AN ABSTRACT OF THE THESIS OF

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Biological decolorization of pulp-mill effluent using filamentous fungi is a promising technology to limit the impact of this wastewater on the natural environment. In this thesis, we evaluated using *Sagenomella striatispora*, a hyphomycete, for color removal from pulp-mill effluent.

S. striatispora removed 74 % of initial color in pulp-mill effluent samples containing 10 g glucose/L without any pH adjustment at 25 °C. Color removal occurred without any apparent lag phase and without nitrogen starvation. However, color removal did not increase beyond 75 %.

In contrast to *Phanerochaete chrysosporium*, *S. striatispora* did not show any laccase, lignin or manganese-dependent peroxidases activity. Nitrogen starvation did not trigger the production of these enzymes.

Analysis of molecular weight fractions in the initial and treated pulp-mill effluent showed that this *S. striatispora* selectively degraded the high molecular weight fraction (> 3 kDa) while *P. chrysosporium* was less specific and reduced all molecular fractions with similar efficiencies. Attempts to decolorize pulp- mill effluent *in vitro* using extra-cellular fluid from *S. striatispora* cultures were not successful.

Using a stirred tank reactor in batch and continuous regimes, we were able to consistently obtain 65 % color removal at two day hydraulic retention time.

The color sorption to the biomass reached a maximum that remained relatively constant around 20 % of initial effluent color. Nevertheless, the accumulation of color on the biomass did not appear to influence the ability of mycelial pellets to remove color or

their growth and respiration activities. In both successive batches and continuous regimes, a significant amount of biomass was lost. There were also problems with foaming particularly when operating the reactor in continuous flow.

A combined model for pellet growth, color removal and color sorption was developed and was found to satisfactorily simulate the experimental data. Analysis of the data from the simulation revealed that glucose as well as oxygen were in excess in all experiments. The simulation also showed that increases in pellets diameter above 1.8 mm may create problems of oxygen limitation.

Pulp-mill Effluent Color Removal Using *Sagenomella striatispora*

by

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INTRODUCTION

The pulp and paper industry annually discharges billions of gallons of brown colored effluent into the natural environment. These chromophores result from fragmentation of lignin contained in the wood chips used to produce pulp. This effluent not only changes the aesthetics of natural streams but also discharges many highly recalcitrant organic aromatics that may accumulate in aquatic plants and animals. One of the most efficient biological treatments for color removal from this effluent involves using lignolytic filamentous fungi. *Phanaerochaete chrysosporium* and *Trametes versicolor* are two species of basidiomycetes that have been extensively evaluated for pulp-mill effluent treatment. Despite more than twenty years of research on these species, the effluent is still discharged without any treatment. Furthermore, very little is known about the physiology and the potential of other fungal groups to remove color from pulp-mill effluent. Among these microorganisms, aquatic fungi have received very little attention although they may be more adapted to color removal from pulp-mill wastewater than the more terrestrial basidiomycetes. In this work, we evaluated the suitability of an aquatic hyphomycete, *Sagenomella striatispora,* for pulp-mill effluent color removal. This thesis is organized into three chapters treating the physiology of color removal; identification of the enzymatic system; and decolorization kinetic analyses using fungal pellets.

In chapter 1, our objective was to define the optimal conditions and limitations of color removal by *S. striatispora*. First, we screened the important variables for color removal from pulp-mill effluent by *S. striatispora*. Then, we investigated the optimal levels for each of these important variables and then analyzed the kinetics of color removal under optimal levels for all variables. The extent and limitation of decolorization were also investigated using pulp-mill wastewater amended with different nutrients in gas-tight serum bottles flushed with pure oxygen and inoculated with blended fungal biomass.

In chapter 2 we examined the lignin degradation enzymes of *S. striatispora* and *P. chrysosporium*. The tests included activity measurements of lignin peroxidase, manganese peroxidase and laccase and their relation to carbon and nitrogen utilization as well as *S. striatispora* growth patterns. Tests of *in vitro* degradation of pulp-mill effluent were also conducted. Extra-cellular culture fluid were concentrated and added to pulp-mill effluent samples with and without *S. striatispora* cultures. The relationship between enzyme production, biomass growth and substrate uptake was assessed by measuring enzyme activities and substrate uptake in chemically defined media.

In chapter 3, the suitability and feasibility of using *S. striatispora* grown as pellets were investigated using a 1.5 L completely-stirred bioreactor operated under batch and continuous conditions. Quantitative relationships between chromophore degradation, sorption to the biomass, fungal pellet growth and nutrient uptake were derived for batch cultures. Simulated data were compared to experimental results in order to optimize pulp-mill color removal using *S. striatispora* grown in the form of pellets.

This thesis gives data on the feasibility and optimal conditions of using *S. striatispora* to remove color from pulp-mill wastewater. It also contributes to the understanding of the physiology and biochemistry of lignin degradation by this hyphomycete.

CHAPTER 1

Optimization of color removal from pulp-mill wastewater using a hyphomycete: *Sagenomella striatispora*

1.1 Abstract

Biological decolorization of pulp-mill effluent using filamentous fungi is a promising technology to limit the impact of this wastewater on the natural environment. *Phanerochaete chrysosporium* is most often evaluated for this purpose but other fungi may also be suitable. *Sagenomella striatispora*, a hyphomycete isolated from a log pond, significantly decolorized pulp-mill effluent. To optimize culture conditions for this fungus, we evaluated the effect of a number of nutrients, pH and temperature on pulp-mill wastewater decolorization using a factorial fractional experimental design. Then, we optimized each significant variable separately. *S. striatispora* removed 74 % of initial color in pulp-mill effluent samples amended with glucose (10 g/L) without any pH adjustment (initial pH of pulp-mill wastewater was 7.6) at 25 °C with 12 % color adsorbed to the biomass. The maximum color removal was obtained without adding nitrogen, Mn or veratryl-alcohol. Furthermore, decolorization occurred immediately without any apparent lag phase and without nitrogen starvation (24 mM NH4Cl). However, color removal did not increase beyond 75 % even with prolonged incubation (1 month) or *de novo* addition of glucose and nitrogen to the batches.

1.2 Introduction

The pulp and paper industry annually produces billions of gallons of dark brown colored effluent that is discharged into the surrounding surface waters. The color results from fractionation and dissolution of lignin from wood chips used in the Kraft process (Krinstad and Lindstrom, 1984). Many groups of microorganisms have been reported to degrade native and transformed lignin (Hartig and Lorbeer, 1991). Some species of these groups of microorganisms can also decolorize and detoxify pulp-mill effluent (Boman et al., 1991; Fukui et al., 1992).

Microorganisms that can attack lignin have been identified in most major microbial groups including gram negative bacteria (Hartig and Lorbeer, 1991), actinomycetes (Zimmermann, 1991), hyphomycetes (Zare-Maivan and Shearer, 1988) and ascomycetes (Prasad and Joyce, 1991). Nevertheless, most investigations on pulp-mill effluent decolorization have used two basidiomycetes: *Phanerochaete chrysosporium* and *Trametes* (*Polyporus*) *versicolor* (Norton, 1992).

Current knowledge on the physiology and biochemistry of decolorization of pulp-mill wastewater in particular and lignin degradation in general is almost entirely based on studies that have used *Phanerochaete chrysosporium* (Shoemaker and Leisola, 1990). Lignin degradation by this microorganism results from secondary metabolism triggered under carbon or nitrogen limitations, in presence of thiamine, veratryl-alcohol and manganese (Kirk et al., 1987). This species requires oxygen for growth and also for lignin degradation. Optimal lignin degradation is obtained at relatively high temperatures (39°C) under acidic conditions (pH 4.5).

Bench-scale and pilot-reactor experiments for color removal and lignolytic enzyme production using *P. chrysosporium* have revealed problems related to the physiological and cultural characteristic of this species. Campbell (1983) noted oxygen transfer limitation and short biomass lifetime when evaluating the MyCoR (Mycelial Color Removal) process. Instability and deactivation of lignin peroxidase production also occurred in chemically defined media (Fiechter, 1993). Prouty (1990) reported problems

with foaming and biomass washout of *P. chrysosporium*'s mycelial pellets with mechanically agitated bioreactors. These physiological and cultural properties of *P. chrysosporium* constitute an obstacle for full-scale application. Attempts to express lignin degradation enzymes in other microorganisms using genetic engineering are very promising but successful applications have not yet been developed (Holzbauer et al., 1991). In this study we evaluated and optimized color removal from pulp-mill effluent by an aquatic hyphomycete: *Sagenomella striatispora* isolated from a log pond (Lesley, 1993). This fungus significantly decolorized pulp-mill wastewater in stationary cultures at 25°C without any pH adjustment or aeration (Lesley, 1993).

To optimize color removal by *S. striatispora*, we evaluated the effect of temperature, pH, aeration and addition of glucose, nitrogen, Mn, veratryl alcohol, yeast extract, thiamine and minerals to the pulp-mill effluent. Then, we investigated the effect of each significant factor on color removal at different levels. Using optimal levels for each variable, we followed the kinetics of decolorization during a two-week incubation period then estimated the percent of color degraded and the color adsorbed on the biomass.

1.3 Materials and methods

1.3.1 Fungal culture

Sagenomella striatispora slant tubes stored at 4°C were subcultured into 250 ml Erlenmeyer flasks containing 50 ml of 1.6 % malt extract broth. The flasks were incubated at 28 °C for two weeks. They were then centrifuged (2,000 g) and washed twice in distilled water containing 0.9 % NaCl. The resulting concentrated inoculum was used to inoculate the pulp-mill wastewater samples in 110 ml serum bottles. Then, the bottles were sealed with aluminum seals and flushed with hydrated oxygen or air at 5 psi for 3 minutes every three days.

1.3.2 Preparation of pulp-mill effluent samples

Pulp-mill wastewater was collected after secondary treatment from a local pulp-mill plant in Halsey, Oregon. The plant uses oxygen and peroxide treatment as well as chlorine bleaching. This sample was stored at 4°C in the dark for further use in decolorization tests. Aliquots of 30 to 50 ml were transferred into 125 ml serum bottles after pH adjustment. The bottles were then autoclaved at 120 °C for 15 min. Glucose was added as a readily available source of carbon at four different concentrations (1, 5, 10 and 20 g/L). We compared four different forms of nitrogen (NH_4Cl, KNO_3, urea and di-ammonium tartrate) at low (2.4 mM nitrogen) and high (24 mM nitrogen) concentrations. The effects of buffer and pH on pulp-mill wastewater decolorization were evaluated using acetate, tartrate, malonate, succinate, citrate and phosphate as buffers at pH's ranging from 2.5 to 7.6. All effluent samples were adjusted to the desired pH using concentrated solutions of NaOH or HCl before adding the buffers. Minerals and micronutrients were provided as recommended by Michel et al. (1991) from a stock solution at the following final concentrations: 1.45 g/L $MgSO_4.7H_2O$, 0.132 g/L $CaCl_2.2H_2O$, 0.07 g/L of NaCl, 0.007 g/L $FeSO_4.7H_2O$, 0.013 g/L $CoCl_2.7H_2O$, 0.07 g $ZnSO_4.7H2O$, 1.1 mg/L $CuSO_4.5H_2O$, 0.7 mg/L $AlK(SO_4).12H_2O$, 0.7 mg/L, H_3BO_4 and 0.7 mg/L $Na_2MoO_4.2H_2O$. Manganese ($MnSO_4.2H_2O$) was added at 10, 20 or 100 mg/L Mn. We also investigated the effects of adding 4 mM veratryl alcohol, 10 mg/L yeast extract or 1mg/L thiamine hydrochloride. Solutions of glucose as well as organic buffers, veratryl alcohol, yeast extract and thiamine were filter-sterilized through 0.2 µm sterile membranes. Solutions of CaCl2 and MgSO4 were autoclaved separately and added before inoculation with the other nutrients.

1.3.3 Analytical methods

Oxygen and CO_2 were monitored in the vial head space. The gas composition was analyzed using an HP 5890II Gas Chromatograph equipped with a Carboxen 2000 (60/80) packed column. Chromosorb 102 was used when only CO_2 measurements were needed because it requires a shorter analysis time. After gas measurements, 5 ml samples of treated effluent were withdrawn to measure pH and color. Color was measured on a Hach 2400 spectrophotometer at 465 nm. Samples were first adjusted to pH 7.6 in 1 M phosphate buffer then filtered through 0.8 μm filters as recommended by NCASI (1971). Results were expressed in Platinum-Cobalt Color Units (PCU) using the linear equation:

$$\text{Color PCU} = 3080 \times \text{Absorbance at 465nm}$$

Colored material adsorbed on the biomass was extracted using 0.1 N NaOH in two to three consecutive washes (Martin and Manzaranes, 1994). After centrifugation at 2000 g, microbial biomass was transferred to a volumetric cylinder to measure the volume. Then 5 ml of 0.1 M NaOH was added and left to react for 30 min. This operation was repeated twice. The supernatants were combined then color was measured as described above. The final results were expressed as absorbed color per biomass dry weight after accounting for dilution.

1.3.4 Biomass dry weight

Fungal biomass was filtered on tarred fiberglass GF/C membranes and dried overnight at 105°C prior to weighing.

1.3.5 Experimental design

In order to screen the important variables in pulp-mill effluent decolorization by *S. striatispora*, we used a resolution IV fractional factorial design with 9 variables in 32 plots (Montgomery, 1991). Each variable was tested at two levels as shown in Table 1. 1.

Table 1.1. Variable levels evaluated for optimization of pulp-mill effluent color removal by *S. striatispora*.

Variable	Low level	High level
Glucose (% w/v)	0.1	1
Nitrogen (mM)	2.4	24
pH	4.5	7.6
Aeration with air	none	every 3 days
Temperature (°C)	25	30
Yeast extract (mg/L)	0	10
Thiamine (mg/L)	0	1
Minerals	none	basic level
Veratryl alcohol (mM)	none	4

Effect estimates for each variable were calculated using Yates Algorithm (Montgomery, 1991). The effect estimates were then analyzed in a normal probability plot. The variables associated with effect estimates that significantly diverged from the normal probability line were thereafter examined in complete factorial designs. Variables examined in these tests were pH (4.5 and 7.6), nitrogen (2.4 and 24 mM), glucose (1 and 10 g/L) and oxygen versus air. All experiments were run in triplicate.

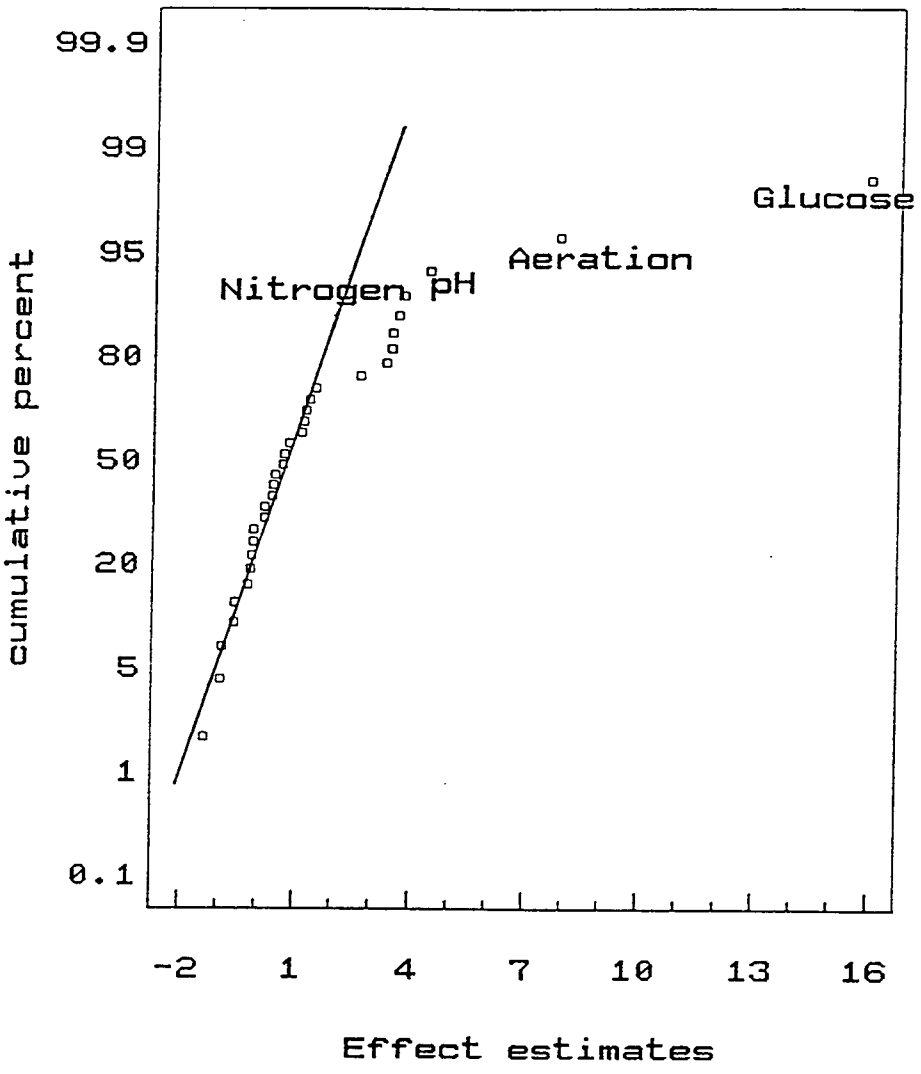

Figure 1.1 Normal probability plot for pulp-mill color removal by *S. striatispora*.

