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ALGINATE ENCAPSULATION OF PHANEROCHAETE CHRYSOSPORIUM
FOR THE FUNGAL BIOCATALYSIS OF ENDOCRINE DISRUPTING
COMPOUNDS IN WASTEWATER

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ABSTRACT

Endocrine disrupting compounds (EDCs), which include certain classes of pesticides and pharmaceuticals, are known to disrupt hormone functions in humans and other animals. These contaminants often make their way into wastewater, pass through traditional wastewater treatment plants (WWTPs), and are discharged along with treated effluent into surface waters where they can threaten both human health and ecosystem well-being. Interception of these contaminants at WWTPs offers the best means of preventing their release into the environment.

One method of removing EDCs is passive biological treatment using white rot fungus (WRF). WRF, with their powerful extracellular oxidative enzymes, have been found capable of mineralizing many common EDCs; yet, the development of an engineered fungal bioreactor for EDC removal has not yet been accomplished. This study investigates the feasibility of utilizing WRF entrapped in an alginate matrix for the removal of EDCs from wastewater.

Calcium alginate beads containing different amounts of fungal inoculum (5% and 15%) and formed under different conditions (in the presence or Na⁺, or without) were applied in batch airlift bioreactors containing wastewater from the Penn State WWTP under sterile conditions. The mechanical stability of the beads and the release of enzymes into reactor solution were monitored using enzyme assays, visual inspection, bead diameter measurements, and rheological data.

Alginate beads were found to be stable in wastewater over the course of the experiment, as measured by bead diameter and visual inspection, though rheological data were inconclusive due to human error in conducting tests. While enzymes were not detected in solution, further investigation is necessary to determine whether enzymes were being produced, yet remained trapped in the alginate beads, where EDC degradation may, in fact, take place.
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Chapter 1

Background and Introduction

As the world population approaches 7 billion people, the demand for potable water continues to intensify. With this heightened demand, it becomes increasingly important to protect existing water resources from contamination due to the industrial and agricultural activity that accompanies population growth. In particular, trace organic pollutants have recently been identified as a threat to both human health and ecosystem well-being.

1.1 Emerging Pollutants

In the past few decades, the development of new analytical methods has allowed for the investigation of trace contaminants in surface waters throughout the United States and elsewhere in the world. During 1999 and 2000, the U.S. Geological Survey conducted a study of 139 streams in 30 states in order to determine the presence of organic wastewater contaminants (OWCs) in surface waters downstream of intense urbanization and agricultural activity. OWCs were detected in eighty percent of sampled streams (Kolpin et al., 2002). Of these OWCs, many were classified as endocrine disrupting compounds (EDCs) and are of particular interest in the present study.

The EPA defines EDCs as exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (USEPA, 1997). EDCs are found in a variety of common products such as pharmaceuticals and pesticides and, as a result, are also located in most wastewater streams. Often, EDCs bypass the traditional
wastewater treatment processes and are discharged with the treated effluent into surface waters, exposing aquatic ecosystems and the human population. In some cases, potable water withdrawal occurs downstream from discharge locations, resulting in a heightened risk of drinking water contamination. Concentration of EDCs in the residual sludge from wastewater treatment can also lead to the contamination of soil and landfill sites when the sludge is spread as fertilizer or buried.

Because EDCs are commonly funneled through wastewater treatment facilities before being released into the environment, a logical strategy is to intercept the contaminants at this point by modifying the treatment process to more adequately remove them. Currently, physical, chemical, and biological options exist, or are being pursued, for the removal of EDCs from wastewater. In selecting the appropriate option, cost, efficiency, and sustainability must all be considered.

1.2 Physical, Chemical, and Biological Removal of EDCs

Physical and chemical EDC removal methods include adsorption by activated carbon and membrane separation (physical), as well as chemical oxidation. These methods often exhibit high removal efficiencies; however, they tend to be expensive and demanding in terms of energy and chemical input. In this section, the current status of these techniques, as applied to EDC removal, is presented.

Rejection of EDCs by semi-permeable membranes is strongly related to the physiochemical properties of the compound of interest, as well as the properties of the membrane, and rejection usually occurs due to size exclusion, charge repulsion, and adsorption (Liu et al., 2009). Additionally, such separations often occur at very high pressures. A recent review shows that membrane separation is capable of EDC rejections ranging from 10% to over 99.9% for the
limited number of EDCs tested. However, problems associated with membrane separation technologies include high cost and the potential for membrane fouling (Auriol et al., 2006).

The potential for EDCs to adsorb onto particulates is high, due to their non-polar and hydrophobic nature. This offers the opportunity to exploit these characteristics through the use of granular or powdered activated carbon (GAC, PAC) to remove EDCs from wastewater. In fact, activated carbon has been shown to have a high capacity for removing a broad range of representative EDCs from both artificial and real wastewater (Liu et al., 2009). The downfall of activated carbon, however, is that EDCs are not destroyed, but concentrated, leading to further handling and disposal issues.

Treatment of EDCs by chlorination has been investigated and shown to have a high removal efficiency for some EDCs (Liu et al., 2009). However, estrogenic activity persists in chlorinated by-products. Other oxidation processes such as ozonation, combined UV-ozone, and some metal oxide catalyzed reactions have also been found to exhibit high removal efficiencies; yet, there is a lack of information regarding the metabolites produced through such treatment processes. Further, the production and use of chlorine compounds, ozone, and UV light has a negative impact on the cost effectiveness and overall sustainability of these processes (reviewed in Auriol et al., 2006).

Passive biological removal of organic contaminants is an alternative to the aforementioned physical and chemical techniques. Biological methods typically involve enzymatic catalysis of the pollutant, either by exposure to whole living cells which are actively producing catalytic enzymes, or by exposure to concentrated, immobilized enzymes. By providing an appropriate environment, living microorganisms (e.g., bacteria or fungi) can be employed to remove pollutants via assimilation or enzymatic bio-catalysis.

This technique has been applied since the early 20th century to reduce the biological oxygen demand (BOD) of domestic wastewater using a host of microorganisms in various
treatment processes (e.g., activated sludge treatment, trickling filter). These early biological treatment schemes are still widely applied in wastewater treatment plants and are indeed capable of removing some EDCs, along with the targeted BOD, from wastewater with varying efficiency depending on the type of treatment process and the particular EDC in question. However, the wide range of EDC removal efficiencies reported in the literature for activated sludge (1-100%) does not support reliance on such systems as the sole EDC removal technique (Liu et al., 2009). Additionally, removal pathways in wastewater treatment plants remain uncertain. Though some transformation and degradation by biological oxidation is likely, organic pollutants also leave the system through adsorption to microbial flocs and volatilization during aeration, leading to concentration in waste sludge and/or redistribution of the contaminant as an air pollutant (Auriol et al., 2006).

1.2.1 Fungi in EDC Removal

Despite the inadequacy of existing biological treatment systems in removing EDCs, biological treatments using certain types of fungi show promise. Recently, fungi have received increased attention for their ability to degrade recalcitrant organic compounds (Cabana et al., 2007). This inherent ability is linked to their ecosystem role as wood degraders.

The polymers lignin, cellulose, and hemi-cellulose are the three major constituents of woody cell walls. Fungi have adapted to degrade these components. Fungal cells form filamentous structures called hyphae, which collectively form a complex branching network referred to as mycelia. This mycelia network can be extended into decaying wood, where extracellular enzymes are secreted to attack the various components of the wood matrix. Different fungi exhibit selectivity for the major wood polymers and this leads naturally to three categories of wood degrading fungi - white rot, brown rot, and soft rot (Rayner and Boddy, 1988).
The fungi of interest for EDC removal, lignin degraders, are considered white rot fungi (WRF) and produce several types of extra-cellular, lignin modifying enzymes (LMEs) that are capable of depolymerization and mineralization of the highly recalcitrant lignin. It is the extracellular nature, low substrate specificity, and powerful oxidative capacity of these enzymes that give the WRF an advantage in EDC removal over the bacteria and protozoa typically present in activated sludge and other traditional biological wastewater treatment systems (Cabana et al., 2007). However, further research is needed to ensure that harmful metabolites are not generated in instances where complete mineralization of EDCs does not occur.

