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INVESTIGATING THE FEASIBILITY OF USING MESOPOROUS SILICA PARTICLES TO DELIVER GLUCOSE DURING ANAEROBIC FERMENTATION

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
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By

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ABSTRACT OF THESIS

INVESTIGATING THE FEASIBILITY OF USING MESOPOROUS SILICA PARTICLES TO DELIVER GLUCOSE DURING ANAEROBIC FERMENTATION

The study presented herein investigated a potential low-energy method to separate and concentrate glucose from a lignocellulosic hydrolysate. The motivation for this method was two-fold: 1) to provide the fermentation microorganism an optimal glucose concentration and 2) to supply a fermentation media free of inhibitory compounds. Two sizes of porous silica particles (with 7 nm and 2.3 nm pores) were synthesized and their ability to adsorb glucose from solution confirmed. Next, the ability of two different microorganisms, *Saccharomyces cerevisiae* and *Streptococcus bovis*, to utilize sugars adsorbed to the porous silica particles was investigated.

Both the 7 nm and 2.3 nm pore-size particles were capable of adsorbing glucose from solution. Fermentations with glucose provided adsorbed to nanoparticles were compared to fermentations with glucose dissolved in media. The success of the fermentation was assessed by using high performance liquid chromatography to compare the concentration of fermentation products at harvest time. Results indicated that although the fermentation with soluble glucose produced significantly more end-products, *S. bovis* demonstrated some ability to metabolize the glucose adsorbed to the 7 nm pore silica particles. No evidence was detected that *S. cerevisiae* could metabolize glucose adsorbed to silica particles of either pore size.

KEYWORDS: facilitated diffusion, *Streptococcus bovis, Saccharomyces cerevisiae,* biomass, hydrolysate, silica particles

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1 Introduction

1.1 Lignocellulosic Biomass Conversion

The United States Department of Energy's goal is for 30% of the nation's gasoline demand to be met by biofuels by the year 2030 (Viikari, Vehmaanpera, & Koivula, 2012). To date most biofuels are produced from sugar or starch-based plant material, and these fuels are known as first generation biofuels. Traditional first generation biofuels are produced with food crops such as cereal grains and sugar crops, and have been moderately profitable to produce. Biofuels, such as bioethanol, are widely used as octane enhancers and are blended with gasoline (Nigam & Singh, 2011). First-generation biofuels have been widely criticized for creating a competition between food and fuel and driving fuel and food costs up. There is also concern that the increasing demand on sugar and starch-based crops will have a negative impact on the environment as non-farm land is converted to cropland for growing more first generation feedstock (Galbe, 2002). As a result, the focus of government-sponsored research has changed to cellulose-based biofuel production, because cellulose is obtained from an agricultural residue or an energy crop, and as such is not in competition for use as food. These biofuel source materials are termed second-generation feedstocks.

Second-generation biofuels are produced from lignocellulosic biomass such as agricultural, industrial and forest residues which account for the majority of the world's biomass (Viikari et al., 2012). Despite great promise as an alternative to first generation biofuels, lignocellulosic biofuels have yet to achieve widespread commercial success. Cellulosic biomass has a more complicated composition than starch rich plant materials, making second-generation biofuels more expensive to produce than first generation

biofuels. Recent work at the University of Kentucky focused on finding solutions to the challenges faced when producing biofuels with lignocellulosic sources. One such challenge is that low solids content is the most efficient conversion configuration for enzymatic hydrolysis, however this results in dilute concentrations of glucose in the hydrolysate (Modenbach, 2013). Typically lignocellulosic hydrolysate contains a lower concentration of glucose (between 0g/L and 10 g/L) than what is ideal for *S. cerevisiae* (typically 20 g/L). Work by Dr. Alicia Modenbach (Modenbach, 2013) investigated the ability of silicon mesoporous particles to adsorb glucose directly from lignocellulosic enzymatic hydrolysate as a method for concentrating the glucose, in order to provide the fermentative organism, *Saccharomyces cerevisiae* (yeast) with closer to optimal glucose concentration. The silica particles have been shown to preferentially adsorb glucose from the hydrolysate. These particles can then be physically removed from the hydrolysate and re-suspended in new fermentation broth such that the glucose would be more concentrated in the broth. However, the work done in our laboratory showed that glucose was tightly bound to the particles, and was not able to be easily removed from the particles after absorption (Modenbach, 2013). The current study explores the feasibility of leaving the glucose adsorbed to the silica particles in the fermentation broth to determine if the fermentative microorganism can utilize the glucose directly from the silica particle, eliminating the need for the glucose desorption step.

1.2 Project Objectives

The overall goal of this project was to determine if two commonly-studied ethanol-producing microbes have the ability to utilize glucose which has been adsorbed

onto silica particles. The organisms studied were the fungus *Saccharomyces cerevisiae* and the bacterium *Streptococcus bovis*. The specific study objectives were:

1a). To determine if *Saccharomyces cerevisiae* (mesophilic facultative anaerobe, fungus) can metabolize the glucose adsorbed to silica particles when the organism is cultured anaerobically.

1b). To determine if diameter of the particle or the size of the pores within the particle affected *S. cerevisae*'s ability to metabolize glucose from the silica particle.

2a) To determine if *Streptococcus bovis* (Gram-positive anaerobic rumen bacteria) has the ability to desorb and utilize glucose adsorbed to silica particles.

2b) To determine if diameter of the particle or the size of the pores within the particle affected *S. bovis'* ability to metabolize glucose from the silica particle.

1.3 Organization of the Thesis

This thesis is organized into chapters. The first chapter provides an introduction to the topic and sufficient background information necessary to understand the motivation for this work. The chapter also presents the objectives for the study.

The second chapter is the literature review pertaining to this thesis. The literature review is intended to provide sufficient background information to this topic so that a technically-literate person without experience in this area can read and understand the thesis.

The third chapter describes the silica particles used in this study. The methods for the creation of the silica particles and the methods for characterization of the particles are detailed. Data taken to confirm the particles' ability to adsorb glucose are shown, and isothemal calorimetry (ITC) data are presented. This chapter is formatted to include an abstract, introduction, materials and methods, and results and discussion for this set of experiments.

The fourth chapter discusses the findings of the first objective; determining the ability of *Saccharomyces cerevisiae* to metabolize adsorbed sugars when cultured anaerobically. This chapter includes an abstract, introduction, materials and methods, and results and discussion for this set of experiments.

Chapter 5 discusses the findings of the second objective; determining the ability of *Streptococcus bovis* to overcome the adsorption forces of the particles and utilize the adsorbed sugars. Chapter 5 includes an abstract, introduction, materials and methods, and results and discussion for this set of experiments.

Chapter 6 summarizes the lessons learned and proposes future work for the continuation of this project.

A complete list of references is included at the end of the thesis.

2 Literature Review

2.1 Lignocellulosic Biomass Chemistry

Second-generation biofuels are produced from lignocellulosic biomass such as agricultural, industrial and forest resources as an alternative to first generation biofuels, such as corn-derived ethanol. Unlike first-generation biofuels, second-generation sources do not compete directly with food sources (Viikari et al., 2012). The national estimate of annual biomass available without any change in land use or forest and agricultural management practices in the United States is approximately 1.3 billion tons, which could supply energy equivalent to 30% of the annual petroleum consumption (Kurian, Raveendran, Hussain, & Raghavan, 2013). In addition, biomass derived fuels offer the benefits of greenhouse gas mitigation, near carbon neutrality, a decrease of dependence on fossil fuels, and an improvement of our nation's energy security (Kumar, Karimi, & Horvath, 2016). Despite great promise as an alternative to first generation biofuels, lignocellulosic biofuels have yet to achieve commercial success. Cellulosic biomass contains monomeric sugars that require more complex pre-treatment methods to release than those in starch-based grains, making cellulosic biofuels more difficult to produce than first generation biofuels.