1.4 Results

1.4.1 Culture characteristics of *Sagenomella striatispora*

Sagenomella striatispora produced individual 2 to 4 mm diameter yellow-brownish colonies on agar after 1 week. In liquid media, the pellets were fluffy. The fungus growth rate was very low. We incubated the cultures for at least two weeks prior to any experiment. The fungus was not able to grow at temperatures above 30 ^{o}C.

1.4.2 Nutritional factors screening

Effect estimates for the variables and their interactions were graphed using normal probability plots (Figure 1.1). The effect estimates corresponding to glucose, aeration, pH, nitrogen and their interactions significantly diverged from the normal probability line. These variables exhibited the most significant effect on color removal by *S. striatispora*. Addition of veratryl alcohol, yeast extract, thiamine and minerals did not significantly improve decolorization of pulp-mill wastewater. Comparable color removals were observed at 25^{o}C and 30^{o}C.

Analysis of variance (Appendix B) of the experiment with the four significant variables given above showed that the four factors have significant (1% level) interactions. A combination of 10 g/L glucose and low nitrogen (2.4 mM nitrogen) under acidic conditions in presence of pure oxygen produced the best color removal (51% after 6 days of incubation).

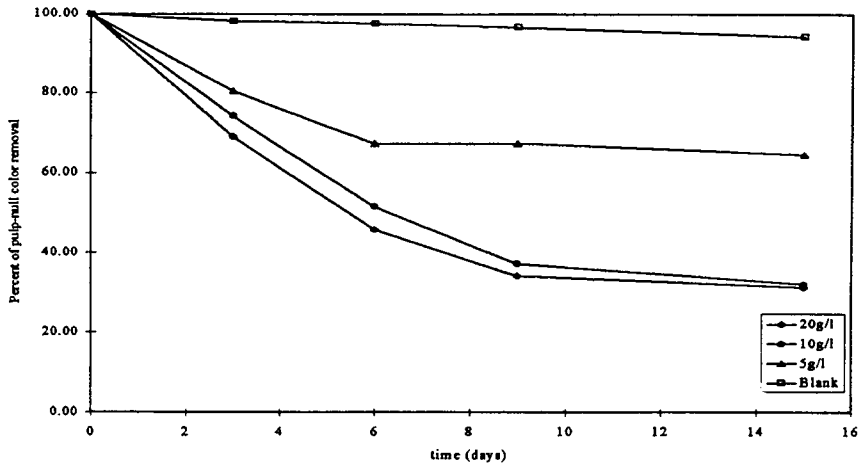

Figure 1.2: Effect of glucose concentration of pulp-mill color removal by *S. striatispora*

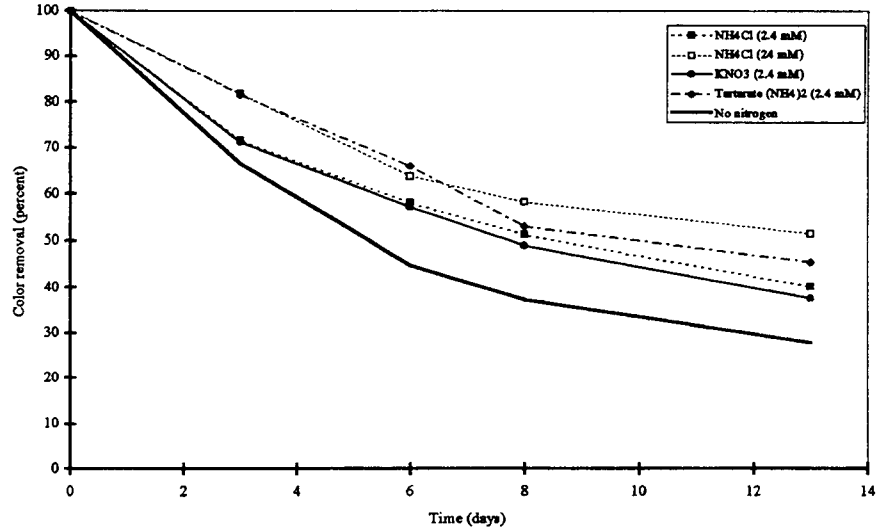

Figure 1.3: Effect of different sources of nitrogen on pulp-mill color removal by *S. striatispora*.

1.4.3 Optimal levels for nutritional factors

Carbon source: Increasing glucose concentration from 5 g/L to 10 g/L enhanced color removal by more than 10 % (Figure 1.2). Color removal was not significantly affected at higher glucose concentrations.

Nitrogen source: All four nitrogen sources supported significant color removal at the lower level (2.4 mM), with NH_4Cl and KNO_3 producing slightly higher color removals (Figure 1.3). Cultures without additional nitrogen gave the best decolorization results. The pH and respiration rate, as measured by CO_2 production, were similar for all treatments.

Manganese: Addition of Mn did not affect the level of decolorization, pH or respiration rate of *Sagenomella striatispora* (Table 1.2).

pH: *S. striatispora* decolorized pulp-mill wastewater better in presence of either tartrate or malonate buffers. Although acetate and succinate showed better buffering capacity, they appeared to inhibit the microoganism's activity as measured by CO_2 production. Color removal by *S. striatispora* in malonate buffer was optimal between pH 4 and 5 (Table 1.3). Decolorization began on the first incubation day even if the starting pH corresponded to the initial pulp-mill effluent pH (7.6). *S. striatispora* cultures significantly decolorized the pulp-mill samples with phosphate, citrate or malonate buffers at pH 7.6 but the pH dropped in all the replicates to around 4.

Table 1.2: Effect of minerals and Mn addition on pulp-mill color removal by *S. striatispora*.

Treatments	Percent color removal after 6 days (average and standard deviation)
Minerals base	49 (3)
Mn (10 ppm)	50 (4)
Mn (20 ppm)	49 (4)
Mn (100 ppm)	52 (6)
No minerals	50 (3)
Without fungus	0

Table 1.3: Effect of starting pH on pulp-mill color removal and final pH of culture medium (average and standard deviation after 6 days incubation) by *S. striatispora*.

Starting pH	Percent color removal	Final pH
2.5	36 (12)	2.48 (0.4)
3	40 (6)	2.96 (0.8)
4	54 (4)	3.33 (0.8)
5	51 (5)	3.59 (0.6)
6	46 (6)	3.90 (0.7)
7	39 (9)	4.17 (1.1)

1.4.4 Color removal under optimal conditions

S. striatispora significantly decolorized the pulp-mill wastewater during the first 6 days of incubation (Figure 1.4 A). *S. striatispora* decreased color without a lag phase. The best color removals after 20 days approached 74 % . After accounting for the amount of color adsorbed on the biomass (Table 1.4), *S. striatispora* degraded 62 % of initial pulp-mill effluent color. Addition of glucose (10 g/L) and ammonia (45 mg/L) to pulp-mill effluent that had been incubated for ten days with *S. striatispora* did not markedly improve color removal.

Table 1.4. Estimates of sorbed pulp-mill effluent color exposed for 15 days to cultures of *S. striatispora* and *P. chrysosporium* at starting pH 4.5 and 7.

Fungal species	Starting pH	Color removed (%)	Color sorbed (%)
P. chrysosporium	4.5	18 (6)	88
S. striatispora	4.5	15 (8)	72
P. chrysosporium	7	16 (6)	84
S. striatispora	7	12 (8)	74

S. striatispora decolorized pulp-mill effluent at similar rates for the two starting pHs (4.5 and 7.6). This species decreased the pH of the culture medium by more than two pH units in less than 24 hours (Figure 1.3B).

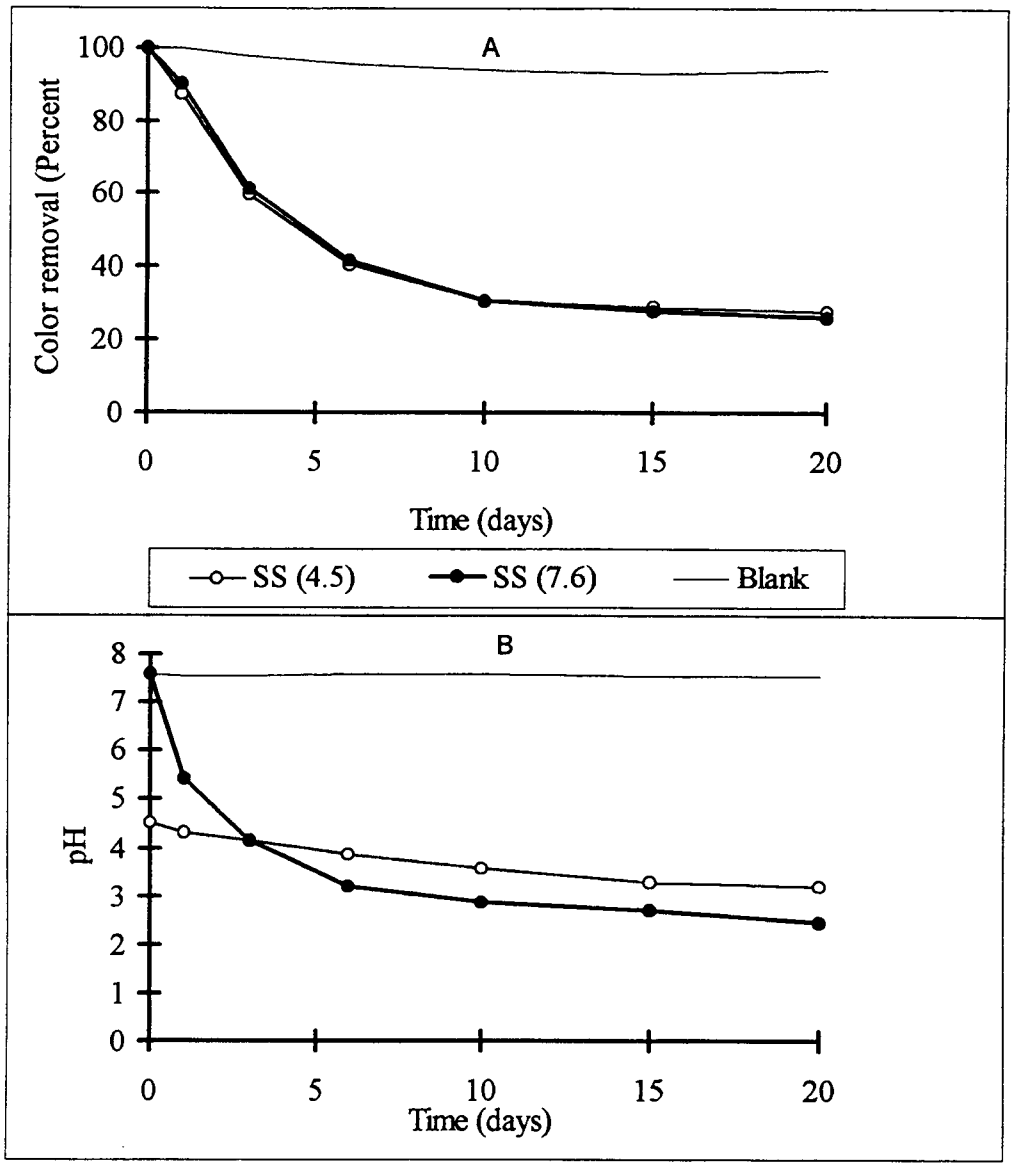

Figure 1.4. Pulp-mill color removal (A) and pH change (B) under optimal conditions by *S. striatispora* at pH 4.5 and 7.6.

1.5 Discussion

Previous results on *Sagenomella striatispora* produced decolorization haloes on agar plates containing Remazol Brilliant Blue (Sigma) and Poly-R 478 (Sigma) (Lesley, 1993). This species appears to be the first representative of the genus that was reported to have lignin degradation potential. This species is characterized by slow growth (Domsch et al., 1980) in the form of pellets in total absence of any agitation. These cultural properties of *S. striatispora* may be advantageous for application to treat pulp-mill wastewater. Biomass wasting may be reduced in the treatment process because of the slow growth. The pelletic growth may allow utilization of completely mixed reactors with mild agitation.

The best color removal rates with *S. striatispora* were obtained by addition of 20 g/L glucose in an oxygen saturated environment. Adding supplemental nitrogen reduced the level of color removal. Addition of veratryl alcohol, yeast extract, thiamine, minerals and manganese did not significantly affect color removal from pulp-mill wastewater. Literature reports on nutritional requirements for lignin and lignin related compounds degradation are diverse depending on the microorganism investigated (Kirk, 1987). Nevertheless, it is generally accepted that lignin degradation occurs during secondary metabolism and requires the presence of a co-metabolisable carbon source.

S. striatispora's ability to remove color was very significantly enhanced by addition of glucose at concentrations up to 10 g/L. Similar results were described for a strain of *Trametes versicolor* by Martin and Manzaranes (1994). Most pulp-mill color treatment investigations using *Trametes versicolor* (Royer et al., 1991) and *Phanerochaete chrysosporium* (Lankinen et al., 1991) have been performed using glucose as co-substrate. However, Duran et al. (1994) obtained 73 % color removal in 5 days using *Lentinus edodes* without any additional carbon source addition. The effect of carbon source concentration on lignin degradation microorganisms is complex. Carbon source limitation triggers lignolytic activity by *Phanerochaete chrysosporium* (Jefferies et al., 1981) but it also increases release of proteases that can degrade lignolytic enzymes

(Dosoretsz et al., 1990). Dosoretsz et al. (1990) maintained lignin peroxidase activity beyond the eighth day of incubation by adding glucose to the culture medium to inhibit the production of proteases. For *S. striatispora*, color removal seems to be stimulated at high glucose concentrations.

The triggering effect of low nitrogen levels on lignin degradation is well documented for many fungi (Kirk and Farrel, 1987). *Sagenomella striatispora* removed color significantly better at low nitrogen concentration (2.4 mM NH_4Cl) than at high nitrogen levels (24 mM NH_4Cl). Moreover, the best color removal rates by *S. striatispora* were obtained for the batches where no nitrogen was added. The pulp-mill effluent already contained nitrogen as soluble NH_4 at very low concentration (0.7 mM) which could be used by the microorganism.

Iron, copper, zinc and particularly manganese are essential elements for lignin degrading microorganisms (Kirk et al., 1986; Kirk and Farrel, 1987; Haapala and Linko, 1993). Although the role of these metals is not yet fully understood, manganese has been reported to trigger peroxidase activity (Pease and Tien, 1992) and contribute to the catalysis cycle of manganese dependent peroxidases (Perez and Jefferies, 1992). Color removal by *S. striatispora* was not enhanced by addition of Mn. Other investigators (Michel et al., 1991) have reported higher color removal by *P. chrysosporium* after Mn concentration was increased to 12 mg/L. The differences observed between Michel et al (1991) and our results could be due to differences of enzymes produced by the two microorganisms or the difference between the pulp-mill effluents (Appendix A).

S. striatispora significantly decolorized pulp-mill effluent under a wide pH range (3-7.6). *S. striatispora* performed well without any pH adjustment or buffer addition even at the initial pulp-mill effluent pH (7.6). This microorganism was able to lower the initial pH of pulp-mill effluent by more than 2 units in 24 to 48 hours. The ability to lower pH has been reported in other lignin degrading microorganisms (Martin and Manzaranes, 1994). Many wood degrading enzymes have higher activities under acidic conditions (Gold et al., 1989, Kirk et al., 1990). Organic acids are also necessary chelators in the manganese-dependent peroxidase catalysis cycle (Perez and Jefferies, 1992). Malonate and tartrate buffers were associated with higher color removals than acetate, citrate or

succinate buffers. Acetate and citrate are known to be good buffers for *P. chrysosporium* (Michel et al., 1990) and *C. versicolor* (Royer et al., 1991), respectively.