1.2.2 Phanerochaete chrysosporium

The prominent enzymes produced by WRF are lignin peroxidases (LiPs), manganese peroxidases (MnPs), versatile peroxidases, and laccases. Together these enzymes make up the ligninolytic system. The WRF Phanerochaete chrysosporium has been thoroughly studied in terms of its ligninolytic enzyme system and its ability to degrade a wide range of industrial pollutants, including some EDCs (Tien and Kirk, 1988; Cabana et al., 2007; Sing and Chen, 2008). In general, P. chrysosporium is considered a model species for the fungal bioremediation of recalcitrant compounds, leading to its selection for the current study.

Optimization of enzyme production is essential for the efficient use of P. chrysosporium in remediation applications. Of the four types of LMEs, the two most relevant to EDC removal are LiP and MnP. It is well known that LiP and MnP are produced by P. chrysosporium during secondary metabolism, induced by nutrient limitations. Typically, nitrogen starvation is employed in order to induce LME production (Tien and Kirk, 1988). In addition to the requisite nutrient limited conditions, LiP and MnP production is aided by high levels of dissolved oxygen. On the other hand, agitation is detrimental to the production of these LMEs, presumably due to
the shear forces acting on the fungus and enzymes alike. In addition, environmental parameters such as temperature, pH, and the presence of a readily available carbon source can all have an effect on the production of LMEs and the subsequent mineralization of EDCs by \textit{P. chrysosporium} in wastewater (Cabana et al., 2007).

1.2.3 Fungal Bioreactors

In order to improve the efficiency of EDC removal by \textit{P. chrysosporium}, engineered systems must be designed which allow the manipulation of the environmental parameters discussed above, in order to maximize enzyme production. There exist several modes of incorporating fungal mycelia into engineered bioreactors. In general, bioreactors can employ either “free” or “immobilized” cells. Free cells are simply suspended in the reactor medium, whereas immobilized cells can be either \textit{attached} to a surface, or \textit{entrapped} within a support matrix.

Suspended (free) growth systems are commonly utilized in activated sludge treatments where bacterial cells are exploited in order to reduce the BOD of the wastewater. Such systems require that suspended organisms exhibit good settling properties so that they can be separated from the treated wastewater. Often, settled biomass is recycled back to the reactor in order to increase the mean cell retention time and support a higher cell concentration. Without cell recycle, there exists a greater risk of cell washout at high dilution rates. Such suspended growth configurations are not suitable for fungus, as the filamentous nature of mycelia yields poor settling properties.

Immobilization is another means of reducing the likelihood of cell washout. Examples of \textit{attached}, immobilized systems commonly used in wastewater treatment include trickling filters, rotating biological contactors, and biological filters. In addition to preventing cell washout,
immobilization has been shown to enhance enzyme production in white rot fungi, and it has been shown that the choice of immobilization material can also affect the production of enzymes (reviewed in Gao et al., 2010). However, even attached fungi are exposed to shear forces generated by agitation and aeration. To protect against this, cell immobilization by entrapment may be employed.

Immobilization by entrapment often involves the incorporation of mycelia into a gel bead. The gel serves as a support matrix for the mycelia, protecting them from shear forces, while theoretically allowing the transfer of nutrients, enzymes, substrate, and metabolites throughout. Examples of gels which have been investigated for the immobilization of living cells in bioreactors include polyvinyl alcohol, polyethylene glycol, κ-carageenan, agar, and Ca-alginate (Vogelsang et al., 2000). These gels differ in terms of gelling mechanism and source, as well as structural properties. Calcium alginate gel has been selected as the immobilization matrix for this study, due to its highly renewable source material (kelp) and established protocol for immobilization.

1.3 Alginate as an Immobilization Matrix

Alginate collectively refers to a family of polysaccharides, which are derived from marine brown algae (kelp) and some bacteria. Alginate accounts for approximately 40% of the dry matter in marine brown algae, providing mechanical strength and flexibility, and acting to hold water and prevent the desiccation of tissue exposed to the air. Commercial alginates are mainly extracted from the brown algae species Laminaria hyperboria, Macrocystis pyrifera, Laminaria digitata, Ascophyllum nodsum, and Laminaria japonica, among others. The properties of a particular alginate vary according to the plant species from which it is derived.
Worldwide annual production of alginate is estimated to be around 30,000 metric tons, only 10% of the biosynthesized material. These figures suggest the continued availability of this highly renewable polysaccharide, derived from fast-growing macro-algae. In addition to being used as an immobilizing gel, alginate is often applied as a stabilizing, viscosifying, and gelling agent and has applications in a wide range of industries from textile printing to food and medical applications (Donatti and Paoletti, 2009).

1.3.1 Physical Characteristics of Alginate and Gelling Mechanism

Alginate is composed of two discrete monomer units: α-L-guluronic acid (G) and β-D-mannuronic acid (M). These carboxylic acid monomers comprise a randomly sequenced heteropolymer, with varying proportions of G and M monomers depending on the plant species from which the alginate was isolated (Klein et al., 1983). Typically, alginates are referred to as “high-G” or “low-G.” Figure 1-1 depicts a portion of a typical alginate polymer molecule.

![Chemical structure of Na⁺ alginate with guluronic acid (G) and mannuronic acid (M) monomers noted](image)

**Figure 1-1.** Chemical structure of Na⁺ alginate with guluronic acid (G) and mannuronic acid (M) monomers noted (Smidsrød and Skjåk-Bræk, 1990).

Gelling is known to occur by a process referred to as ionotropic gellation (Klein et al., 1983). The egg-box model, which attempts to explain the ionotropic gellation of alginate with
multivalent cations, was proposed by Smidsrød (1974) and Morris and Rees (1978). In the egg-box model it is proposed that diaxially linked G-monomers form a cavity into which divalent ions are tightly bound in a chelate type binding (Donatti and Paoletti, 2009). Figure 1-2 shows a hemi-spherical portion of such a cavity and its possible interaction with a divalent calcium ion.

Figure 1-2. Egg-box model for binding of divalent cations to homopolymeric G-blocks (Smidsrød and Skjåk-Bræk, 1990).

Where multiple such cavities are adjacent to one another over some length of the alginate polymer, the unfavorable binding of an initial divalent cation may lead to the more favorable binding at subsequent, adjacent binding sites. This has been referred to as the zipper effect (Donatti and Paoletti, 2009). A macro-perspective of alginate polymerization according to the egg-box model is depicted in Figure 1-3.

Figure 1-3. Egg-box model for gel formation. Buckled portions of the polymer represent diaxially linked G-monomers (Smidsrød and Skjåk-Bræk, 1990).
For purposes of immobilization, Na-alginate powder is typically dissolved to 1 or 2% in distilled water and blended with cells. The resulting solution is then extruded dropwise into a calcium-containing bath (usually CaCl$_2$). When the droplet of Na-alginate solution comes in contact with the bath solution, gellation first occurs at the surface followed by the migration of Ca$^{2+}$ into the interior of the bead and the migration of Na-alginate from the center of the bead outward. Due to the migration of Na-alginate from the center outward, gel beads can be inhomogeneous, with a higher polymer concentration at the surface. By adding a counter ion such as Na$^+$ to the hardening bath, this effect can be minimized and more homogeneous beads formed. Figure 1-4 depicts the bead formation process.

![Figure 1-4](image)

**Figure 1-4.** Schematic of bead formation and gelling mechanism (Draget et al., 2005).

As with many gels used for immobilization, alginate gels formed by ionotropic gellation are viscoelastic materials. This means that the ratio of elastic to viscous properties depends on
the time scale of deformation (Martins dos Santos et al., 1997). Ca-alginate gel is typically elastic over short time scales of deformation and exhibits a strong viscous component over longer time scales.

1.3.2 Current Applications

To date, alginate has been studied as a fungal bio-carrier with several intended applications. These include a delivery and storage mechanism for terrestrial application, dye de-colorization in aqueous bioreactors (using nutrient rich media), and the adsorption of heavy metals (Bennet et al., 1996; Loomis et al., 1997; Arica et al., 2001). Alginate has also been explored as a bio-carrier for nitrifying bacteria in domestic wastewater treatment systems (Leenan et al., 1996) and the literature contains studies on the properties of alginate gels independent of any particular application (Martinsen et al., 1989; Smidsrød and Skjåk-Bræk, 1990; Martinsen et al., 1991). Through these studies, a proven method for bead formation and mycelia immobilization at the laboratory scale has been developed. Scale up of bead production has also been investigated (Klein and Vorlap, 1983). However, there is nothing in the literature that points to the use of alginate beads as a bio-carrier for fungi in the removal of EDCs from wastewater.

