OPTIMIZATION OF A NOVEL TWO-STAGE WHOLE-CELL ENZYME PRODUCTION AND LIGNOCELLULOSE HYDROLYSIS REACTOR DESIGN

by

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ABSTRACT

Production of lignocellulosic biofuels is one mechanism by which we can reduce our dependence on fossil fuels and enhance the carbon neutrality of transportation fuels. Current biochemical platforms for lignocellulosic biofuel production are not economically viable due to the high costs of hydrolytic enzymes for the conversion of lignocellulose to fermentable sugars. One solution to reducing these costs is the incorporation of on-site enzyme production for consolidated bioprocessing of lignocellulose to fermentable sugars. I have optimized and incorporated a whole-cell encapsulation approach into a novel two-stage fed-batch bioreactor consisting of a nursery reactor and hydrolysis reactor. This design spatially separates enzyme production and lignocellulose hydrolysis, allowing for simultaneous enzyme and sugar production for extended time periods while simplifying enzyme-cell separation. The integrated two-stage design was tested at the bench (250 ml) and pilot (70 L) scales. Encapsulated enzyme production was similar to unencapsulated nursery reactors over four consecutive 72-hour batch runs. Reducing sugar concentrations of hydrolysis reactors containing crude enzymes from encapsulated nursery reactors were similar to, or higher than, reactors containing unencapsulated nursery enzymes. Enzymes from encapsulated treatments produced nearly two times more fermentable sugars during hydrolysis than unencapsulated controls. Pilot scale runs yielded less enzymes in the nursery reactor though hydrolysis performance was only minimally affected. We have established a whole-cell encapsulation approach that enables both high levels of enzyme production

and simplified catalyst recovery for extended periods of time. These results serve to illustrate that an integrated on-site enzyme production and hydrolysis reactor system can be self-maintained and operate at high levels of productivity over time. These results also suggest that this system has the potential to be successfully scaled to an industrially relevant size.

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INTRODUCTION

In response to concerns about the anthropogenic effects on climate change, fossil fuel availability, and rising energy prices, the role of liquid biofuels as a viable transportation fuel source has been in the spotlight. Currently, these fuels are produced, for the most part, through microbial metabolism (i.e., the alcoholic fermentation of starch-based sugars). Although these alternative fuels may beneficially impact energy independence, rural economic development, carbon emissions, and fossil fuel demand, they are not without their own set of obstacles that must be overcome.

The most abundantly produced microbial biofuel is ethanol. Currently, commercial-scale ethanol is derived primarily from renewable food crops such as corn and sugar cane but is also in direct competition with the food supply chain. Corn is the largest U.S. crop accounting for nearly 25% of all croplands in the US, 30 million hectares, which is about 1/3 larger than the state of Idaho. About 13% of the approximately 13 billion bushels produced annually currently is slated for ethanol production (EERE 2010; USDA 2011). With increasing demand for ethanol fuel, diverting these crops from use as a dedicated food source runs the risk of increased food prices while applying additional pressure to convert natural land into farmland to alleviate the elevated demand on the food supply.

The Renewable Fuel Standards (RFS) legislation of 2005 requires liquid biofuel production to be ramped up to 15 billion gallons (BG) annually by 2015 (EPA 2010a).

The EPA determined this volume of biofuel could be produced from corn without negatively affecting the U.S. food supply. In 2007, the RFS was amended to increase the amount of biofuels to be produced including a change that mandates increased levels of lignocellulosic biofuel production (derived from inedible plant material) annually starting in 2010. Ideally, cellulosic biofuels would become commercialized and contribute to the alternative fuels pool, supplementing 30% (70 billion gallons per year) of the nation's gasoline by the year 2030. There are currently several dozen cellulosic plants either planned or under construction that could yield 364 BG/year (RFA 2011). However, due to the costs associated with conventional lignocellulose saccrification, none are currently producing lignocellulosic biofuels on a large scale (Dwivedi, Alavalapati et al. 2009).

Lignocellulose, the structural constituent of plant cell walls, is the most abundant source of organic carbon on earth with 1.3 billion tons produced in the U.S. annually (Zhang 2008). Its availability and renewable nature make it an attractive feedstock source for microbial biosynthesis applications. Lignocellulose has three main components: cellulose, hemicelluloses, and lignin. Cellulose and hemicelluloses make up a majority of the compound and are comprised of long chains of 5- and 6-carbon sugars. Cellulose is a large glucose polymer consisting of up to 27,000 subunits held together by β-1,4 glycosidic bond that are packed into elementary fibrils, which are in turn attached to each other by hemicellulose. Hemicellulose is a complex, web-like polysaccharide of 5-carbon sugars, primarily xylose, and six carbon sugars. Lignin is a phenolic matrix that acts as a glue to hold the macro-fibrils together.

Though the process for enzymatic hydrolysis of lignocellulose is well known, economically converting this inexpensive feedstock into fermentable sugars has several

challenges. The primary hurdle to overcome is reducing the cost of producing lignocellulose degrading enzymes. The current practice is to buy these hydrolytic enzymes from a third-party purveyor. However, up to 40% of total biofuel production costs can be attributed to enzyme purchases (Wyman 2007; Lynd, Laser et al. 2008; Yang and Wyman 2008). Furthermore, the high costs of enzymatic catalysts is compounded by the fact that these hydrolytic enzymes can have relatively low specific activity levels (DOE 2006; Wyman 2007), can unproductively adsorb to their substrates (e.g., 60 -70% of enzymes can permanently adsorb to lignin) (Singh, Kumar et al. 1991; Lu, Yang et al. 2002; Jørgensen, Kristensen et al. 2007), and inhibited by product accumulation (Bezerra and Dias 2004; Andrić, Meyer et al. 2010a; Andrić, Meyer et al. 2010b).

Approaches for improving biochemical conversion of lignocellulose to fermentable sugars have largely been dependent on optimization of biological catalysts via genetic modification of known organisms to enhance cellulase expression and activity, discovery of novel enzymes/organisms, or optimization of catalyst application via advanced packaging conditions (e.g. enzyme mixtures, concentration, shelf life, activity, etc.) (Wyman 2007). In this work, we focus on whole-cell encapsulation of enzyme producing microorganisms to improve the lignocellulose hydrolysis productivity.

Encapsulation of microbial catalysts, either whole cells, individual enzymes or enzyme cocktails, has long been used in the pharmaceutical industry as a means for enhancing viability, activity, and longevity of organisms and enzymatic catalysts (Jen, Wake et al. 1996; Park and Chang 2000; Chang and Prakash 2001). This process typically involves mixing the desired catalyst or enzyme excreting organism with a liquid form of the encapsulation matrix and then entrapping the organism within the matrix

during a polymerization step. Encapsulation matrix properties vary widely (i.e., for porosity, reactivity, surface area to volume ratios, solubility, durability, etc.). Specific matrices are chosen typically based on desired process parameters, suitability for incorporation of the desired microbial catalyst, and cost. Encapsulated organisms can be recycled for multiple reactor runs to produce prolonged metabolic activity and facilitate continuous reactor operation (Park and Chang 2000; Kar, Mandal et al. 2008). For example, microbial cells encapsulated in an alginate hydro-gel matrix can be protected from harsh environmental conditions such as pH, temperature, organic solvent, and high agitation rates, thereby increasing the ease of culture handling and enhancing viability over long durations of use. By restricting growth to the encapsulation matrix, the excreted hydrolytic catalysts can be more easily removed and used in downstream processes, providing freedom of bioreactor design and facilitating continuous flow operation (Park and Chang 2000; El-Katatny, Hetta et al. 2003). This approach can further enhance bioreactor systems by protecting cells from impeller damage and reduce clogging of plumbing and sensor maintenance inherent in high-density cell cultures.

Sodium alginate is a widely employed encapsulation matrix that is inexpensive to produce, non-toxic, and amenable for use with biological samples (Park and Chang 2000; Chang and Prakash 2001). Alginate is made from a modified algal polysaccharide that can be converted from a soluble form to an insoluble one by substituting Ca^{2+} for Na^{+}, resulting in the formation of a hydro-gel. Variation in alginate concentration can control matrix pore size and therefore diffusion of substrates into and out of the matrix (Klein, Stock et al. 1983; Smidsrod and Skjakbrk 1990). The alginate hydro-gel can be amended with a variety of compounds to alter its physical and chemical properties as a means to

optimize the encapsulation environment for a given organism or process. For example, this approach has been used extensively for protein characterization, optimization of fermentation processes (Steenson, Klaenhammer et al. 1987; Park and Chang 2000; Talebnia, Niklasson et al. 2005), bioremediation of nitrogen during wastewater treatment (van Ginkel, Tramper et al. 1983; Kokufuta, Yukishige et al. 1987), and production of monoclonal antibodies for research and medical applications.

Trichoderma reesei strain RUT C-30 is a well-described cellulolytic organism that produces large quantities of extracellular cellulases and xylanases (Mach and Zeilinger 2003; Stricker, Mach et al. 2008; Ahamed and Vermette 2009). In fact, this fungal species is one of the major sources of cellulases used in a variety of commercial enzyme preparations (Nevalainen, Suominen et al. 1994; Kar, Mandal et al. 2008). However, the filamentous nature of this organism and the specialized cultivation and separation technology necessary to grow *T. reesei* and recover extracellular enzymes from the culture milieu has traditionally limited the utility of on-site *T. reesei* cultivation and enzyme production on industrial scales. As a result, the production and dissemination of cellulases and xylanases has become the rate-limiting step in the overall scheme of biomass conversion to ethanol (DOE 2006). One of the hurdles for using *T. reesei* in an industrial application is the affect of mixing on its fragile mycelium. For example, impellers used in large bioreactors can damage hyphae thereby limiting growth and consequently cellulase production (Larroche and Gros 1997). However, less vigorous mixing leads to inadequate oxygen availability and can decrease growth and enzyme production rates (Lejeune and Baron 1995; Marten, Velkovska et al. 1996; Domingues, Queiroz et al. 2000). Toward a solution, immobilization of *T. reesei* on a

variety of substrates has been explored and effects on hydrolytic enzyme production studied (Linkoa, Haapalaa et al. 1996; El-Katatny, Hetta et al. 2003; Kar, Mandal et al. 2008; Zhang, Lo et al. 2009; Hui, Amirul et al. 2010). For example, encapsulation of *T. reesei* in a Ca-alginate matrix or immobilization of the fungi on polyurethane foam helped enhance retention of fungal biomass during separation and recovery of extracellular enzymes and enabled multiple reactor runs without reinoculation (Zhang, Lo et al. 2009). Here I propose a novel strategy for optimizing the encapsulation matrix to maintain enhanced cellulase and xylanase production by *T. reesei* RUT C-30. My goal is to simplify enzyme production and integrate this process into a unified saccharification system. This approach has the potential to lower enzyme production costs while increasing saccharification productivity.

CHAPTER 1: DEFINING OPTIMAL CULTURE PARAMETERS OF CALCIUM ALGINATE ENCAPSULATED TRICHODERMA REESEI RUT C-30 FOR CELLULASE AND XYLANASE PRODUCTION

Abstract

Lignocellulosic biofuels production is a mechanism that can help reduce our dependence on fossil fuels and enhance the carbon neutrality of transportation fuels. Current biochemical platforms for lignocellulosic biofuel production are not economically viable due to the high costs of hydrolytic enzymes for the conversion of lignocellulose to fermentable sugars. One solution to reduce these costs is incorporation of on-site enzyme production for consolidated bioprocessing of lignocellulose to fermentable sugars. I optimized whole-cell encapsulation parameters that allow for prolonged enzyme production, ease of catalyst separation, and enable incorporation of on-site catalyst production into existing biochemical lignocellulose transformation platforms. *Trichoderma reesei* RUT-C30 was encapsulated in calcium alginate hydro-gel beads and grown in continuously stirred 250 ml bioreactors containing purified cellulose. Physical and environmental parameters including agitation, reaction volume, and hydro-gel diameter and concentration were modulated and compared with unencapsulated controls for cellulase and xylanase activity. The encapsulation matrix retained 90% of the *T. reesei* biomass. *Trichoderma reesei* in optimized encapsulation matrices produced similar filter paper units of cellulase activity as unencapsulated controls over a 144-hour period while producing significantly higher xylanase activity than unecapsulated *T. reesei*

over the same time period. Recycling of encapsulated organisms during successive 3-day batch runs yielded similar filter paper activity to the unencapsulated organism by the end of the second batch. When crude enzyme liquor was used to hydrolyze purified cellulose, encapsulated *T. reesei* effluent yielded nearly two times more fermentable sugars than unencapsulated effluent.

Introduction

Lowering the cost of lignocellulose saccharification processes has renewed importance in light of revisions made to the U.S. Renewable Fuel Standards (RFS2) in 2007. With efforts to limit greenhouse gas emissions, reduce reliance on fossil fuels, and increase domestic security these EPA mandates require lignocellulose to play an increasingly dominant role in the liquid biofuels arena. The new RFS2 cellulosic ethanol production goals call for 0.5 billion gallons (BG) to be produced in 2012 and 1.0 BG in 2013 (EPA 2010a). Although recent advances in biocatalyst synthesis have dramatically reduced the cost of enzymatic saccharification and thermochemical conversion of biomass, lignocellulosic biofuels are still not economically feasible at large scales, impeding achievement of the RFS2 targets (EERE 2010). Indeed, the U.S. EPA estimated a best case scenario for cellulosic ethanol production in 2012, which included facilities not yet in operation, to be a mere 15.7 million gallons and possibly as low as 3.6 million gallons (EPA 2010b). Advancements in biochemical conversion strategies can facilitate reaching the goals of the RFS2.