Furthermore, *S. striatispora* hyphae adsorbed only 12 % of initial color. Adsorption of chromophoric compounds can affect the activity and color removal abilities of the microorganism. Martin and Manzaranes (1994) observed a progressive darkening of the biomass exposed to pulp-mill effluent in three simultaneous batches. They reported an adsorption of 50 % of initial color. Duran et al. (1994) found a 13% color adsorption on the biomass using *L. edodes*.

Color removal by *S. striatispora* never exceeded 75 %. This result does not seem to be related to carbon or nitrogen source limitation as has been reported for *P. chrysosporium* (Dosoretsz et al., 1990). Even after sterilization of the decolorized pulp-mill effluent and *de novo* inoculation with fresh cultures of *S. striatispora*, pulp-mill effluent decolorization did not increase beyond 75 %. More likely, *S. striatispora* selectively degrades some chromophoric compounds.

In conclusion, *Sagenomella striatispora* showed a good potential for pulp-mill effluent decolorization removing 74 % of initial color after two weeks incubation with glucose and oxygen as the only nutrients added to the effluent. The main advantage of this species relates to its ability to decolorize pulp-mill effluent without pH adjustment or addition of other nutrients such as nitrogen, Mn or veratryl-alcohol. Furthermore, this species exhibited different physiological patterns when compared to the standard white rot fungus *Phanerochaete chrysosporium*. Color removal occurs without lag phase under neutral pH.

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CHAPTER 2

Peroxidase activity and pulp-mill effluent color degradation by *Sagenomella striatispora* and *Phanerochaete chrysosporium*

2.1 Abstract

Production of lignin degrading enzymes (laccase, lignin peroxidase and managanese peroxidase) by *Sagenomella striatispora* was compared to the standard lignolytic basidiomycete *Phanerochaete chrysosporium*. In contrast to *P. chrysosporium*, *S. striatispora* did not produce any known lignin degradation enzymes. Enzyme activity measurements did not show any laccase, lignin or manganese-dependent peroxidase activity. Nitrogen starvation did not trigger the production of these enzymes. Furthermore, *S. striatispora* significantly reduced the pulp-mill effluent color without a lag phase or adjustment of the wastewater pH to 4.5. Analysis of molecular weight fractions in the initial and treated effluent showed that this species selectively degraded the high molecular weight fraction (> 3 kDa) while *P. chrysosporium* was less specific and reduced all molecular fractions with similar efficiencies. Attempts to decolorize pulp- mill effluent *in vitro* using extra-cellular fluid from *S. striatispora* cultures were not successful.

2.2 Introduction

Using whole microbial cell cultures to remove color from pulp and paper-mill effluent involves addition of nutrients and oxygen under sterile conditions. This can be very costly because of the high volumes of wastewater produced every year from pulp and paper mills. In addition, experiments using *Phanerochaete chrysoporium* cultures in bioreactors revealed other problems such as: short fungal biomass life and biomass wasting (Eaton et al., 1980), contamination by other microorganisms and oxygen limitation (Messner et al., 1990) and foaming in agitated systems (Prouty, 1991). More recent research has explored the use of isolated ligninases instead of whole cell systems (Ferrer et al., 1991; Presnell et al., 1992). This is an attractive alternative especially if the enzymes can be recycled or immobilized to improve the economics of the process (Presnell et al., 1994).

However, *in vitro* lignin degradation experiments using purified lignin degrading enzymes have produced conflicting results. Haemmerli et al. (1986) were the first to report *in vitro* repolymerization of lignin in experiments using purified enzymes. Odier et al., (1988) have described similar results on lignin repolymerization rather than depolymerization by *P. chrysosporium* peroxidases. These findings raise questions concerning the role of the two main groups of lignin peroxidases: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) isolated from *P. chrysosporium* extracellular culture fluid (Tien, 1989; Gold et al., 1989). Nevertheless, Leisola et al. (1988) obtained significant increase of lignin mineralization in cultures of *P. chrysosporium* that were washed then amended with purified LiP and veratryl alcohol. More recent studies have demonstrated *in vitro* lignin depolymerization by LiP (Hammel and Moen, 1991) and MnP (Wariishi et al., 1991).

The roles of each group of enzymes in lignin degradation or pulp-mill effluent decolorization are poorly understood, although there is increasing evidence that MnP plays a more important role in pulp-mill effluent decolorization. Michel et al. (1991) obtained similar color removals using the wild type *P. chrysosporium* and a mutant that

lacked LiP but still produced MnP. Lackner et al. (1991) reported degradation of high molecular weight chlorolignins (> 30kDa) using purified MnP. The importance of MnP in lignin transformation has also been described by Archibald (1992) and Kondo et al. (1994) for *Trametes versicolor* in experiments of wood biobleaching. Other microbial strains degrade lignin but do not produce LiP (Paice et al., 1993).

Whether these two peroxidases are the only or main components of lignin degrading systems in fungi remains to be demonstrated. In this work, we compared the degradation of different molecular weight fractions in pulp-mill effluent using *Sagenomella striatispora* and *Phanerochaete chrysosporium*. *S. striatispora*, a hyphomycete isolated in our laboratory removed a significant amount of color without acidification of the pulp-mill effluent. We also investigated peroxidase and laccase activity along with the possibility of pulp-mill effluent decolorization *in vitro* using the two species.

2.3 Material and Methods

2.3.1 Fungal culture

Cultures of *Sagenomella striatispora* and *Phanerochaete chrysosporium* were grown on 1.6 % malt extract then blended and washed in distilled water. Cultures of *S. striatispora* were added to 125 ml serum bottles containing 30 ml of culture medium containing (per liter) : KH_2PO_4 g, NaCl 0.1 g, $CaCl_2$-$6H_2O$ 10 ml, $MgSO_4$-$7H_2O$ 10 ml, yeast extract 10 mg, glucose 10 g, NH_4Cl 45 mg, and 1 ml of a mineral solution containing per 100 ml: Dissodium EDTA 1.58 g, $ZnSO_4$-$7H_2O$ 0.7 g, $MnSO_4$-$4H_2O$ 0.30 g, $FeSO_4$-$7H_2O$ 0.16 g, $CoCl_2$-$6H_2O$ 0.052 g, Na_2MoO_4-$2H_2O$ 0.047g, $CuSO_4$-$5H_2O$ 0.047 g. Cultures of *P. chrysosporium* were inoculated in the same medium but with di-ammonium tartrate as a nitrogen source in acetate buffer (20 mM at pH 4.5).

Pulp-mill effluent samples (30 ml) were prepared in 125 ml serum bottles then autoclaved at 120°C for 15 min. prior to fungal inoculation

2.3.2 Molecular weight fractionation of pulp-mill effluent

Fractionation of the original and treated pulp-mill effluent was carried out using Amicon Centrip-Prep tubes with molecular cut-offs of 3 kDa and 10 kDa. Color adsorbed on biomass was extracted using NaOH (0.1 N) and fractionated in a similar way.

2.3.3 *In vitro* pulp-mill decolorization assays

Three different tests were undertaken to check if color removal can occur without the presence of whole cells.

In the first test, *Sagenomella striatispora* was grown in a culture medium containing pulp-mill wastewater that was filtered through 0.22 µm pores. Then, six samples (4 ml) were withdrawn from the culture tubes and aseptically filtered (0.22 µm) in sterile tubes. Three of the six tubes were used for reference measurement and the other three were incubated for an additional 24 hours. Along with the cultured pulp-mill effluent, we used a blank non-cultured pulp-mill wastewater to account for non-biological color removal.

In the second test we used *S. striatispora* concentrated extra-cellular fluid. One liter of three day old cultures was centrifuged at 10,000 g for 15 min at 4°C to remove the mycelial material. Phenyl Methyl Sulfonyl Fluoride (at a final concentration of 0.1 mM) was added to inhibit the proteolytic activity as recommended by Dosoretz et al. (1990). The supernatant was then concentrated to 50 ml using Amicon Stir-Cell equipped with 10 kDa molecular cut-off membranes(YM-10). Aliquots of 2 ml and 4 ml of the extra-

cellular fluid concentrate were added to 2 ml pulp-mill effluent samples. The reaction was initiated by adding H_2O_2 (25 mM) and malonate buffer at pH 4.5 (20 mM). Three replicates were prepared for each extra-cellular fluid dilution and incubated for 24 hours at 25 °C. At the end of the experiment, measurements of light absorbance at 465 nm of pulp-mill samples amended with extra-cellular fluid were compared to tubes containing similar volumes of pulp-mill effluent with distilled water instead of extra-cellular fluid.

Enzyme assays for *S. striatispora* revealed the presence of a small peroxidative activity related to the 10 kDa extra-cellular fluid filtrate. To verify if this peroxidative activity was related to pulp-mill effluent decolorization, we used sterile dialysis tubes with 10 kDa molecular weight cutoff (Serva-Por) packed with *S. striatispora* fungal mycelium. The dialysis tubes were placed in 250 ml serum bottles containing the high molecular weight fraction (> 10 kDa) of pulp-mill effluent amended with the culture medium given above. Concentrated samples of the high molecular weight fraction (> 10 kDa) were prepared using the Amicon Stir-Cell equipped with YM-10 membranes. The fraction was washed with distilled water until the filtrate absorbance at 465 nm was equal to zero and amended with the culture medium given above, then sterilized by filtration using 0.45 µm sterile membranes. After five days incubation, dialysis tubes were removed and color was measured and compared to a blank prepared using dialysis tubes filled with distilled water instead of mycelium. The dialysis tubes were inspected under a microscope for evidence of damage. After removing the dialysis tubes, the high molecular weight pulp-mill wastewater samples were incubated for an additional week in malt extract to check for microbial contamination.

2.3.4 Fatty acids profile

Cultures of *Sagenomella striatispora* were centrifuged at 10,000 g for 10 min to remove mycelial material. The supernatant was then filtered using Amicom YM-10 membranes to remove proteins. Fatty acids were purified using a liquid-liquid extraction with di-chloromethane and solid-liquid extraction using strong anion exchange carriers

(Liebich, 1990). For liquid-liquid extraction, 20 ml of the filtrate was adjusted to pH 1.5 then mixed with 10 ml di-chloromethane. The fatty acids were then re-extracted in a 10 ml solution of NaOH at pH 7 to reduce interference from non-acidic compounds. Finally, the last portion was adjusted to pH 1.5 then extracted in 5 ml di-chloromethane. For solid-liquid extraction, the filtrate was adjusted to pH 6.5 with Tris-HCl buffer then loaded onto a Q-Sepharose (Pharmacia) column. The acids were eluted using 0.5 mM sulfuric acid. The eluted fraction was then extracted with di-chloromethane. The free acids were injected in a GC-MS system equipped with a capillary Nukol silica column (Supelco).

2.3.5 Enzyme assays

Activities of LiP, MnP and laccase were measured in the culture fluid after removal of mycelial material by centrifugation (10,000 g for 15min). The enzyme activities were also measured in concentrated supernatant of the culture medium described above. To verify if the presence of pulp-mill wastewater influenced the enzyme production by *S. striatispora*, the enzyme activities were measured in the culture medium (25 ml) described above containing pulp-mill effluent (5 ml).

2.3.6 Lignin peroxidase

LiP activity was determined by measuring absorbance changes at 310 nm (Kirk et al., 1990) of the following mixture: 2 ml extracellular fluid, 2 nM veratryl alcohol, 50 mM tartrate (pH 3). Hydrogen peroxide was added at a final concentration of 0.4 mM to start the reaction. The mixture was incubated at 37°C for 5 min. One unit activity corresponded to the oxidation of 1 µmol of veratryl alcohol to veratryl-aldehyde. The molar extinction coefficient of veratryl-aldehyde is 9300/ M.cm (Kirk et al., 1990).

2.3.7 Manganese peroxidase

MnP was determined by monitoring vanillyl acetone oxidation at 336 nm for 3 min (Paszynski et al., 1988). The reaction mixture contained 2 ml extracellular fluid, 100 mM sodium malonate (pH 4.5), 100 μM vanillylacetone, 100 μM MnSO4 and 100 μM H_2O_2 to start the reaction. The blank contained the same reagents except H_2O_2. Unit activity of MnP was calculated with an extinction coefficient for vanillylacetone of 18,300/ M.cm (Paszynski et al., 1988).

2.3.8 Laccase

Laccase activity was determined by following the oxidation of 2,6 dimethoxyphenol to an orange complex with an extinction coefficient of 10,000/ M.cm (Paszynski et al., 1988) at 468 nm. The reaction mixture contained 2 ml extracellular fluid, 50 mM dimethoxyphenol in malonate buffer (20 mM at pH 4.5).

All enzyme activities were measured in 3 ml volume cuvets. Absorbance was measured using a Spectronic 501 specrophotometer.

2.3.9 Analytical techniques

Glucose was measured by the dinitrosalycilic acid method using D-glucose as a standard (Ghose, 1987). Ammonium concentrations were determined using an Orion ammonia electrode (Model 95-12) with NH_4Cl as standard. Total proteins were estimated using Biuret reagent using Biorad Test kits with bovine serum albumin in tartrate buffer as standard. Oxygen and CO_2 were monitored in the vials headspace using gas

chromatography. Biomass was measured as dry solids after filtration on tared fiberglass GF/C membranes that were dried overnight at 105°C prior to weighing.

2.4 Results

2.4.1 Effect of pH and color removal kinetics for *P. chrysosporium* and *S. striatispora*

Both fungal species significantly decolorized the pulp-mill wastewater during the first 6 days of incubation (Figure 2.1 A). During two weeks of incubation, *P. chrysosporium* removed 85 % of the initial color while *S. striatispora* removed 73 %. *S. striatispora* decreased pulp-mill effluent color without any apparent lag phase with similar efficiency at either initial pH 7.6 and 4.5. In contrast, *P. chrysosporium* did not remove color during the first 24 hours of incubation. This species decreased pulp-mill effluent color more efficiently in the samples buffered at pH 4.5. Furthermore, *S. striatispora* changed the pulp-mill wastewater pH from 7.6 to around 4 during the first 48 hours of incubation (Fig. 2.1 B). Although *P. chrysosporium* acidified the pulp-mill effluent, the pH change was slower.

In addition to the residual color in the liquid portion, 12 % of the initial color for *S. striatispora* and 14 % for *P. chrysospoium* were adsorbed on the mycelial biomass.