In general, the literature to date provides some useful data and conclusions that can serve as a foundation for determining the appropriateness of alginate for the intended application as a bio-carrier for *P. chrysosporium* in wastewater. The following categories provide a framework within which the critical factors for success can be fitted, the state of the art analyzed, and the focus of further experimentation determined:

- Cell survival and growth
- Alginate bead durability
- Mass transfer characteristics
1.3.3 Cell Survival and Growth

Whereas dead cells are sufficient for the removal of heavy metals via adsorption, survival of the mycelial inoculum through the immobilization procedure is imperative for enzyme production and EDC removal. Alginate and the associated immobilization procedure have been shown to meet this requirement in numerous studies (Bennet et al., 1996; Loomis et al., 1997; Arica et al., 2001). Subsequent mycelial colonization, by contrast, may or may not be necessary for enzyme production and EDC removal, as LME production is part of a secondary metabolic pathway for *P. chrysosporium*. In the nutrient poor conditions which induce secondary metabolism, the fungus would be unlikely to exhibit substantial growth. Mycelial growth, if unnecessary for enzyme production and subsequent EDC removal, may be undesirable due to the potential for structural alteration of the alginate bead. Ramsay et al. (2005) showed that growth of the WRF *T. versicolor* in Ca-alginate beads caused a reduction in hardness of the bead, as measured by the force necessary to compress the bead by 35%. Additionally, beads with no subsequent colonization could be compressed indefinitely, while colonization led to breakage of the bead at about 40% compression. In the same experiment, beads for which extended colonization by *T. versicolor* had been allowed did show a much faster decoloration rate (5x), but this was proposed, by the authors, to be due to the increased biomass alone and not the change in bead structure due to colonization. The authors suggest that a high initial biomass concentration (during immobilization) and the prevention of colonization may provide a balance between reaction rate and bead strength.
1.3.4 Alginate Bead Durability

In addition to the above considerations, the structural integrity of the alginate matrix depends on the interaction between the alginate, fluid, and fluid constituents in which it is immersed. This interaction could be chemical, physical, or biological.

Prominent chemical factors include the effect of gelling (Ca$^{2+}$) and anti-gelling cations (Na$^+$), and the presence of chelating compounds such as citrate and phosphate, which can sequester the gelling cation and increase solubility (Smidsrød and Skjåk-Bræk, 1990). This increase in solubility reduces the structural integrity of the hydrogel and causes it to become more susceptible to the physical stresses noted below, or to dissolve completely. The presence of significant amounts of anti-gelling compounds will require the addition of Ca$^{2+}$ in order to counteract these negative effects. Leenan et al. (1996) found that Ca-Ba-alginate beads dissolved completely in wastewater, with a lifetime of 3-40 days, depending on the guluronic acid content of the alginate (with high-g alginate lasting longer). This rapid dissolution was determined to be due to phosphate and citrate levels in the wastewater. Two factors led to this conclusion. The Na$^+$/Ca$^{2+}$ ratio was found to vary between 5/1 and 9/1 in the geographical area of study, which is well below the 30/1 ratio documented as the maximum sodium to calcium ratio tolerable for alginate stability by Martinsen et al. (1989). Also, biodegradation was ruled out by repeating the experiments in the presence of the biocide, sodium azide (NaN$_3$), and reaching the same results.

In the current study, the effects of chelating agents are not expected to be substantial. This presumption is based on preliminary experimentation by the author, which has found that the effects of citrate and phosphate are suppressed in the low pH environment required for growth and enzyme production by *P. chrysosporium*.

Physical processes that will affect durability and compromise the effectiveness of the treatment system include abrasion and breakage due to compression. The risk of compression
breakage can be greatly reduced or eliminated by employing a fluidized bed reactor. This type of reactor would also be superior to a stirred tank reactor in terms of abrasion, which has been reported to affect κ-carrageenan beads (Hunik and Tramper, 1993).

Finally, biological effects on bead durability include the biodegradation of alginate under microbial attack. As mentioned, Leenan et al. (1996) ruled out biodegradation in their study, which was conducted in wastewater at the secondary treatment stage where the microbial population is quite high. However, they advocate that organisms with alginate degrading enzymes do exist (mainly in marine environments) and that there is at least some potential for biodegradation.

1.3.5 Mass Transfer Characteristics

Mass transfer through the alginate matrix is important, as insufficient mass transfer will likely inhibit the removal of EDCs. As such, a diffusion coefficient comparable to that in water is desirable, ensuring that mass transfer through the matrix will not act to severely limit the rate of EDC removal. In many cases mass transfer is determined by pore size and structure. For alginate gels, it has also been shown that high-g alginate not only forms the most stable gels, but exhibits the largest pore sizes and highest rates of diffusion (Martinsen et al., 1989). For some important constituents, mass transfer appears to be very good. Leenan et al. (1996) point out that the mass transfer rates for \( \text{O}_2 \) and glucose in high-g alginate (2% w/v) have been reported to be as high as 75-80% and 98% of that in water, respectively.

In older literature, Tanaka et al. (1983) conducted a study of molecular diffusion into and out of Ca-alginate beads. Their results showed that for substances with molecular weight (MW) of less than \( 2.0 \times 10^4 \) (where MW is used as a proxy for size) diffusion coefficients (both into and out of the bead) were comparable to those for water, and that the concentration of the \( \text{CaCl}_2 \) bath
and the concentration of alginate in the bead forming solution had little effect on this result. They also tested substances with MW greater than $6.5 \times 10^3$ and reached very different conclusions. For the larger molecules, there was no diffusion into the bead. Diffusion out of the bead was achieved but it was found that the alginate concentration in the bead forming solution had a major effect on the rate (a higher alginate concentration led to a lower diffusion rate). The reasoning for diffusion out of and not into the bead is that, for the diffusion-out experiment, the bead was formed with the substance incorporated in the interstices of the matrix, thereby changing the structural properties of the bead. It is important to note that the average MW of LiPs is $3.9-4.3 \times 10^4$. This range was not covered by Tanaka et al., so these LMEs could lie on either side of the threshold.

Mass transfer characteristics are also the most important factor controlling the formation of defined environmental conditions within the beads (created for example, by “spiking” them with different nutrients). However, based on the above mass transfer coefficients for small molecules, it is not likely that the major environmental parameter of concern, pH, can be controlled within the bead, as protons should migrate freely through the alginate matrix.

**1.4 Scope of Study**

Although reactor systems consisting of alginate encapsulated microorganisms in nutrient rich media have been studied on multiple occasions, only one case of alginate encapsulated microorganisms in wastewater has been reported (Leenan et al., 1996). As a result, the stability of alginate gels in wastewater remains uncertain. Additionally, the ability of LMEs to diffuse through the alginate matrix has not been established. This study uses treated secondary effluent from the Penn State Wastewater Treatment Plant in bench-scale, simulated airlift bioreactors to
investigate the stability of fungi-inoculated alginate beads against abrasion, and the ability of LMEs to migrate from the gel beads into the reactor solution.
Chapter 2

Materials and Methods

Treatments for this study were selected following a full $2^2$ factorial experimental design. The independent variables of interest were the concentration of mycelia in the alginate beads and the presence of Na$^+$ during bead formation. The dependent variables of interest were bead diameter, enzyme production, and bead rheological properties. Two sets of controls were run in addition to the four factorial treatments – a “blank” control without mycelia, and a “dead” control, for which mycelia was killed via autoclave after encapsulation. In both controls, the beads were formed in the presence of Na$^+$. A summary of the conditions for each treatment is presented in Table 2-1, below.

Table 2-1. Factorial Design of the Experiment.