Lignocellulosic biomass is the only known sustainable feedstock for biorefineries to use to meet the increasing global energy demands. However, the unit operations required in the current lignocellulosic biofuel production process makes second-generation cellulosic biofuels more expensive to produce than first generation biofuels. Lignocellulosic biomass contains cellulose (composed of β-1,4-linked chains of glucose molecules), hemicellulose (composed of 5 and 6 carbon sugars), and lignin (composed of

three major phenolic compounds)(Balan, 2014). Historically, the return on investment is low for lignocellulose-based fuels, which makes it difficult for cellulosic ethanol to compete with corn-based ethanol (Sims, Warren, Saddler, & Taylor, 2010).

The basic steps required to convert cellulosic biomass into ethanol are as follows:

1) Biomass size reduction

2) Biomass pretreatment

3) Hydrolysis of pretreated biomass to fermentable sugars

5) Fermentation of sugars to ethanol

6) Ethanol recovery

Pretreatment is the second step in breaking down these complex polymers (lignocellulose) into monosaccharaides for subsequent fermentation. Popular methods of pretreatment include chemical, physical, physio-chemical, and biological pretreatment (Balan, 2014). Pretreatment processes work by disrupting hydrogen bonds in cellulose, breaking down the crosslinked matrix present in hemicellulose and lignin, and increasing the porosity and surface area of lignin. However, pretreatment may also release or form compounds that inhibit fermentation. Inhibitors generated during pretreatment are mainly weak acids, furan derivatives (furfural and hydroxymethylfurfural), and phenolic compounds (such as vanillin and syringaldehyde) (Delgenes, 1996; Galbe, 2002; Wood, Orr, Luque, Nagendra, & Berruti, 2015). Even fructose, a sugar with a structure similar to glucose, can act as an inhibitor during fermentation (Moysés, Reis, de Almeida, de Moraes, & Torres, 2016). Inhibitors decrease cell growth and metabolism necessary for fuel production during fermentation (K. Zhang, Agrawal, Harper, Chen, & Koros, 2011).

The pretreated biomass next undergoes hydrolysis, which depolymerizes the complex sugars into simple sugars. Currently, the prevailing method of hydrolyzing low-solids biomass into fermentable sugars yields a low concentration of sugar (Balan, 2014). Ideally the hydrolysate can then be used as a media for fermentation, but the resulting concentration of ethanol is very low due to the low starting concentration of glucose in the hydrolysate (Y. Q. Zhang, Li, Wang, et al., 2015). In addition, large amounts of energy are required to recover low concentrations of ethanol from the fermentation broth after fermentation which significantly increases the cost of ethanol production (Y. Q. Zhang, Li, Want, et al., 2015).

Much of the recent work in the development of lignocellulosic biofuel production processes has focused on finding solutions to low glucose and/or high inhibitor concentrations in the hydrolysate. Scientists have developed methods to simultaneously concentrate and detoxify lignocellulosic hydrolysates using vacuum membrane distillation (Y. Q. Zhang, Li, Wang, et al., 2015). This process uses heat and a glucose impermeable membrane to remove inhibitors and water from the hydrolysate. However, the research looked only at the two most common inhibitors, acetic acid and furfural. The actual hydrolysate can be very complex with dozens of inhibitors, meaning the membrane would often foul and have to be replaced. This method is also quite energy intensive because of the energy required to maintain the heat gradient and the energy required to run the vacuum pump used to drive the separation.

Another proposed solution is the use of activated carbon particles to remove inhibitors. A study in 2011 demonstrated that activated carbon could successfully remove most furfural from a hydrolysate media (K. Zhang et al., 2011). However, this method

has only been shown to work on a few inhibitors, and it fails to solve the problem of low glucose concentrations after hydrolysis.

Another project investigated the ability of mesoporous silica particles to preferentially adsorb glucose from the hydrolysate (Joshi, Rao, Lehmler, Knutson, & Rankin, 2014; Modenbach, 2013). Once the glucose was adsorbed to the silica particles, the particles were physically separated from the hydrolysate, and re-suspended in fermentation broth. This method has the advantage of removing glucose from the hydrolysate inhibitors, allowing for an inhibitor-free fermentation. Adsorption on to particles also allows the glucose to be used at the desired concentration, as glucose adsorbed on the particles is added to the appropriate volume of fermentation media to achieve the desired glucose concentration. The particles also have the advantage of being inexpensive to produce and of being reuseable. The process of adsorbing the glucose to the particles and removing the particles from the hydrolysate requires little energy. However, there is currently no energy efficient method to remove the glucose from the particles for subsequent fermentation (Modenbach, 2013). Overcoming this impediment would provide a viable low-energy method of glucose concentration and inhibitor mitigation during the production of second-generation biofuels.

2.2 Mesoporous Silica Particles

Previous work involving the use of particles and nanoparticles in biorefineries has largely been focused on immobilizing pretreatment enzymes. In order to reduce costs, it is important to recycle enzymes for future use, and immobilizing enzymes onto nanoparticles of high surface area kept the enzymes effective over several re-use cycles (S. Ansari & Qayyum, 2012).

Mesoporous silica particles (MSP) have been studied for their ability to adsorb proteins and carbohydrates (Joshi et al., 2014; Schlipf, Rankin, & Knutson, 2013). MSPs are capable of withstanding high temperatures, and are robust. The particles have been shown to contain well-ordered hexagonal closely-packed pores (Meynen, Cool, & Vansant, 2007a). The particle pore diameter can be adjusted between 5 and 20 nm by adjusting the aging temperature (Schlipf et al., 2013). Increasing the aging temperature increases the final pore size of the particle after the surfactant is rinsed away. When adsorbing proteins, the negative charge of the nanoparticles allows for good adsorption between the particles and generally positively charged proteins (Schlipf et al., 2013). Glucose is also capable of being adsorbed onto the particles (Joshi et al., 2014). These particles have been shown to preferentially over other sugars found in a hydrolysate solution. When previously investigated, liquid chromatography separation techniques were not able to remove glucose from the silica mesoporous particles (Modenbach, 2013).

2.3 Saccharomyces cerevisiae

Yeast is the most commonly used microorganism to ferment sugars to ethanol, and its glucose uptake systems and metabolic processes have been well studied (Chen, Cheung, Feng, Tanner, & Frommer, 2015; Otterstedt, Larrson, M.B., & Stahlberg, 2004; Santangelo, 2006). Glucose is the preferred carbon source of the yeast *S. cerevisiae*. In the yeast cell, glucose is metabolized to pyruvate via glycolysis. The enzyme pyruvate decarboxylase then transforms pyruvate into CO_2 and ethanol, generating two adenosine triphosphates (ATPs). In the presence of oxygen, cellular respiration could use O_2 to generate three ATPs (Johnston & Kim, 2005), however *S. cerevisiae* prefers to ferment

glucose (De Deken, 1966). Glucose must be transported into the cell at a high enough rate to supply glycolysis and generate a sufficient amount of ATP for cell processes.

The transport of glucose into the yeast cell through the cell membrane is the rate-limiting step in fermentative growth (Cirillo, 1962). Glucose is transported across the membrane via glucose transporter proteins. Yeast is known to have 18 distinct hexose transporters (Hxts), at least 6 of which are specific to glucose transport. The diversity in transporter proteins help yeast survive in the large range of glucose concentrations present in their natural environments. These transport proteins differ in their affinity for glucose, and different Hxts are expressed depending on the concentration of glucose in the extracellular environment. Hxt 1 is a low affinity transporter that is most useful in solutions with high glucose concentrations. Hxt 2 and 4 have a high-affinity for glucose and are expressed when yeast is grown in environments with low glucose concentrations. Hxt 3 encodes for a glucose transporter with an intermediate glucose affinity which are expressed in solutions with both low and high glucose concentrations. Finally, Hxts 6 and 7 are high affinity glucose transporters expressed at low glucose concentrations in the presence of non-fermentable carbon sources such as ethanol (J.-H. Kim, Roy, Jouandot II, & Cho, 2013; Reifenberger, Boles, & Ciriacy, 1997; Roy, Kim, Cho, & Kim, 2014). The distribution of these transporters in the yeast cells changes as the glucose concentration in the yeast's environment changes.