Research aimed at further reduction in biocatalyst costs can be, for the most part, divided into two camps: increasing production rates and/or catalytic activity of lignocellulytic enzymes or improving hydrolysis efficiencies and enzyme recovery for

reuse (Banerjee, Mudliar et al. 2010; Kambam and Henson 2010; Chandel and Singh 2011). The dichotomy of these approaches reflects the current biofuel production model, which spatially and temporally separates enzyme synthesis and saccharification with the biofuel producer typically purchasing enzymatic catalysts from a third party.

Enzyme purchases represent one of the major operating costs for biochemical cellulosic ethanol production. Typically, commercial enzyme preparations are provided by only a few suppliers (MacLean and Spatari 2009) and must be bought on a continuous basis. Though recent advancements have lowered the cost of enzymes from $2.00/gallon of finished product in 2007 to $0.69 - $0.50/gal 2009; such purchases are still estimated to comprise 30-45% of cellulosic biofuel production costs assuming $1.62/gallon of finished product (2007$)(EERE 2010; Kazi, Fortman et al. 2010; Humbird, Davis, et al. 2011). These cost estimates also fall short of the National Renewable Energy Lab's (NREL) 2008 State of Technology report targeted costs of $0.12/gallon by 2012 (Humbird and Aden 2009).

On-site enzyme production can lower the cost of enzymatic hydrolysis by minimizing costs associated with third-party purchases, preservation, transportation, and storage. For example, a recent NREL report predicts that on-site enzyme production can lower catalyst cost to $0.34/gal (Humbird, Davis, et al. 2011). Moreover, Slade, Bauen, and Shah(2009) estimate that 50%-60% of greenhouse gas (GHG) emissions generated from cellulosic ethanol production can be attributed to enzyme production. They conclude that on-site enzyme production could reduce total GHG emissions by 5-6%, helping ethanol production facilities meet the 2007 Energy Independence and Security Act threshold of 60% lifecycle GHG reduction for cellulosic biofuels (EPA 2010a). The

current costly and energy intensive, off-site production model endures because several hurdles make on-site enzyme production impractical (e.g., capital costs associated with enzyme production/separation and expense/availability of enzyme inducers). Based on the 1999 NREL process design Kazi, Fortman et al. (2010) estimated that on-sight enzyme production would require an additional $434 million in capital expenses while increasing feedstock and energy demands, and thus, increasing ethanol costs by about 4%. Barta, Kovacs et al. (2010) added that the economic feasibility of on-site enzyme production can be achieved but relies heavily on the type of feedstock used for enzyme production. Humbird, Davis, et al. (2011) suggested that innovative process design that simplify or streamline the lignocelluloses to biofuel process can significantly lower overall production costs. Therefore, a cultivation strategy that optimizes growth and extracellular enzyme production while minimizing the complexity of enzyme recovery is needed to make on-site enzyme production a cost-efficient option.

Lignocellulytic enzymes from the filamentous fungus *Trichoderma reesei* are commonly found in commercially available enzyme preparations (Nevalainen, Suominen et al. 1994; Kar, Mandal et al. 2008). *Trichoderma reesei* strain RUT C-30 produces large quantities of extracellular cellulases and xylanases that can hydrolyze the cellulose and hemicellulose fractions of lignocellulosic biomass (Mach and Zeilinger 2003; Stricker, Mach et al. 2008; Ahamed and Vermette 2009). The highest titer of hydrolytic enzymes are produced during the stationary growth phase (Pakula 2005), suggesting that maintenance of high-density stationary phase cultures is necessary for continuous cellulase and xylanase production. However, stationary phase cultures of *T. reesei* also commonly support large amounts of hyphal biomass that complicate separation and

purification of extracellular hydrolytic enzymes (Weber and Agblevor 2005; Patel, Choy et al. 2009).

Encapsulation of microorganisms allows for high-density populations to be maintained in a bioreactor for prolonged and efficient bio-synthesis (Park and Chang 2000; Kar, Mandal et al. 2008) while simultaneously simplifying separation of biomass from extracellular products (Kar, Mandal et al. 2008). Commercial scale up and effective use of calcium alginate whole cell encapsulation has been proven and extensively used in the pharmaceutical industry (Jen, Wake et al. 1996; Park and Chang 2000; Chang and Prakash 2001). Therefore, encapsulation of a lignocellulosic microorganism could enhance on-site catalyst production by facilitating maintenance of stationary phase cultures and potentially simplifying catalyst-biomass separation.

Immobilization and/or encapsulation of *T. reesei* can affect hydrolytic enzyme production (El-Katatny, Hetta et al. 2003; Kar, Mandal et al. 2008; Zhang, Lo et al. 2009; Hui, Amirul et al. 2010). For example, immobilization of *T. reesei* on polyurethane foam increased relative cellulase activity by 71% (from 0.07 FPU/mg cells to 0.12 FPU/mg cells) while retaining a majority of the fungal biomass over four days (Zhang, Lo et al. 2009). However, reuse of the immobilization substrate over multiple reactor runs was not evaluated. Xylanase activity of *T. reesei* in a Ca-alginate matrix has been shown to be similar to unencapsulated *T. reesei* (approximately 0.8 U/ml) (Kar, Mandal et al. 2008). Furthermore, significant xylanase production of encapsulated *T. reesei* in repeated batch experiments was observed for 22 days, suggesting that prolonged enzyme production is achievable with an encapsulated microorganism. Their study, however, did not evaluate cellulase production nor did it measure fungal migration out of the hydro-gel, therefore

enzyme activity could have arisen from hyphae that grew out of the encapsulation matrix during incubation. This study investigates both cellulase and xylanase activity of a Ca-alginate encapsulated *T. reesei* and its ability of the hydro-gel to retain fungal cells over multiple reactor runs

In an attempt to simplify and ultimately reduce costs of on-site enzyme production, I have optimized a whole-cell encapsulation approach that enhances bio-catalyst synthesis and separation over current models by simplifying costly enzyme separation and purification steps and consequently reducing associated capitol and processing costs. This process utilizes a nursery reactor containing *Trichoderma reesei* RUT-C30 encapsulated in a calcium alginate hydro-gel for semi-continuous production of lignocellulose hydrolyzing enzymes (e.g., cellulase and xylanase) while minimizing the need for complicated downstream catalyst separation and purification steps. Here I demonstrate that encapsulation of *T. reesei* produces enzymes with consistently high activity and requires minimal post-production purification. Furthermore, lignocellulose hydrolysis reactions driven by an enzyme cocktail recovered from an encapsulated *T. reesei* bioreactor releases approximately two times as much fermentable sugar from purified cellulose than the enzyme cocktail recovered from the unencapsulated control reactors. This new approach could provide a simple way to reduce the overall cost of lignocellulosic biofuel by decreasing the costs of catalyst production.

Materials and Methods

Strain and Media Preparation

Trichoderma reesei RUT-C30 (NRRL 11460, ATCC 56765) was obtained with permission from the Department of Agriculture's ARS Culture Collection. The culture

medium (used for liquid cultures and agar plates) was as follows (amounts for each ingredient are indicated in per liter values): KH_2PO_4 (2g), $MgSO_4 \cdot 2H_20$ (0.3g), $CaCl_2 \cdot 2H_20$ (0.3g), $FeSO_4 \cdot 7H_2O$ (0.005g), $MnSO_4 \cdot 4H_2O$ (0.0016g), $ZnSO_4 \cdot 7H_20$ (0.0014g), $CoCl \cdot 6H_2O$ (0.002g), $(NH_4)_2 SO_4$ (1.4g), Urea (0.3g), Peptone (1.0 g), Tween 80 (2 ml). This media was filter sterilized and added to sterile carbon substrates. Minimal media was supplemented with Avicel PH-101 (Sigma 11365) (10.0g l^{-1}) for 2% agar plates and optimization experiments.

Subculture Preparation

Trichoderma reesei was grown on 2% agar plates containing 1% Avicel at 30°C for 3-4 days and stored at 4° C for up to 3 weeks. Subcultures were prepared by adding an agar plate plug to 250 ml Erlenmeyer flasks containing 200 ml Avicel and incubated at 30° C on a 200 RPM orbital shaker for four days. Subcultures were concentrated to 40 ml by centrifugation at 7000 RPM for 5 min. After centrifugation, the fungal pellet was rinsed in fresh basal media three times, centrifuging as described above after each rinse.

Encapsulation Matrix Preparation

Calcium alginate beads were produced using 1.0%, 1.5%, or 2% sodium alginate solutions with pH either unmodified (7.4) or adjusted to 5.7 with 1M HCl. Bead diameter was varied using 16 gauge (Ga.) or 27.5 Ga. needles. Sodium alginate solutions for hydro-gel encapsulation of *T. reesei* were prepared by adding 6 g of Na-alginate (Fisher Scientific NC9676930) to 286 ml of 100° C distilled water, while continuously stirring. The solution was allowed to cool to room temperature and stirred until the Na-alginate completely dissolved. Final pH of the alginate solution was adjusted to 5.7 (using approximately 0.5 ml 1N HCl). Distilled water was added to bring the total volume to

288 ml before autoclaving. After adding 12 ml of subculture concentrate to cooled, sterile Na-alginate solution, hydro-gel beads were formed by adding the solution drop-wise (using a peristaltic pump and needle) to a continuously stirred solution of 0.2 M $CaCl_2$ at 4° C. Hydro-gel beads were rinsed three times with an equal volume of basal media to remove excess $CaCl_2$ and used immediately for experiments.

Culture Conditions

All encapsulation optimization experiments were carried out at 30° C in 1% Avicel media. Agitation rates, reactor volume, and reaction volume were adjusted in that order to achieve optimal cellulase expression by *T. reesei*. Each new modulation experiment built upon the optimized parameters determined in the previous experiment. Both treatments were prepared in 250 ml Erlenmeyer flasks and incubated on an orbital shaker at 400 RPM.

Encapsulation Matrix Reuse

The longevity of the encapsulated organism and the encapsulation matrix performance were assessed in two batch culture experiments that differed in incubation times, specifically either 144 hour or 72 hours. Media was removed from encapsulated treatments by pipette or after 5 minute 7000 RPM centrifugation of unencapsulated treatments and reusing the encapsulated organism in an additional round of incubation. Treatments were refreshed with new media and incubated under optimum culture conditions described previously with 1% w/v Avicel for 144-hour batches and 0.5% w/v Avicel for 72-hour batches.

Cellulase Activity Measurements

Cellulase activity was determined using the standard colorimetric Filter Paper Assay adapted for micro-plate use (Xiao, Storms et al. 2004). Briefly, an aliquot of enzyme preparation was added to a defined mass of known quality cellulose paper and incubated at 50°C in 0.2M citrate buffer at pH 4 for one hour. After incubation, a dinitrosalicylic acid (DNS) reagent (Xiao, Storms et al. 2004) was added and samples were incubated at 95°C for 5 minutes. Absorbance was measured at 540 nm, reducing sugars released were determined by comparison to a glucose standard, and enzyme activity reported as Filter Paper Units (FPU)/ml. One FPU is defined as the amount of enzyme that will liberate 1 µmol of glucose equivalents from the filter paper in 1 minute. Enzyme activity is expressed as glucose equivalents because this assay detects not just glucose but all reducing carbohydrates.

Xylanase Activity Measurement

Xylanase activity measurements were determined using a colorimetric assay similar to the cellulase filter paper assay. As described by Bailey, Biely et al. (1992), an aliquot of enzyme preparation was added to a 1% xylan solution (pH 5.2) and incubated at 50°C for 5 minutes. After incubation, DNS reagent was added to develop color and stop the reaction, After a 20 minute incubation at 100^{o} C, 50 µl of cooled reaction was added to a 96-well microplate containing 150 µl distilled water and absorbance was measured at 540 nm. Reducing sugars released were determined by comparison to a xylose standard, and enzyme activity reported as Xylanase Activity Units (XAU)/ml. One XAU is defined as the amount of enzyme that will liberate 1 µmol of Xylan equivalents in 1 minute.

Biomass Determinations

With the exception of yeast, fungi are generally not comprised of discrete cells making conventional cell counting methods impractical. Therefore, total fungal biomass levels were determined using a standard Bradford (coommasie blue) assay kit (Thermo-Fisher 23200) for total soluble protein. Hyphal pellets from unencapsulated treatments were collected by centrifuging two milliliter aliquots at 5000 RPM for 5 minutes. After washing the pellet in minimal media three times, hyphae were lysed by adding a 0.1 M NaOH to the pellet and incubating at 4° C overnight. One ml of pellet lysate was added to 0.5 g silicon beads (BioSpec Products Cat. #11079101z) and subjected to bead beating using a Fastprep FP 120 at a setting of 6.5 for 45 seconds. Samples were put on ice for 5 minutes between each round of bead beating. Bead beating was repeated 3 times and followed by a 20 minute, 100^{o} C incubation. Fungal biomass from encapsulated treatments was first liberated by dissolving the Ca-alginate matrix in a 0.1M Na-citrate buffer. The mycelia mass was then lysed using the procedure described for unencapsulated treatments. The resulting soluble proteins were measured colorimetrically by adding 250 µm of room temperature Bradford reagent to 5 µm of prepared sample. After a 10-minute room temperature, incubation in the dark absorbance was measured at 540 nm. Total protein levels were converted to estimates of Cell Dry Weight (CDW) as describe previously (Zhang, Lo et al. 2009).