<table>
<thead>
<tr>
<th>Color I.D.</th>
<th>Na$^+$</th>
<th>Mycelia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>Red</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>White</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>Blue</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Yellow</td>
<td>+</td>
<td>Absent</td>
</tr>
<tr>
<td>Pink</td>
<td>+</td>
<td>15, Dead</td>
</tr>
</tbody>
</table>

An initial experimental run was hindered by unexpected pH fluctuations due to the combined effect of residual alkalinity in the wastewater and CO$_2$ sparging due to aeration. The experiment was terminated and a second attempt made with the pH adjusted as described below to ensure that all alkalinity was removed.
2.1 Water Source and Quality

Wastewater used in this study was obtained from the Wastewater Treatment Plant (WWTP), owned and operated by The Pennsylvania State University (University Park, PA). Treated effluent (pre-chlorination) was selected for use in the study and, as such, the wastewater was collected from the overflow weir of the secondary clarifier (Appendix A). Wastewater was collected into each of 4 2-L media bottles (approx. 1-L per bottle) and used within 8 hours of the time of collection.

Several adjustments and amendments were made to the wastewater prior to use. A surfactant, Tween 80 (Sigma, St. Louis, Missouri), was added to a final concentration of 0.05%, in order to help stabilize any enzymes in solution. Glucose (10 g/L, EMD, USA) was added as a carbon source for *P. chrysosporium*. The pH of the wastewater was adjusted down to 3.5 using 1M hydrochloric acid (EMD, USA) and back up to 4.3 using 1M sodium hydroxide (J.T. Baker, Phillipsburg, New Jersey) over a period of 3 hrs, while subjected to vigorous mixing to assure that all alkalinity was removed from the wastewater. Finally, the system was lightly buffered at pH 4.3 using 50 mM trans-aconitic acid (TAA) (TCI, Tokyo, Japan). After all the water quality adjustments were complete, the wastewater was sterilized via autoclave (1hr, 120 °C, 20 psig) to avoid interferences from other organisms (e.g., bacteria) at this stage in the experiment.

2.2 Fungus, Alginate, and Encapsulation

2.2.1 Alginate Preparation

High-G (65-75%), high viscosity, sodium alginate (LF200FTS) was obtained as a 500 g sample from FMC Biopolymer (Philadelphia, PA). Sodium alginate solution 1% w/v was
prepared by dissolving 10 g sodium alginate to a total volume of 1-L in each of two volumetric flasks under stirred and heated (60 °C) conditions.

2.2.2 Fungal Encapsulation

_P. chrysosporium_ (BKM-F-1767) was obtained from Forest Products Laboratory (Madison, Wisconsin). _P. chrysosporium_ was maintained via subculture on YMPG media agar slants, where media was prepared according to Tien and Kirk (1988). See Appendix D for the full media recipe.

A spore solution was prepared by scraping the fungal mat off of the agar within several slant tubes using a sterile 10–mL glass serological pipette tip, suspending it in sterile distilled deionized (DDI) water, and then filtering the solution through sterile glass wool, allowing only the spores to pass into an Erlenmeyer flask (500-mL). The spore-containing filtrate was then diluted to an absorbance of 0.5 at 650 nm, as measured by a UV-Spectrophotometer (Shimadzu, UV-1601). The resulting spore solution was used (10-mL, each) to inoculate 2-L Erlenmeyer flasks containing 90-mL of low nitrogen (LN) media, as adapted from Tien and Kirk (1988). See Appendix D for the full media recipe. Flasks were incubated under stationary conditions at a temperature of 29 °C, for 5 days.

Following the incubation period, residual LN media was decanted from the fungal mat under sterile conditions in a laminar flow hood (LABCONCO, Purifier II Biosafety Cabinet). The mat was then blended into the 1% w/v sodium alginate solution using a handheld blender (OSTER, Model 2605) which had been dipped in ethanol, flame sterilized, and allowed to cool prior to use. Mycelia/alginate solutions were prepared at varying concentrations of mycelia – 5% w/v and 15% w/v (wet weight mycelia).
Also, in the laminar flow hood under sterile conditions, the mycelia/alginate solutions were drawn into sterile 20-mL syringes equipped with 18G, sterile needles. An infusion pump (Harvard Apparatus, PhD 2000 Infusion), situated in the laminar flow hood, was used to extrude the alginate/mycelia solution from the syringes at a constant rate of 6 mL/min. Droplets formed at the needle tip and were allowed to fall 20 cm to the surface of a sterile, stirred bath solution containing either 0.1M CaCl$_2$, or 0.1M CaCl$_2$ and 0.2M NaCl. The resulting alginate beads (approx. 3 mm dia.) were allowed to harden for 1 hour in the stirred bath solution before being removed and rinsed with 200-mL of sterile, DDI water. Blank beads were prepared in a similar manner. A portion of the beads containing 15% mycelia were placed in LN media after hardening and rinsing, and were autoclaved for a 15 minute cycle (120 °C, 20 psig). All beads (including dead mycelia and blank controls) were stored in sterile LN media for 48 hrs until bioreactor setup.

### 2.3 Batch Bioreactor Setup

The bioreactors were assembled in sterile, 50-mL disposable glass centrifuge tubes (Kimble, USA) by adding alginate beads to sterile, Tween 80 amended, and pH adjusted (pH 4.3) wastewater at a ratio of 21% v/v (total volume of 38-mL). Portioning of alginate spheres was accomplished by volume displacement in a sterile graduated cylinder in the laminar flow hood as follows.

- Beads were filtered from the LN media storage solution under vacuum and rinsed with 200-mL of sterile DDI water. After rinsing, the vacuum was left on for approximately 15 seconds in order to remove excess water.
- Sterile, amended wastewater (30-mL) was added to a sterile 50-mL graduated cylinder in the laminar flow hood.
• Alginate beads were then added to the same graduated cylinder until the liquid level reached 38-mL (21% w/v).

• The entire contents of the graduated cylinder were then transferred to a sterile 50-mL glass centrifuge tube.

Once the wastewater and alginate beads were combined in each reactor, they were sealed with a sterile air delivery apparatus consisting of a black one-hole rubber stopper (No. 2) with pass-through aeration tubing terminating at a 20G needle (see Figure 2-1 and Appendix B for reactor photographs). The opposite terminal end of the aeration tubing was also sealed to maintain sterile conditions within the reactor. Once all reactors were assembled, they were removed from the laminar flow hood and transported to the lab bench, where the air delivery apparatus was connected to the aeration manifold through sterile, syringe filters (0.2 µm, VWR, USA). The filters separated the non-sterile manifold from the sterile air delivery apparatus and acted to filter any contamination from the lab air. Sterile 20G needles were then pushed through the rubber stoppers to vent the system.

Figure 2-1. Photographs of the bioreactor configuration.
Lab air (0.4 scfh, per reactor) was passed through a humidification apparatus constructed from 2 1-L media bottles in series in order to prevent loss of reactor solution by evaporation (see Appendix B for humidification apparatus photograph). On day 11, airflow was reduced to 0.2 scfh per reactor. On day six, sterile veratryl alcohol (TCI, Tokyo, Japan) was added to each of the reactors to a final concentration of 0.4 mM.

### 2.4 Sampling and Analytical Methods

In addition to time 0 measurements, reactors from each treatment were sacrificed in triplicate at each of 6 time points (day 1, 3, 6, 11, 17, and 22). Air was shut off to each of the sacrificed reactors and the total air flow was adjusted to maintain constant flow to the remaining reactors. 13-mL of reactor solution was immediately removed and frozen at -20 °C in 15-mL slant tubes until enzyme tests could be completed. Enzyme assays were conducted on thawed samples as described by Tien and Kirk (1988), using the UV-Spectrophotometer specified in section 2.2. pH was measured directly in each reactor using a bench-top electrode connected to a pH meter (SympHony, SP70P, VWR). Beads were then filtered from the remaining reactor solution, rinsed three times with DDI water, and stored in DDI water at 4°C until further testing. A digital photograph of one representative bead from each treatment was recorded (Canon 40D, EF 100-mm Macro Lens, Canon 250D close-up lens), and the diameters of ten beads from each treatment were measured using digital calipers (Digimatic, Mitutoyo). Rheological properties of the beads were determined as discussed in section 2.5.
2.5 Rheological Tests

Force-compression data were obtained using a rheometer (ARES Rheometer, Colby labs, PSU MatSE). Rheometer tests were conducted by an undergraduate Civil Engineering student, Andrew Strubilla. For rheometer testing, three beads from each treatment were examined. Each bead was placed on the lower platen of the rheometer (parallel-plate geometry) and the upper platen was then lowered to contact the bead. It should be noted that there was some difficulty in determining the point where the platen had just contacted the bead due to the adhesion of water from the still wet bead to the upper platen. While an effort was made to ensure consistency in choosing a starting point for each compression, results indicate the possibility of a systematic error, as will be discussed. The diameter of each bead was recorded as the gap between plates at the start of compression, and beads were compressed from this relaxed state to a gap of 1.0 mm (0.03 mm·s⁻¹ step rate). When compression was ceased, the platens were held in this fixed position as the material began to relax up to an elapsed time of 140 seconds.