Comparison between molecular fractions in the initial and treated pulp-mill wastewater (Fig. 2) revealed that the two species degraded different fractions. *S. striatispora* preferentially degraded the high molecular weight fractions while *P.*

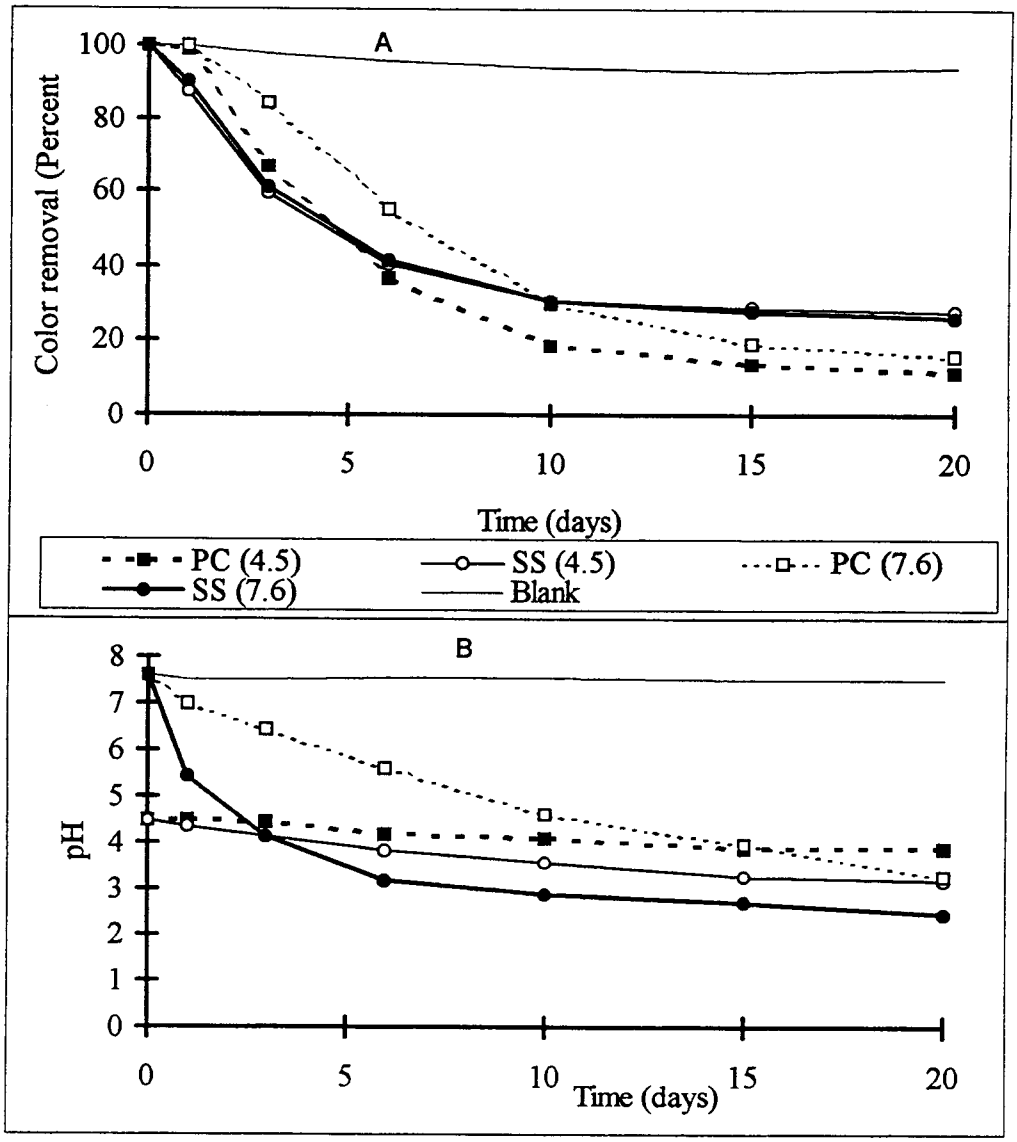

Figure 2.1. Pulp-mill color removal (A) and pH changes (B) during a 2 week incubation using *P. chrysosporium* (PC) and *S. striatispora* (SS) with starting pH 7.6 and 4.5.

chrysosporium significantly degraded all three fractions. *S. striatispora* removed more than 80 % of the high molecular weight fraction (> 10 kDa) but less than 10 % of lowest molecular weight fraction (< 3 kDa). In the pulp-mill effluent samples treated with *S. striatispora*, the sum of color remaining due to low molecular weight fraction chromophores (< 3 kDa) in liquid effluent and sorbed to the biomass combined for more than 100 % of the initial effluent low molecular weight color.

2.4.2 Growth and substrate utilization

In chemically defined media, *S. striatispora* was characterized by a slow growth phase that extended to the tenth day of incubation. During this growth phase, glucose and ammonia uptake rates were 1.2 g/L.day and 5 mg/L.day, respectively. After the seventh day, the culture was nitrogen limited. However, even when all the external ammonia was consumed, organic nitrogen as extra-cellular protein was still present in the culture fluid. Total extra-cellular proteins increased from 10 mg/L to around 80 mg/L (Fig. 2.3) over the incubation period.

2.4.3 Enzyme activity measurement

Very low levels of laccase and manganese peroxidase were detected in the course of two-week incubation for *S. striatispora* (Figure 2.4). We did not detect any lignin peroxidase activity. The production of the enzymes did not seem to correlate with any substrate uptake although a small increase in MnP concentration was observed after the seventh day when the concentration of glucose and ammonia were relatively low. To separate the effects of glucose and nitrogen decrease on the enzyme production, two more trials with low nitrogen (1.2 mM NH4Cl) and no nitrogen in the culture medium were

performed (Figure 2.5). Although the concentration of MnP and laccase were still very low, we observed slightly higher levels of activity in both later trials compared to the first one with 2.4 mM nitrogen.

In contrast, activities as high as 350 µmole MnP /min.l and 50µmole LiP /min.l were detected in *P. chrysosporium* extra-cellular fluid. Enzyme activities peaked after all of the soluble nitrogen was consumed (Figure 2.4 A).

2.4.4 *In vitro* color removal

After filtering the pulp-mill effluent to remove the fungal biomass, no decolorization was observed when only extra-cellular fluid from *S. striatispora* remained in contact with the pulp-mill effluent (Figure 2.6). In contrast, there was a significant but low daily decolorization of the pulp-mill effluent in contact with the extra-cellular fluid from *P. chrysosporium*.

Concentrated extra-cellular fluid (filtration with 10 kDa molecular cut-off) of *S. striatispora* cultures did not produce any detectable pulp-mill effluent color removal. In addition, laccase and peroxidase activities were not significantly different in concentrated and filtered fractions. The small peroxidative activity observed in *S. striatispora* cultures was still detected in the 10 kDa filtrate. No color removal was observed in the high molecular weight fraction (Table 2.1) inoculated with *S. striatispora* mycelium packed in dialysis tubes (10 kDa molecular weight cut-off).

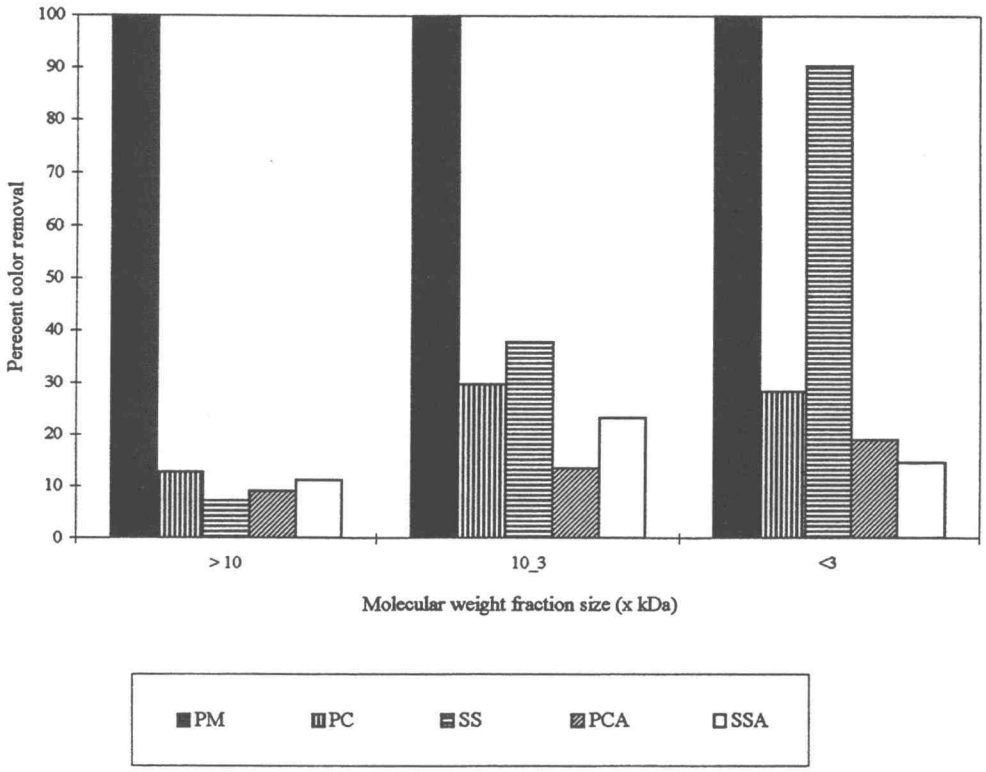

Figure 2.2. Results of molecular fractionation of pulp-mill effluent (PM) treated and sorbed to the biomass by two fungal species: *P. chrysosporium* (treated PC and sorbed PCA), and *S. striatispora* (treated SS and sorbed SSA).

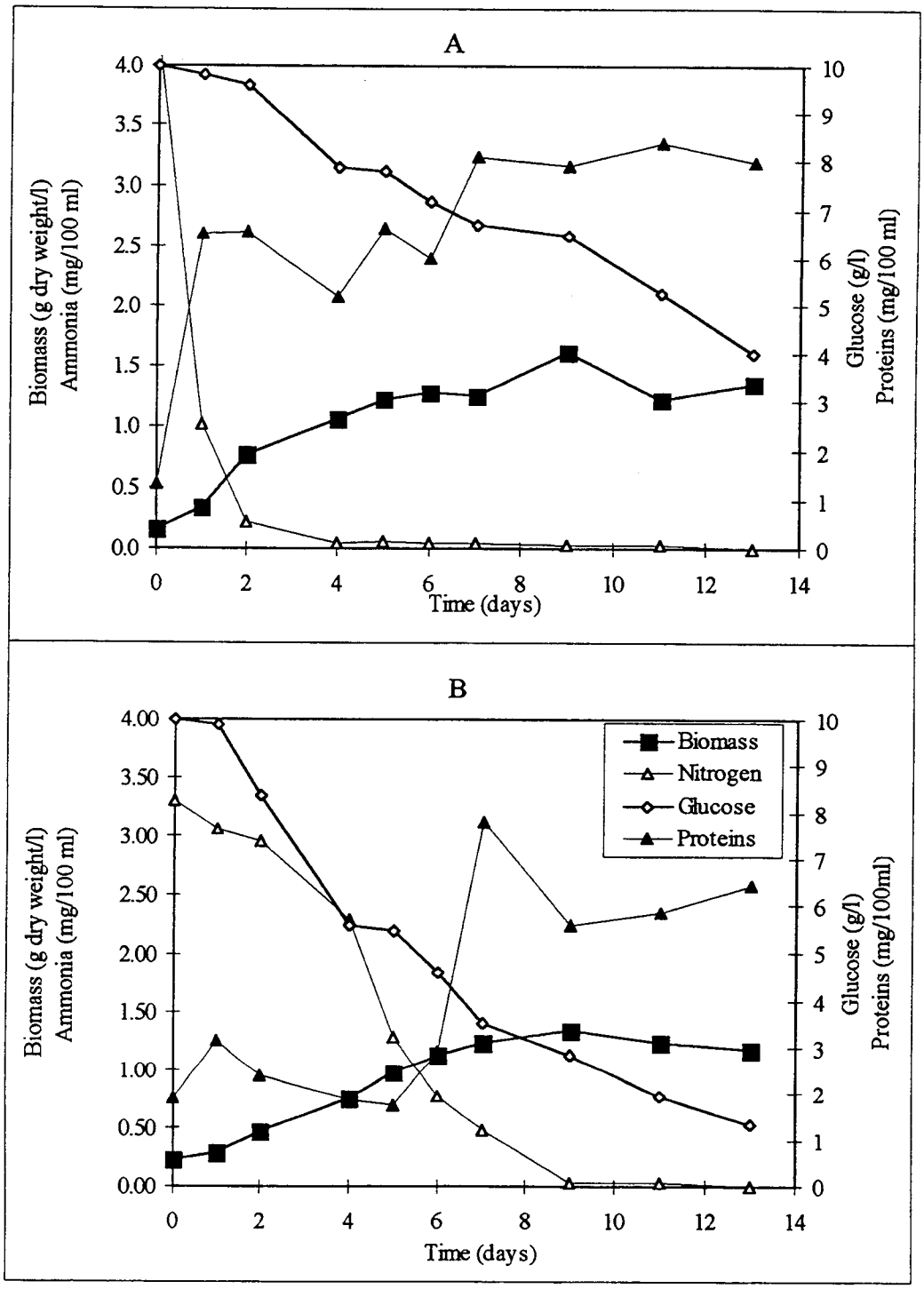

Figure 2.3. Growth and substrate consumption by cultures of *P. chrysosporium* (A) and *S. striatispora* (B) in chemically defined culture media.

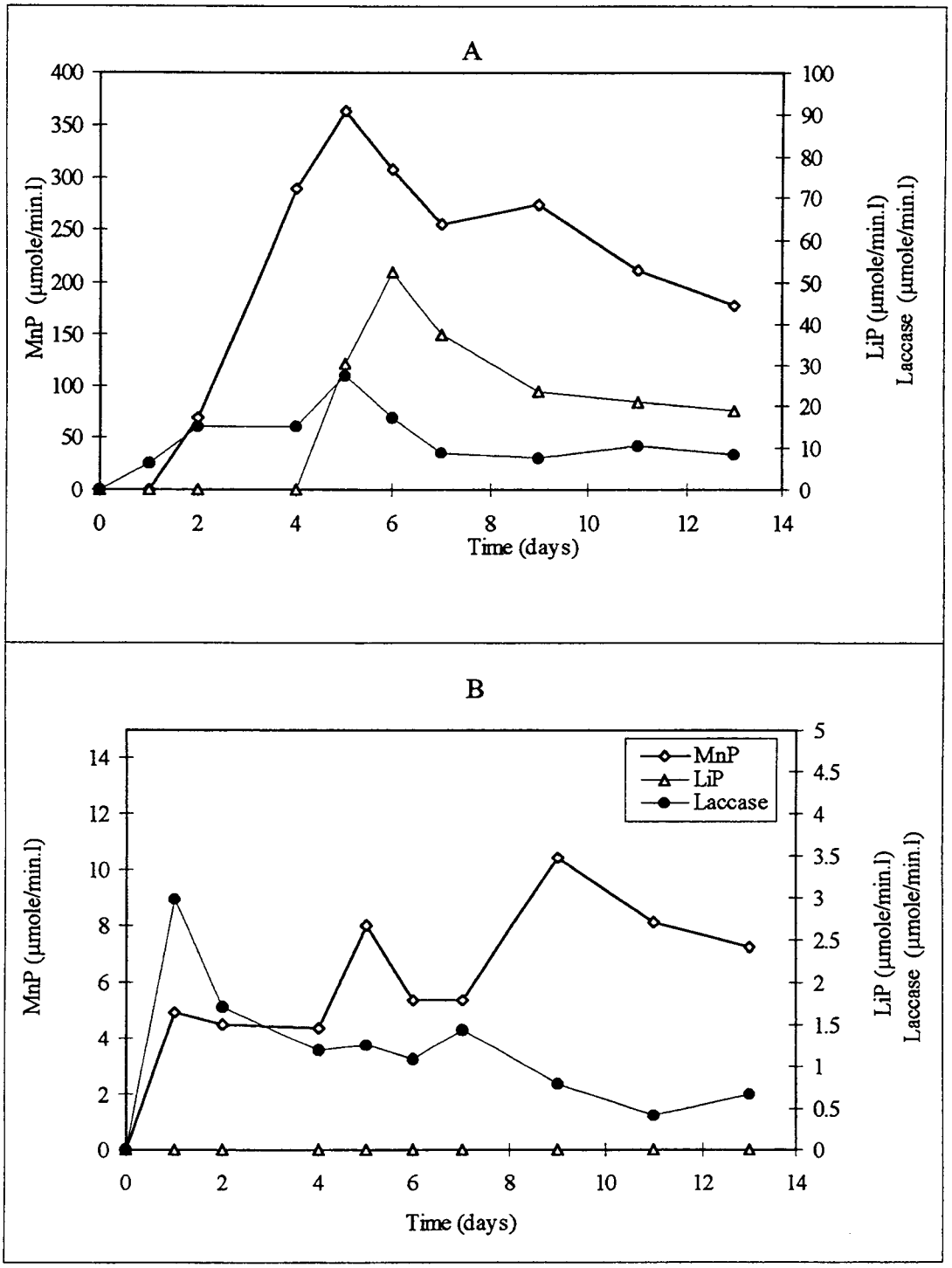

Figure 2.4. Lignin peroxidase (LiP), Manganese peroxidase (MnP) and Laccase activities in cultures of *P. chrysosporium* (A) and *S. striatispora* (B).

Table 2.1. Pulp-mill color removal by *S. striatispora* packed in dialysis tubes (10 kDa) after 6 days of contact.

Treatment	Color removal (%)
S. striatispora in culture	63
S. striatispora packed in dialysis tubes	0
P. chrysosporium packed in dialysis tubes	16

2.5 Discussion

The pH of pulp-mill effluent affected the two species in different ways. *S. striatispora* showed similar efficiency of color removal at starting pHs 4.5 and 7.6, while color removal by *P. chrysosporium* was significantly better under acidic conditions. *S. striatispora* performed similarly at the two starting pHs because of its ability to change the pH very rapidly. Analysis of the fatty acid profile of *S. striatispora* revealed a mixture of myristic, palmitic and stearic acids. Other investigators have reported acidification of the pulp-mill effluent by other fungal species (Martin and Manzaranes, 1994). The significance of fatty acid production and pH decrease on the pulp-mill effluent decolorization process is not yet clearly understood. Nevertheless, pH affects the activity of the degrading enzymes that is optimal at acid pH (Gold et al., 1989, Kirk et al., 1988). Furthermore, more recent evidence shows that certain fatty acids such as malonate used as buffers in lignin degrading microorganisms cultures are also important chelator agents for manganese dependent peroxidase catalysis cycle (Perez and Jefferies, 1992, Wariichi et al., 1992). However, there is no evidence yet that microorganisms use their fatty acids as chelating agents for lignin degradation in natural systems.

