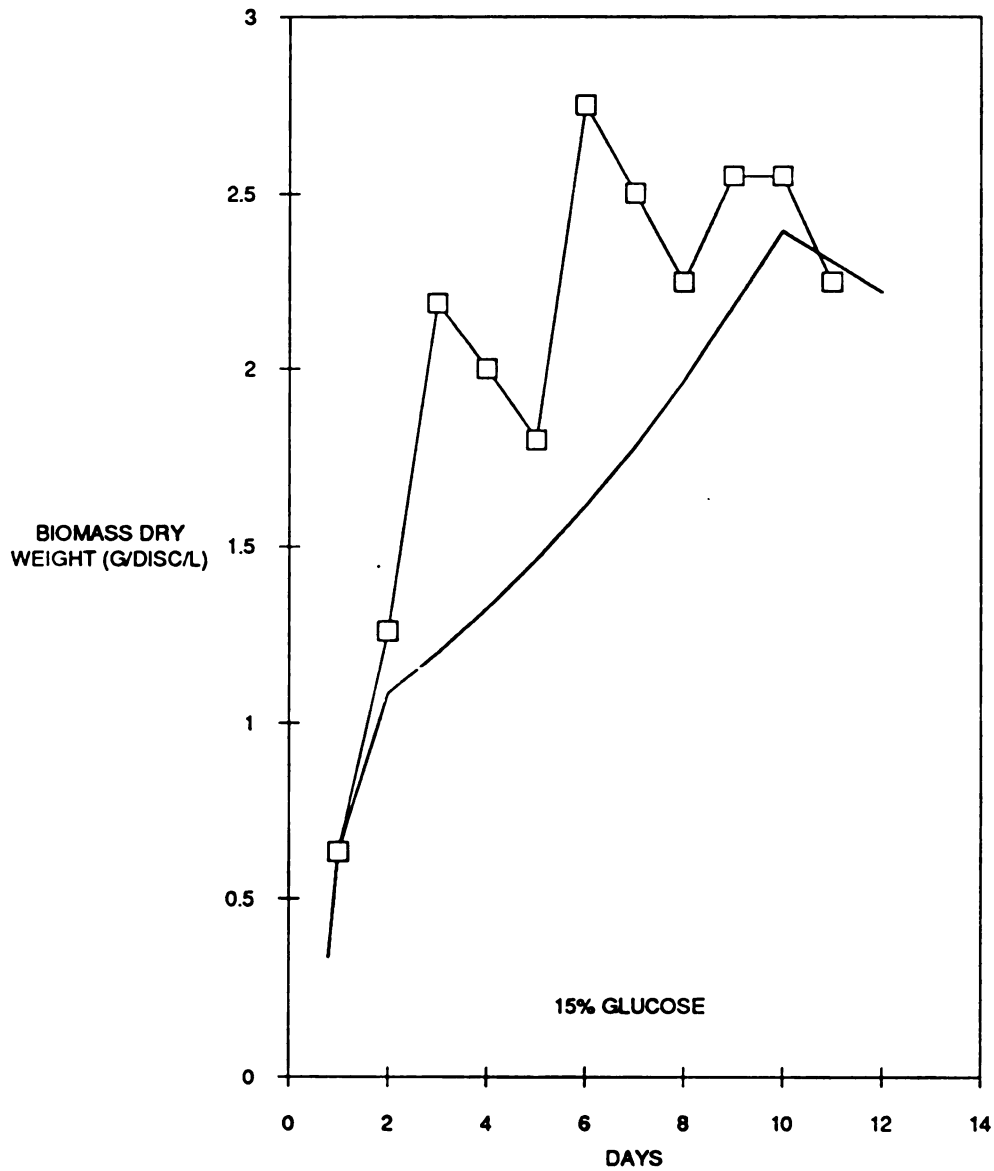


Figure 5.5 Comparison of biomass growth predicted by Michel model to experimental RBC data for 15 g/l initial glucose concentration.

between days 2-10. This indicates that a difference exists between film and pellet morphologies. Higher glucose concentration in the RBC most likely results in higher polysaccharide formation which would account for the increased biomass production.