In order to sense the glucose concentration in the surrounding media, *S. cerevisiae* utilizes both cell-membrane receptors and changes in the cell's metabolism. Glucose binding to receptors on the yeast cells cause conformational changes in the cell, which leads to changes in the protein expression in the cell, including the expression of glucose

transporters. All Hxt transporters are uniporters, which means they facilitate the transport of glucose along the glucose gradient across the membrane (Chen et al., 2015). The driving force of the transport of glucose in yeast cells is the glucose concentration gradient. No ATP is consumed when glucose is transported by any of the transport proteins used by *S. Cerevisiae. S. cerevisiae* can utilize both anaerobic and aerobic metabolism, but the kinetics of glucose transport are the same regardless (Reifenberger et al., 1997).

2.4 Streptococcus bovis

Streptococcus bovis is a bacterium found in the rumens of animals that ingest cellulose (J. M. Chow & J. B. Russell, 1990). The ideal conditions for growth of *S. bovis* are similar to conditions in the rumen of a cow. The optimal temperature is 39°C and the environments is anaerobic. *S. bovis* is of interest to researchers due to its role in rumen acidosis in cattle (Mantovani & Russell, 2002; F. N. Owens, D.S. Secrist, W.J. Hill, & D.R. Gill, 1998b). *S. bovis* can grow in both very low and very high glucose concentrations due to multiple glucose uptake methods (Cook & Russell, 1994). *S. bovis* is a Gram-positive bacterium whose glucose uptake is regulated by the activity ratio of lactate dehydrogenase to pyruvate formate lyase. In the wild, *S. bovis* operates in a low-sugar concentration environment in the rumen of the cow. If the glucose concentrations are high, *S. bovis* produces lactate, which can cause acidic conditions and lead to rumen acidosis in the animal (F. N. Owens, D. S. Secrist, W. J. Hill, & D. R. Gill, 1998a). If the carbohydrate and ethanol concentrations are low, *S. bovis* produces acetate, formate, and ethanol. *S. bovis* has been shown to uptake glucose both by facilitated diffusion, and the phosphoenol pyruvate (PEP) dependent glucose phosphotransferase (PTS) active uptake system, an ATP driven transport (Bond & Russell, 1996). Facilitated diffusion requires

no energy input and is driven by the difference in glucose concentration across the cell membrane. The PEP-PTS uptake system does require energy, and it is the same uptake system *S. bovis* uses for to uptake cellobiose, maltose and sucrose (J.M. Chow & J.B. Russell, 1990). The PEP-PTS uses phosphoenol pyruvate as its energy source. The system involves enzymes in the plasma membrane and cytoplasm and transfers PEP to imported sugars by several proteins. The glucose-specific PTS transport system is unique to enteric bacteria. Glucose forms glucose-6-phosphate upon entering the cell.

When glucose is abundant, *S. bovis* transports glucose using a low-affinity facilitated diffusion, as this is a more rapid means of glucose uptake (Cook & Russell, 1994). Glucose excess cultures exhibit ATP spilling that parallels lactate production. Lactate in *S. bovis* is fructose 1,6 disphosphate (FDP)-dependent (Bond & Russell, 2000). If glucose levels are high, all glucose is converted to lactate. The L-lactate created in high glucose conditions has many applications in food, pharmaceutical, and polymer industries (Mazumdar, Blankschien, Clomburg, & Gonzalez, 2013).

3 Mesoporous Silica Particle Characterization and Properties

3.1 Abstract

Two different mesoporous silica particles were produced. One which contained a 7 nm pores (SBA-15) and one which contained 2.3 nm pores (MCM-48). Both particles adsorbed glucose, although the SBA-15 particle adsorbed 38% more than the MCM-48 particle per gram of particle.

ITC data supported the hypothesis that glucose adsorbed to the particle by displacing a water molecule previously adsorbed to the particle.

3.2 Introduction

Zeolites, a class of minerals consisting of hydrated aluminosilicates, are the most well known porous material and have the advantages of uniform pore size, good stability, and selectivity (Meynen et al., 2007a). However, industries like biological and pharmaceuticals require bulkier materials with larger pore sizes than those of which zeolites are capable. These limitations have led to a surge in research on the synthesis of mesoporous materials. Several techniques were developed to create siliceous mesoporous materials. Overall, these particles are synthesized by dissolving a template molecule into a solvent before adding the template source. The particle will condense around the template molecule, which can then be washed off leaving a pore on the surface of the particle. One such mesoporous silica particle is Santa Barbara Acids (SBA-15). SBA-15 contains highly ordered and tunable mesopores (4-14nm). Compared to other similar particles, SBA-15 have high thermal stability (Meynen, Cool, & Vansant, 2006). Another popular mesoporous silica particle is Mobil Composition of Matter No. 48 (MCM-48)

particles have there-dimensional cubic pore structure similar to the structure of SBA-15 particles but with thinner walls and smaller pore diameter range (S. Kim, Ida, Guliants, & Lin, 2005; Morey, O'Brien, Schwarz, & Stucky, 2000).

Mesoporous silica particles (MSP) have been studied for their ability to adsorb proteins. (Schlipf et al, 2013). MSPs are capable of withstanding high temperatures and are robust. MSP materials contain well-ordered hexagonal closely packed pores. The pore diameter can be adjusted between 5 and 20 μm by adjusting the aging temperature (Meynen et al., 2007a). Increasing the aging temperature increases the final pore size of the particle after the surfactant is rinsed away. When adsorbing proteins, the negative charge of the nanoparticles allows for good adsorption between the particles and generally positively charged proteins (Schlipf et al., 2013). Recently, several studies investigated the ability of mesoporous silica particles can also adsorb saccharides (Joshi, Lehmler, Knutson, & Rankin, 2017; Joshi et al., 2014; Meynen et al., 2007a; Modenbach, 2013).

3.3 Materials and Methods

3.3.1 Material Synthesis of SBA-15 Particles

Spherical SBA-15 (Santa Barbara Acids-15) particles were made using a modified procedure from Schlipf et al., 2013 (Schlipf et al., 2013). First, 3.10 grams of Pluronic P123 triblock copolymer (P123; purchased from Sigma Aldrich) was heated in a round bottom flask in a 100°C oven until the liquid phase was achieved. Cetyltrimethylammonium bromide (CTAB; 98% pure, obtained from Research Organics, Sigma-Aldrich Corporation, Cleveland, OH) in the amount of 0.465 grams was solubilized in 20 mL of deionized water. This solution was placed in a 30°C water bath

and stirred. To this solution was added 7.8 mL of 200 proof ethanol (purchased from Decon Labs, King of Prussia, PA) and 45.9 mL of 1.5 M HCL. Once the CTAB had completely dissolved in solution, 10 mL of Tetraethyl orthosilicate (TEOS; >98% pure; Acros Organics, Thermo-Scientific, New Jersey) was added in a drop-wise fashion. The solution was mixed for two hours, then transferred to a Parr 4748 Teflon-lined reactor which had been acclimated to the aging temperature. The solution was held at the desired aging temperature for 3 days. After the three days, the solution was removed from the reactor and stirred for 10 minutes to homogenize the mixture. The mixture was then filtered and rinsed with 100 mL of deionized water. The washed sample was placed into a single walled Whatman cellulose extraction thimble, and the surfactants were removed by washing with 200 mL of 200 proof ethanol which was recycled through the material for 24 hours. The sample was then dried in a 90°C oven for 4-24 hours to remove ethanol used for rinsing.