Rheometer data were obtained as a time series of normal force measurements and saved in text format. Raw data were manipulated in order to determine the Young’s modulus for each of the three beads tested from each treatment at every time point using the method described below.

By multiplying the step rate of compression by the time at which each force measurement was recorded, data were converted from force/time to force/gap format. Gap values were further converted to Hencky strain \( \varepsilon_H \) as follows:

\[
\varepsilon_H = \ln \left( \frac{h_0 + \Delta h}{h_0} \right)
\]

(1)
Where $h_0$ is the initial diameter of the gel bead and $\Delta h$ is the change in diameter of the gel bead at the corresponding time.

Force values were also converted to stress ($\sigma$) values, where stress is defined as:

$$\sigma = \frac{F}{A}$$  \hspace{1cm} (2)

Here, $F$ is the normal force at a particular measurement point and $A$ is the bearing area between the upper platen and the gel bead at that same measurement point. While the force was directly recorded by the rheometer, the bearing area changes continually over time and must be calculated. The following expression was used to relate the change in radius of the bearing area to the degree of compression (Martins dos Santos et al., 1997):

$$r^2 = r_0^2 - (r_0 - c)^2$$  \hspace{1cm} (3)

Where $r_0$ is the radius of the bead prior to compression and $c$ is the change in radius at the point when the force measurement was taken.

After determining the stress as a function of strain, as described above, the slope of a linear fit to the data was determined and reported as the Young’s modulus for a particular bead. Manipulation of rheological data was performed using Matlab. Relevant code can be found along with sample data in Appendix C.
Chapter 3

Results and Discussion

3.1 Wastewater Characterization

Wastewater quality analysis, as presented in section 2.1, resulted in wastewater characterization as summarized in Table 3.1.

Table 3-1. Characterization of wastewater taken from the effluent of the secondary clarifier at the Penn State Wastewater Treatment Plant. All parameters were tested in triplicate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.51 ± 0.07</td>
</tr>
<tr>
<td>Alkalinity (mg/L as CoCO$_3$)</td>
<td>246 ± 10.0</td>
</tr>
<tr>
<td>PO$_4^{3-}$ (mg/L as P)</td>
<td>0.48 ± 0.009</td>
</tr>
<tr>
<td>Na$^+$ (µg/L)</td>
<td>62.9 ± 3.37</td>
</tr>
<tr>
<td>Ca$^{2+}$ (µg/L)</td>
<td>165.5 ± 38.4</td>
</tr>
</tbody>
</table>

The metals data in Table 3.1 yields a molar Na$^+$/Ca$^{2+}$ ratio of approximately 4.5/1, which falls well within the acceptable range for alginate gel stability, suggesting that the alginate beads will not be destabilized by Na$^+$ ions. Phosphate levels are also low and should not interfere with gel stability. Initial wastewater pH is well outside the acceptable range for enzyme stability and production by *P. chrysosporium* (pH 4.0-4.5), necessitating a pH adjustment down to 4.3 with care taken to ensure that the high level of alkalinity is entirely eliminated, as described in section 2.1.
3.1 Enzymes

No enzymes were detected over the course of the experiment. This result was unexpected, as the conditions here were nearly identical to those in previous experiments within our research group where *P. chrysosporium* did produce enzymes. There are two obvious explanations for the lack of enzymes in solution: 1) the fungus did not produce enzymes; or 2) produced enzymes were not able to escape the alginate matrix.

3.2 Sterility

Despite efforts to maintain sterile conditions throughout the experiment, visual signs of potential contamination began to appear on day three, when the solution in some reactors started to take on a cloudy, whitish appearance. While it has not been proven that this cloudy appearance is a direct result of contamination, experiments using non-sterile wastewater conducted within the research group have developed very similar characteristics, usually accompanied by fluctuations in pH. In previous cases, we have assumed that rapid growth of wastewater microorganisms, fueled by glucose amendments, has been the cause of the cloudy appearance, and that their production of CO$_2$ and/or fatty acids has been the cause of pH fluctuations. However, with such a complex community of microorganisms present in wastewater, it is difficult to rationalize these effects with a high degree of certainty without more detailed analysis.

From day three until day six, additional reactors became cloudy and previously affected reactors became increasingly opaque. By day six, it was apparent that the green and blue treatments (both with 15% mycelia) were exhibiting the highest level of apparent contamination. Interestingly, this is also reflected in the pH data presented in section 3.2. Examples of clear, cloudy, and opaque reactor solutions are presented in Figure 3-1.
Figure 3-1. Photographs of reactor solutions exhibiting varying degrees of cloudiness on day 11.

From day six until day eleven, the reactors were closely monitored for further signs of contamination. On day nine, dark colored colonies of microorganisms were found to be present inside the centrifuge tubes, above the liquid level and also on some beads. In addition, some reactors were found to have reddish contamination within the reactor medium and on some beads. Because the spores and mycelia of *P. chrysosporium* are white, the development of dark and colored areas within the reactors was taken to be a sure sign of contamination. Two examples of contaminated beads are shown in Figure 3-2.
Figure 3-2. Photographs of contaminated alginate beads taken from airlift bioreactors. The black bead on the left was taken from the blue treatment (15% mycelia, + Na) after 11 days, while the red bead on the right was taken from the red treatment (5% mycelia, - Na) after 11 days.

No visible signs of contamination were present in the controls until day fifteen, when dark colonies were discovered within two of the dead mycelia controls. No visible contamination was detected in the blank control. Interestingly, the reactor solution in the blank control remained clear throughout the experiment, and the reactor solution in the dead mycelia control became cloudy around day seventeen.

3.3 pH

During the experiment, pH was observed to decrease in each of the four treatments from the initial (adjusted) value of pH 4.3 to stabilize between pH 3.4 and 3.6 (Figure 3-1). Though the final data point for the red treatment appears to be outside this range, it should be noted that the red treatment was sacrificed only in duplicate (due to the breakage of the third reactor) at this time point, with one reactor exhibiting an unusually high pH and the other behaving as expected. The downward trend in pH began around day three, with stabilization occurring around day 11. Since the solution was buffered with TAA (an organic acid), it is possible that either the buffering
capacity was exceeded by day three due to acid production by fungus or foreign microorganisms, or that the buffer itself was consumed by fungus or foreign microorganisms. In contrast to the four treatments, the pH in both controls remained near the initial (adjusted) value, showing only a slight increase. The presence of contamination exhibiting color did not appear to have an effect on the pH.

Figure 3-3. pH variation in batch airlift reactors containing alginate beads in sterile wastewater. Data points are triplicate averages; error bars represent one standard deviation. Legend reflects the experimental factors presented in Table 2-1.

Changes in pH appear strongly correlated with the cloudiness of the reactor solution. While the cloudy solution may, in fact, have been due to the growth of wastewater microorganisms, these results cannot be compared directly to those from previous non-sterile
experiments with wastewater. Wastewater in this study had been autoclaved, so one would expect a different community of microorganisms to be present if the reactors had indeed been contaminated with airborne microbes or microbes from laboratory surfaces. It is possible that the wastewater was not completely sterile after autoclaving, yet if that were true it would be likely that all reactors would become cloudy either simultaneously, or in a random fashion. This was not the case. Blue and green treatments (with 15% mycelia) became cloudier and exhibited a larger drop in pH earlier than red and white treatments (with 5% mycelia), while the blank controls never became cloudy and dead mycelia controls became cloudy only in final week of the experiment.