[CDW] (g/ml) = [intracellular protein] (g/ml) X 8.0.

Quantities of culture media proteins (e.g., extra-cellular proteins) were determined by using the initial supernatant from the CDW pellet directly with the Bradford method.

Results/Discussion

To determine the feasibility of Ca-alginate as an effective encapsulation matrix for cellulase production, I first examined the ability of the alginate hydro-gel to successfully encapsulate *T. reesei*. To increase hydrolytic activity of the encapsulated organism, different encapsulation matrix and reactor configurations were explored. All experimental results from alginate encapsulated treatments were compared to unencapsulated controls and/or values published in the literature. Lastly, hydro-gel properties including encapsulation matrix diameter, pH, and alginate concentration were modulated and evaluated for their effect on cellulase and xylanase activity.

All data presented in the figures are the mean of 3 treatment replicates ± standard error. Statistical analysis was performed using R build 2.13.

Effect of Culture Agitation Rate on Encapsulated Biomass Retention and Enzyme Activity

Initial alginate encapsulation experiments were performed to determine viability and retention of *T. reesei* within the Ca-alginate matrix. Viability was determined based on biomass production within the alginate matrix and retention was evaluated by comparing biomass production within the alginate matrix and culture supernatant, respectively. Retention of the fungal mycelium was directly influenced by the culture agitation rate. Experiments conducted at an agitation rate of 100 RPM revealed that fungal growth was not restricted to the encapsulation matrix. Within 196 hours, 96% of the total fungal biomass was observed in the culture supernatant (Figure 1.1a). At this agitation rate, relative cellulase activity between encapsulated and non-encapsulated *T.*

reesei (0.035 FPU/mg CDW and 0.038 FPU/mg CDW) showed no treatment effect, likely due to cell outgrowth from the alginate beads.

High agitation rates can negatively affect filamentous mycelia growth (Cui, van der Lans et al. 1997; Grimm, Kelly et al. 2005; Ahamed and Vermette 2010). Therefore, we hypothesized that at higher agitation rates the hydro-gel would afford the encapsulated organism protection while inhibiting growth in the supernatant. In an attempt to reduce outgrowth from the encapsulation matrix, we performed an experiment in which the agitation rate was increased to 400 RPM. Increased agitation improved retention within the encapsulation matrix. Encapsulated treatments did not significantly increase overall biomass. However, mycelium distribution shifted appreciably; favoring the hydro-gel. Fungal growth within the encapsulation matrix increased more than 10 fold with approximately 91% of the total biomass being contained within the encapsulation matrix (Figure 1.1a). Although, the total biomass of encapsulated treatments did not surpass unencapsulated treatments, *T. reesei* did grow to a higher density (6.54 mg/ml CDW) within the encapsulation matrix relative to that found in the media of unencapsulated controls (3.52 mg/ml CDW) (Figure 1.1b). Increased agitation also had a positive effect on enzyme activity of both treatments with cellulase activity increasing 171% (0.235 FPU to 0.401 FPU) and 259% (0.314 FPU to 0.814 FPU) for encapsulated and unencapsulated treatments, respectively (Figure 1.1c). The lower relative enzyme activity of the encapsulated treatments (0.12 FPU/mg CDW) compared to the unencapsulated (0.23 FPU/mg CDW) suggests that factors within the hydro-gel may have inhibited enzyme activity, enzyme synthesis, or diffusion of enzymes out of the encapsulation matrix possibly due to sorption to the encapsulation material.

To determine if enzyme adsorption to the encapsulation matrix impeded catalytic function, we evaluated the activity of encapsulated cellulase. Calcium alginate beads loaded with a commercially available enzyme preparation (Sigma 8546-5KU) showed similar enzyme activity to unencapsulated enzymes (data not shown). These data suggest that oxygen and/or nutrient diffusion into the hydro-gel may be the cause of the lower cellulase activity observed, and not enzyme diffusion or sorption to the alginate matrix.

Reactor Size and Reaction Volume

Reactor configuration was modulated to examine how surface area to volume ratios between the air-media interface affect bioreactor productivity. Working volumes of 250 ml reactors were adjusted to 125 ml and 62.5 ml. Additionally, 50 ml reactor with a 15.6 ml volume were evaluated. The reactors had surface area to volume ratios (mm^2:ml) of 19.47, 53.45, and 1.67, respectively. Reducing the reaction volume from 125 ml to 62.5 ml increased the surface area to volume ratio almost 3-fold and resulted in a nearly two-fold increase of FP activity for both treatments (Figure 1.2). At a 62.5 ml reaction volume, both encapsulated relative filter paper (FP) activity (0.406 FPU/mg CDW) and specific filter paper activity (2.94 FPU/mg extracellular protein) were similar to the unencapsulated reactor (P= 0.52 and P= 0.64, respectively). Dissolved oxygen (DO) levels directly affect *T. reesei* cell growth (Domingues, Queiroz et al. 2000; Weber and Agblevor 2005; Patel, Choy et al. 2009) and cellulase production (Reczey, Szengyel et al. 1996; Domingues, Queiroz et al. 2000). Increasing volumetric surface area may allow for higher levels of DO in the media and consequently increase fungal growth and cellulase production within the hydro-gel. Similarly, a correlation between surface area and productivity can be seen in the unencapsulated treatments.

Since *T. reesei* cellulase production increases during stationary growth phase (Reese and Mandels 1980; Mohagheghi, Grohmann et al. 1988), the increased DO may have permitted rapid fungal growth, reaching stationary phase more quickly and resulting in increased levels of cellulase expression during the same incubation period as more slowly growing cultures. The 15.6 ml reactor volume resulted in lower overall performance activity for both treatments when compared to the 62.5 ml reaction volume. Reaction volumes of 62.5 ml were used for the remaining experiments.

Effect of Bead Diameter

The disparity between cellulase activity of encapsulated and unencapsulated treatments along with the presumed positive effect of increased aeration via higher agitation rates suggests that oxygen diffusion may play an important role in cellulase production. Mass transfer and oxygen diffusion limitations within hydro-gel matrices, specifically those with a diameter above 1mm, are well documented (Margaritis, Bajpai et al. 1981; Prüsse, Bilancetti et al. 2008). Furthermore, Domingues, Queiroz, et al. (2000) report that under certain growth conditions, *T. reesei* will form tightly aggregated mycelia in liquid culture. Within these tightly aggregated mycelia, the cells in the center of the aggregation have low levels of metabolic activity that is thought to be due to oxygen limitation within the aggregate. I hypothesized that similar conditions were occurring in the alginate beads. Dominguez, Queiroz, et al. (2000) found that at the end of a 144-hour incubation, cultures with mycelial aggregates produced approximately 1 FPU while non-aggregated cultures had about 1.3 FPU activity, mirroring the difference in cellulase activity within our own encapsulated and unencapsulated treatments. To limit the potential for metabolic inhibition within our encapsulation matrix, I modulated the

encapsulation approach to increase the surface area:volume ratio of the alginate hydro-gel by reducing its diameter from approximately 3.25 mm to approximately 2.0 mm. Our results indicate that high levels of biomass can be maintained within smaller diameter beads and that this decrease in surface area:volume ratio resulted in a significant increase (P= 0.05) of 17.6% in FP activity, from 0.91 FPU to 1.07 FPU (Figure 1.3). These results imply an inverse relationship between bead diameter and filter paper activity and that further size reduction may obtain higher levels of cellulase activity by the encapsulated cells.

Adjusting Bead pH

Trichoderma reesei cellulase production is directly affected by the pH of the medium in which it is cultured. Cellulase production and corresponding FPU activity is enhanced at a pH near 5 (Juhász, Szengyel et al. 2004). However, adjusting the pH of the alginate solution from its natural pH of 7.3 to a pH of 5.7 (5.5 to 5.6 after autoclaving) during the encapsulation procedure did not significantly increase FP activity (Figure 1.4). Any further decrease in pH resulted in misshaped beads. Therefore, the initial pH of the encapsulation environment is less important than matrix diameter and surface area:volume ratio of the reactor for cellulase production by *T. reesei*.

Alginate Concentration

The concentration of alginate used during hydro-gel formation influences mean pore size and therefore diffusion of extracellular products from an encapsulated organism to the culture medium, as well as mechanical integrity of the encapsulation matrix (Smidsrod and Skjakbrk 1990). The optimum alginate concentration can vary widely as it is dependent upon the application and organism being encapsulated. Alginate

concentrations ranging between 3% and 1% are most common for the production of low molecular weight molecules. Higher alginate concentration reduces pore size (Park and Chang 2000) and consequently reduces enzyme production while lower alginate concentrations result in cell leakage from the hydro-gel (Kar, Mandal et al. 2008; Varma and Gaikwad 2009). We evaluated the encapsulation capabilities of 1%, 1.5%, and 2% alginate and found that 1% alginate beads effectively encapsulated the organism at high agitation rates. However, this concentration allowed migration of fungal growth out of the bead (Figure 1.5a). At the end of a 144-hour incubation period, 44% of the total biomass was in the culture media. Additionally, these beads began showing signs of fatigue after just 48 hours of incubation. As seen in the 100 RPM agitation experiments, the higher FP activity may be attributed to cell growth in the culture media (Figure 1.5b). The performance of the 1.5% beads were similar to the 2% concentration beads with respect to FP activity ($P = 0.97$) and encapsulation capability. It appears that there is a tradeoff between the integrity of the alginate encapsulation matrix and cellulase production by the encapsulated fungus. While the 1.5 and 2% alginate encapsulation matrices resulted in decreased FPU/ml relative to the 1% alginate ($P<0.05$), the enhanced integrity of the higher alginate concentration matrices may make them more desirable for prolonged periods of use.

Enzyme Activity Under Optimized Encapsulation Conditions

Trichoderma reesei encapsulated in 2 mm diameter 2% Ca-alginate hydro-gel beads at optimal reactor conditions (62.5 ml reaction and 400 RPM agitation) were used to evaluate cellulase and xylanase activities. Encapsulated treatments had similar cellulase activity for the first 96 hours (Figure 1.6a). Although a Repeated Measure

ANOVA found no significant difference in FP activity over 144 hours (P= 0.07), pairwise time point comparisons detected higher cellulase activity for encapsulated treatments (P<0.05) at 120 and 144 hours. Conversely, Repeated Measure ANOVA analysis showed significantly higher xylanase activity of encapsulated *T. reesei* over control treatments (P= 0.001) (Figure 1.6b).

Reuse of Encapsulated *T. reesei* for Multiple Reactor Runs

To be advantageous, an encapsulation strategy must simplify the process of enzyme/organism separation while allowing for continuous or semi-continuous reactor operation. Biomass-enzyme separation in our experiments was achieved quickly and efficiently by merely stopping agitation of the reactor. Within seconds, the alginate encapsulated mycelium settles to the bottom of the flask and then the enzyme-laden supernatant can simply be decanted from the reactor. Moreover, we have successfully reused the alginate-encapsulated fungus for multiple reactor runs lasting up to three weeks in duration. Three successive 144-hour incubations of encapsulated *T. reesei* produced increasingly higher levels of filter paper activity. Cellulase activity increased from 1.04 FPU/ml during the initial run to 1.46 FPU/ml and 1.51 FPU/ml at the end of two subsequent runs. However, cell growth accumulation in the supernatant approached unencapsulated levels by the end of the third incubation. This gradual increase of unencapsulated biomass becomes less of an issue if the culture supernatant is removed after shorter periods of incubation (i.e., the dilution rate is increased or batch feeding period decreased).

Considering that 91% of total cellulase activity of encapsulated reactors is achieved within 72 hours, and enzyme activity is similar to unencapsulated reactors,

shorter 72-hour fed-batch reactions were performed and evaluated for enzyme activity and supernatant cell growth. Decreasing incubation to 72 hours improved reactor productivity (i.e., volumetric cellulase activity and FPU/gof Avicel), while at the same time, limiting cell proliferation in the culture supernatant. Although initial cellulase activity of the encapsulated 72 hour fed-batch treatment was approximately 20% - 40% lower than the highest activity observed in the unencapsulated control, by the third batch similar cellulase activity (1.61 FPU/ml) was being observed in both reactors (P= 0.97) (Figure 1.7a). This level of cellulase activity was the same as the unencapsulated fed-batch counterpart and also similar to activity seen in the second and third runs of the 144-hour batch reactions (P= 0.79 and P= 0.45, respectively).

Shorter batches also limited fungal accumulation outside the encapsulation matrix. Over the course of four 72-hour batch experiments, free biomass in the encapsulated reactor did not exceed 2.4 mg CDW/ml. This concentration of biomass is 65% less than an unencapsulated reactor (Figure 1.7b) and 23% less than an encapsulated 144-hour batch reactor during the same time period.

Reducing the batch run time yielded over two times more FPU/liter·hour while receiving less Avicel than the longer batches (Table 1.1). As with the longer fed-batch treatments, the first 72-hour run received 1% w/v Avicel. However, Avicel was reduced to 0.5% w/v for each subsequent batch. This resulted in a total of 55% more FPU/g Avicel for the shorter runs.