3.3.2 Materials synthesis of MCM-48 Silica Nanoparticles

Particles with smaller diameter pores (monodispersed spherical mesoporous silica particles with controlled particle size (MCM-48)) were prepared using a modified Stöber particle technique described by Kim et al (T.-W. Kim, P.-W. Chung, & V. S. Y. Lin, 2010). Cetyl trimethylammonium bromide (CTAB) was used as the templating compound. Tetraethyl orthsilicate (TEOS) and the tri-block copolymer Pluronic F127 were used as the silica source and dispersing agent, respectively. Initially 0.5 g of CTAB and 2.05 g of F127 were dissolved in 96 mL of DI/UF water, followed by the addition of 43.1 ml of ethanol and 11.9 ml of NH_4OH solution (29.3 wt%). The solution was stirred until complete dissolution of solutes was achieved. Then, TEOS (1.9 mL) was added to

the solution and the solution was stirred vigorously by hand for exactly 1 min at room temperature. The solution was then aged at room temperature for 24 h without any stirring to ensure complete silica condensation. The particles were removed from the solution by ultrahigh speed centrifugation (Sorvall Evolution) at 17,000 rpm for 5 minutes and were washed three times with 50mL DIUF water with repeated centrifugation then dried at 80ºC in oven overnight. Finally, template-free silica particles were obtained by washing in 200 ml acidic ethanol (HCl, 1.5 M) for 24 hours followed by repeated centrifugation and then washing with DIUF water and ethanol. Template free particles were dried overnight at 84ºC.

3.3.3 Characterization of particles

The morphology of the particles were characterized using a Hitachi S-4300 Scanning Electron Microscope (SEM). The samples for SEM characterization were prepared by dispersing the particles onto a double-sided carbon tape attached on a 15mm aluminum mount. Samples were held in a desiccator for 24 hours after excess silica materials were blown off with dry nitrogen gas. Prior to analysis, the samples were coated with gold-palladium alloy using an Emscope SC400 with gold-palladium alloy sputtering.

Average pore diameter, pore size distribution and particle surface area were estimated from nitrogen sorption tests conducted at 77 K using a TriStar 300 purchased from Micrometrics. Samples were degassed at 120ºC for a minimum of 4 h under flowing dry N_2 gas before conducting the nitrogen sorption experiment. The specific surface area was estimated using the Brunauer, Emmett and Teller (BET) isotherm, and average pore diameter and pore size distribution were estimated by the method of Barrett, Joyner and

Halenda (BJH) (Barrett, Joyner, & Halenda, 1951; Brunauer, Emmett, & Teller, 1938). Micropore volume and external surface area were estimated using the method described in (Jaroniec & Kruk, 1999). Low angle x-ray diffraction (XRD) analysis was performed with a Bruker-AXS D8 Discover diffractometer using an x-ray wavelength of 1.54 Å to determine the degree of mesostructural ordering. Particles were scanned at 0.5 °/min in 2θ increments of 0.02º from 1.5 to 6º. Samples were prepared in a powder sample holder and tapped flat with a spatula. Data analysis was performed with the Bruker Diffrac-Suite software (Joshi et al., 2014).

3.3.4 Loading Glucose onto Particles

Particles were loaded with glucose to confirm their ability to adsorb glucose from solution. Particles were added to a 7mL Wheaton vial along with 4mL of deionized water and stirred for 24 hours. After 24 hours, the solution was transferred to a 15mL centrifuge tube and centrifuged for 30 minutes at 4500 rpm to separate the water from the particles. The supernatant was then removed and 4mL of a 10 g/L and a 250g/L glucose solution was added to different treatments of the SBA-15 particles, and a 250 g/L glucose solution was added to the MCM-48 particles. The glucose/particles solution was transferred to 7mL Wheaton vials and stirred for 24 hours. After 24 hours, the solution was transferred to 15mL centrifuge tubes and centrifuged at 4500rpm for 30 minutes. The supernatant was then collected into 1.5mL micro centrifuge tubes. The glucose concentration of the collected supernatant and the original stock of glucose solution were measured on a YSI 2900D glucose analyzer to confirm that the particles had adsorbed glucose. The difference in the glucose concentration of the original solution and the supernatant was used to calculate the amount of glucose absorbed onto the particles in moles of

glucose/gram of particles. These particles could then be used as a glucose source for the microorganism.

3.3.5 Isothermal Calorimetry (ITC)

In order to better predict the energy needed to overcome the adsorption forces of the glucose onto silica, the heat of adsorption was measured using an isothermal titration calorimeter (ITC). The Nano ITC Low Volume was purchased from TA Instruments. The temperature was held at 25°C, the syringe size was 50µL and the cell size was 300µL. Each injection volume was 2.5µL. A 111mM glucose solution was titrated into the 16.7mM solution of silicon particles which was stirred at a rate of 250rpm. The baseline was determined by first calculating the heat of reaction of diluting glucose into water.

3.4 Results and Discussion

3.4.1 Particles

The particle characteristics determined by the nitrogen sorption experiments are shown below in Table 3-1. The pore size and pore diameter depend on the relative pressure. The peaks in Figure 3.1 and Figure 3.2 below represent the point at which capillary evaporation can no longer be delayed in the particle's pores, indicating the average pore diameter in nm. The SBA 15 particles had an average pore diameter of 7nm while the MCM-48 particles had an average pore diameter of 2.3nm. The SBA-15 particles had a surface area of $620m^{2/}g$ and a diameter range of 5-15µm while the MCM-48 particles had a surface area of $840m^2/g$ and a diameter range of 200-230nm.

Table 3-1: Particle Physical Characteristics.
Diameter, surface area and pore diameter of the SBA-15 particles and the MCM-48 particles.

Characteristic	SBA-15	MCM-48
Diameter	5-15µm	200-230nm
Surface Area	620m^2/g	840m^2/g
Pore Diameter	7nm	2-3nm

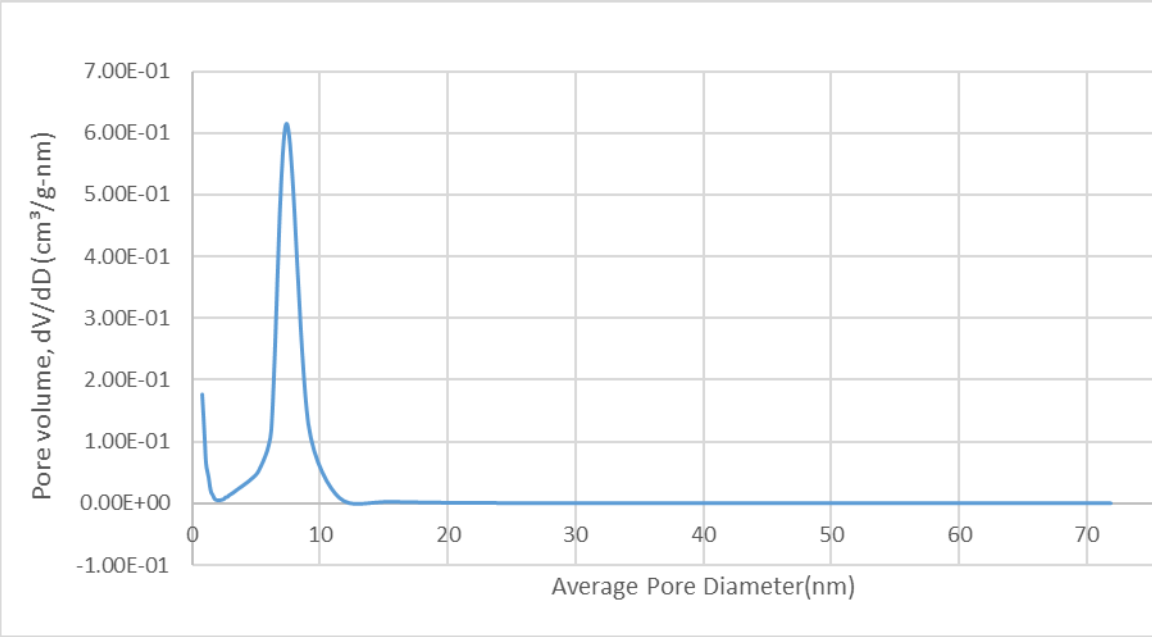

Figure 3.1: Pore size distribution of SBA 15 silica particles.
The peak at 7 nm corresponds to an average pore diameter of 7 nm (Table 3-1).