To explain these circumstances, it is proposed that the fungi themselves may have been responsible for the change in pH and the cloudy appearance of the reactor solution, either by spore production or spore germination in solution. This hypothesis is supported by the fact that those treatments with a higher initial biomass concentration became cloudy first. It is also supported by the behavior of the controls. The major difference between the controls and the treatments was the presence of living fungus. Where living fungus was not present (the controls) there was no measurable change in pH and apparent cloudiness. The slight cloudiness in the dead mycelia control at the end of the experiment could be due to the survival of spores through the autoclaving process and their delayed germination and growth.

3.4 Mechanical Stability of Alginate

3.4.1 Abrasion

Overall, photographs of the beads and measurements of bead diameters show good mechanical stability for alginate beads in wastewater. Photographs of the bead surfaces showed
no noticeable abrasion occurring throughout the three week experiment. Photographs (Figure 3-4) also appear to show increased opacity of the beads with time, indicating internal fungal growth.

Figure 3-4. Photographs of abrasion effects on 5% mycelia, -Na treatment, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 3 mm in diameter.

All treatments showed a similar resistance to abrasion as indicated by photographs (see Appendix E for complete set of photographs). To put this into context, Figure 3-5 shows an analogous presentation of agar and κ-carageenan beads after exposure to similar physical stresses.
Figure 3-5. Comparison of abrasion in 1.5% carrageenan (top) and 2.6% agar (bottom) gel beads at day 0, 10, and 17 in an airlift bioreactor. Beads are approximately 3 mm in diameter (Martins dos Santos et al., 1997).

From visual comparison of the photographs in Figures 3-4 and 3-5, it is apparent that the alginate beads in the present study held up much better than the carrageenan beads and slightly better than the agar beads tested by Martins dos Santos et al. (1997).

3.4.2 Rheological Properties

Young’s moduli were calculated for three beads from each time point of each treatment (Figure 3-6). It should be noted that Young’s moduli calculated at 5% strain are most representative of the material, and those calculated at higher strains can be more representative of the compression itself. In the present study, however, scatter in the rheometer data at such low strains yielded very low $R^2$ values for the linear fit to stress strain data and, consequently, highly variable and unreliable Young’s moduli. Due to this constraint, moduli were calculated around
25% strain (as the slope of stress-strain regression between strain values of 0.2 and 0.3). It is assumed that moduli calculated at this strain are still useful in determining trends in alginate stiffness.

Initial results appeared to show an upward trend in Young’s moduli for all treatments over time. Such a trend would be quite interesting, as it would indicate that the beads were hardening with time. This is unlikely, however, as Ramsay et al. (2005) have shown that fungal growth inside alginate beads tends to reduce their strength over time. These unexpected results prompted a review of the data in search of errors.

Figure 3-6. Young's moduli for alginate beads after undergoing physical stresses in batch airlift bioreactors containing sterile wastewater reactor solution. Data points are triplicate averages. Legend reflects the experimental factors presented in Table 2-1.
As mentioned, the calculation of Young’s modulus performed in this study depended on the transformation of time values to strain values and the transformation of force values to stress values. Both of these conversions relied heavily on the initial recording of the bead diameter based on the starting point for compression (i.e. initial gap). This starting point was difficult to choose, as discussed, and a closer examination of the bead diameters obtained from the rheometer (Figure 3-7) reveals the likelihood of a systematic error in the choice of starting point over the course of the rheometer tests.

**Figure 3-7.** Diameters of alginate beads from batch airlift bioreactors. Measurements were obtained from gap readings during rheometer testing. Data points are triplicate averages. Legend reflects the experimental factors presented in Table 2-1.
Figure 3-7 shows that bead diameters, as measured by the initial rheometer gap, decreased steadily as measurements progressed. Comparing these diameters to a separate set of bead diameters measured using digital calipers, it is clear that the decreasing trend in rheometer determined diameters is due to error, as a similar trend was not observed in caliper measurements (Figure 3-8). This suggests that the upper platen was either out of contact with the beads at the start of tests performed early on, or that beads were under slight compression at the start of later tests, or both.

![Figure 3-8](image)

**Figure 3-8.** Diameters of alginate beads from batch airlift bioreactors. Measurements were obtained using digital calipers. Data points are triplicate averages; errors bars represent one standard deviation. Legend reflects the experimental factors presented in Table 2-1.
If the beads were to start under compression, higher force measurements at a particular (false) strain would lead to larger Young’s moduli. Similarly, if there was free travel at the start of the test, before the upper platen contacted the bead, stress values would be artificially low at any particular strain (i.e. calculated strains would be artificially large), leading to smaller Young’s moduli.

In an attempt to draw a correlation between the two data sets (rheometer bead diameters and Young’s moduli), data were plotted and regression lines and the slopes of those lines were added to the plots (Figures 3-6 & 3-7). The regression equations were color coded and ranked in order from the largest magnitude slope to the smallest magnitude slope. It is apparent from Figures 3-6 and 3-7 that there is a correlation between the magnitude of the decrease in bead diameter over time and the increase in Young’s modulus over time. Those treatments with the largest rate of decrease in measured bead diameter over time showed the largest rate of increase in Young’s moduli over time. Due to this error, the rheometer data is inconclusive, although a large decrease in Young’s modulus may be viewed as unlikely.
Chapter 4

Conclusions and Future Work

4.1 Conclusions

- The levels of phosphate present in treated effluent from the Penn State WWTP were not sufficiently high to cause deterioration of alginate beads under the conditions and duration of this experiment.
- The sodium to calcium ratio present in treated effluent from the Penn State WWTP was indeed low enough to prohibit sodium destabilization in alginate beads under the conditions and duration of this experiment.
- Abrasion was not a concern for alginate spheres inoculated with fungal mycelium and agitated by aeration under the conditions and duration of this experiment.
- Fungal growth will likely occur inside alginate spheres under the conditions present in this experiment.
- Enzymes do not appear to be present in solution under the conditions maintained in this experiment. If enzymes are produced under these conditions, they appear to be trapped within the alginate bead, requiring EDC transfer into the bead in order for enzymatic catalysis to occur.
4.2 Future Work

4.2.1 Critique of Experimental Set-up

Several problems were encountered during the course of the experiment, demanding an overall critique of the experimental setup. The following issues should be noted:

- The number of reactors (84) and the tediousness associated with making one liter of beads using 20-ml syringes and distributing those beads to each reactor leads to a very large time investment on the front end (> 40 hrs) and limits adaptability in the face of unexpected challenges.
- The nature of the set-up procedure, with beads being transferred multiple times between solutions and into reactors, increases the likelihood of contamination dramatically.
- Aeration caused large volumes of reactor solution to be lost throughout the experiment, which leads to a distortion of the measured concentration of enzymes in solution, if present. Water loss occurs despite the use of humidification apparatus.

Together, these problems may be sufficient to force consideration of an alternative experimental set-up if experimentation with alginate encapsulated fungus is to be continued.

4.2.2 Suggestions for Improvements to the Experimental Set-up

If further experimentation with alginate beads is to be conducted, a more efficient means of making beads must be used. As an alternative to using the syringe pump, beads could be made by allowing alginate solution to drip, under gravity, from a machined manifold with many ports. Such an apparatus exists, but is quite expensive ($7000, Nisco, Switzerland). The simple design, however, would allow for custom fabrication using resources available to the Brennan lab.
In terms of sterility, consultation with research group members who are more experienced with aseptic technique may reveal possible avenues for contamination and lead to a better overall plan for preparation of batch bioreactors. Finally, a tall column-like humidification apparatus may be developed to increase bubble residence time in the water within the humidifier and, hence, increase the transfer of water to lab air.

As an alternative to using many batch bioreactors, a larger bench scale experimental set-up could be used. This would decrease the up-front set-up time and allow for greater adaptability to unexpected experimental challenges. Currently in our lab a graduate student is developing an up-flow column reactor to test alternative substrates for fungal growth in a packed bed configuration. The design includes aeration from the bottom of the column, and could possibly be converted to a continuous flow, airlift bioreactor by replacing the packed bed with alginate beads suspended via aeration. Of course, some further justification of the effectiveness of alginate encapsulated fungi in EDC removal would be necessary before investing time in scale-up efforts.