Our data also suggest that the low levels of fungal cells in the reactor supernatant may positively affect hydrolysis reactor performance. We believe that low-levels of

fungal biomass migration to the culture supernatant during crude enzyme transfer may be beneficial in a two-stage reactor design. To assess the influence of biomass production in the culture supernatant after prolonged periods of encapsulation, we fed crude nursery reactor effluent to a cellulose hydrolysis reactor. Cellulose is hydrolyzed into cellobiose (i.e., a glucose dimer) by the action of endo- and exo-gluconases (i.e., cellulases) (Jørgensen, Kristensen et al. 2007). Cellobiose is then converted to glucose monomers by the hydrolytic activity of ß-glycosidase (Jørgensen, Kristensen et al. 2007). In the absence of ß-glycosidase, cellobiose can accumulate and act as an inhibitor of cellulose activity (Bezerra and Dias 2004; Bezerra and Dias 2005; Andrić, Meyer et al. 2010b). In *T. reesei,* ß-glycosidase is for the most part, an intracellular enzyme and its activity is considered to be the rate-limiting step of cellulose hydrolysis (Slade, Bauen et al. 2009). I hypothesized that the increased temperature from 30°C in the nursery reactor to the optimal saccharification temperature of 50° C in the hydrolysis reactor would facilitate autolysis and ß-glycosidase release from the incidental mycelia transfer. This would, in turn, result in more conversion of cellobiose to monosaccharides, which can be fermented to liquid biofuels. A twelve-hour hydrolysis with nursery reactor liquor from encapsulated *T. reesei* yielded nearly two times more fermentable sugars than unencapsulated effluent with 3.25 g/L, 0.30 g/L, and 0.70 g/L of glucose, galactose, and cellobiose, respectively. In contrast, unencapsulated nursery effluent produced 1.49 g/L, 0.175 g/L, and 1.14 g/L of the same sugars, respectively (Table 1.2). CDW analysis of the hydrolysis reactor cell pellets revealed lower biomass density at the end of 72 hours, suggesting a loss of soluble proteins through cell lysis, thus supporting this hypothesis.

Conclusion

Reducing enzyme cost is imperative for biochemical conversion of lignocellulose to cellulosic biofuels to become economically viable. On-site enzyme production is one possible strategy to achieve this goal. We have established a whole-cell encapsulation approach that enables both high levels of enzyme production and simplified catalyst recovery for extended periods of time. Effluent from encapsulated treatments produced nearly 2 times more fermentable sugars from Avicel despite having lower filter paper activity in the nursery reactor. Extended enzyme production, ease of separation, and enhanced hydrolysis activity may facilitate on-site catalyst production for biochemical hydrolysis of lignocellulosic biomass for biofuel production.

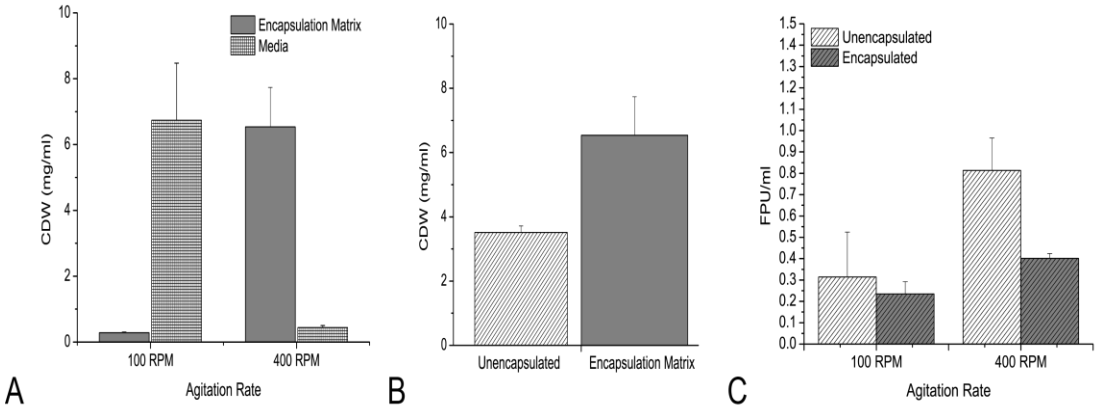

Figure 1.1. The Effect of Agitation Rate on: A) Biomass Distribution, B) Total Biomass Accumulation, and C) Cellulase Production/Activity.

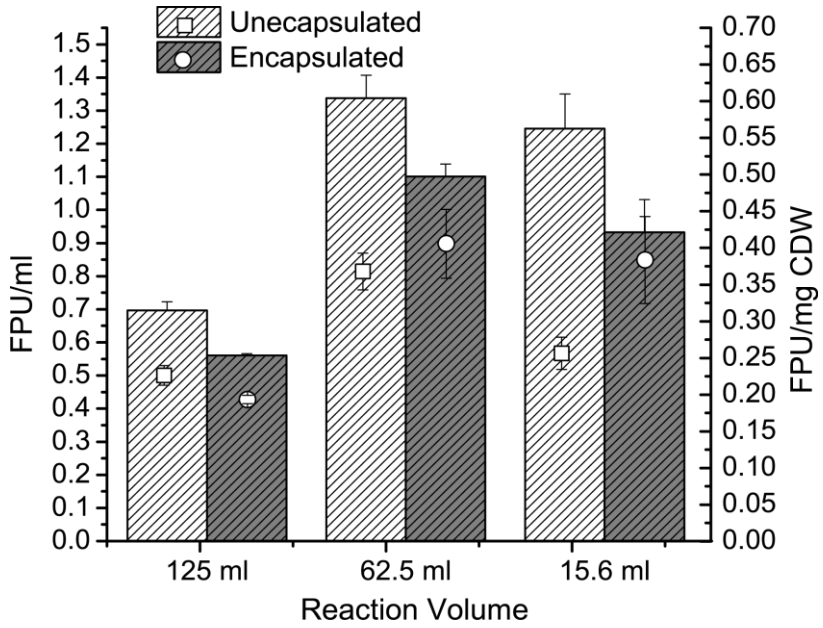

Figure 1.2. Measure of Absolute and Relative Cellulase Activity as a Function of Reaction Volume. Absolute Cellulase Activity (FPU/ml) Is Indicated by Columns. □and ○ Represent Relative Cellulase Activity (FPU/CDW).

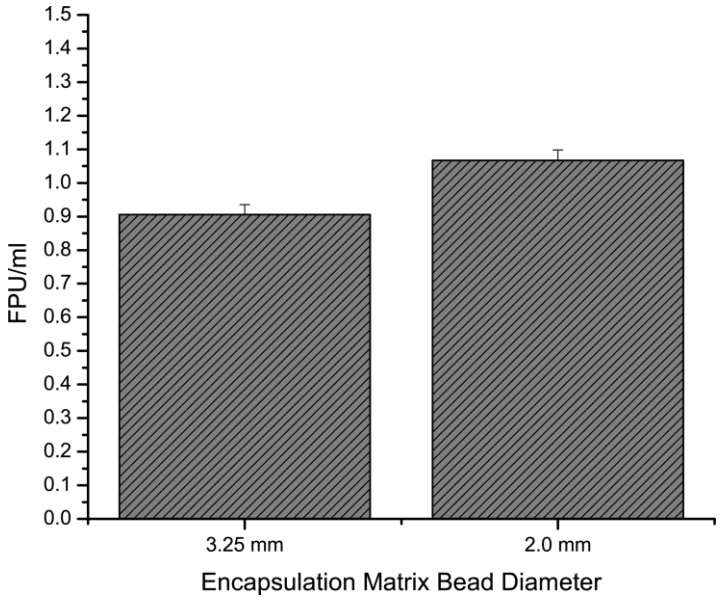

Figure 1.3. The Effect of Decreasing the Encapsulation Matrix Bead Diameter on Cellulase Activity.

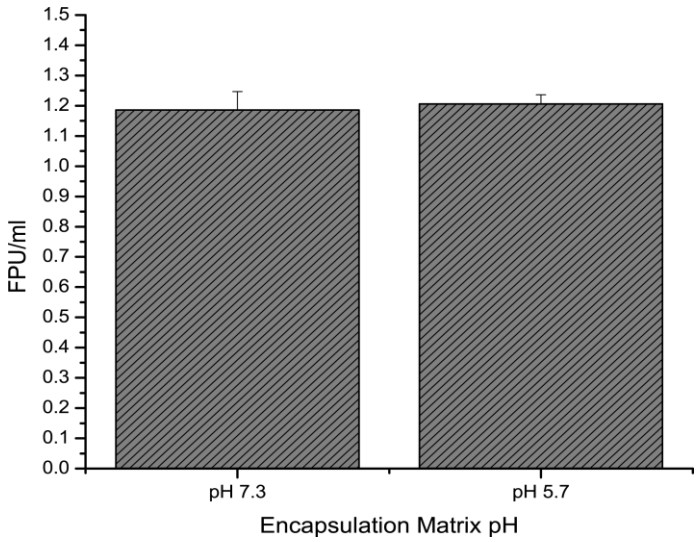

Figure 1.4. The Effect of Lowering the pH of the Sodium Alginate Solution on Cellulase Activity.

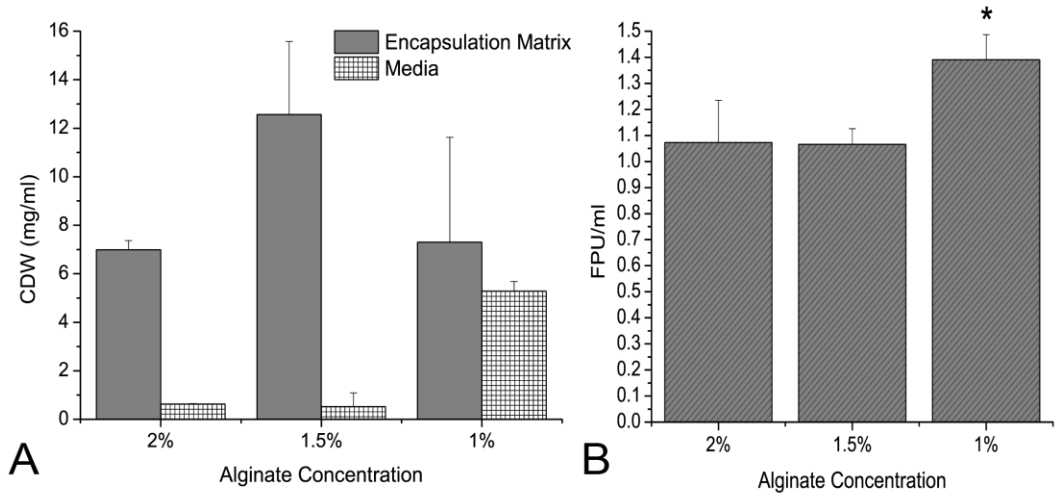

Figure 1.5. The Effect of Alginate Concentration: A) Biomass Retention of the Calcium Alginate Hydro-Gel, and B) Filter Paper Activity. The Increased FP Activity of the 1% Alginate Encapsulation Matrix Is a Result of Increased Mycelia Growth Outside the Encapsulation Matrix. Asterisk Indicates a Significant Difference (Alpha = 0.05).

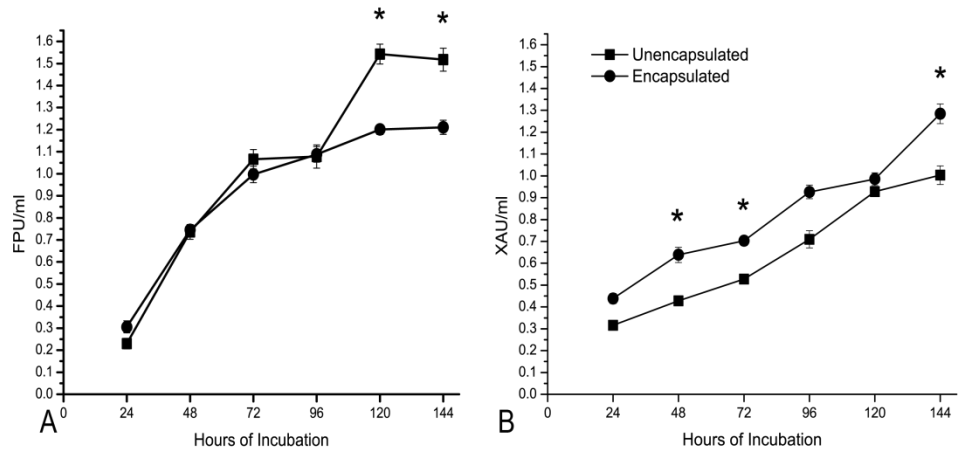

Figure 1.6. Time Course Measurement: A) Cellulase Activity, and B) Xylanase Activity for *T. reesei* Growing on Avicel Under Optimized Reactor Conditions. Repeated-Measures ANOVA Indicated That There Was No Significant Difference in Cellulase Activity Over 144 hours (n=18, P= 0.07), but Reveal Significantly Higher Xylanase Activity from the Encapsulated Treatments (n= 12, P= 0.001). Asterisks Denote Significant Changes During Pairwise Comparisons (alpha = 0.05).

Table 1.1. A Comparison of Bioreactor Productivity Parameters for 144- and 72-Hour Reactors.