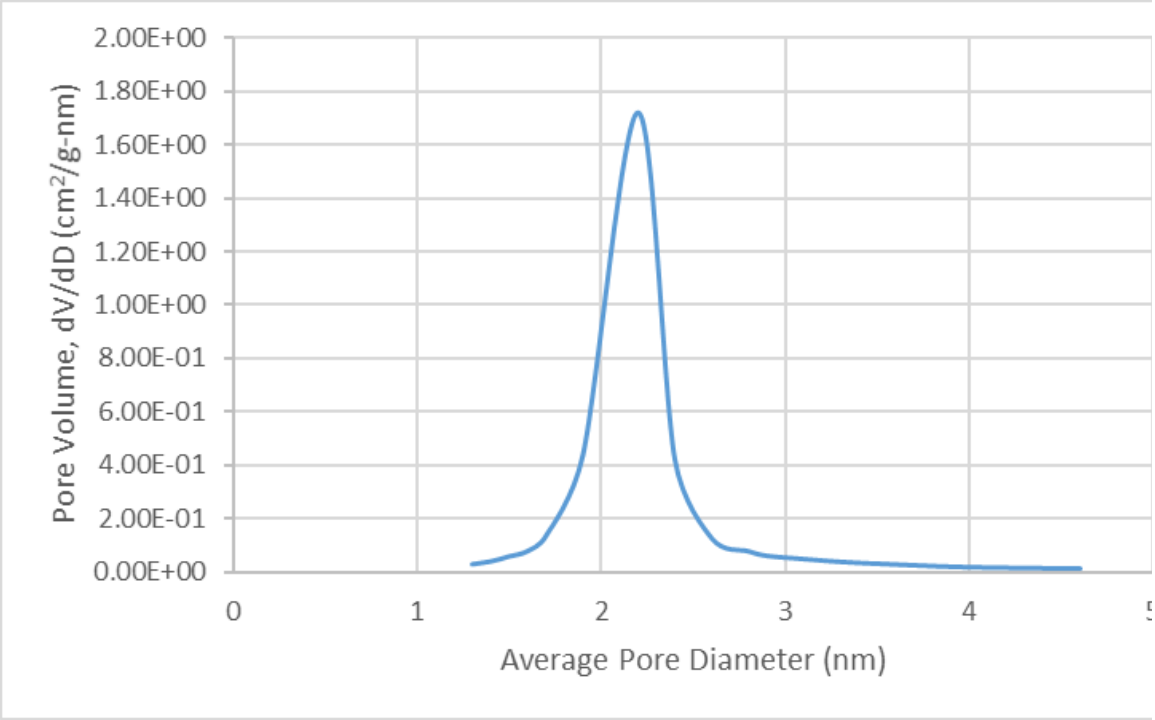

Figure 3.2: Pore size distribution of MCM-48 silica particles.
The peak at 2.3 nm corresponds to an average pore diameter of 2.3 nm (Table 3-1.)

An image captured using the Hitachi S-4300 Scanning Electron Microscope is seen below in Figure 3.3. The particles below have a pore diameter of 2.3 nm.

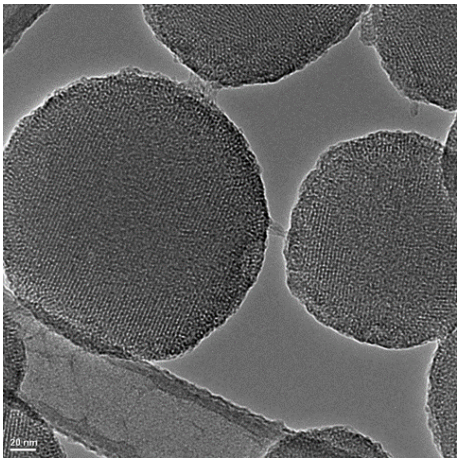

Figure 3.3: TEM image of MCM-48 silica particle.
Both the pore and the silica matrix are visible (Image courtesy of Arif Khan).

3.4.2 Adsorption of Glucose

Both sizes of particles were able to adsorb glucose from solution (Table 3-2). The smaller particles were capable of adsorbing more moles of glucose per gram of particles, which is likely a result of their larger pore size.

The adsorption experiments showed that glucose was successfully adsorbed onto both of the particle types. The highest adsorption was achieved by SBA 15 particles in the high concentration of glucose. A Joshi et. al publication used particles similar to this experiment but imprinted with a glucose template. They used a similar technique to determine glucose adsorbed, measuring glucose concentration before and after incubation with particles. They measured 0.006 mol/g of particles, or 0.006 moles of glucose adsorbed per gram of particles used. Our number of 0.004 mol/g is comparable (Joshi et al., 2017). Modenbach did similar work but used actual hydrolysate to measure glucose adsorption capabilities of the particles. The particles adsorbed a much smaller amount of glucose, 3.77×10^{-5}mol/g. This is likely due to the fact that the glucose concentration of the hydrolysate is fairly low (less than 20g/L), and the hydrolysate contains many other compounds adsorbed by the nanoparticles, such as xylose.

Table 3-2: Glucose adsorbed to particles
as a function of particle type and initial glucose concentration.

	SBA 15 *10 g/L Glucose*	**SBA 15 *250 g/L Glucose***	**MCM-48 *250 g/L Glucose***
Average g glucose/g particles	0.17	0.80	0.58
Average mg glucose/m^2 particles	0.26	1.30	0.61
Average mol/g particles	0.001	0.004	0.003

3.4.3 ITC Data

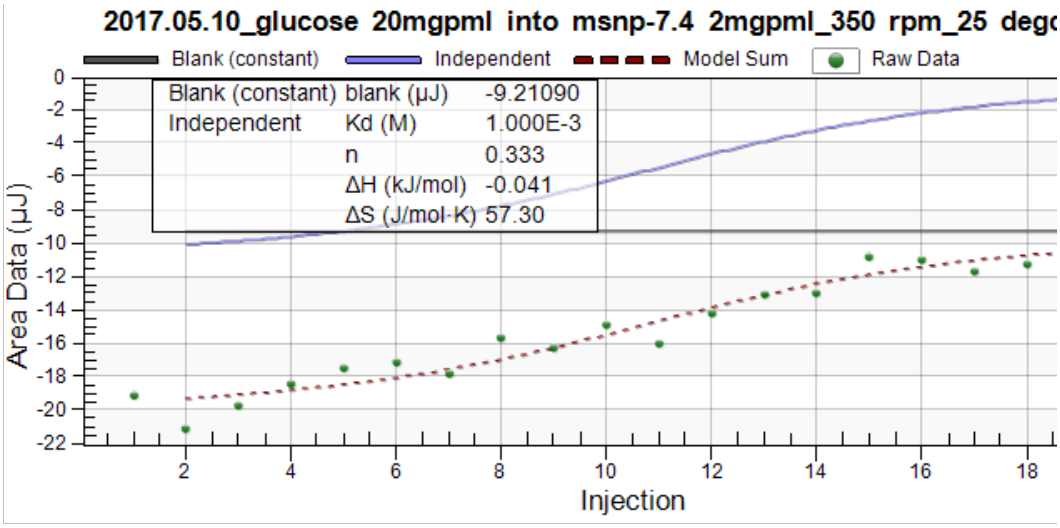

Figure 3.4: Isothermal titration Calorimetry Data
The baseline (adding glucose to water) is the flat black line. The model sum is the amount of energy released when a glucose solution was added to a solution of particles adjusted for the amount of energy released when glucose is added to water. The model sum line never crosses the baseline, meaning no measurable heat of reaction was given off.