5.6 Discussion of Growth Modelling Experiments

Two models have been applied to biomass growth data and were found to approximately describe fungal growth in the RBC. The major obstacle to further modelling in the RBC is the scatter in the biomass data within an experiment and the reprodicibility of the data between different reactor fermentations. This is due in part to differences in the disc surfaces. Surface roughness plays a major role in biomass adhesion. Although an attempt was made to prepare the discs as identically as possible, some differences exist which have an effect on adhesion. Futher modelling experiments would require a new set of discs to be prepared.

Another factor which affects the biomass data is the attachment of mycelia from the medium to the discs. The mycelia in the culture fluid were removed after 24 hours in order to prevent this, but the effect was not totally eliminated. Another consideration in performing growth modelling experiments in the RBC is the limited number of experiments which can be run. Unlike shake flask experiments where duplicate flasks can be easily cultured, RBC experiments are much more time-consuming. In spite of these factors, a first attempt at

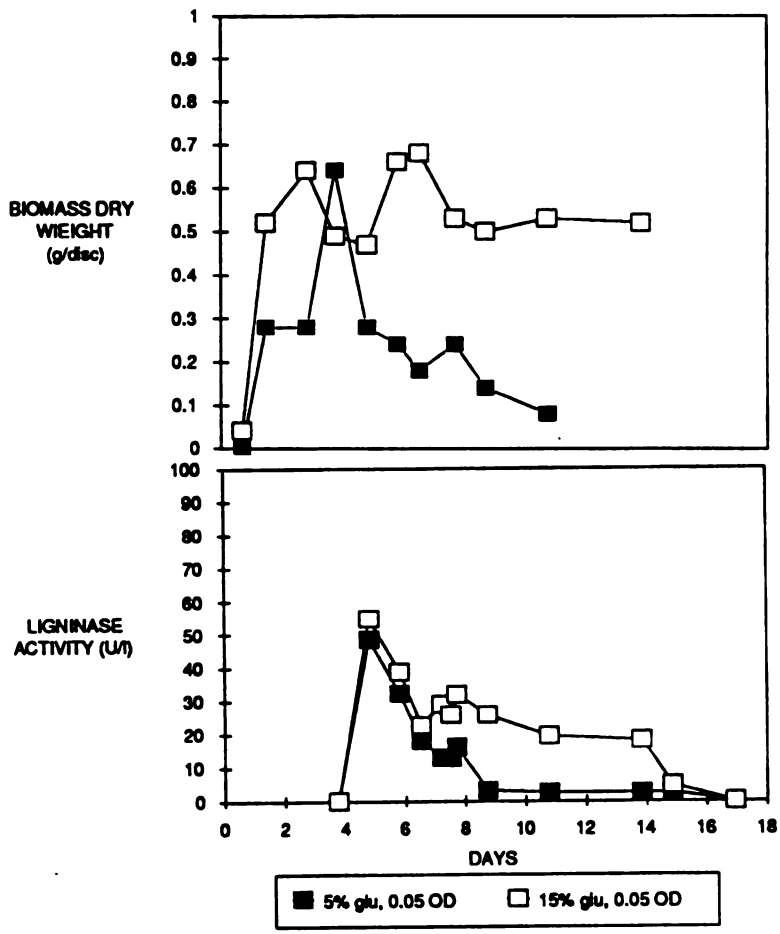


Figure 5.6 Lignin peroxidase production and biomass growth for 5 g/l and 15 g/l initial glucose concentration.

modelling RBC growth kinetics has shown that surface growth on the discs resembles pellet growth in submerged culture in several aspects.