Fed-Batch Regime	Reactor Productivity			
Unencapsulated Treatment	*Total Hours of Operation*	*Total FPU Produced*	*FPU / liter hour^{-1}*	*FPU/g Avicel*
72 Hours Batches (X 4)	288	354.33	19.68	141.7
Encapsulated Treatments				
72 Hours Batches (X 4)	288	321.9	17.88	128.7
144 Hours Batches (X 3)	432	250.6	9.14	83.5

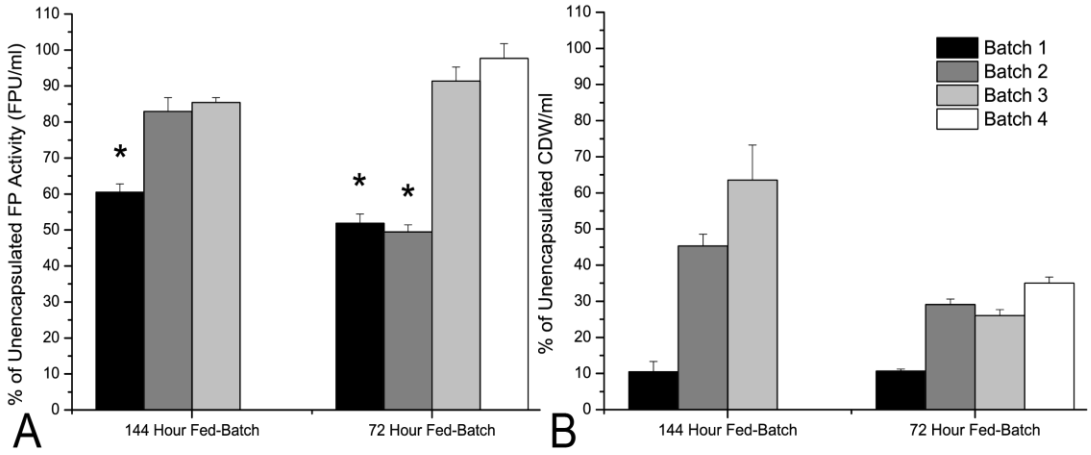

Figure 1.7. The Effect of Varying Encapsulated Fed-Batch Reactor Cycle Length. Fed-Batch Runs (144 hour and 72 hour) Were Evaluated: A) FP Activity and B) CDW Accumulation in the Media. Measurements Are Expressed as a Percentage of the Most Productive Unencapsulated 72-Hour Batch Run (Batch 3). Asterisks Indicate a Significant Difference from the 72Hour Batch Unencapsulated Comparison.

Table 1.2. HPLC Analysis of Sugars Released After Using a Crude Enzyme Cocktail Taken at the Twelfth Hour of the Third Consecutive 72-Hour Fed-Batch Reactor Run.

Crude Enzyme Source	Hydrolysis Product (g / l)							
	Glucose	±	*Cellobiose*	±	*Galactose*	±	*Unidentified*	±
Unencapsulated Nursery	1.49	0.06	1.14	0.13	1.75	0.11	0.37	0.05
Encapsulated Nursery	3.25	0.02	0.70	0.06	0.30	0.11	0.84	0.14

CHAPTER 2: OPTIMIZATION OF A NOVEL TWO-STAGE FED-BATCH LIGNOCELLULOSE HYDROLYSIS REACTOR

Abstract

Whole-cell encapsulation allows for efficient enzyme production while simplifying enzyme-cell separation. We have optimized operational parameters at both bench and pilot scales for a novel two-stage fed-batch lingocellulose hydrolyisis bioreactor consisting of a nursery reactor and hydrolysis reactor. This design spatially separates enzyme production and lignocellulose hydrolysis, allowing for simultaneous process optimization and operation for extended time periods. Nursery bioreactors were inoculated with either calcium alginate encapsulated or unencapsulated *Trichoderma reesei* RUT-C30 and monitored for enzyme production using purified cellulose (Avicel) as the growth and enzyme induction substrate. Encapsulated *T. reesei* facilitated biomass-enzyme separation. Nursery reactor fed-batch intervals and volumes were varied to optimize hydrolytic enzyme production. Enzymes from the nursery reactor were used to optimize hydrolysis reactors containing Avicel or ball-mill pretreated sawdust as the hydrolysis substrate. The integrated two-stage design was tested at the bench (250 ml) and pilot (70 L) scales. Lastly, hydrolysis reactor products (e.g., soluble sugars) were tested as enzyme inducers for continual operation of the nursery reactor. Encapsulated enzyme production was significantly higher by 25% over unencapsulated nursery reactors during the 4th batch of consecutive 72-hour batch runs. Reducing sugar concentrations of Avicel hydrolysis reactors containing crude enzymes from encapsulated nursery reactors

were similar to or higher than reactors containing unencapsulated nursery enzymes. Sawdust hydrolysis activity was similar for encapsulated and unencapsulated treatments. Encapsulated hydrolysis reactors contained half the cellobiose and approximately twice the glucose of unencapsulated treatments. Xylanase production of encapsulated nursery reactors was two times greater than their unencapsulated counterparts. Enzyme activity of the pilot scale nursery reactor was lower than bench-top reactors, however sugar production was only slightly lower (5.5%) than that observed in bench scale reactors (P= 0.018). Effluent from enzymatic hydrolysis of both ball-milled sawdust and Avicel produced higher enzyme activity per gram of carbon than 1% Avicel in the nursery reactor. Specifically, sawdust hydrolysate produced approximately 80% more cellulase activity and 67% more xylanase activity in the nursery reactor while Avicel hydrolysate produced 27.5% and 15% more cellulase and xylanase activity, respectively, than unhydrolyzed Avicel powder. Preliminary economic analyses suggest that feeding the nursery reactor sawdust hydrolysate resulted in the least expensive cellulase and xylanase production.

Introduction

Improving the economics of lignocellulose saccharification processes has new importance in light of revisions made to the U.S. Renewable Fuel Standards (RFS2) in 2007 that require lignocellulose to play an increasingly dominant role in the liquid bio-fuels arena (EPA 2010a). Biochemical conversion of lignocellulose to sugar for the production of alcohol-based liquid biofuels and other economically relevant bio-molecules has gained appreciable interest due to its lower energy requirements and environmental impact (i.e. reactions occur at mild pressure, temperature, and pH)

compared to thermo-chemical approaches (Dwivedi, Alavalapati et al. 2009; Jones 2009; Piccolo and Bezzo 2009). Although recent advances in biocatalyst synthesis have dramatically reduced the cost of enzymatic saccharification, lignocellulosic biofuels are still economically infeasible at large scales (Wyman 2007; Dwivedi, Alavalapati et al. 2009; EERE 2010; Somma, Lobkowicz et al. 2010). Therefore, additional advancements in biochemical conversion strategies are necessary to reach current lignocellulosic biofuel production goals.

The majority of commercial systems, employing enzymatic lignocellulose hydrolysis for liquid biofuels production, purchase saccharification enzymes from a third party (Humbird and Aden 2009; Banerjee, Mudliar et al. 2010; Kazi, Fortman et al. 2010; Humbird, Davis, et al. 2011), accounting for $0.50 - $0.69/gallon of end product (assuming $1.60/gallon of finished product) (EERE 2010; Kazi, Fortman et al. 2010; Humbird, Davis et al. 2011). Furthermore, sensitivity analyses indicate that, along with feedstock costs, fluctuations in enzyme prices can greatly contribute to the cost of lignocellulosic biofuels. Adding to expenses associated with hydrolytic enzyme purchases is the fact that cellulase and xylanase can permanently adsorb to the solid substrate and lose catalytic activity over time (Singh, Kumar et al. 1991; Lu, Yang et al. 2002; Lynd, Weimer et al. 2002; Tu, Pan et al. 2009), making enzyme recovery and reuse expensive and impractical.

Several recent techno-economic analyses have determined that combining enzyme production and hydrolysis within the same facility can lower the cost of enzymatic hydrolysis by minimizing costs associated with third-party purchases, preservation, transportation, and storage (Piccolo and Bezzo 2009). For example, a recent NREL report

predicts that on-site enzyme production can lower catalyst cost by 31% - 51% to $0.34/gal ethanol (EERE 2010; Kazi, Fortman et al. 2010; Humbird, Davis et al. 2011). Piccolo and Bezzo (2009) estimates on-site enzyme production could lower biofuel production costs by 18%, while Barta, Kovacs et al. (2010) estimates are more conservative at 2%. Furthermore, Iogen, a commercial provider of cellulase and xylanase has demonstrated the feasibility of on-site enzyme production at a 40-ton/day lignocellulosic ethanol plant (Lawford and Rousseau 2003) .

The current costly and energy intensive off-site production model endures because several hurdles make on-site enzyme production impractical. Commonly, to implement on-site enzyme production, additional equipment is required in the form of seed reactors and enzyme/biomass separation systems, costing 18.3-24 million dollars (MM) (Kazi, Fortman et al. 2010; Humbird, Davis, et al. 2011). Additionally, the need for a separate feedstock or enzyme inducing molecules, not only add to costs but also increases overall system complexity. For example, the 1999 NREL cellulosic ethanol production model for on-site enzyme production utilizes the same pre-treated feedstock stream for both enzyme production and saccharification (Wooley, Ruth et al. 1999). At first glance, the ability to use the same low-cost material for catalyst feedstock and hydrolysis substrate would seem to favor on-site enzyme production. Yet, solid substrates present many challenges, especially if periodic or continuous enzyme removal is desired. In addition to non-productive binding of enzymes (Juhász, Egyházi et al. 2005; Jørgensen, Kristensen et al. 2007), solid substrates also decrease bioreactor efficiency by increasing the energy needed for mixing and diminishing oxygen transfer for organism respiration (Szengyel, Zacchi et al. 1997). The revised 2010 NREL model, using

Trichoderma reesei as the on-site enzyme producing organism, describes using glucose as the initial feedstock for enzyme production. Although a soluble carbon source eliminates the non-productive binding of enzymes to the substrate, this approach requires an additional carbon input (i.e., glucose) from food crops. Furthermore, glucose is a cellulase inhibitor and must first be converted to sophorose, a cellulase expression inducer (Ilmén, Saloheimo et al. 1997; Lo and Ju 2009). This can be accomplished by treating glucose with *T. reesei* cellulase at 65^{o}C for three days (Mitchinson 2004). However, this step requires a 10% increase in cellulase production to support sophorose production (Barta, Kovacs et al. 2010; Kazi, Fortman et al. 2010) and increases energy inputs while adding additional steps and a higher potential for contamination. Over the past twenty years, innovative process design has been attributed to lowering the cost of biofuels (Wyman 2001; Humbird, Davis et al. 2011). Using this same line of reasoning, instituting a cultivation strategy that optimizes growth and extracellular enzyme production while minimizing the complexity of culture maintenance and enzyme recovery is needed to make on-site enzyme production a cost-efficient option. Although met with limited success, much of the current lignocellulosic biofuel research is focused on genetically modified organisms, thermo-tolerant species, and consolidating hydrolysis and alcohol production as a means to reduce cost. The approach described here focuses on optimizing reactor processes through enhanced control of biomass distribution, a strategy that has the potential to be used with current and future microorganisms.

Whole-cell encapsulation in a porous hydro-gel matrix such as calcium alginate is one approach used in other bioconversion industries (e.g., pharmaceuticals, municipal waste and food production) to optimize bioreactor parameters (Kokufuta, Yukishige et al.

1987; Steenson, Klaenhammer et al. 1987; Park and Chang 2000; Talebnia, Niklasson et al. 2005). Encapsulation of microorganisms allows for high-density populations to be maintained in a bioreactor for prolonged and efficient bio-synthesis (Park and Chang 2000; Kar, Mandal et al. 2008), while simultaneously simplifying separation of biomass from extracellular products (Kar, Mandal et al. 2008).

Trichoderma reesei strain RUT C-30 produces large quantities of extracellular cellulases and xylanases and are commonly found in commercially available enzyme preparations (Nevalainen, Suominen et al. 1994; Mach and Zeilinger 2003; Kar, Mandal et al. 2008; Stricker, Mach et al. 2008; Ahamed and Vermette 2009). The highest titer of hydrolytic enzymes are produced during the stationary growth phase (Pakula 2005), suggesting that maintenance of high density stationary phase cultures is necessary for continuous cellulase and xylanase production. However, stationary phase cultures of *T. reesei* commonly support large amounts of hyphal biomass that complicate separation and purification of extracellular hydrolytic enzymes (Weber and Agblevor 2005; Patel, Choy et al. 2009).

To address the challenges associated with integrating on-site enzyme production for lignocellulosic biofuel production, I have optimized operational parameters for a novel two-stage, semi-continuous bio-reactor. This design segregates on-site enzyme production and hydrolysis activities to separate reactors, allowing for simultaneous process optimization and a semi-continuous fed-batch operation of both reactors for extended time periods. I investigated the utility of several soluble carbon sources including hydrolysis reactor effluent to maintain nursery culture growth and enzyme production. I demonstrated that the novel two-stage reactor design produces enzymes

with consistently high levels of activity, shortened enzyme production times, and enhanced ease of catalyst separation. Utilization of a single feedstock for enzyme and fermentable sugar production increased hydrolysis throughput, diminished overall system complexity, and reduced the number/size of bioreactors and their associated capital costs.

Materials and Methods

Strain and Media Preparation

Trichoderma reesei RUT-C30 (NRRL 11460, ATCC 56765) used in this study was obtained with permission from the Department of Agriculture's ARS Culture Collection. Composition of the culture medium used for liquid cultures and agar plates was as follows (amounts for each ingredient are indicated in per liter values): KH_2PO_4 (2g), $MgSO_4 \cdot 2H_2O$ (0.3g), $CaCl2 \cdot 2H_2O$ (0.3g), $FeSO_4 \cdot 7H_2O$ (0.005g), $MnSO_4 \cdot 4H_2O$ (0.0016g), $ZnSO_4 \cdot 7H_2O$ (0.0014g), $CoCl \cdot 6H_2O$ (0.002g), $(NH_4)_2SO_4$ (1.4g), Urea (0.3g), Peptone (1.0 g), Tween 80 (2 ml). This media was filter sterilized and added to sterile carbon substrates. Minimal media was supplemented with Avicel PH-101 (sigma 11365) (10.0g/L) for 2% agar plates and enzyme production experiments.