The black line in Figure 3.4 shows the heat released when glucose was added to water and the glucose diluted. The dotted line represents the model sum and is the actual heat released per injection when a glucose solution is injected into a water and particle solution. The blue line is the difference between the heat released when the glucose is diluted and the heat released when glucose is added to a solution of particles in water. The blue line represents the actual heat evolved from the glucose adsorbing to the particle. The change in enthalpy (ΔH) from the model fitting is close to zero, thus there is no measurable change in enthalpy during the reaction. However, the change in entropy (ΔS) is 57.3J/mol-K, meaning that the adsorption process is entropy-driven. The reaction is therefore an entropic exchange, with a water molecule on the surface of the silica being replaced with a molecule of glucose, yielding no measurable heat of reaction.

The isothermal calorimeter data confirmed that particles should be washed with water prior to glucose adsorption, as done by Modenbach (Modenbach, 2013). Since a water molecule adsorbed to the surface of the silica nanoparticle is replaced with a glucose particle, it is important to soak the particles for 20 minutes before attempting to adsorb glucose to the particles to achieve maximal glucose adsorption.

4 Investigating the Ability of *Saccharomyces cerevisiae* to Metabolize Glucose Adsorbed onto Silica Particles

4.1 Abstract

The objective of this experiment was to determine if *Saccharomyces cerevisiae* (yeast) could utilize glucose adsorbed to particles and, if so, to determine if glucose uptake was a function of the size of the particle or particle pore size. The following study compared the *Saccharomyces cerevisiae* fermentation to produce ethanol when the glucose was provided two different ways, in solution (dissolved in the media) and adsorbed onto silica particles.

There was no evidence that *S. cerevisiae* was been able to utilize the glucose adsorbed onto the SBA-15 (7 nm pores) particles or the glucose adsorbed to the MCM-48 particles (2.3 nm pores). This result is likely due to *S. cerevisiae* relying solely on passive diffusion to uptake glucose. We hypothesize that without an energy-utilizing source of glucose uptake, *S. cerevisiae* is unable to overcome the adsorptive force holding glucose to the particle.

4.2 Introduction

Currently, the hydrolysis of biomass solids into fermentable sugars during the manufacturing of biofuels yields a low concentration of sugar. In addition, there are many potential inhibitors present in the hydrolysate that may lower fermentation yields (Delgenes, 1996; Galbe, 2002). Low glucose concentration yields a low ethanol concentration, makes ethanol recovery expensive and the

return on investment low for the biorefinery. In this study we investigated a novel technique to remove glucose directly from the hydrolysate, enabling the desired glucose concentration to be established in the microbial growth media, and potentially limiting the number of inhibitors present during fermentation.

4.3 Particles

Previous work involving the use of nanoparticles in biorefineries has largely involved pretreatment enzymes. In order to keep costs down, it is important to recycle enzymes for future use, and immobilizing them onto particles of high surface area keeps enzymes effective and makes them easy to remove from solution after pretreatment (S. H. Ansari, Qayyum, 2012). In a similar way, particles have been produced that will selectively adsorb glucose from lignocellulosic hydrolysate, resulting in a simple, less energy intensive method to concentrate glucose.

Mesoporous silica particles (MSP) have been studied for their ability to adsorb proteins (Meynen, Cool, & Vansant, 2007b; Schlipf et al., 2013). MSPs are capable of withstanding high temperatures and are robust. These materials contain well-ordered hexagonal close packed pores. The pore diameter can be adjusted between 5 and 20 μm by adjusting the aging temperature between 80 and 125 °C (Meynen et al., 2007b). Increasing the aging temperature increases the final pore size of the particle after the surfactant is rinsed away. Glucose is also capable of being adsorbed onto these particles (Joshi et al., 2014). Particles have been shown to prefer glucose to other carbohydrates when in a mixed-sugar solution (Joshi et al., 2017).

The use of these particles is a potential solution for separating glucose from other inhibitory compounds found in the hydrolysate. However, there is currently no energy

efficient method of separating glucose from the particles once glucose has adsorbed to them. When previously studied, liquid chromatography separation was incapable of removing the glucose from the particle (Modenbach, 2013). What is not known is if some or all microorganisms have the ability to directly utilize the glucose adsorbed to particles. It is possible that microorganisms can pull the glucose from the particle directly, eliminating the need for the removal step. This study investigates the ability of *Saccharomyces cerevisiae* to utilize glucose directly adsorbed to the particle.

4.3.1 Saccharomyces cerevisiae

Yeast has a long history of use in fermentation. *Saccharomyces cerevisiae* is the most commonly used microorganism to ferment sugars to ethanol, and its glucose uptake systems and metabolic processes have been well studied. Glucose is the preferred carbon source of the yeast *S. cerevisiae* (Roy et al., 2014). Yeast is unusual because it prefers to ferment rather than oxidize glucose, even in the presence of abundant oxygen, when glucose is abundant (De Deken, 1966).

In the yeast cell, glucose is metabolized to pyruvate via glycolysis. The enzyme pyruvate decarboxylase then transforms pyruvate into CO_2 and ethanol, creating two adenosine triphosphates (ATPs). In the presence of oxygen the enzyme pyruvate dehydrogenase can use O_2 to generate three ATPs (Johnston 2004). Though aerobic metabolism produces a much higher number of ATPs, *S. cerevisiae* prefers to ferment glucose. The fermentation of glucose is very inefficient, so glucose must be transported into the cell at a high rate to feed glycolysis and generate a sufficient amount of energy for cell maintenance. The transport of glucose into the cell through the cell membrane is the rate-limiting step in fermentative growth (Cirillo, 1962). Glucose is

transported across the membrane via a glucose transporter protein. Yeast is known to have 18 distinct hexose transporters (Hxts), at least 6 of which are specific to glucose transport. The large number of transporter proteins help yeast to survive in the large range of glucose concentrations present in their natural environments. These transport proteins differ in their affinity for glucose, and different Hxts are expressed depending on the glucose concentration in the extracellular environment. The kinetics of glucose transport are the same regardless of whether the *S. cerevisiae* is metabolizing aerobically or anaerobically (Reifenberger et al., 1997).

The type of Hxt proteins expressed will depend on how well the yeast can detect the adsorbed glucose. Ideally, glucose adsorbed onto the particles will be able to bind to the glucose receptors on the outer membrane of the cell.

4.4 Materials and Methods

4.4.1 Synthesis of silica particles

4.4.1.1 Material Synthesis of SBA-15

Spherical SBA-15 (Santa Barbara Acid) particles were made using a modified procedure from Schlipf et al, 2013. First, 3.10 grams of Pluronic P123 triblock copolymer (P123; purchased from Sigma Aldrich) was heated in a round bottom flask in a 100°C oven until the liquid phase was achieved. Cetyltrimethylammonium bromide (CTAB; 98% pure, obtained from Research Organics, Sigma-Aldrich Corporation, Cleveland, OH) in the amount of 0.465 grams was solubilized in 20 mL of deionized water. The solubilized CTAB was placed in a 30°C water bath and stirred. To this solution was added 7.8 mL of 200 proof ethanol (purchased from Decon Labs, King of Prussia, PA)

and 45.9 mL of 1.5 M HCL. Once the CTAB had completely dissolved in solution, 10 mL of Tetraethyl orthosilicate (TEOS; >98% pure; obtained from Acros Organics, Thermo-Scientific, New Jersey) was added in a drop-wise fashion. The solution was mixed for two hours, then transferred to a Parr 4748 Teflon-lined reactor which had been acclimated to the aging temperature. The solution was held at the desired aging temperature for 3 days. After the three days, the solution was removed from the reactor and stirred for 10 minutes to homogenize the mixture. The mixture was then filtered and rinsed with 100 mL of deionized water. The washed sample was placed into a single-walled Whatman cellulose extraction thimble, and the surfactants were removed by washing with 200 mL of 200 proof ethanol which was recycled for 24 hours.