In contrast to *P. chrysosporium*, *S. striatispora* preferentially removed color from the high molecular weight fraction (> 10 kDa). *P. chrysosporium* removed color from all three molecular weight fractions. Similar results were described by Sundman et al. (1986)

and Fukui et al. (1992) for pulp-mill color degradation by *P. chrysosporium*. This is in accordance with the non-specific degradation of lignin by *P. chrysosporium* peroxidases (Shoemaker, 1991).

More than 90 % of initial color from the low molecular weight fraction (< 3 kDa) remained in the pulp-mill effluent treated by *S. striatispora* while more than 80 % of color from the high molecular weight fraction was removed. These results obtained for *S. striatispora* reflect a more specific depolymerization of the high molecular weight compounds to smaller molecular fragments that are not further degraded or are degraded at a slower rate.

The differences observed above for pH and color removal for the the two fungal species were also observed in substrate uptake and enzyme activity. Ammonia uptake by *S. striatispora* was slower than *P. chrysosporium*. This result is probably related to the relatively slow growth of *S. striatispora*. *P. chrysosporium* biomass increased dramatically during the two first days of incubation. During this period almost all soluble nitrogen as ammonia was removed from the culture medium. Rapid uptake of nitrogen at a concentration of 2.4 mM is well documented for *P. chrysosporium* (Michel et al., 1991).

The three groups of enzymes (laccase, LiP and MnP) were detected in the extra-cellular fluid of *P. chrysosporium* cultures. However, negligible levels of laccase and MnP activities were detected in *S. striatispora* cultures. No LiP activity was detected in *S. striatispora* cultures. Measured activities of laccase and MnP for *S. striatispora* did not significantly increase in culture media with lower nitrogen concentration (1.2 mM NH4Cl) or no ammonia added. After filtering *S. striatispora* extra-cellular fluid through 10 kDa molecular cut-off membranes, enzymatic activity was detected in both the filtrate and the concentrate. This indicated that the enzymatic activity observed is not due to any of the above-mentioned enzymes because their molecular weighs are greater than 10 kDa (Gold et al., 1989). Furthermore, color removal experiments using dialysis tubes with molecular cut-offs of 10 kDa revealed no color removal in *S. striatispora* cultures. Hence, it is very unlikely that the enzymatic activity detected in *S. striatispora* extra-cellular fluid is related to pulp-mill effluent color removal. The relationship between color removal and the

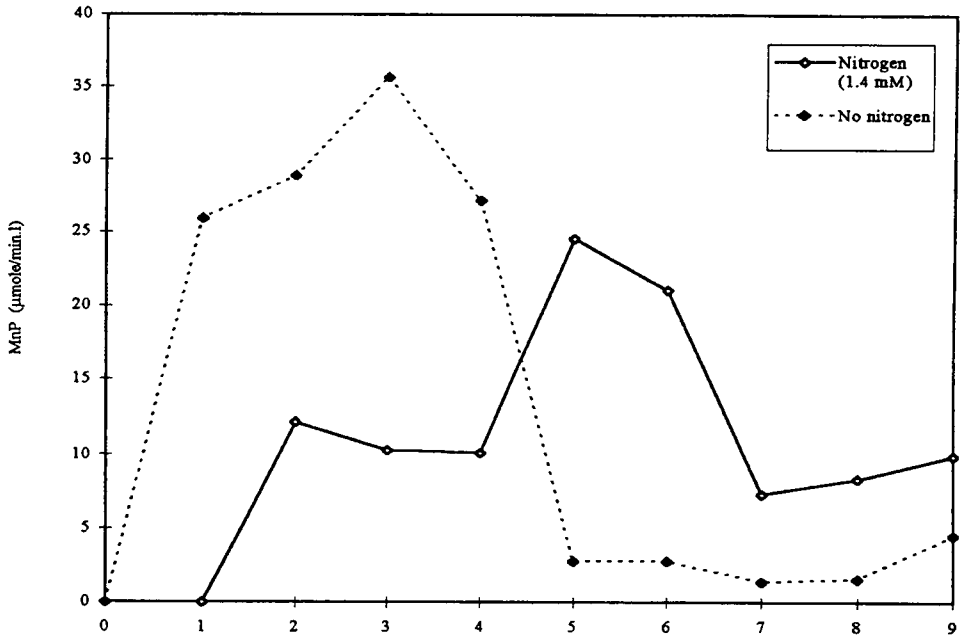

Figure 2.5. Manganese peroxidase activity by *S. striatispora* in nitrogen limited culture medium.

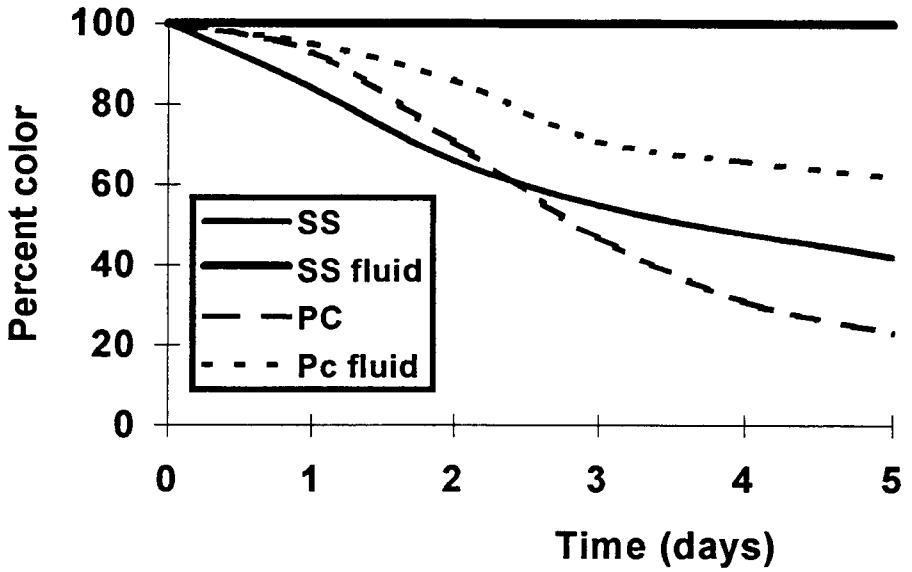

Figure 2.6. Pulp-mill color removal using cultures and culture fluids of *S. striatispora* (SS) and *P. chrysosporium* (PC).

presence of *P. chrysosporium* laccase and peroxidases is very well established (Michel et al., 1991). However, many lignin degrading fungi do not produce LiP (Paice et al., 1993). Davis and Burns (1990) were able to decolorize pulp-mill effluent using free and immobilized purified laccase in the absence of the peroxidases. Furthermore, lignin degrading bacteria do not produce any *P. chrysosporium* enzymes (Zimmermann, 1990). Similarly, no laccase, LiP or MnP activity was detected in the extra-cellular fluid of *S. striatispora* cultures.

In vitro experiments using filtered cultures and concentrated enzymes did not reveal any pulp-mill effluent decolorization in the absence of contact with *S. striatispora* mycelium. Similar experiments with *P. chrysosporium* showed that the culture extra-cellular fluid contained all ingredients needed for color removal although higher color removal rates were observed in the trials using the mycelial biomass. Successful but limited pulp-mill effluent color removal was reported for laccase (Davis and Burns, 1990), MnP (Lackner et al., 1991) and horse-radish peroxidase (Ferrer et al., 1990). However, many cases of enzyme precipitation and inactivation in pulp-mill effluent were also reported (Presnell et al., 1994, Davis and Burns, 1992). Furthermore, higher degradation rates were observed in the presence of mycelial biomass than with concentrated culture fluid or pure enzymes (Archibald et al., 1992). The results obtained for *S. striatispora* strongly suggest that pulp-mill color removal requires the contact with the fungal mycelium.

In conclusion, the process of pulp-mill effluent decolorization by the hyphomycete *S. striatispora* does not correspond with the standard lignin degrading basidiomycete *P. chrysosporium*. *S. striatispora* did not produce any of the known lignin degradation enzymes laccase, lignin peroxidase or manganese peroxidase. Furthermore, nitrogen starvation did not trigger the production of these enzymes in *S. striatispora* cultures. Growth and nitrogen uptake by *S. striatispora* were significantly slower than *P. chrysosporium*. In addition, *S. striatispora* removed color from the high molecular weight fraction (> 10 kDa) more efficiently than from the low molecular weight fraction (< 3 kDa), while *P. chrysosporium* non-specifically removed color from all molecular fractions with similar efficiency. Although *P. chrysosporium* showed higher color removal

efficiencies, *S. striatispora* decreased pulp-mill effluent color at a starting pH 7.6 without adjustment of the pH to 4.5. *S. striatispora* acidified the culture medium very rapidly (3 pH unit in 48 hours). Using three different techniques we could not obtain *in vitro* color removal in absence of direct contact between fungal biomass and pulp-mill effluent.

2.6 References

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CHAPTER 3

Pulp-mill effluent decolorization using fungal pellets of *Sagenomella striatispora*: evaluation and analysis.

3.1 Abstract

Using a stirred tank reactor in batch and continuous regimes, we were able to consistently obtain 65 % color removal at two day hydraulic retention time. Before starting the reactor experiments, we analyzed the effects of pulp-mill effluent color concentration and pH on the sorption of color to the biomass. The amount of pulp-mill effluent color sorbed to the biomass increased with time, color level and pH decrease until it reached a maximum then remained constant aftre the first batch. Color removal by sorption did not account for more than 250 CU/ g biomass (20 % of initial color) after the first batch. This accumulation of color on the biomass did not appear to influence the ability of mycelial pellets to remove color or their growth and respiration activities. In both successive batches and continuous regimes, a significant amount of biomass was lost. There were also problems with foaming particularly when operating the reactor in continuous flow. Using a low stirring force helped reduce foaming but there was still a thin layer of foam due to oxygen bubbling. We proposed an integrated color removal and fungal pellets growth model to predict and analyze the interactions of the different processes. The combined model satisfactorily simulated the experimental data. Analysis of the data from the simulation revealed that glucose and oxygen were in excess in all experiments. The simulation also showed that an increase of the pellets diameter above 1.8 mm may create problems of oxygen limitation.

3.2 Introduction

The most effective biological treatment for reducing color from pulp-mill effluent involves utilization of filamentous lignolytic fungi. Successful results have been obtained using the white rot filamentous basidiomycetes *Phanerochaete chrysosporium* and *Trametes versicolor* in immobilized or free pellets systems (Norton, 1992). The first patented process for pulp-mill effluent color treatment used immobilized oxygenated *P. chrysosporium* cultures (Eaton et al., 1980). The process, known as MyCoR (Mycelial Color Removal), used the basidiomycete *P. chrysosporium* fixed on rotating biological contactors. Messner et al. (1990) developed a trickling filter reactor using *P. chrysosporium* immobilized on polyurethane foam cubes. Cammarota and Sant (1992) tested a glass packed-bed air-lift reactor for pulp-mill decolorization with biomass immobilized on glass. The work of Prouty (1990) is among the first attempts to use free agitated pellets of *P. chrysosporium* for pulp-mill decolorization in a continuous stirred tank reactor. Royer et al. (1991) also obtained successful decolorization of pulp-mill effluent in a bubble column reactor using fungal pellets of *Trametes versicolor*.

These laboratory and pilot scale studies have revealed many problems for continuously using fungal biomass to decolorize pulp-mill effluent in both immobilized and agitated pellet reactors. Immobilized cell designs suffer from short biomass lifetime and oxygen transfer limitations (Campbell, 1983). In all aerated systems, detergents like Tween 80 are used to protect the biomass and enzymes from inactivation (Jager et al., 1985, Venkatadri et al., 1990). These detergents increase foaming in systems using fungal pellets (Prouty,1990; Royer et al., 1991). Royer et al. (1991) have also reported loss of biomass decolorization activity for pulp-mill effluent with concentrations higher than 5000 CU/l. Martin and Manzaranes (1994) could not use *T. versicolor* biomass for more than three consecutive batches. They also observed a progressive darkening of the biomass due to color sorption on fungal mycelia. Pulp-mill effluent chromophores sorption to the biomass was also reported by other investigators (Momohara et al., 1989; Duran et al., 1994). Duran et al. (1994) found that 13% of total initial color was adsorbed to the

mycelium of *Lentinus edodes* after 5 days incubation. The effect and importance of chromophores bio-sorption on biomass activity in pulp-mill decolorization is still unclear. The sorption process accounts for a significant part of biological removal of many pulp-mill compounds such as high molecular weight organo-chlorines (Yan and Grant, 1994). These high molecular weight compounds are also responsible for color in pulp-mill effluent (Krinstad and Lindstrom, 1984). Momohara et al. (1989) found that the sorption of high molecular weight compounds to fungal biomass is an important step in their removal. Hence, sorption affects color removal as well as the degrading microorganism activity.

In this study, we investigated the potential of using *Sagenomella striatispora* pellets for pulp-mill effluent decolorization. We also analyzed the effect of color sorption on the activity of fungal pellets during 3 successive batches. Finally, we developed a model to investigate the relationship between color degradation, color sorption and fungal pellets growth and respiration. In the experimental part we focused on analyzing the characteristics of the pellets and the kinetics of color sorption during long contact times (batch incubation of 7 days) between pulp-mill effluent and the fungal pellets. We also investigated the effect of certain environmental factors such as pH and color level on color sorption to *S. striatispora* pellets. Then, we used a completely mixed reactor to analyze the effect of color sorption in a few successive batches on pellets activity as well as the relationship between color sorption and biomass growth and color degradation in a stirred reactor.

3.3 Theoretical considerations for kinetic model

3.3.1 Pellet growth modeling

Mycelial pellets are formed by the entanglement of filamentous fragments and the branching growth characteristic of filamentous fungi (Metz and Kossen, 1977). Metz and Kossen (1977) developed an unstructured model that was later modified by Van Suijdam et al. (1982) for *Penicillium chrysogenum* growth as pellets. This model was successfully used by Michel et al. (1991) to model *P. chrysosporium* life-cycle and lignin peroxidase production.

In this work we used the Van Suijdam et al (1982) model to analyze kinetic data of biomass growth, effluent color removal and substrate consumption by *S. striatispora*. This model derives an effectiveness factor for the nutritional limiting factor oxygen, defined as the ratio of the observed limiting factor uptake rate over theoretical uptake rate without nutrient limitation. This factor is then used to correct for inactive biomass under oxygen limitation. The model assumes that pellets are perfect spheres where oxygen diffuses at the same rate within every part of the pellet. The model also assumes that oxygen is the growth limiting factor for fungal pellets, and that liquid-solid mass transfer coefficient for oxygen is negligible.