Subculture Preparation

T. reesei was grown on 2% agar plates at 30^oC containing 1% Avicel for 3-4 days and stored at 30^o C for up to 3 weeks. Plugs from these plates were used as an innoculum for each experiment by aseptically introducing the agar plug into the media described above. Subcultures with a working volume of 200 ml were incubated in 250 ml Erlenmeyer flasks at 30° C on a 200 RPM orbital shaker for four days. Subcultures were concentrated to 40 ml by centrifugation at 7000 RPM for 5 min. After centrifugation, the

fungal pellet was rinsed in sterile basal media three times, centrifuging as described above after each rinse.

Hydro-Gel Preparation

Calcium alginate bead production was performed using 2% sodium alginate solutions. Sodium alginate solutions for hydro-gel encapsulation of *T. reesei* were produced using the following method: 6 g of Na-alginate (Fisher Scientific NC9676930) was slowly added to 286 ml of 100° C continuously stirred distilled water. The solution was allowed to cool to room temperature and stirred until the Na-alginate dissolved. pH of the alginate solution was adjusted to 5.7 using approximately 0.5 ml 1N HCl. Distilled water was added to bring the total volume to 288 ml before autoclaving. Twelve ml of subculture concentrate was added to the sterilized and cooled Na-alginate solution. The hydro-gel was then polymerized by adding the solution drop-wise to a continuously stirred solution of 0.2 M $CaCl_2$ at 4° C using a peristaltic pump and a 27½ Ga. needle. Hydro-gel beads were rinsed three times with an equal volume of basal media to remove excess $CaCl_2$ and used immediately for experiments.

Culture Conditions

Nursery Reactor Experiments

Enzyme synthesis experiments consisted of 62.5 ml of media and 20 g of calcium alginate beads. Unencapsulated treatments were prepared using 62 ml media and 0.5 ml subculture concentrate. A half milliliter of subculture concentrate and 20 g of *T. reesei* laden calcium alginate beads contained the same amount of *T. reesei* biomass, thus normalizing the initial biomass across encapsulated and unencapsulated treatments. Both treatments were prepared in 250 ml Erlyenmeyer flasks and incubated on an orbital

shaker at 400 RPM. Carbon substrates evaluated included purified cellulose (Avicel PH-101), lactose, lodge pole pine sawdust ball-milled to <100 mesh size, concentrated acid hydrolysate of sawdust, and enzymatic hydolysate of Avicel or sawdust. All carbon substrate evaluations were carried out using unencapsulated *T. reesei* at 1% w/v or 1% v/v carbon concentrations except where noted. Nursery reactor runs consisted of up to four 72 hour batches. After each batch, the crude enzyme liquor was separated from the organism and retained for hydrolysis experiments. Nursery flasks were replenished with additional carbon substrate media after removal of the enzyme-laden supernatant. For encapsulated treatments, the enzyme cocktail was simply drawn off using a serological pipette. To separate unencapsulated *T. reesei* from the supernatant, treatments were transferred to 50 ml tubes and centrifuged at 5000 RPM for 10 minutes.

Hydrolysis Reactor Experiments

Fifty ml centrifuge tubes containing 35 ml of nursery reactor effluent, 5 ml citrate buffer (pH 4) and 0.4g Avicel or ball-milled sawdust were incubated on a 200 RPM orbital shaker at 50^{o}C for 72 hours. After the 72-hour incubation samples were collected and a fraction was preserved for HPLC analysis by boiling for 10 minutes to stop enzymatic activity and stored at -20^{o} C.

Pilot Reactor Experiments

The pilot reactor consisted of two 52 liter stirred tank reactors. Nursery and hydrolysis reactions were carried out simultaneously. Nursery incubations were scaled to 46 liters. At the end of 72 hours, nursery effluent was transferred to the hydrolysis reactor and the nursery was replenished with fresh 1% Avicel media. Hydrolysis reactions were

scaled to 38 liters and were incubated for 72 hours in 1% Avicel at a pH of 4.8 and a temperature of 50^{o} C.

Cellulase Activity Measurements

Cellulase activity was determined using the standard colorimetric Filter Paper Assay adapted for micro-plate use (Xiao, Storms et al. 2004). Briefly, an aliquot of enzyme preparation was added to a defined mass of known quality cellulose paper and incubated at 50°C in 0.2M citrate buffer at pH 4 for one hour. After incubation, DNS reagent (Xiao, Storms et al. 2004) was added and samples were incubated at 95°C for 5 minutes. Absorbance was measured at 540 nm, reducing sugars released were determined by comparison to a glucose standard, and enzyme activity reported as Filter Paper Units (FPU)/ml. One FPU is defined as the amount of enzyme that will liberate 1 µmol of glucose equivalents from the filter paper in 1 minute. Enzyme activity is expressed as glucose equivalents because this assay detects, not just glucose, but all reducing carbohydrates.

Xylanase Activity Measurement

Xylanase activity measurements were determined using a colormetric assay similar to the cellulase filter paper assay. As described by Bailey (1992), an aliquot of enzyme preparation was added to a 1% xylan solution (pH 5.8) and incubated at 50°C for 5 minutes. After incubation, to stop the reaction and to develop the color, DNS reagent (Xiao, Storms et al. 2004) was added and samples were boiled for 20 minutes. Fifty microliters of reaction was added to a 96-well microplate containing 150 µl distilled water. Absorbance was measured at 540 nm, reducing sugars released were determined by comparison to a xylose standard, and enzyme activity reported as Xylanase Activity

Units (XAU)/ml. One XAU is defined as the amount of enzyme that will liberate 1 µmol of xylan equivalents in 1 minute.

Biomass Determinations

With the exception of yeast, micro-fungi are not comprised of discrete cells making conventional cell counting methods impractical. Total biomass levels were determined using a standard Bradford (coommasie blue) assay kit (Thermo-Fisher 23200) for total soluble protein. Aliquots were taken from unencapsulated treatments and hyphal pellets were collected by centrifugation at 5000 RPM for 5 minutes. Hyphal pellets were washed in sterile minimal media three times, centrifuging after each wash as described above. Fungal hyphae were lysed by adding a 0.1 M NaOH to the pellet and incubating at 4° C overnight. One milliliter of pellet solution was added to 0.5 g silicon beads (BioSpec Products Cat. #11079101Z) and subjected to bead beating using a Fastprep FP 120 (MP Biomedicals, Solon, OH) at a setting of 6.5 for 45 seconds. Samples were put on ice for 5 minutes between each round of bead beating. Bead beating was repeated 3 times and followed by a 20-minute incubation in a 100^{o} C water bath. Biomass from encapsulated treatments was determined by dissolving the Ca-alginate matrix in a 0.1M Na-citrate buffer. The liberated mycelia mass was then lysed using the procedure described for unencapsulated treatments. The resulting soluble proteins were measured colorimetrically by adding 250 µl of commassie blue reagent at room temperature to 5 µl of prepared sample. After a 10-minute incubation in the dark at room temperature, absorbance at 540 nm was measured. Total protein levels were converted to estimates of Cell Dry Weight (CDW) as describe previously (Zhang, Lo et al. 2009).

Quantities of culture media proteins (e.g. extra-cellular proteins) were determined by using the initial supernatant from the CDW pellet directly with the Bradford method.

Results and Discussion

Integrated Reactor Design Fed-Batch Cycle Optimization

An advantageous integrated enzyme production and hydrolysis reactor system must simplify the process of enzyme/organism separation while allowing for continuous or semi-continuous reactor operation. Biomass-enzyme separation in our experiments was achieved quickly and efficiently by merely stopping agitation of the reactor. Within seconds, the alginate-encapsulated mycelium settles to the bottom of the reactor and then the enzyme-laden supernatant can simply be decanted or pumped from the reactor. Moreover, we have successfully reused the alginate-encapsulated fungus for multiple reactor runs lasting up to three weeks in duration.

Optimization of Nursery Reactor Fed-Batch Intervals

Initial nursery fed-batch experiments were counducted at 144-hour batch intervals. Three successive incubations of encapsulated *T. reesei* produced increasingly higher levels of filter paper activity. Cellulase activity increased from 1.04 FPU/ml during the initial run to 1.46 FPU/ml and 1.51 FPU/ml at the end of two subsequent runs. However, cell growth accumulation in the supernatant approached unencapsulated levels by the end of the third incubation (Figure 2.1a and Figure 2.1b). During the 144-hour reactor runs, approximately 91% of total enzyme activity was achieved by 72 hours. Therefore, shorter 72-hour nursery fed-batch reactions were performed and evaluated for enzyme activity and supernatant cell growth. Although initial cellulase activity of the encapsulated treatment was approximately 20 - 40% lower than its unencapsulated

control, by the third batch similar cellulase activity (1.61 FPU/ml) was observed in both reactors (P= 0.97) (Figure 2.1c). At the end of the fourth batch, encapsulated reactor cellulase activity (1.76 FPU/ml) was significantly higher (P= 0.03) than unencapsulated reactors (1.30 FPU/ml).

Shorter batches also limited cell growth outside the encapsulation matrix. Over the course of four 72-hour batch experiments free biomass in the encapsulated reactor did not exceed 2.4 mg CDW/ml. This concentration of biomass was 65% less than observed in unencapsulated reactors (Figure 2.1d) and 23% less than encapsulated 144-hour batch reactors during the same time period.

Optimization of Nursery Reactor Volume

Nursery reactor configuration was modulated to examine how surface area to volume ratios between the air-media interface affect bioreactor productivity. Reactors with surface area to volume ratios (mm^2:ml) of 19.47 and 53.45 were evaluated. Prior work by our group determined that these ratios influence *T. reesei* enzyme production rates (Deis, Bala et al. 2011). Preliminary nursery reactor experiments consisting of one 144-hour run revealed that at 19.47 mm^2:ml, the unencapsulated reactor yielded 0.70 FPU/ml and 0.56 FPU/ml for the encapsulated reactor. Increasing the ratio to 53.45 mm^2:ml doubled FP activity for both unencapsulated and encapsulated treatments (1.36 FPU/ml and 1.12 FPU/ml, respectively). Additional experiments were conducted to assess if this pattern held true for multiple short reactor runs (i.e., four 72-hour batches) when the *T. reesei* innoculum was being reused. Reactor volume had no significant effect on cellulase activity over the course of 4 batches for both unencapslated (P= 0.78) and encapsulated treatments (P= 0.08) (Table 2.1). Additionally, there was no significant

difference in overall reactor productivity (FPU/L˙hour) between unencapsulated and encapsulated at 53.45 mm^2:ml (P= 0.035). It is noteworthy that the 19.47 mm^2:ml unencapsulated reactor reached a peak activity of 1.52 FPU/ml at the end of the 2^{nd} cycle and 1.76 FPU/ml at the end of the 3^{rd} cycle of reactions while encapsulated cellulase activity continued to increase over the entire run at both volumes. Although 53.45 mm^2:ml reactors had higher mean productivity, crude enzymes from the larger 19.47 mm^2:ml nursery reactors were used to ensure that there was enough effluent to evaluate performance of purified cellulose (Avicel) and ball-milled sawdust hydrolysis reactors.

Evaluation of Purified Cellulose and Ball-Milled Sawdust as C-Substrates for Growth and Cellulase/Xylanase Production in the Hydrolysis Reactor

Reducing sugars released by crude enzymes from encapsulated nursery reactors were similar to or higher than reactors containing unencapsulated nursery enzymes despite lower nursery cellulase activity (Figure 2.2a) when the hydrolysis reactors contained Avicel. Although reducing sugars released from ball-milled sawdust in hydrolysis reactions containing enzymes from encapsulated nursery reactors trended lower for each batch, hydrolysis activity was only significantly different between encapsulated and unencapsulated effluent fed reactors during the first batch (Figure 2.2b).

To determine sugar composition of the hydrolysis reactor output, HPLC analysis was performed on 12-hour samples from the third hydrolysis batch. Interestingly, similar sugar composition trends between encapsulated and unencapsulated hydrolysis reactors were observed regardless of substrate. For example, both Avicel and sawdust, encapsulated hydrolysis reactors contained half the cellobiose (glucose dimers) and approximately twice the glucose of unencapsulated treatments (Table 2.2). Additionally,

though not optimized for xylanase production, encapsulated nursery enzymes produced twice the xylose of their unencapsulated counterparts.

Increased output of easily fermentable monosacchrides (i.e., glucose and xylose) may have been caused by the low levels of fungal cells detected in the encapsulated nursery reactor supernatant. Cellulose is hydrolyzed into cellobiose by the action of endo- and exo-gluconases (i.e., cellulases) (Jørgensen, Kristensen et al. 2007). Cellobiose is then converted to glucose monomers by the hydrolytic activity of ß-glycosidase (Jørgensen, Kristensen et al. 2007). Xylose is hydrolyzed by xylanases in the same manner. In the absence of ß-glycosidase, cellobiose can accumulate and act as an inhibitor of cellulose activity (Bezerra and Dias 2004; Bezerra and Dias 2005; Andrić, Meyer et al. 2010b). In fact, a 60% reduction in cellulase activity can be observed at a cellobiose concentration of 6 g/L (Nag 2008). Similarly, the unencapsulated effluent fed Avicel hydrolysis reactor (1.14 g cellobiose/L) produced nearly 38% less reducing sugars than the encapsulated fed hydrolysis reactor. The lower cellobiose concentrations of the sawdust hydrolysis reactors may exhibit less inhibition and in turn account for the relative difference in encapsulated reactor performance compared to unencapsulated (i.e., higher hydrolysis rates for Avicel and similar rates for sawdust).