4.4.1.2 Material synthesis of MCM-48 Silica Particles

Particles with smaller diameter pores (monodispersed spherical mesoporous silica particles with controlled particle size (MCM-48)) were prepared using a modified Stöber particle technique described by Kim et al., 2010 (T.-W. Kim et al., 2010). Cetyl trimethylammonium bromide (CTAB) was used as the templating compound. Tetraethyl orthsilicate (TEOS) and the tri-block copolymer Pluronic F127 were used as the silica source and dispersing agent, respectively. Initially 0.5 g of CTAB and 2.05 g of F127 were dissolved in 96 mL of DI/UF water, followed by the addition of 43.1 ml of ethanol and 11.9 ml of NH_4OH solution (29.3 wt%). The solution was stirred until complete dissolution of solutes was achieved. Then, TEOS (1.9 mL) was added to the solution and stirred vigorously for exactly 1 min at room temperature. The solution was then aged at room temperature for 24 h without any stirring to ensure complete silica condensation. The particles were removed from the solution by ultrahigh speed centrifugation Sorvall

Evolution centrifuge at 17,000 rpm for 5 minutes and were washed three times with 50mL DI/UF water with repeated centrifugation and dried at 80ºC in oven overnight. Finally, template-free silica particles were obtained by washing in 200 ml acidic ethanol (HCl, 1.5 M) for 24 hours followed by repeated centrifugation and washing with DIUF water and ethanol. Template free particles were dried overnight at 84ºC.

4.4.2 Characterization of particles

Particles were characterized as detailed in Chapter 3.

4.4.3 Loading glucose onto particles

Particles were loaded with glucose as detailed in Chapter 3. These particles could then be used as a glucose source for investigated microorganisms. Each of the fermentations were provided with 1g of loaded silica particles which equated to approximately 20g/L of glucose provided in the vessel. The glucose adsorption was determined from YSI data taken at the end of each glucose loading step.

4.4.4 Experimental Design

Four treatments were used. Treatments are described Table 4-1. In the first treatment, glucose was adsorbed onto particles and added to the fermentation vessels so that 20g/L of glucose was available in the media. Treatment 2 consisted of particles with no glucose adsorbed. The third treatment included 10g/L of glucose dissolved in media (no particles present). Finally, the fourth treatment contained no glucose or particles. Three replications of each treatment were conducted for both particle sizes.

Table 4-1: Experimental Design

Summary of treatments used in the experiment to determine glucose uptake by *S. cerevisiae.*

	Particles	**No Particles**
Glucose	Treatment 1 (3 reps)	Treatments 3 (3 reps)
No Glucose	Treatment 2 (3 reps)	Treatment 4 (3 reps)

4.4.5 Fermentation

All samples were fermented in anaerobic conditions. The growth media for all samples was YPD media, containing 20g/L of proteose-peptone and 10g/L of yeast extract). The pH was adjusted to 5.5 prior to sterilization. For each sample, 4mL of growth media was added to a 4mL Wheaton vial and a stir bar was placed inside. Each sample was inoculated with equal amounts of washed pure culture *S. cerevisiae*. Each sample was then flushed with nitrogen for 10 minutes before being sealed with a rubber septum to ensure an anaerobic fermentation. Samples were incubated on a stir plate at 29°C for 72 hours. After 72 hours, the media was transferred to a micro centrifuge tube and stored at -30°C for subsequent analysis.

The amount of glucose adsorbed to the 0.2 grams of particles in a glucose solution was more than enough to provide the fermentation with 20g/L of glucose, so the exact amount of particles to add to each fermentation vessel was calculated using YSI data. An example calculation is provided below. This calculation is based on the SBA15 particle being used.

The exact amount of glucose in each vessel can be determined from the total amount of glucose adsorbed and the total weight of particles added:

$$0.720624\frac{g\ glucose}{g\ particle} * \frac{0.2g\ particle}{vessel} = 0.144g\ glucose\ adsorbed\ in\ vessel$$

The amount of glucose needed per vessel is also calculated from the desired concentration of 20 $\frac{g\ glucose}{L}$ and the volume of liquid in the vessel (4 mL).

$$\frac{20\ g\ glucose}{L} = \frac{0.08\ g\ glucose}{4mL}$$

Finally the wet weight of the particles is taken into account because the overall weight of particles is higher due to water adsorption occurring. For example, if the particles now weigh 0.3 g instead of 0.2 g:

$$\frac{0.3\ g\ particles}{0.144g\ glucose} = \frac{0.167\ g\ particles}{0.08\ g\ glucose}$$

In this example, we would need 0.17g of particles in the fermentation vessel to ensure the media contained 20g/L of glucose.

4.4.6 Analyses

Fermentation products (ethanol and lactic acid) and residual glucose were quantified by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column with a differential refractive index detector. An aqueous solution of 5 mM H2SO4 was used as the solvent.

4.4.7 Statistics

Data were analyzed using JMP software from SAS®, Research Triangle Park, NC. A one way ANOVA was performed to determine statistical significance of the product concentrations by treatment.

4.5 Results and Discussion

4.5.1 Particle Physical Parameters

Nitrogen adsorption studies confirmed the particles contained the desired pore size. Table 4-2 details the physical parameters of each type of particle, as quantified by the nitrogen adsorption studies (repeated from Chapter 3 for the reader's convenience).

Table 4-2: Physical Characteristics of the Two Particles Used.
Physical characteristics of the two particles studied, Table repeated here from Chapter 3 for the reader's convenience.

Characteristic	SBA-15	MCM-48
Diameter	5-15μm	200-230nm
Surface Area	620m^2/g	840m^2/g
Pore Diameter	7nm	2.3nm

4.5.2 Fermentation Products

In fermentations where glucose was provided on larger SBA-15 particles, no fermentation products were detected in the harvested liquid material by HPLC (Figure

4.1**).** Ethanol concentrations were about half what was expected in fermentations with soluble glucose in YPD media. The theoretical ethanol concentration was 5.11 g/L. The reduced ethanol concentration may have been due to the increased fermentation time (3 days) allocated to overcome a potential lag time for the treatments with glucose supplied on silica particles (Treatment 1). After all sugar was converted to ethanol by the *S. cerevisiae,* the microorganism may have undergone a diauxic shift and started consuming ethanol as a carbohydrate source (Turcotte, Liang, Robert, & Soontorngun, 2010). Ethanol in the unloaded treatment (Treatment 2) indicates that there was residual ethanol present from the rinsing step of particle synthesis. Also, because the ethanol produced from the glucose loaded particles was not significantly greater than the ethanol present in the unloaded particles treatment, one must be conclude that the yeast were not able to use the glucose adsorbed to the particles.

Ethanol was produced in fermentations with glucose provided on the MCM-48 particles (Figure 4.2). However, ethanol levels were not significantly higher than those detected on fermentations with unloaded particles provided no glucose. This indicated that yeast were unable to use the adsorbed glucose on the MCM-48 particles, and also that more care must be taken to ensure all ethanol used in the particle preparation protocol is removed prior to conducting the experiments.

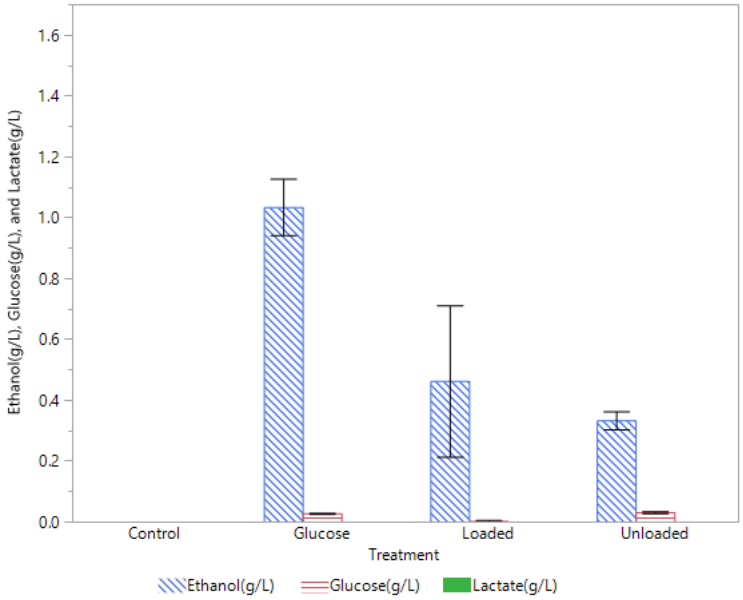

Figure 4.1: Ethanol, glucose, and lactate concentrations for the SBA-15 particle treatment. Concentrations are at harvest in *S. cerevisiae* fermentations using glucose adsorbed to SBA-15 particles as the carbon source for the loaded treatments, no carbon source for the unloaded treatment. Control and Glucose treatments contained no particles, but did contain the microorganism.