One goal of developing a kinetic model is to relate fungal growth and product formation to culture conditions for use in process design. The first growth model (Section 5.1) used in this work provides a fit to data for the lag, exponential growth, and stationary phase of the fungus. The model does not account for the onset of a death phase. More complete modelling efforts would need to include this term.

Figure 5.6 shows biomass growth and lignin peroxidase production for initial glucose concentrations of 5 g/l and 15 g/l. This figure indicates that an optimum biomass thickness may exist for enzyme production and that higher amounts of biomass do not significantly contribute to enzyme activity.

CHAPTER 6. CONCLUSIONS

6.1 Summary of Results

P. chrysosporium was immobilized on polymer discs in a bench-scale rotating biological contactor and used for lignin peroxidase production. Biofilm adhesion was tested on various polymer surfaces and was found to be enhanced by the use of roughened, high surface energy discs. The influence of several factors on biomass growth and enzyme production was studied. These factors involved reactor operating conditions as well as media composition. Spore inocula and inocula prepared from mycelial mats were both found to be effective inocula resulting in enzyme activity in the RBC. This may be an important consideration in large-scale reactor design since it would be desirable to avoid more time-intensive inocula preparation. Lignin peroxidase was consistently expressed in the RBC although the activities measured were 3-4 times lower than enzyme production reported for agitated shake flask cultures.

A mechanistic growth model for surface growth was applied to biomass data obtained from the RBC. The specific growth rate of the fungus and the carrying capacity of the discs were determined using the model. The specific growth rate determined for film growth was similar to that reported for pellet growth under equivalent fermentation conditions. A second growth model, developed for pellet growth, was applied to RBC biomass

data. The close fit of the model to the data indicates that film growth in the RBC is similar to pellet growth in submerged culture. Lignin peroxidase production was not found to be correlated to biomass growth.

6.2 Directions for Future Work

Further work needs to be done to better understand the behavior of this fungus in different morphologies in order to accomplish successful process scale-up. Immobilized reactor systems offer unique advantages for industrial fermentation systems as well as for laboratory studies. Continued studies involving the RBC would provide a greater depth of understanding of biofilm growth and fungal product formation.

The adhesion of biomass to surfaces is an area which has significant importance in industrial reactor design. In some cases, unwanted biofouling occurs on reactor components and piping. In other systems, adhesion is encouraged in order to immobilize biomass to surfaces for growth. The mechanisms associated with fungal adhesion and the factors which influence this occurrence need to be better understood. Surface science techniques can be applied to study the interactions which exist between the biomass, liquid medium, and solid support surface. The RBC provides a low-shear, high surface area environment which is well-suited for studying bioadhesion. The separate compartments of the RBC would allow various conditions to be tested simultaneously.

The study of fermentation conditions in the RBC should be continued in order to increase lignin peroxidase production. A study of polysaccharide formation, glucose levels, and protease production in the RBC may be useful in determining the quantity of lignin peroxidase enzyme that is degraded by proteases. This may allow certain conditions to be altered in the RBC which would lead to increased enzyme activity.

Experiments to lengthen the lifetime of the fungus in the RBC would be useful in making this biosystem more viable in industry. Attempts to extend fungal lifetime in the RBC during bleach plant effluent decolorization experiments have been reported. Decolorization was observed for over 30 days when the medium was supplemented with glucose, nitrogen, and other components. The bench-scale RBC offers the flexibility of batch and continuous modes of operation. This would be useful for doing extended biomass lifetime studies and bringing this system closer to the ultimate goal of a continuous process.

The diffusion of oxygen into fungal mycelia is a critical factor for biomass viability. Microprobe techniques could be used to determine the oxygen diffusion profile in the biofilm. In addition, various staining techniques could be used to find the regions of lignin peroxidase production and to measure metabolic activity within the mycelia. These results could be used to determine the optimum biomass thickness on support surfaces. They would also provide insight into the transport rates of nutrients in the film and indicate further similarities and differences between surface and pellet fungal growth. These

findings could then be incorporated into future kinetic modelling.

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