T. reesei ß-glycosidase is, for the most part, an intracellular enzyme and its activity is considered to be the rate-limiting step of cellulose hydrolysis (Slade, Bauen et al. 2009). We hypothesized that the increased temperature from 30° C in the encapsulated nursery reactor to the optimal saccharification temperature of 50° C in the hydrolysis reactor would facilitate autolysis of any transferred *T. reesei* hyphae and subsequent ß-glycosidase release. This would, in turn, result in more conversion of cellobiose to

monosaccharides, which can be fermented to liquid biofuels. CDW analysis of the hydrolysis reactor cell pellets revealed lower biomass density at the end of 72 hours, suggesting a loss of soluble proteins through cell lysis, supporting this hypothesis.

Pilot Reactor Scale-Up

To evaluate performance of the integrated reactor design at larger volumes, 72-hour fed-batch reactions were scaled up by three orders of magnitude using an integrated two-stage stirred-tank pilot reactor (Figure 1.3). The nursery reactor (left), with a working volume of 46 liters, and hydrolysis reactor (right), with a working volume of 38 liters were controlled for pH and temperature. Crude enzyme liquor from the nursery reactor was pumped aseptically into the hydrolysis reactor leaving the encapsulated *T. reesei* behind. Fresh media and Avicel were added to the nursery at the beginning of each batch.

Nursery reactor agitation was initially set at 1400 RPM. At the end of the first 72-hour batch, cellulase activity (0.2 FPU/ml) was 6 times lower than the bench top experiments while xylanase activity (1.03 XAU/ml) was similar to levels observed in the bench top-scale reactors (Figure 2.4). Since high sheer stress from aggressive agitation can significantly lower cellulase production (Lejeune and Baron 1995; Ahamed and Vermette 2010) and activity (Gunjikar, Sawant et al. 2001; Jørgensen, Kristensen et al. 2007; Ahamed and Vermette 2010), agitation was reduced to 700 RPM for the second batch. At 700 RPM, specific cellulase activity (FPU/g extracellular protein) decreased slightly but yielded the highest cellulase production and activity (0.42 FPU/ml). Absolute xylanase activity (XAU/ml) and specific activity (XAU/mg extracellular protein) dropped 38% and 77%, respectively. Further reduction of agitation in the 3rd batch resulted in the

lowest reactor productivity for both enzymes. Agitation in the nursery reactor greatly affects enzyme production and activity (Lejeune and Baron 1995; Patel, Choy et al. 2009; Ahamed and Vermette 2010). The close similarity of specific cellulase activity at 1400 and 700 RPM suggests that the higher agitation rate was affecting cellulase production and not enzyme activity (i.e., enzyme deactivation). The higher xylanase activity at 1400 RPM agree with Lejeune and Baron (1995) who observed that xylanase production increases in relation to cellulase production at higher agitations when Avicel is used as the carbon source. Other factors could have potentially contributed to higher xylanase production at 1400 RPM as well. During a comparison of 500 ml shake flasks and 10 L stirred tank reactors, Antonio Rocha-Valadez, Estrada et al. (2006) found that production of 6-pentyl-α-pyrone by *Trichoderma harzianum* occurred earlier in the stirred tank reactor than in the shake flasks, suggesting that hydro-mechanical forces influence expression characteristics of secondary metabolites. It is possible that a similar process is responsible for the increased xylanase production at 1400 RPM in our system. Lastly, xylanase synthesis is favored at a pH of 6 while cellulase production increases at a pH of 5. The pH of the first batch ranged from 5.0 to 5.69 where the 2nd batch ranged from 5.0 to 3.98. Furthermore, the drop in total enzyme activity at 350 RPM suggests that aeration was limiting enzyme production. These results underscore the important role agitation and other environmental factors have on reactor productivity characteristics.

Overall fungal density reached levels seen in bench-top nursery reactors, though a larger fraction was detected in the culture media. This is not surprising as the beads showed signs of degradation at the end of batch 2, likely due to high mechanical shear forces during the first nursery reactor run. The increased fungal biomass transferred to the

hydrolysis reactor did not exhibit growth nor did it seem to negatively impact the saccharification step.

Despite lower nursery productivity in the 46 L reactors relative to the bench-top scale, hydrolysis activity was not greatly attenuated. A majority of the hydrolysis occurred within the first 36 hours during batch 2, similar to bench-top experiments (Figure 2.5). Additionally, sugar production was only 5.5% less than that observed in flask reactors (P= 0.018). The relatively similar activity of the hydrolysis reactor at both large and small scales may be due the non-linear relationship between enzyme loading and hydrolytic activity (i.e., diminishing returns at higher enzyme loading) (Wyman, Grohmann, Lastik 1986; Kim, Irwin et al. 1998; Bezerra and Dias 2004; Zhang, Su et al. 2010). Although the pilot scale nursery reactor enzyme output was 65% lower than the bench-top scale at the end of batch 2, the hydrolysis reactor was being dosed with 42 FPU/g cellulose, twice the amount determined sufficient in the NREL saccharification model (Kazi, Fortman et al. 2010). This observation is in agreement with finding from other investigations that suggest increasing enzyme concentration beyond 35-50 FPU does not substantially improve hydrolysis yield (Wyman, Grohmann, Lastik 1986; Zhang, Su et al. 2010).

Nursery Carbon Source Evaluation

Encapsulated *T. reesei* produces significant quantities of lignocellulose hydrolyzing enzymes when grown on purified cellulose (Avicel) and on less expensive, and therefore, more industrially relevant feedstocks. Several soluble carbon sources have been identified as inducers of cellulose, such as cellobiose, lactose, and sophorose. However, these inducers are either not as potent as cellulose (e.g., lactose) or are very

expensive (e.g., sophorose). Lo, Zhang et al. (2005) demonstrated that hydrolysate from concentrated acid treated lignocellulose induced cellulase production. Here I evaluated several feedstocks for their potential as growth and cellulolytic enzyme production substrates for *T. reesei* in the bench-scale nursery reactors including effluent from the hydrolysis reactor. Cellulase induction results for lactose and acid hydrolyzed sawdust were similar to results reported in the literature but less than Avicel. Interestingly, effluent from enzymatic hydrolysis of both ball-milled sawdust (0.11% reducing sugars) and Avicel (0.57% reducing sugars) produced activity (FPU/g carbon substrate) higher (80% and 27.5% respectively) than 1% Avicel (Table 2.3). These high levels of enzyme activity:carbon substrate ratios were also observed for xylanase activity. Specifically, the sawdust and Avicel hydrolysate produced approximately 67% and 15% more xylanase per gram of carbon than Avicel treatments. Our analysis also examined substrate cost. Based on a sawdust price of $50/ton, sawdust hydrolysate produced the least expensive enzymes. It is important to note that expenses associated with feedstock processing (e.g., ball-milling, acid neutralization, and sophorose conversion) were not taken into account and would attribute to enzyme production cost. Due to the nature of the integrated reactor design, hydrolysis effluent used for maintaining the nursery would require minimal if any processing. Results of these studies suggest that using a fraction of the hydrolysis reactor output to sustain growth and enzyme production in the nursery reactor is a simple and cost-effective method for prolonged and continuous enzyme production.

Economics of an Encapsulated Nursery Reactor vs. NREL Base Case

An economic-sensitivity analysis was conducted to determine how reactor and encapsulation parameters affect enzyme production costs at a commercial scale over 10

years. The intent of this evaluation was not to determine feasibility of the currently proposed encapsulated reactor system as much as it was to identify critical areas of focus that could most influence cost reduction during commercial-scale development of this enzyme production approach.

Enzyme production costs were calculated as kg extracellular protein/initial equipment cost + encapsulation reagents. To evaluate the affect of reaction characteristics on enzyme costs, several assumptions were used to provide a straightforward and simplified model for comparison. One major assumption used in this model is that the large, commercial-scale encapsulated reactor design is in an n^{th} plant (i.e., optimized reactor parameters) and enzyme production performance is similar to the NREL base case (Humbird, Davis et al. 2011). Considering the lack of general information on encapsulation equipment prices, $150,000 was used for hydro-gel production equipment and consumables were estimated to be $0.30/kg of finished encapsulation matrix. Additional operational parameters of both NREL and encapsulation base models are listed in Table 2.4.

Equipment costs for the encapsulated nursery model ranged from $750,000 or 9% below the NREL base case when the enzyme production reactor size was kept the same This reduced cost was due to the elimination of multiple pre-enzyme production seed reactor trains needed to inoculate the shorter reactor runs of the NREL model. Increasing encapsulated reactor size to compensate for the liquid fraction displaced by the encapsulation matrix increased capital costs by $1.4 million or 16% over the NREL base case. Although these differences in costs may not have a dramatic affect on long term enzyme costs, they may be of importance when acquiring initial capital.

Alternative reactor scenarios were based upon a combination of our experimental data and published literature. For instance, the NREL model describes a 96-hour enzyme production period while my results reveal maximum enzyme production has occurred by 72 hours. Additionally, I found that reducing the sodium alginate solution from 2% to 1.5% has no significant affect on encapsulation matrix performance. Reducing the hydro-gel diameter has also been shown to increase mass-transfer and enzyme production while fluidized bed reactors may provide additional encapsulation matrix longevity. Although many of the encapsulated scenarios suggest higher annual enzyme yield and a cost benefit during the first year, there is an increase in enzyme cost over the remaining nine years that can be attributed to the cost of encapsulation matrix reagents. Reducing the amount of alginate needed (e.g., sodium alginate concentration and hydro-gel volume in the reactor) or increasing encapsulation matrix shelf-life had more impact on enzyme costs than did increased reactor volume or reactor productivity. Decreasing the encapsulation cost of a 390 kiloliter reactor producing 0.24 kg protein/l from $14,040 to $7,000 per run could lower the cost of enzyme production from $0.038/kg (NREL base case) to $0.037/kg resulting in a savings of $330,000 to $475,000 over 10 years depending on annual enzyme yields. This cost reduction could be realized by extending the encapsulation matrix shelf life to 45 days, reducing hydro-gel volume in the reactor to 11% or a combination of these two approaches.

Conclusion

On-site enzyme production is one possible strategy to lower the cost of lignocellulosic biofuels. We have established a whole-cell encapsulation approach that

enables both high levels of enzyme production, simplified catalyst recovery, and efficient hydrolysis for extended periods of time.

Decreasing the nursery reactor incubation period from 144 hours to 72 hours improved reactor productivity (i.e., volumetric cellulase activity and FPU/gram of Avicel) during fed-batch reactions. Encapsulated nursery reactors reached productivity levels (FPU/L·hour) similar to unencapsulated reactors when surface area:volume ratios were increased. Even when encapsulated nursery reactor productivity was lower than unencapsulated reactors, hydrolysis performance, for the most part, was similar or higher when encapsulated nursery enzymes were used. Furthermore, higher quality sugars (i.e., glucose and xylose) were being produced in these reactors. At pilot scale, the nursery reactor produced less enzymes though hydrolysis performance was only slightly affected.

Enzymatic hydrolysate of sawdust, when used as a carbon source in the nursery reactor, produced higher enzyme activity than other traditional, more expensive cellulase inducing substrates. These results serve to illustrate that an integrated on-site enzyme production and hydrolysis reactor system can be easily maintained and operate at high levels of productivity for extended periods of time. These results also suggest that this system has the potential to be successfully scaled to an industrially relevant size.

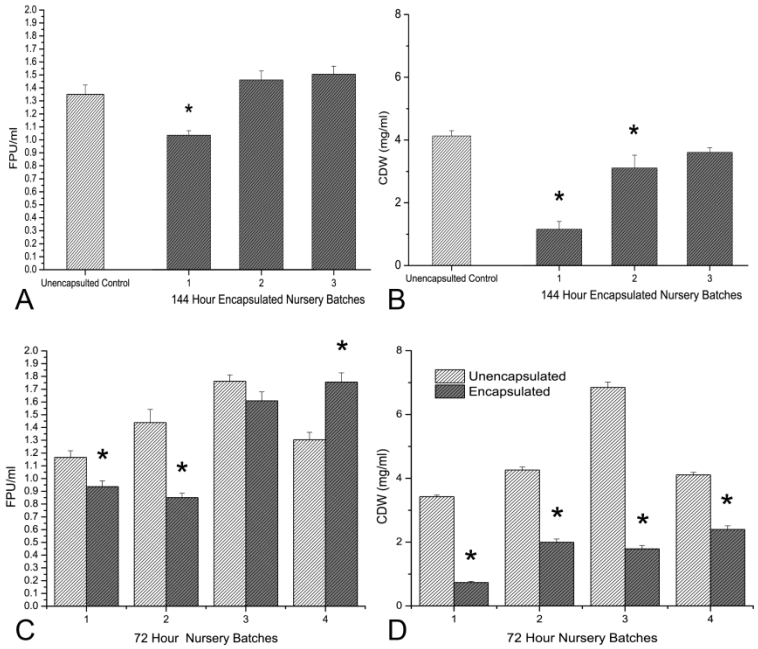

Figure 2.1. Filter Paper Activity and Unencapsulated Hyphal Growth as a Function of Fed-Batch Retention Time. A) FP Activity and B) Unencapsulated Mycelia Accumulation of 3 Successive 144 Hour Encapsulated Fed-Batch Runs Compared to a Single 144 Hour Unencapsulated Batch. C) Cellulase Activity and D) Unencapsulated Mycelial Accumulation in Encapsulated and Unencapsulated Reactors During Four Successive 72 Hour Fed-Batch Runs. * Denote Significant Difference in FP Activity When Compared to the Unencapsulated Treatment ($P<0.05$).