4.2.3 Avenues for Further Investigation

While the analysis of rheometer data was inconclusive for the reasons outlined above, it may be useful to re-run the stored alginate bead samples (4 °C, DDI water) on the rheometer using greater care to ensure consistency throughout testing. In re-testing the beads it may be advantageous to choose the starting point to be the point where some minimum force is registered on the rheometer, rather than trying to choose the starting point as some point just before any force is registered. Of course, the force threshold for this technique would have to be very small so as not to disturb the material and distort the Young’s moduli calculated from the data.
While the effects of abrasion were shown to be negligible under the conditions and duration of the experiment, a much longer period of stability is necessary for application of alginate encapsulated fungi at full-scale (stability on the order of months). In order to establish the maximum period of stability for alginate beads under the airlift bioreactor conditions necessary for EDC removal, a larger-scale column-type airlift reactor (as described in 4.2.2) could be employed with the sole focus of determining the length of this period of stability.

Finally, more data must be collected regarding the effectiveness of alginate encapsulated fungi in either enzyme production or EDC removal before any scale-up can be considered. If in fact enzymes are being produced by the encapsulated fungi and are simply trapped in the bead, it remains possible that EDCs are migrating into the beads where they will be destroyed by the enzymes. It would be ideal if the beads could be dissolved and the resulting solution tested for enzyme activity; however, as mentioned, the effectiveness of typical dissolution methods (e.g. dissolution by phosphate buffer) are not effective at the pH required to maintain enzyme stability (pH 4.3). Therefore, it may be necessary to test for EDCs directly in future experiments, comparing treatments with entrapped living fungus to controls with dead fungus and no fungus in order to quantify the adsorption of EDCs onto both the alginate and the mycelia itself.

Currently, quantification of EDCs at the concentrations typically found in wastewater requires the use of specialized analytical techniques (e.g. Liquid Chromatography – Mass Spectrometry, LC-MS/MS). At these low concentrations (ppb - ppt) samples must be sent out from the lab for quantification and the associated cost is quite high ($58/sample at the Huck Institutes of the Life Sciences, Penn State). In light of this constraint, reactors could be spiked with EDCs in order to increase EDC concentrations to those measurable with standard analytical techniques (e.g. High Performance Liquid Chromatography), which can be performed in-house at low cost.
References


Appendix A

Wastewater Collection Site

Figure A-1. Schematic of entire Penn State Wastewater Treatment Plant
Figure A-2. Schematic of wastewater collection location
Appendix B

Photographs of the Experimental Set-up

Figure B-1. Photograph of bead extrusion apparatus in the laminar flow hood.

Figure B-2. Photograph of a single bank of 24 reactors.
Figure B-3. Photograph of a single port of the reactor manifold.

Figure B-4. Photograph of the humidification apparatus.
Appendix C

Data Handling

C.1 Sample Rheometer Output

<table>
<thead>
<tr>
<th>time</th>
<th>G(t)</th>
<th>Normal Force</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>Pa</td>
<td>g</td>
</tr>
<tr>
<td>0</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>0.03</td>
<td>-12.194</td>
<td>0.09433</td>
</tr>
<tr>
<td>0.08</td>
<td>11.3481</td>
<td>-0.2155</td>
</tr>
<tr>
<td>0.13</td>
<td>-5.2441</td>
<td>-0.0182</td>
</tr>
<tr>
<td>0.18</td>
<td>1.09620</td>
<td>0.06966</td>
</tr>
<tr>
<td>0.23</td>
<td>-4.8113</td>
<td>0.31349</td>
</tr>
<tr>
<td>0.28</td>
<td>-16.618</td>
<td>-0.0567</td>
</tr>
<tr>
<td>0.33</td>
<td>-42.164</td>
<td>0.24010</td>
</tr>
<tr>
<td>0.38</td>
<td>-25.956</td>
<td>-0.0948</td>
</tr>
<tr>
<td>0.43</td>
<td>-34.437</td>
<td>0.03840</td>
</tr>
<tr>
<td>0.48</td>
<td>-7.8011</td>
<td>0.24866</td>
</tr>
<tr>
<td>0.53</td>
<td>17.7205</td>
<td>0.24793</td>
</tr>
<tr>
<td>0.58</td>
<td>-9.3882</td>
<td>0.19518</td>
</tr>
<tr>
<td>0.63</td>
<td>-13.228</td>
<td>-0.0723</td>
</tr>
<tr>
<td>0.68</td>
<td>19.3958</td>
<td>-0.0965</td>
</tr>
<tr>
<td>0.73</td>
<td>50.2558</td>
<td>0.22344</td>
</tr>
<tr>
<td>0.78</td>
<td>7.17201</td>
<td>-0.0187</td>
</tr>
<tr>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
</tbody>
</table>
% Read filenames into myfiles and determine the number of files

myfiles = textread('f_names.txt', '%q');
numberoffiles = length(myfiles);

% Load bead diameters
bead_diameters = load('rheometer_diameters.txt');

% CONSTANTS
pi = 3.14;

% ADJUSTABLE PARAMETERS
step_rate = 0.03; % mm.s^-1, fixed at 0.03 for this data
lower_limit = 0.05; % lower stress limit for Young's mod calc.
upper_limit = 0.15; % upper stress limit for Young's mod calc.
strain_index = 0.3; % set the strain at which normal force is
% of interest

% Define a loop with number of iterations equal to the size of myfiles.
% Within the loop, stress and normal force at the strain_index defined
% above will be calculated and stored in a vector. Young's modulus
% will also be calculated between the strain limits defined above and
% stored in a vector.

% RELEVANT OUTPUT VECTORS are:
% Stress
% Force
% Youngs

% These vectors can be copied and pasted into the relevant Excel Sheets
% which are set up to calculate mean and standard deviation of each
% time point for each treatment and plot trends.

for k = 1:numberoffiles
    disp(k)                  %display the number of each iteration
    filetoload = char(myfiles(k)); %convert data in myfiles to string
    data = load(filetoload);    %load data from an individual file
    disp(filetoload)           %Show file name in print out as
                              % associated with index in
                              % myfiles
% Define bead diameter and radius for each iteration
bead_dia = bead_diameters(k);
bead_rad = bead_diameters(k)./2;

% Assign time and corresponding Normal force data to parallel % arrays for each iteration
Time = data(:,1);
NF = data(:,3);

% Calculate the contact area as a function of time and assign to % array. This will be used to determine stress.
Area = pi.*((bead_rad.^2)-(bead_rad-Time.*step_rate./2).^2);

% Calculate Hencky strain
H_strain = log((bead_dia+(Time.*step_rate))/bead_dia);

% NOTE: this value will exceed one at long time

% Calculate stress (N/m^2)
Stress = (NF./Area).*9800;

% Find 30% strain index
z = abs(strain_index-H_strain);
[i]= find(min(z)==z);

% Find normal force and stress at the predefined strain (defined % above)
z(i);
stress(k) = Stress(i);
force(k) = NF(i);

% Find indices for limits of Young's modulus calculation
z = abs(upper_limit-H_strain);
[i]= find(min(z)==z);

w = abs(lower_limit-H_strain);
[j]= find(min(w)==w);

% Determine parameters of linear fit to stress/strain data
coef = polyfit(H_strain(j:i),Stress(j:i),1);

% Generate an array containing slope of polyfit above = Youngs mod
youngs(k) = coef(1);

end

% Rotate to generate vectors to be copied to Excel
Stress = rot90(stress,3);
Force = rot90(force,3);
Youngs = rot90(youngs,3);
Appendix D

Media Recipes and Instructions for Preparation

D.1 YMPG Media

Slant and liquid media for growing

*Phanerochaete chrysosporium* conidiospores

Note: The following recipe is for 1-L of media; however it can be made in any volume by adjusting the mass of ingredients accordingly.