3.3.2 Pellet growth model derivation

Effectiveness factor. A steady state oxygen balance for a spherical shell of thickness dr and radius R leads to :

$$D\left(\frac{d^2O}{dr^2} + \frac{2}{r}\frac{dO}{dr}\right) = k \qquad (1)$$

With the boundary conditions : at $r = 0$, $\frac{dO}{dr} = 0$ and at $r = R$, $O = Ol$

If we assume zero-order kinetics for oxygen intra-pellet transfer, equation (1) is analytically solved (Bailey and Ollis, 1986) to derive the effectiveness factor (η) for oxygen diffusion in a fungal pellet as:

$$\eta = \frac{\text{actual reaction rate}}{\text{rate if there was no diffusion}} = 1 - \left(1 - \frac{6D\,O_l}{R_p{}^2 k}\right)^{\frac{3}{2}} \qquad (2)$$

Biomass growth. Results of *S. striatispora*'s growth, given in chapter 2, show that this fungus grows slowly with a growth phase extending to more than one week. The best fit for our data was obtained using the linear and logistic equation with a growth rate depending on the concentration of glucose in the culture medium. We used the linear model.

$$\frac{dX}{dt} = \mu_e \qquad (3)$$

where μ_e is the specific growth rate corrected for effectiveness

$$\mu_e = \eta\mu_m \left(\frac{G_l}{G_l + K_g}\right) \qquad (4)$$

3.3.3 Glucose balance

The fungal pellets use glucose for both organic matter synthesis and respiration as expressed in the following mass balance:

$$V_l \frac{dG_l}{dt} = - Y_{gx} \frac{dX}{dt} V_l - V_l Y_{go} R_{o2} \quad (5)$$

Rate of change of glucose in reactor	Rate of glucose uptake for biomass growth	Rate of glucose uptake for respiration

3.3.4 Oxygen balance

The resistance to oxygen transfer from the liquid phase to the surface of the pellets is negligible compared to gas-liquid and intra-pellet transfer of oxygen (Van Suijdam et al., 1982, Michel et al., 1992). The total oxygen mass balance in the reactor can be written as :

$$V_l \frac{dO_l}{dt} + V_{hs} \frac{dO_g}{dt} = R_{o2} V_l \quad (6)$$

Change in liquid phase	Change in gas phase	Uptake by microorganisms

The concentration of oxygen in the gas phase can be related to the equilibrium oxygen concentration in the liquid phase using Henry's law:

$$O_l = \frac{O_g}{H} \quad (7)$$

The gas phase concentration O_g can be calculated from the gas partial pressure equation:

$$O_g = \frac{P_{o2}}{RT} M_{o2} \quad (8)$$

Expressions for oxygen change in the two phases as a function of the microbial oxygen uptake can be derived from the oxygen balance and Henry's law:

$$\frac{dO_l}{dt} = R_{o2}\frac{V_l}{V_l + HV_{hs}} \quad (9) \quad \text{and} \quad \frac{dO_g}{dt} = R_{o2}\frac{V_l}{V_{hs} + \frac{V_l}{H}} \quad (10)$$

The microbial oxygen consumption rate of the pellet is proportional to the biomass growth rate:

$$R_{o2} = \mu_e X Y_{ox} \quad (11)$$

3.3.5 Color removal

Color is removed from the pulp-mill effluent by two main processes: degradation and sorption.

Color removal by biodegradation. We did not observe any biomass growth of *S. striatispora* in pulp-mill effluent without a growth substrate such as glucose. Hence, we assumed color compounds did not support fungal growth. The transport of chromophores inside the pellets was also assumed to be limited by diffusion because of the relatively high concentration of color. The initial color was assumed to be composed of a degradable and a residual non-degradable fraction. Based on our data from the first and second chapter, we assumed the residual fraction to be 30 % of initial color. Color removal of the degradable fraction (Cd) was modeled using a first order kinetics model.

$$-\frac{dC_d}{dt} = k_C X C_d \quad (12)$$

Color sorption. Color sorption expresses the accumulation of chromophores from the pulp-mill effluent on the biomass due to both adsorption and absorption. In the absence of a mechanistic model for this type of non-equilibrium time-dependent sorption (Skopp, 1986), we used an apparent sorption rate based on the experimental data. Results of color extraction from biomass revealed a saturation-type kinetics for the bio-sorption of

chromophores on *S. striatispora* pellets. Color sorption is also dependent on the available surface area on the fungal pellets (Zhou and Banks, 1993). The color sorption reaches a maximum when all the available sites on the biomass are used. The rate of sorption was derived using a first order term of the difference between the maximum amount of color that can sorb on the biomass and the actual color sorbed. From our experimental data the maximum sorbed color (C_{smax}) was found around 240 CU/ g biomass.

$$R_{cs} = \frac{k_{cs}}{X}(Cs_{max} - C_s) \qquad (13)$$

And the mass balance for sorbed color is :

$$V_l \frac{dC_s X}{dt} = V_l X \frac{k_{cs}}{X}(Cs_{max} - C_s) \qquad (14)$$

or after taking the derivative of the product Cs x X and rearranging

$$\frac{dC_s}{dt} = \frac{k_{cs}}{X}(Cs_{max} - C_s) - \frac{C_s}{X}\frac{dX}{dt} \qquad (15)$$

The color remaining in the pulp-mill effluent is obtained by substracting the sum of degraded and sorbed color from the the initial color.

Effluent Color = Initial color - Color degraded - Color sorbed to the biomass

3.4 Materials and Methods

3.4.1 Microorganisms and pulp-mill effluent

Cultures of *Sagenomella striatispora* were started from slant tubes stored at 4°C. They were sub-cultured in 250 ml flasks containing 1.6 % malt extract broth for one to 2 weeks. They were then centrifuged (2,000 g) and blended for 1 min. Blended cultures were used to inoculate 250 ml Erlenmeyer's containing 1.6 % malt. The flasks were incubated on a gyratory shaker (Lab-Line Instruments Inc.) for 72 hours at 25 °C.

Pulp-mill wastewater collected from a local pulp-mill was filtered on 1.2 μm porosity membranes to remove solid impurities. Citrate buffer was used to prepare solutions at pH 3, 4, 5, 6 and 7 for the experiments on the effect of pH on bio-sorption. Aliquots of 50 ml effluent were autoclaved at 120 °C for 15 min. Glucose solution was filter-sterilized separately then added to the effluent right before fungal inoculation.

Experiments on the effects of pH, color concentration and biomass growth were carried out using batch shake cultures using 250 ml Erlenmeyer on a gyratory shaker. These data were used to derive the model parameters. The effect of successive batches on the biomass activity and the validation of the model were carried out in a BIOFLO R (Model C32, 1.5 L volume) New-Brunswick reactor. The reactor was flushed daily with hydrated pure oxygen. We used a mild agitation of 100 rpm.

3.4.2 Analytical methods

Glucose was measured by the dinitrosalycilic method using D-glucose as standard (Ghose, 1987). Oxygen and CO_2 were monitored in the head-space of the reactors by gas

chromatography. After gas measurements, 50 ml samples were withdrawn to measure pH, color and biomass. Color was measured on a Spectronic 500 C spectrophotometer at 465 nm. Colored material sorbed to the biomass was extracted using 0.1 N NaOH in two to three consecutive washes. At the end of the experiment, the biomass was collected after centrifugation at 2000 g then transferred to a volumetric cylinder to measure the volume. Five ml of 0.1 M NaOH was added and left to react for 30 min. The supernatant was removed after centrifugation and additional NaOH was added to the biomass. This operation was repeated until all color was extracted. The supernatants were combined; then color was measured as described above. The final results were expressed as percent absorbed color per volume after accounting for dilution. Biomass dry weight was determined using fiberglass GF/C membranes dried at 105°C (APHA, 1992).

The distribution of pellet size was estimated after separation of pellets using standard screens. Three samples of pellet suspension containing around 30 pellets each were screened through standard screens (American Standard Screens) then each size fraction was counted under dissecting microscope.

3.4.3 Kinetic parameters

Oxygen utilization coefficient k was obtained from respirometer data using the dynamic method (Dunn et al., 1992).

The mean pellet density $\overline{\rho}$ was determined from biomass dry weight assuming that all pellets are perfect spheres and using an average pellet radius $\overline{R}$:

$$\overline{\rho} = \frac{3}{4} \frac{X}{\pi \overline{R}^3 n} \qquad (16)$$

Where n is the number of pellets.

3.5 Results

3.5.1 Pellets formation and characteristics

The pellet size distribution and density are given on Table 3.1 for pellets formed at a biomass concentrations of 1 g/L and agitattion 250 rpm. For higher biomass concentration (2 g/L) and lower agitation forces (100 rpm) the size distribution was less homogeneous with a higher number of large diameter pellets (> 2.5 mm). The size range of 0.8 to 2 mm diameter pellets represented more than 80% of total number of pellets (Table 3.1). They also represented more than 70 % total mass of the whole pellet sample analyzed.

Pellet density was calculated based on the average diameter, weight and number of pellets in each size using equation (17). The estimated pellet density values of the small diameter pellets were slightly higher than larger pellets. The average pellet density for *S. striatispora* was around 85 g/L.

Table 3.1. Size and density of *S. striatispora* pellets obtained using 1 g biomass/L and 250 rpm agitation force

Diameter (mm)	Number (in 40 ml)	Density (g/L)
> 2.00	15	69
1.40-2.00	109	84
0.82-1.40	255	89
0.60-0.82	36	98
	Total[a]: 415 (35)	Average[a]: 85 (12)

[a]Standard deviation given between brackets

3.5.2 Effect of color concentration on color sorption

The amount of color sorbed to the biomass was directly correlated with initial pulp-mill effluent color level (Figure 3.1B). The amount of color extracted from the biomass in contact with the highest color level was more than ten times higher than the amount extracted at the lowest color level. The process of color sorption is also time-dependent. Figure 3.1 shows that color sorption increased with increasing contact time between the effluent and the biomass although the amount sorbed did not change after the fourth day of incubation.

3.5.3 Effect of pH on color sorption

Table 3.2. shows the effect of pH on the sorption of color to the biomass after one and 24 hours contact between the biomass and the pulp-mill effluent. The experiment was terminated after 24 hours because the pH of all samples started decreasing despite the presence of citrate buffer. The amount of color sorbed at low pH (pH = 4) was greater than higher pHs particularly after 24 hours contact between the biomass and the pulp-mill effluent.

Table 3.2. Effect of pH on effluent color sorption (average CU/g ± standard deviation) to S. striatispora mycelial pellets

Time (hours)	pH			
	4	5	6	7
1	63 ± 8	52 ± 6	42 ± 8	36 ± 10
24	136 ± 12	128 ± 14	68 ± 10	54 ± 12

3.5.4 Color removal in continuous and successive batches using mycelial pellets of *S. striatispora*

The same mycelial biomass of pellets was used for four successive batches in a Bioflo reactor under the conditions given in Table 3.3. At the end of each batch, agitation was stopped to allow settling of the pellets. The treated pulp-mill effluent was then removed under sterile conditions and replaced by another batch of untreated pulp-mill effluent.

Despite the accumulation of color on the pellets, color removal (Figure 3.2) and the activity of the microorganisms as shown by growth and respiration (Figure 3.3) were not affected during the whole period of study. The decrease of biomass from one batch to the next was due to the biomass removed with the treated effluent. Similarly, the mycelial pellets removed color with high efficiency during the whole period of four successive batches.

Table 3.3 Initial conditions for successive batch experiments in the Bioflo reactor.

Parameter	Value
Temperature (°C)	25
Liquid phase volume (L)	0.5
Gas phase volume (L)	1
Pellet number (1/L)	6000
Initial effluent color (CU/L)	2050
Glucose (g/L)	10

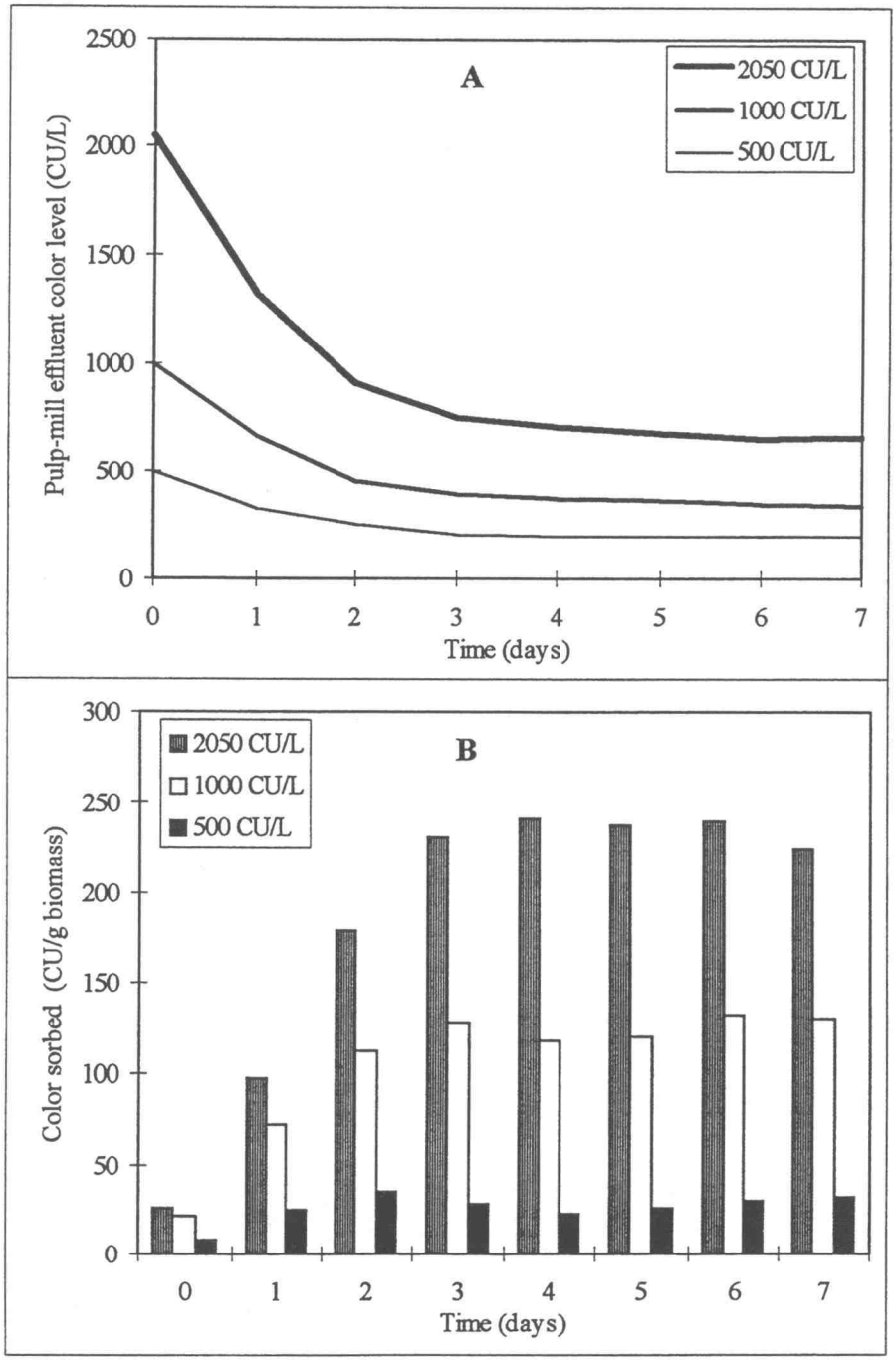

Figure 3.1. Pulp-mill effluent color (A) and sorbed color to the biomass (B) at three different initial color levels (2050, 1000 and 500 CU/L) using *S. striatispora*.

The amount of color sorbed to the biomass increased with contact time during the first batch until it reached a maximum then remained relatively constant at 250 CU/g biomass (Figure 3.2). These results were similar to the findings in shake-flask experiments.

Continuous decolorization of pulp-mill effluent using a two-day retention time for the pulp-mill effluent was investigated in the same reactor but under different conditions. The liquid volume was increased to 1.5 L and pure oxygen was bubbled in the reactor at a rate of 200 ml/min. The effluent was removed by overflow. Stirring was adjusted to 100 rpm with a quiescent zone on the top of the reactor to minimize pellet loss by overflow.

Color removal and sorption results were similar to those obtained for successive batches. Color was consistently removed at a rate of more than 65 % using an effluent with an initial color level of 2050 CU/L (Figure 3.4) . The color sorbed never exceeded a value of 12 % of initial color after the first two turn-overs (Figure 3.4). In this experiment, some biomass was also lost by flowing out in the overflow (Figure 3.5). We also noticed a small layer of foam on the top of the reactor that trapped a significant amount of biomass.

3.5.5 Kinetic parameters

The data from shake flask cultures were fit to the equations given above and used to derive the different kinetic coefficients for growth, color removal and color sorption (Table 3.4).

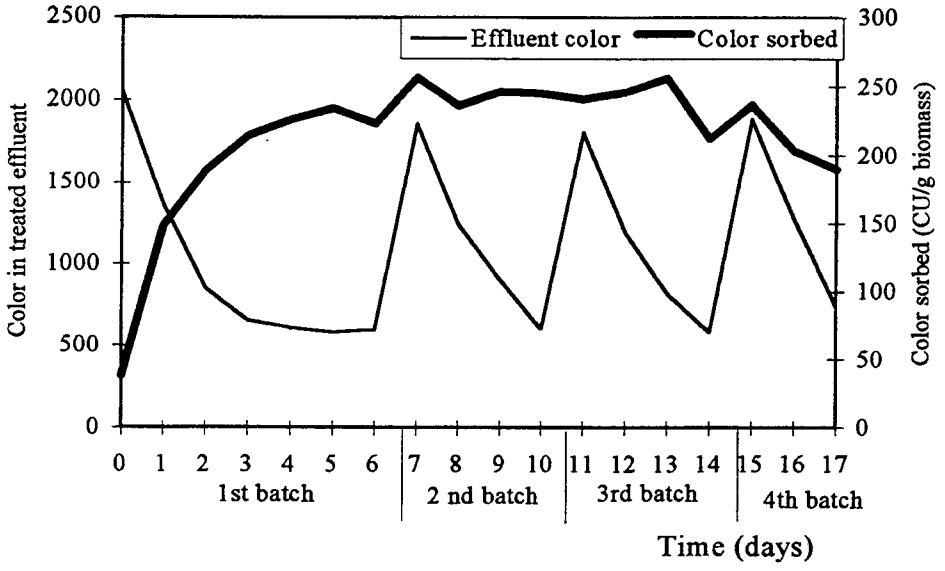

Figure 3.2. Treated effluent color and sorbed color to fungal pellets of *S. striatispora* in pulp-mill effluent in four successive batches.