Table 2.1. The Effect of Reactor Surface Area:Volume Ratio on Nursery Reactor Productivity During Four 72 Hour Fed-Batch Runs. Values Represent the Mean ± Standard Deviation; n=3. * Denotes a Significant Difference Between Treatments (P<0.05).

Surface Area : Volume Ratio	FPU / liter hour^{-1}									
	Batch #									
53.45 mm^2:ml	**1**	**±**	**2**	**±**	**3**	**±**	**4**	**±**	**Total FPU / liter hour^{-1}**	**±**
Unencapsulated Reactor	16.20	2.47	19.98	4.95	24.48	2.37	18.11	2.77	19.69	3.14
Encapsulated Reactor	13.01	2.17	11.81	1.70	22.34	3.33	24.40	3.46	17.89	2.67
19.47 mm^2:ml										
Unencapsulated Reactor	15.39	2.16	21.07	5.85	20.60	4.32	19.86	2.61	19.23	3.74
Encapsulated Reactor	11.39	3.77	16.08	3.51	16.19	2.16	16.97	1.76	15.16 *	2.80

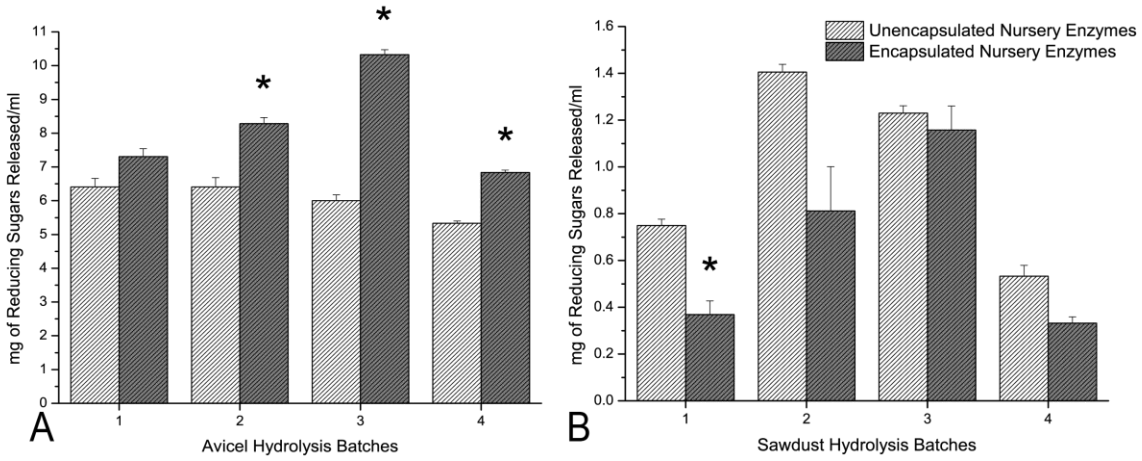

Figure 2.2. Reducing Sugar Production During 4 Successive Hydrolysis Batch Reactions Containing A) Avicel or B) Ball-Milled Sawdust. * Denotes a Significant Difference Between Treatments During the Same Batch (P<0.05).

Table 2.2. A Comparison of Hydrolysis Reactor Sugar Production with Avicel and Sawdust Substrates. Values Represent the Mean ± Standard Deviation; n=3.

Substrate	Crude Enzyme Source	Hydrolysis Product g / l									
Avicel		*Glucose*	±	*Cellobiose*	±	*Galactose*	±	*Xylose*	±	*Unidentified*	±
	Unencapsulated Nursery	1.49	0.06	1.14	0.13	0.18	0.11			0.37	0.05
	Encapsulated Nursery	3.25	0.02	0.70	0.60	0.30	0.11			0.84	0.14
Ball-Milled Sawdust											
	Unencapsulated Nursery	0.26	0.02	0.30	0.03			0.05	0	0.09	0.01
	Encapsulated Nursery	0.39	0.12	0.12	0.01			0.08	0.01	0.08	0.01

Figure 2.3. The Pilot Scale Integrated Nursery Reactor (left) and Hydrolysis Reactor (right) System.

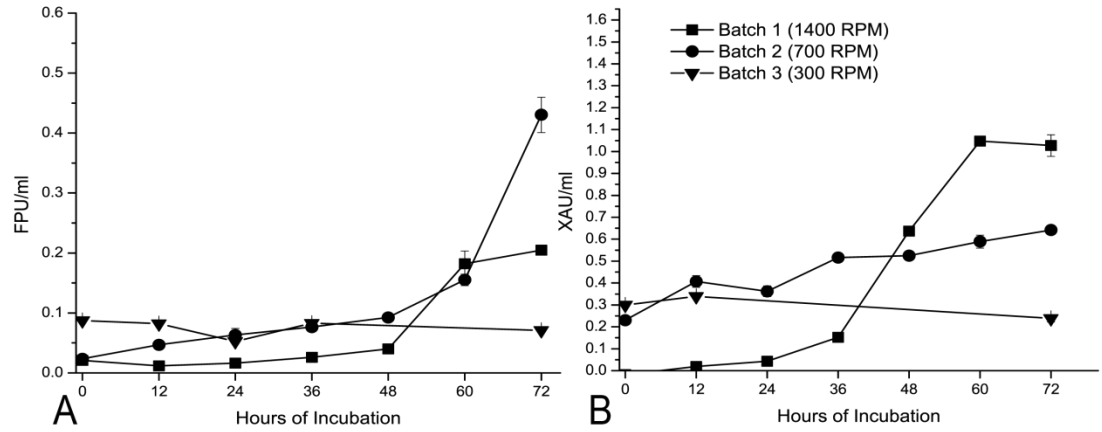

Figure 2.4. A Comparison of A) Cellulase and B) Xylanase Activity as a Function of Time During 3 Successive Pilot-Scale Fed-Batch Reactions.

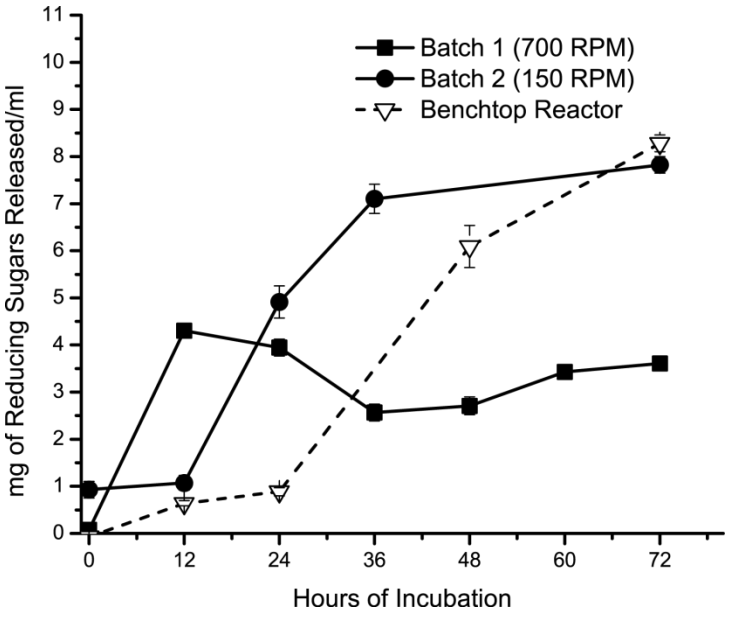

Figure 2.5. Reducing Sugar Production From Avicel as a Function of Time During Pilot-Scale and Bench-Top Scale Hydrolysis Reactions.

Table 2.3. Comparison of Cost and Cellulase Induction Properties of Various Carbon Substrates.

Carbon Substrate	Substrate Cost / g[1]	FPU/ liter	g of Substrate /100 FPU	Substrate Cost / 100 FPU
1% Avicel	$0.13	1400	0.714	$0.09
1% Lactose	$0.03	800	1.25	$0.04
1%[2] Sawdust Hydrolysate from Concentrated Acid Treatment	$0.01[3]	900	1.11	$0.01
0.57%[2] Avicel Hydrolysate from Enzymatic Treatment	$0.13	1100	0.518	$0.07
0.11%[2] Sawdust Hydrolysate from Enzymatic Treatment	< $0.01	760	0.144	< $0.01
Sophorose (Theoretical)[4]	$0.02	940	1.17	$0.02

[1] Prices (except sawdust) are from Fisher Scientific for relative comparison
[2] reducing sugar concentration
[3] Includes cost of sulfuric acid but not neutralization supplies
[4] Based on information from Genencor and NREL

Table 2.4. NREL and Encapsulated Nursery Reactor Base Case Models for Commercial Scale Enzyme Production.

	Pre-Production Seed reactors	Nursery Reactor	Retention Time /Production Stages	Extracellular Protein Production Rate
NREL Base Case	3 reactor X 4 trains	300 kiloliter reactors, 80% working volume x 9 reactors	1 reactor batch for 7 days • Growth - 24 Hours • Enzyme Production – 96 Hours • Draining/Cleaning 48 hours	0.2 kg/l
Encapsulation Base Case	3 reactor X 1 train	300 kiloliter reactors, 80% working volume. 25% of working volume consists of encapsulation matrix	4 reactor batches for 5-7 days • Growth - 24 Hours (Batch 1 Only) • Enzyme Production – 96 Hours • Draining 24 Hours (Batch 1-3) • Draining and Cleaning – 48 Hours (Batch 4 Only)	0.2 kg/l

Table 2.5. Economic Evaluation of Different Commercial Scale Encapsulated Nursery Reactor Scenarios.

Nursery Reactor Scenario	Nursery Reactor Scenario Description	Equipment Cost ($MM)	Liquid Volume/Reactor (kiloliters)	Hydro-gel Volume /Reactor(kiloliters)	Cost of Beads / Reactor	Number of Batches/Year	Enzyme Production (thousand kg/batch)	Year 1 Enzyme Cost/kg	Year 5 Enzyme Cost/kg	Year 10 Enzyme Cost/kg
	NREL Base Case	8.64	240			52	432	$0.385	$0.077	$0.038
S-1	Encapsulation Base Case	7.89	180	60	18000	66	324	$0.494	$0.199	$0.162
S-2	S-1 Modification: • 72 hr Enzyme production period (5 batches)	7.89	180	60	18000	83	324	$0.393	$0.158	$0.129
S-3	S-2 Modification: • Encapsulation matrix reduced to 15% working volume	7.89	204	36	10800	83	36.7	$0.312	$0.104	$0.079
S-4	S-1 Modification: • Working Volume increased by 30%	10.05	234	78	23400	83	421.2	$0.387	$0.157	$0.128
S-5	S-4 Modification: • Encapsulation matrix reduced to 15% working volume	10.05	265.2	46.8	14040	83	477.4	$0.306	$0.103	$0.078
S-6	S-4 Modification: • Encapsulation matrix shelf life extended by 30% (30 days ~ 7 batches)	10.05	234	78	23400	83	421.2	$0.360	$0.130	$0.101
S-7	S-6 Modification: • Encapsulation matrix reduced to 15% working volume	10.05	265.2	46.8	14040	83	477.4	$0.292	$0.089	$0.064
S-8	S-7 Modification: • 20% increased productivity		265.2	46.8	14040	83	572.8	$0.243	$0.074	$0.053
S-9	S-8 Modification: • 1.5% sodium alginate concentration	10.05	265.2	46.8	10530	83	572.8	$0.235	$0.066	$0.045

CONCLUSION

On-site enzyme production is one possible approach to improve the economic viability of lignocellulosic biofuels. To this end, we have developed a novel two-stage enzyme production and hydrolysis reactor. Encapsulating an industry relevant lignocellulosic enzyme producing organism allows for extended enzyme production, ease of catalyst recovery, and enhanced hydrolysis activity. Encapsulated enzyme production was similar to unencapsulated nursery reactors over 4 consecutive 72-hour batch runs. Reducing sugar concentrations of Avicel hydrolysis reactors containing crude enzymes from encapsulated nursery reactors were similar to, or higher than, reactors containing unencapsulated nursery enzymes. Furthermore, effluent from encapsulated treatments produced nearly two times more fermentable sugars from Avicel. Pilot-scale reactions yielded fewer enzymes in the nursery reactor though hydrolysis performance was only minimally affected. Enzymatic hydrolysate of sawdust when used as a carbon source in the nursery reactor produced higher enzyme activity than other traditional and more expensive cellulase inducing substrates.

These results serve to illustrate that an integrated on-site enzyme production and hydrolysis reactor system can be self-maintained and operate at high levels of productivity for extended periods of time. These results also suggest that this system has the potential to be successfully scaled to an industrially relevant size. An on-site biochemical hydrolysis approach, such as this one, that focuses on optimizing the spatial and environmental parameters of the bioreactor may play an increasingly relevant role in

the bio-transformation arena as continued advances in bio-prospecting and molecular biology provide industries with improved strains and more efficient enzymes.

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