**Procedure**

1. To a 2-L autoclavable glass flask, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:
   - D - Glucose, Anhydrous (Dextrose; CAS# 492-62-6) 10g
   - Malt extract (VWR Part# 90001-014) 10g
   - Peptone (VWR Part# 61001-506) 2g
   - Yeast extract (Sigma-Aldrich Part# 70161-100G) 2g
   - L - Asparagine, Anhydrous (CAS# 70-47-3) 1g
   - KH$_2$PO$_4$ (CAS# 7778-77-0) 2g
   - MgSO$_4$ \cdot 7H$_2$O (CAS# 10034-99-8) 1g
3. If making slants, add 20g of agar (CAS# 9002-18-0). (Omit agar if making liquid media.)
4. Remove the stir bar, and take the final volume to 1 L with DDI water.
5. Wrap aluminum foil over the opening of the flask and add a piece of autoclave tape.
6. Place flask in an autoclavable bin and fill with several inches of tap water to aid in equal heating.
7. Autoclave for 30 minutes.
8. Remove from autoclave and allow media to cool until you can place your hands on the sides of the flask without being burnt.
9. Add 1ml of sterile 1mg/ml thiamine hydrochloride (vitamin B1; CAS# 67-03-8) solution to the cooled media to yield a final concentration of 1mg/L, and swirl by hand to mix.
   a. To make a 1mg/ml thiamine solution:
      i. Mix 50mg of thiamine with 50mL of DDI H₂O in a volumetric flask (yields a 1mg/mL solution).
      ii. Invert the flask until the solution is completely mixed.
      iii. Filter sterilize the thiamine solution into a sterile serum bottle (see sterilizing serum bottles and venting procedure).
10. In laminar flow hood with Bunsen burner lit, aliquot out 3mL of media into sterile 2-position snap tubes (17 x 100 mm polypropylene, Dot Scientific Inc, Product # 592-S) using a pipettor bulb and sterile volumetric pipette. Cap to first (loose) position to vent.
11. Place capped tubes at a 20⁰ angle or less to allow for maximum surface area once cooled.
12. Once cooled, push the cap to the bottom position to seal.
13. Place all tubes into a Ziploc bag, and store in refrigerator until ready to streak with fungi.
    If storing individually, wrap the cap with plastic wrap or parafilm.
D.2 Low Nitrogen (LN) Media

Liquid media for production of lignin and manganese peroxidases by

*Phanerochaete chrysosporium*

Note: The following recipe is for 1-L of media; however it can be made in any volume by adjusting the mass of ingredients accordingly.

**Procedure**

1. To a 2-L autoclavable glass flask, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

   - 10X Basal III media 100ml
   - D – Glucose, Anhydrous (Dextrose; CAS# 492-62-6) 10g
   - 0.1M transaconitic acid, pH 4.3 (CAS# 4023-65-8) 100mL
   - Ammonium tartrate (CAS# 3164-29-2) 0.2g
   - Trace elements 60mL

3. Remove the stir bar, and take the final volume to 1 L with DDI water.
4. Wrap aluminum foil over the opening of the flask and add a piece of autoclave tape.
5. Place flask in an autoclavable bin and fill with several inches of tap water to aid in equal heating.
6. Autoclave for 30 minutes.
7. Remove from autoclave and allow media to cool until you can place your hands on the sides of the flask without being burnt.
8. Add 1mL of sterile 1mg/ml thiamine hydrochloride (vitamin B1; CAS# 67-03-8) solution to the cooled media to yield a final concentration of 1mg/L, and swirl by hand to mix.
   a. To make a 1mg/ml thiamine solution:
      i. Mix 50mg of thiamine with 50mL of DDI H₂O in a volumetric flask (yields a 1mg/mL solution).
      ii. Invert the flask until the solution is completely mixed.
iii. Filter sterilize the thiamine solution into a sterile serum bottle (see sterilizing serum bottles and venting procedure).

9. For stationary cultures, add 100mL of sterile 4mM veratryl alcohol (CAS# 93-03-8) on day 3. For agitated cultures, add the veratryl alcohol and 50mL of sterile 1% Tween 80 solution.

10X Basal III Media

For use in fungal culture media

Note: The following recipe is for 1-L of solution; however it can be made in any volume by adjusting the mass of ingredients accordingly.

Procedure

1. To a 1-L glass container, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

   \[
   \begin{align*}
   \text{KH}_2\text{PO}_4 \quad \text{(CAS# 7778-77-0)} & \quad 20\text{g} \\
   \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \quad \text{(CAS# 10034-99-8)} & \quad 5\text{g} \\
   \text{CaCl}_2 \quad \text{(CAS# 10035-04-8)} & \quad 1\text{g} \\
   \text{Trace elements solution} & \quad 100\text{mL}
   \end{align*}
   \]

3. Remove the stir bar, and take the final volume to 1 L with DDI water.
4. Store in refrigerator.
Trace Elements Solution
For use in Basal III media

Note: The following recipe is for 1-L of solution; however it can be made in any volume by adjusting the mass of ingredients accordingly.

Procedure

1. To a 1-L glass container, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add:
   - Nitrilotriacetic acid (NTA; CAS# 139-13-9) 1.5g
   - MgSO\(_4\)•7H\(_2\)O (CAS# 10034-99-8) 3g
   - MnSO\(_4\)•H\(_2\)O (CAS# 10034-96-5) 0.5g
   - NaCl (CAS# 7647-14-5) 1g
   - FeSO\(_4\)•7H\(_2\)O (CAS# 7720-78-7) 0.1g
   - CoCl\(_2\)•6H\(_2\)O (CAS# 7646-79-9) 0.1g
   - ZnSO\(_4\)•7H\(_2\)O (CAS# 7446-20-0) 0.1g
   - CuSO\(_4\)•5H\(_2\)O (CAS# 7758-99-8) 0.1g
   - Al\(_2\)(SO\(_4\))\(_3\)•18H\(_2\)O (CAS# 7784-31-8) 10mg
   - H\(_3\)BO\(_3\) (boric acid; CAS# 10043-35-3) 10mg
   - Na\(_2\)MoO\(_4\)•2H\(_2\)O (CAS# 10102-40-6) 10mg

   *If needed, adjust pH to 6.5 with concentrated NaOH or HCl (~1-2mL)*
   *(some minerals will not go into solution if pH drifts from 6.5 significantly)*

   Continue adding in order, allowing each to dissolve in turn:

3. Remove the stir bar, and take the final volume to 1 L with DDI water.
4. Store in refrigerator.
Appendix E

Photographs of Abrasion Effects on Alginate Beads from Batch Airlift Bioreactors

**Figure E-1.** Photographs of abrasion effects on 15% mycelia, + Na treatment, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 3 mm in diameter.

**Figure E-2.** Photographs of abrasion effects on 5% mycelia, - Na treatment, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 3 mm in diameter.
Figure E-3. Photographs of abrasion effects on 5% mycelia, + Na treatment, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 3 mm in diameter.

Figure E-4. Photographs of abrasion effects on 15% mycelia, - Na treatment, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 3 mm in diameter.

Figure E-5. Photographs of abrasion effects on blank control, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 3 mm in diameter.
Figure E-6. Photographs of abrasion effects on dead mycelia control, in batch airlift bioreactors over time. From left to right: day 0, 11, and 22. Beads are approximately 2.5 mm in diameter.
Academic Vita

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EDUCATION

The Pennsylvania State University
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AWARDS AND SCHOLARSHIPS

- Top Graduate, College of Earth and Mineral Sciences - *Fall 2010 graduating class*
- Evan Pugh Scholar Award, Junior and Senior Year – *Top 0.5% of junior and senior class, respectively*
- President’s Freshman and President’s Sparks Awards – *4.0 G.P.A. 1st and 3rd semester, respectively*
- Alpha Sigma Lambda Honor Society for Adult Learners – *Inducted as a lifetime member (Spring 2008)*
- Fiedoreck Trustee Scholarship – *2009/2010 and 2010/2011 academic years*
- Aplan Centennial Mineral Engineering Scholarship - *2009/2010 and 2010/2011 academic years*

EDUCATIONAL EXPERIENCES

Undergraduate Honors Thesis Research (Fall 2009 - Fall 2010)
- Design and oversee laboratory batch-microcosm tests to evaluate the enzyme production and mechanical stability of alginate encapsulated white rot fungus
- Collaborate with a team of graduate students
- Measure enzyme activity and alginate rheology
- Use SimaPro 7.2 PhD software to begin a comprehensive life cycle assessment of passive techniques for endocrine disrupting compound removal from wastewater

International Service Learning Work – Kenya Biogas Installation (Summer 2010)
- Design and implement a small-scale, household biogas system as a demonstration unit near the town of Nyeri, Kenya
- Lead a team of two other students in the implementation of the design
- Conduct market surveys in the surrounding communities in order to assess entrepreneurial opportunities associated with the dissemination of small-scale biogas
- Educate and empower local youth throughout the installation process