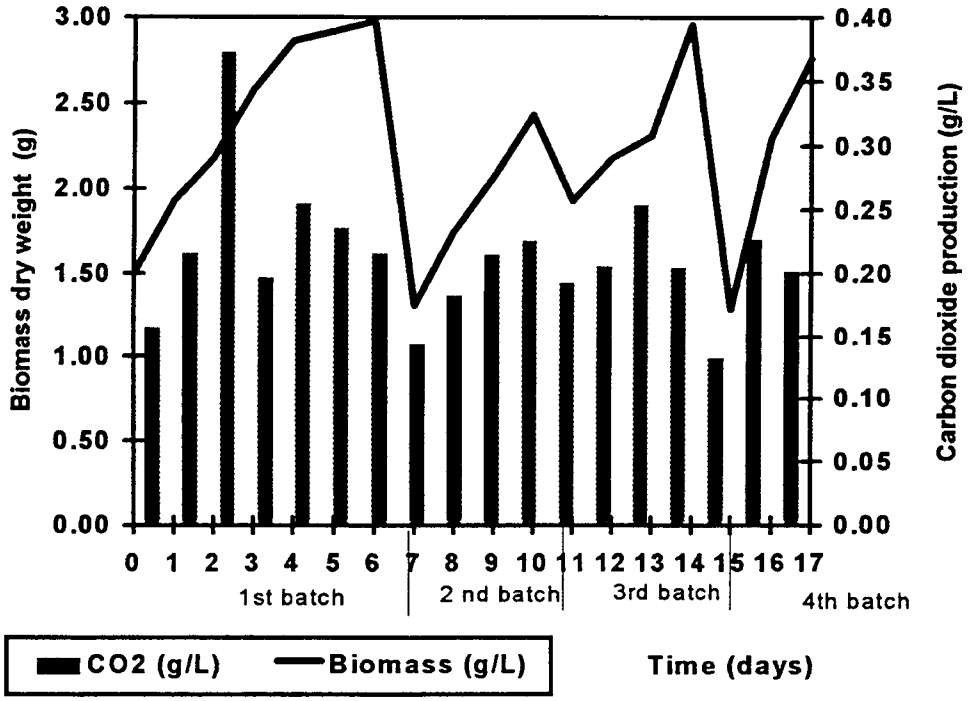

Figure 3.3. Biomass dry weight (lines) and CO2 production by fungal pellets of *S. striatispora* in pulp-mill effluent in four successive batches.

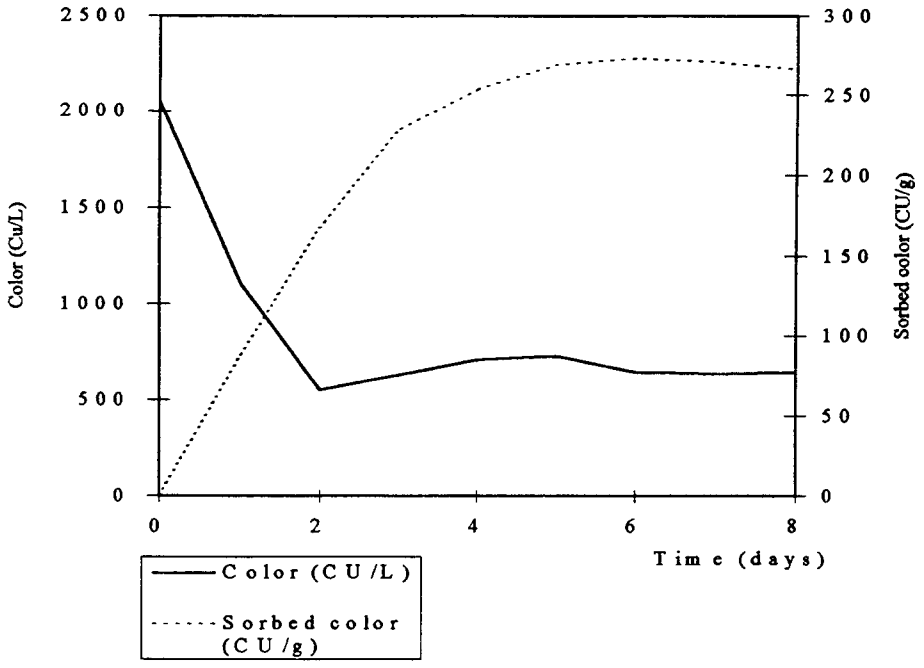

Figure 3.4. Results of color removal and sorption using fungal pellets of *S. striatispora* in continuous flow operation with 2 day retention time.

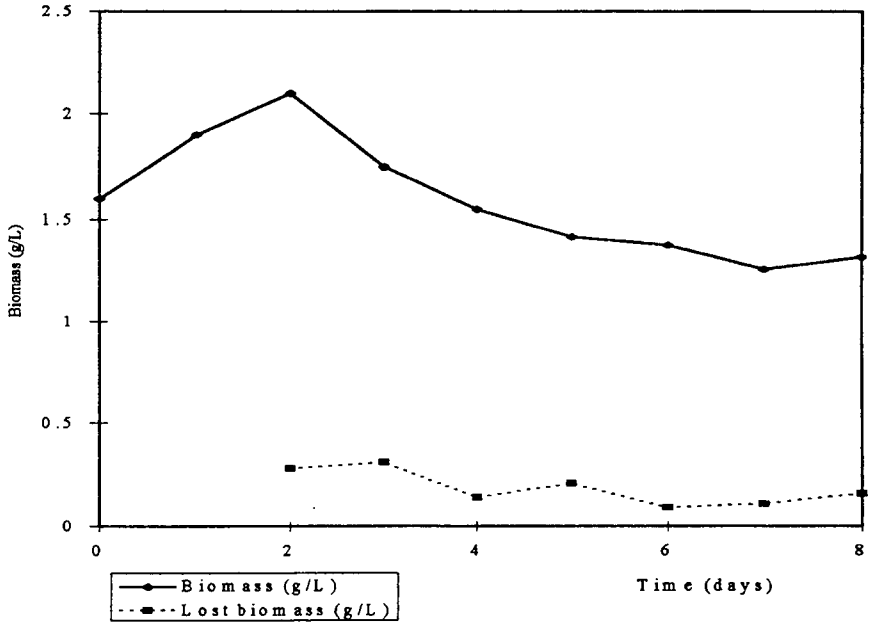

Figure 3.5. Biomass dry weight in the reactor and lost in the effluent for fungal pellets of *S. striatispora* in continuous flow operation with 2 day retention time.

Oxygen uptake rate was derived by fitting oxygen consumption data to a linear model (Figure 3.6). The oxygen consumption rate (0.0088 mg O_2/L.s) was then corrected for the biomass concentration (2.17 g/L) to yield 0.35 g O_2/ g biomass.day. The yield coefficient of oxygen (0.92 g glucose/ g oxygen) was obtained from the aerobic oxidation of glucose assuming that glucose is the only source of carbon in the culture medium. The yield coefficient of biomass from glucose (0.90 g glucose / g biomass) was estimated assuming that 90 % of biomass originates from the sole source of carbon glucose. We used a coefficient yield of oxygen from biomass of 1 g oxygen/ g biomass (Michel et al., 1992; Van Suijdam et al., 1982).

Table 3.4. Kinetic parameters used in the model.

Parameter	Value	Reference
Color removal		
k_c (L/ g biomass.day)	0.48	This work
C_i (CU/L)	2050	This work
k_{cs} (g/L.day)	0.086	This work
Pellet growth		
μ_{max} (1/ day)	0.35	This work
Y_{gx} (g glucose/g biomass)	0.90	This work
Y_{ox} (g oxygen/g biomass)	1	This work
Oxygen respiration		
Oxygen diffusivity (dm/day)	$2.51\ 10^{-2}$	Perry et al., 1984
Henry's constant (atm)	$4.34\ 10^{4}$	Alberty and Silbey, 1992
k (g oxygen/pellet volume L)	18.8	This work
Glucose consumption		
K_g (Half saturation concentration g/L)	1	This work
Y_{go} (g glucose/ g oxygen)	0.92	This work

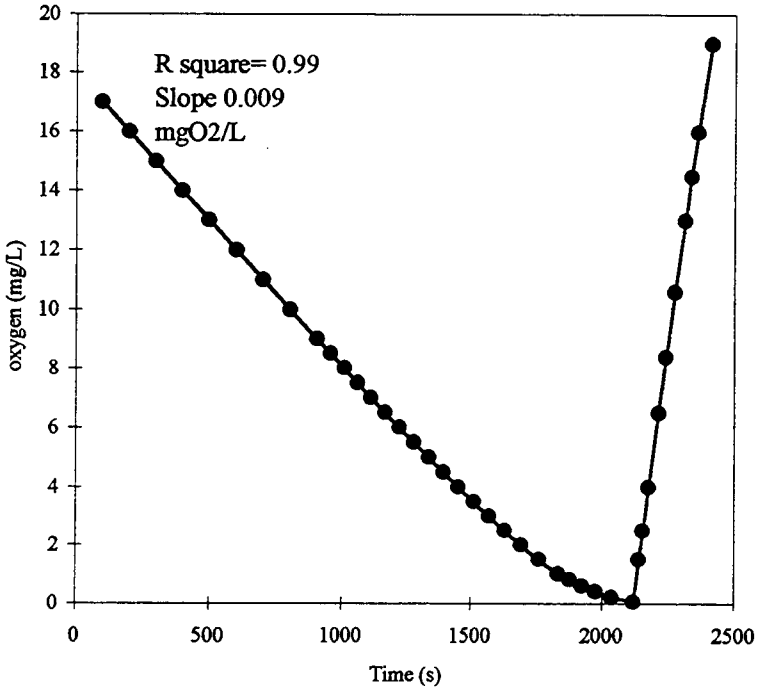

Figure 3.6. Dissolved oxygen concentration changes after gassing-out the pellet culture of *S. striatispora* and regasing with 100 % oxygen.

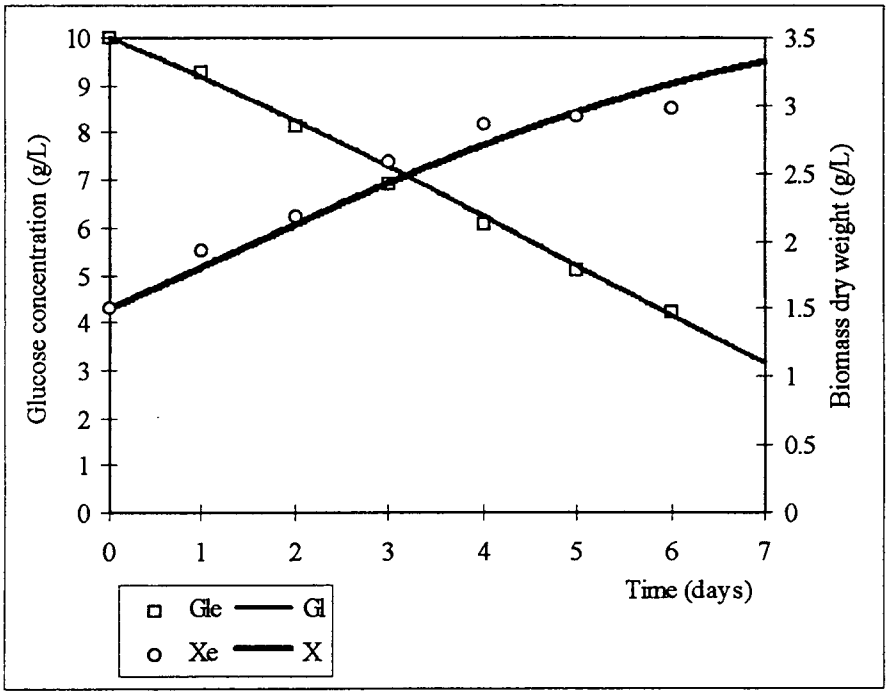

Figure 3.7. Simulated and experimental data for biomass and glucose consumption in batch cultures of *S striatispora*.

3.5.6 Simulation of batch cultures

Based on our experiments in chapter 1, addition of 10 g/L glucose and an oxygen saturated environment were optimal conditions for color removal by *S. striatispora* mycelial pellets cultures. The results of the simulation showed that during the whole batch incubation period glucose concentration was not limiting for the microorganism (Figure 3.7).

Similarly, oxygen did not seem to be limiting during the 7 days of the experiment (Figure 3.8). Assuming that glucose is the only carbon source used by the mycelial pellets, we calculated the amount of oxygen removed from the gas phase using CO_2 (1 mole CO_2/ 1 mole O_2) production data based on the aerobic glucose oxidation reaction:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

The simulated data closely corresponded to the calculated values of oxygen concentration in the gas phase (Figure 3.8). The simulation also showed that the concentration of dissolved oxygen in the medium never decreased below 25 mg oxygen/L. As a consequence, the effectiveness factor was equal to 1 even as critical pellet radius increased from 0.9 mm to 1.2 mm during the simulated time period. Figure 3.9 shows simulated data for the effectiveness factor as a function of pellet radius and dissolved oxygen concentration using equation 1. According to this plot, the pellet radius corresponding to zero dissolved oxygen (g oxygen/ L pellet) is 1.8 mm assuming that the cultuture medium is oxygen saturated at 25°C.

The changes in pulp-mill effluent color removal and sorption were also satisfactorily simulated by the proposed equations (Figure 3.10). Most of the color was removed during the first two days of incubation. By the third day of incubation almost all degradable color was removed. At the end of the batch, color in the treated effluent

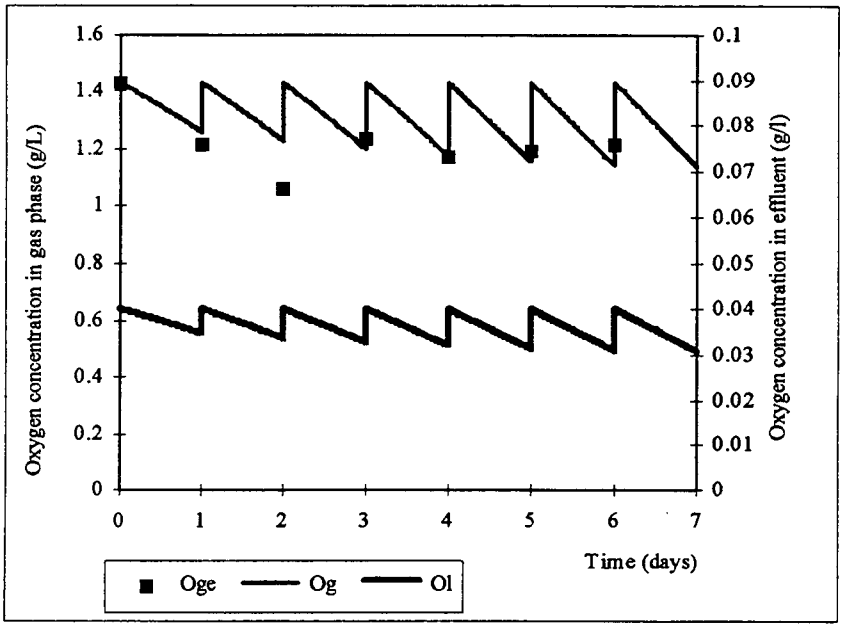

Figure 3.8. Simulated (lines) and experimental (markers) results for oxygen uptake by mycelial pellets of *S. striatispora* in batch culture.

remained relatively constant as observed for experimental data. In contrast, the amount of color sorbed to the biomass increased exponentially until reaching a constant value around 250 CU/g biomass. At the end of the batch, the amount sorbed per unit of biomass started to decrease due to the combined effect of increase in biomass and low color level in the treated effluent.

3.6 Discussion

Mycelial pellets of *S. striatispora* were successfully used for pulp-mill color removal both in batch and continuous regimes. A consistent 65% color removal in two-day effluent residence time was achieved. Mycelial pellets were used for color removal using other microbial species such as *P. chrysosporium* (Prouty, 1990) and *Trametes versicolor* (Royer et al., 1991) but detergents were added to the effluent. In this study, mycelial pellets of *S. striatispora* were used without addition of detergents avoiding additional costs and foaming due to the presence of detergents. The only nutrients supplied to *S. striatispora* cultures were glucose and oxygen without adjusting the effluent pH. However, the experiment revealed some problems that need to be addressed before scaling-up the process.

Color sorption to the biomass contributed to more than 20 % of color removed from the effluent. Color sorption increased with effluent color level, particularly under acidic conditions which causes precipitation of high molecular weight lignins increasing their sorption to the mycelial pellets (Zhou and Banks, 1993). The color sorption increased with increasing contact time with the biomass. Similar results were found by Martin and Manzaranes (1994). However, in our study the amount sorbed did not increase significantly during subsequent batches. Furthermore, the color sorbed to the biomass did not affect their growth or respiratory activity nor their pulp-mill color removal ability.

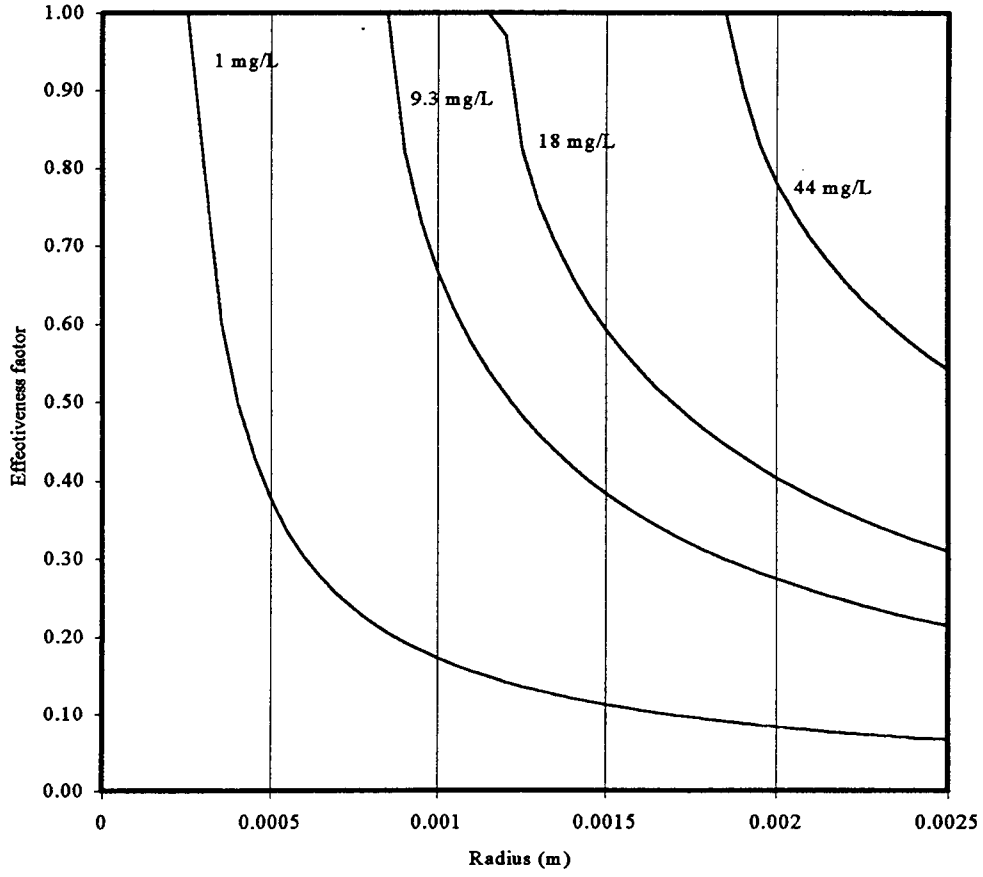

Figure 3.9. Simulated values of effectiveness factor for *S. striatispora* pellet cultures as a function of oxygen concentration in liquid phase and pellet radius.

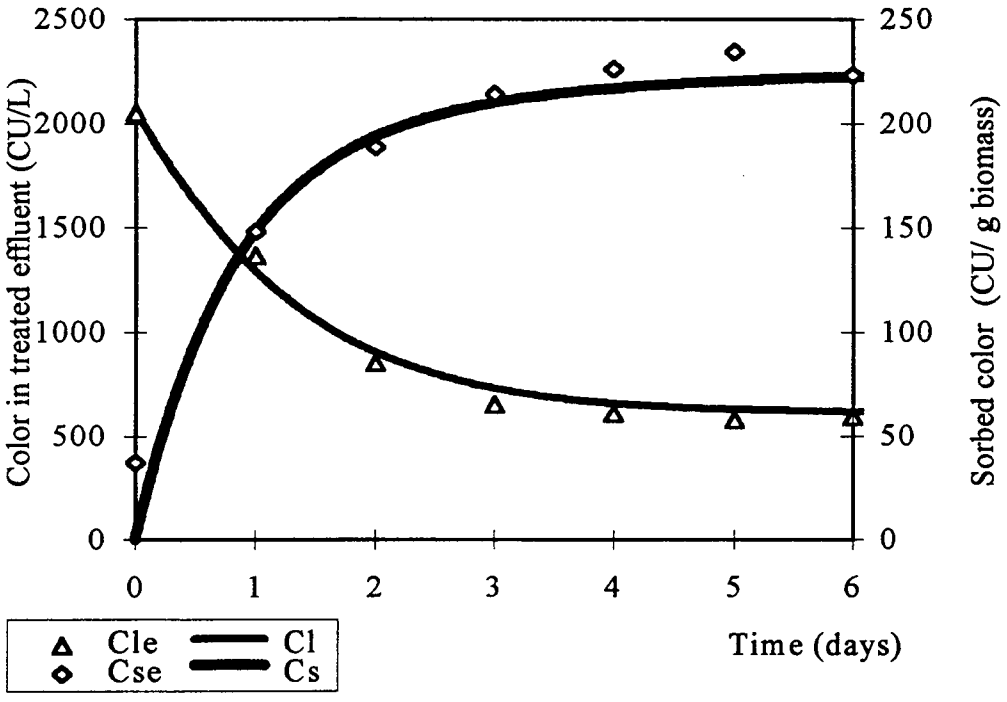

Figure 3.10. Simulated (lines) and experimental data (markers) of pulp-mill color removal and color sorption to the pellets of *S. striatispora* in batch culture.

In both successive batch and continuous regimes, a significant amount of biomass was lost. Despite using a low stirring force of 100 rpm with a quiescent zone on the top of the reactor a significant amount (around 20 %) of the biomass is lost every day in the continuous regime. Some of the pellets were swept by the oxygen bubbles to the overflow zone. Furthermore, oxygen bubbling create a small layer of foam that trapped biomass contributing to the biomass loss. Using lower oxygen flows combined with recycling would probably reduce this biomass loss. The mycelial pellets of *S. striatispora* settle rapidly when agitation ceases making pellet recycling relatively easy. Simulation of oxygen uptake rates showed that in oxygen saturated environments (without bubbling) oxygen is not limiting in the culture medium. A lower bubbling flow than the one we used for this experiment might be sufficient.

In our experiment we used relatively small pellets which improved oxygen transfer for the biomass inside the pellets. The whole biomass in the pellets was active for growth, respiration and color removal. However, if the experiments were followed for longer periods the size of the pellets would increase. Consequently, an increasing fraction of the biomass will be inactive due to oxygen limitation (Shugerl et al., 1988).

Using the pellet growth model developed by Van Suijdam et al. (1982) combined with proposed equations for color removal and sorption, we were able to satisfactorily simulate the experimental data obtained for batch cultures. This model constitutes the basis for optimizing the system and investigating the relationships between the different processes occurring during pulp-mill color removal by mycelial pellets of *S. striatispora*. To our knowledge this is the first attempt to integrate color removal and mycelial pellet growth. More experimental work is needed to test the value of this model. Furthermore, inclusion of the effect of shear force during agitation or bubbling on the biomass growth and the pellet size could be very instrumental before scaling-up the system.

Notation

$\overline{R}$	Average pellet radius (dm)
C_d	Degradable color (CU/L)
C_i	Initial concentration of pulp-mill color (CU/L)
C_l	Color concentration expressed in cobalt units (CU/ L)
C_s	Color sorbed to the biomass (CU/g)
D	Diffusivity of oxygen in pellets (dm^2 / day)
G_l	Glucose concentration (g / L)
H	Henry'sconstant (atm)
k	Maximum oxygen uptake rate (g oxygen/ pellet volume L)
k_c	Color removal rate (L/g biomass.day)
k_{cs}	Adsorption coefficient (g/L.day)
K_g	Half saturation concentration for glucose (g/L)
M_{o2}	Oxygen molecular weight (g/mole)
n	Number of pellets per L
O	Oxygen concentration within the pellet (g / L)
O_g	Oxygen concentration in bulk liquid (g / L)
O_l	Oxygen concentration in bulk liquid (g / L)
P_{o2}	Partial pressure of oxygen (atm)
r	Distance from pellet center (dm)
R	Ideal gas constant (atm L/ mole. °K)
R_p	Pellet radius (dm)
R_{o2}	Rate of oxygen uptake (g oxygen /L.day)
R_{cs}	Rate of color removed by sorption (CU/ L.day)
T	Temperature (°K)
t	Time (day)
V_{hs}	Volume of the gas phase above the liquid (L)
V_l	Culture medium volume (L)
X	Biomass concentration (g / L)
Y_{go}	Yield coefficient for oxygen from glucose (g/g)
Y_{gx}	Yield coefficient for biomass from glucose (g/g)
Y_{ox}	Yield coefficient for biomass from oxygen (g/g)
$\overline{\rho}$	Average pellet density (g dry weight / pellet volume L)
η	Effectiveness factor (dimensionless)
μ_e	Effective growth rate (1/ day)
μ_m	Maximum growth rate (1/ day)

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SUMMARY

Biological decolorization of pulp-mill effluent using filamentous fungi is a promising technology to limit the impact of this wastewater on the natural environment. *Phanerochaete chrysosporium* is most often evaluated for this purpose but other fungi may also be suitable. *Sagenomella striatispora*, a hyphomycete isolated from a log pond, significantly decolorized pulp-mill effluent. The three chapters of this study were devoted to the optimization and analysis of pulp-mill effluent decolorization as it relates to the physiology of *S. striatispora*.

In batch experiments using serum bottles (125 ml), *S. striatispora* removed 74 % of initial color in pulp-mill effluent samples amended with glucose (10 g/L) without any pH adjustment (initial pH of pulp-mill wastewater was 7.6) at 25 °C . Extraction of chromophores from biomass using NaOH (0.1 M) revealed that 12 % of initial color was sorbed to the biomass after 15 days incubation. The maximum color removal was obtained without adding nitrogen, Mn or veratryl-alcohol. Furthermore, decolorization occurred immediately without any apparent lag phase and without nitrogen starvation (24 mM NH4Cl). However, color removal did not increase beyond 75 % even with prolonged incubation (1 month) or *de novo* addition of glucose and nitrogen to the batches. These physiological features of pulp-mill effluent color removal by *S. striatispora* did not correspond to the published data on standard lignolytic fungi such as *P. chrysosporium*.

Measurement of lignin degradation enzymes (laccase, lignin peroxidase and manganese peroxidase) by *S. striatispora* confirmed the difference observed above with the standard lignolytic *Phanerochaete chrysosporium*. In contrast to *P. chrysosporium*, *S. striatispora* did not produce any known lignin degradation enzymes. Enzyme activity measurements did not show any laccase, lignin or manganese-dependent peroxidases activity. Nitrogen starvation did not trigger the production of these enzymes. Furthermore, *S. striatispora* significantly reduced the pulp-mill effluent color without a lag phase or adjustment of the effluent pH to 4.5. Analysis of molecular weight fractions in the initial

and treated effluent showed that this species selectively degraded the high molecular weight fraction (> 3 kDa) while *P. chrysosporium* was less specific and reduced all molecular fractions with similar efficiencies. Attempts to decolorize pulp- mill effluent *in vitro* using extra-cellular fluid from *S. striatispora* cultures were not successful.

Using a stirred tank reactor in batch and continuous regimes, we were able to consistently obtain 65 % color removal at two day hydraulic retention time. Before starting the reactor experiments, we analyzed the effects of pulp-mill effluent color concentration and pH on the sorption of color to the biomass. The amount of pulp-mill effluent color sorbed to the biomass increased with time, color level and pH decrease. Nevertheless, the amount sorbed per weight of biomass reached a maximum stable value after the first batch. Color removal by sorption did not account for more than 250 CU/ g biomass (20 % of initial color) after the first batch. This accumulation of color on the biomass did not appear to influence the mycelial pellets color removal potential or their growth and respiration activities. In both successive batches and continuous regimes, a significant amount of biomass was lost. There were also problems of foaming particularly when running the reactor in continuous flow. Using a low stirring force helped reduce foaming but there was still a layer of few mm due to oxygen bubbling. We proposed an integrated color removal and fungal pellets growth model to predict and analyze the interactions of the different processes. The combined model satisfactorily simulated the experimental data. Analysis of the data from the simulation revealed that glucose as well as oxygen were in excess in all experiments. The simulation also showed that an increase of the pellet diameter above 1.8 mm may create problems of oxygen limitation.

RECOMMENDATIONS FOR FUTURE RESEARCH

Continuous isolation and investigation of new microbial species that degrade complex chromophores are primary to the understanding of complex molecules biodegradation. They can also lead to applications in the fields of hazardous waste treatment. Although *Sagenomella striatispora* showed some potential for pulp-mill color removal there might be still more effective fungi in the natural environment.

Operating a fungal mono-culture reactor to treat pulp-mill effluent color will be an economically and technically challenging task. Utilization of isolated enzymes seems to be the solution for the future. More research is needed for isolating new enzymes, understanding their catalytic cycle and designing technically and economically optimized systems to use these enzymes for color removal. In the case of this research we did not detect any extracellular enzymes. More investigations are needed to confirm these results, then try to locate the sites of color degradation by *S. striatispora* hyphae.

Bio-sorption on live fungal biomass received very little attention from research investigators. Our results showed that this process accounts for a significant amount of color removal. What is the role of the sorption in color removal ? How does color sorption relate to color degradation and biomass growth ? Is bio-sorption primarily absorption or adsorption ? Those questions among others need to be addressed before designing an industrial unit for pulp-mill color removal.

Developing mathematical relationships between the different processes involved in color removal was not extensively investigated. We presented an attempt to capture the growth, substrate uptake, color degradation and color sorption and their interactions. More work is needed to validate the integrated model and improve its predictive performance if necessary. In all our work we used optimal conditions using nutrients in excess to avoid nutrient limitation. Obtaining experimental data under stress conditions and comparing them to model simulated results will help test the limitation of the model.

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APPENDICES

APPENDIX A: Pulp-mill effluent analysis[a]

Parameter	Range
BOD5 (mg/L)	10-30
COD (mg/L)	300-500
TOC (mg/L)	100-300
TSS (mg/L)	15-30
Ammonia (mg/L)	0.5-1.0
pH	7.6
Nitrate/Nitrite (mg/L)	0.1-0.5
Organic N (mg/L)	5-10
Phosphorus (mg/L)	0.5-1.0
Sulfate (mg/L)	50-100
Sulfide (mg/L)	0.1-0.2
Sulfite (mg/L)	0.2-0.5
Fe (mg/L)	1-5
Mg (mg/L)	5-10
Mn (mg/L)	0.5-1.0

[a] Anallysis obtained from POPE & TALBOT, INC. HALSEY, OREGON.

APPENDIX B: Table of variance for the four factors pH, minerals, glucose and oxygen.

Source of variation	Sum of squares	d.f.	Mean square	F-ratio	Level of significance
pH	0.00276	1	0.00276	13.58	<1%
Nitrogen	0.00472	1	0.00472	23.22	<1%
Glucose	0.00715	1	0.00715	35.18	<1%
Oxygen	0.00740	1	0.00740	36.41	<1%
Interactions§					
Glucose/Oxygen	0.01178	1	0.01178	57.96	<1%
pH/Glucose	0.00203	1	0.00203	9.99	<1%
pH/Nitrogen	0.00122	1	0.00122	6.00	5%
Residual	0.00752	37	0.00020		
Total	0.04458	47			

§ only signinficant interactions are reported