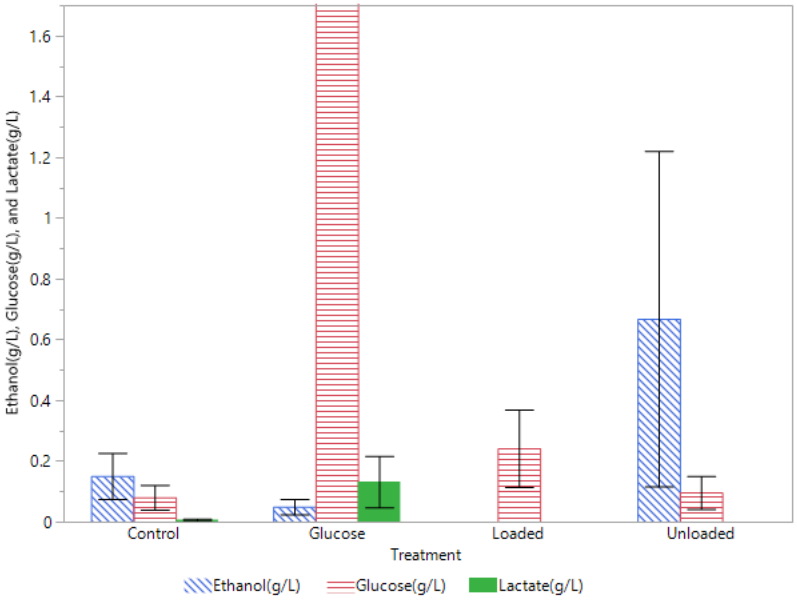

Figure 4.2: Ethanol, glucose, and lactate concentrations for MCM-48 particles. Concentrations are at harvest in *S. cerevisiae* fermentations using glucose adsorbed to MCM-48 particles as the energy source for the loaded treatments, no energy source for the unloaded treatment. Control and Glucose treatments contained no particles. The glucose concentration for the "Glucose" treatment was 2.6 g/L.

Given the comparative size of the particle to yeast, the yeast cell (average 3.4 x 10^{-6} m) was unable to uptake a 5x 10^{-6} m particle, but it may be feasible, from a size standpoint, for the yeast cell to uptake the 0.22-0.23 x 10^{-6} m particle into the cell. However, glucose membrane transport relies on facilitated diffusion, which is driven by the concentration gradient of the soluble glucose.

The analysis of final fermentation products revealed a few problems that need to be remedied in future studies. Firstly, the control treatment, or traditional fermentation of *S. cerevisiae* had lower than expected levels of ethanol and low levels of glucose at harvest. It is possible that *S. cerevisiae* underwent a diauxic shift and switched from using glucose to ethanol as a carbon source after *S. cerevisiae* has used all available glucose (Turcotte et al., 2010). In the future, more frequent sampling would be advisable, so peak ethanol production can be detected. Second, detectable levels of ethanol in the unloaded treatments (particles with no adsorbed glucose) indicates that residual ethanol was present from the final rinsing step of particle synthesis. Particles will need to be washed more thoroughly in the future.

5 Investigating the ability of *Streptococcus bovis* to Metabolize Glucose Adsorbed onto Silica particles

5.1 Abstract

The use of silica mesoporous particles has been suggested as a low-energy method for separating glucose from dilute solutions because of the high affinity of glucose for the particles. The glucose-laden particles can be physically separated from the original solution after glucose has adsorbed to the particles, thereby concentrating the glucose. The hypothesis for this study was that *Streptococcus bovis* could metabolize the adsorbed glucose directly from the silica particles, thereby eliminating the need for a glucose desorption step. To determine the degree to which *S. bovis* could utilize adsorbed glucose, fermentation product concentrations were quantified and compared to *S. bovis* cultures where an equivalent concentration of glucose was provided solubilized in the fermentation media. *S. bovis* can uptake glucose using facilitated diffusion as well as ATP-driven transport. Porous silica particles with two different pore sizes (7 nm and 2.3 nm) were investigated to determine if *S. bovis'* ability to metabolize the adsorbed glucose was a function of pore size. *S. bovis* appears to have metabolized glucose adsorbed onto the silica particles with the larger pore diameter (SBA-15; 7nm pores), but not in for the smaller pore size particles (MCM-48). The production of lactate in addition to ethanol in multiple treatments indicated that *S. bovis'* metabolism was under stress for these fermentation conditions.

5.2 Introduction

One of the challenges of producing biochemicals and biofuels from cellulosic biomass is the difficulty of fermenting low glucose concentrations resulting from the

hydrolysis of pretreated biomass. Solutions have been devised but they are prohibitively energy intensive. Porous silica particles have shown promise as a method of selectively adsorbing glucose in solution (Joshi et al., 2014; Modenbach, 2013). The hypothesis for this study was that *Streptococcus bovis* could metabolize the adsorbed glucose directly from the silica particles, thereby eliminating the desorption step.

Streptococcus bovis is a Gram-positive anaerobic bacterium found in ruminant animals and of interest to researchers due to its role in rumen acidosis in cattle (Mantovani & Russell, 2002; Owens et al., 1998b). *S. bovis* can survive in environments with both very low and very high glucose concentrations due to multiple glucose uptake methods (Cook & Russell, 1994). In environments rich in carbohydrate sources, *S. bovis* uses a low-affinity high-capacity facilitated diffusion carbohydrate uptake mechanism. In environments with low concentrations of carbohydrates, *S. bovis* utilizes a phosphotransferase system (PEP-PTS). The PEP-PTS uptake system is unique to bacteria and uses phosphoenol pyruvate as its source of energy. *S. bovis* uses the same mechanism when uptaking cellobiose, maltose, or sucrose (Narito Asanuma, 2002)

In an environment where carbohydrate uptake is rate limited, the common fermentation products of *S. bovis* are ethanol, formate, and acetate. High rates of carbohydrate uptake typically result in lactate being the primary fermentation product. Lactate, a stronger acid than the volatile fatty acids typically produced in the rumen, quickly lowers the pH of the rumen environment. The lowering of pH can lead to rumen acidosis in the animal, which is a common problem in feedlots where cattle are fed a high carbohydrate diet (Bond & Russell, 2000).

Facilitated diffusion is driven by the difference in concentration across the membrane (Cook & Russell, 1994);(Bond & Russell, 2000). The ideal conditions for growth of *S. bovis* are similar to conditions in the rumen of a cow; 39℃ with anaerobic conditions. The medium should include salts, 0.5 g yeast extract/L, and 4 g glucose/L.

Glucose-excess cultures have ATP spilling that parallels lactate production. If glucose levels are high, the "extra" glucose is converted to lactate). The L-lactate formed has many applications in food, pharmaceutical, and polymer industries (Mazumdar et al., 2013).

5.2.1 Particles

Particles with two different pore sizes were used in this study. The 7nm pore size particles were synthesized with a method (described below) adapted from Schlipf et al, 2013. The particles with the 2.3 nm pore size were synthesized using a method by Kim et al, 2010, adapted as described below (T. W. Kim, P. W. Chung, & V. S. Y. Lin, 2010).

5.3 Materials and Methods

5.3.1 Synthesis of silica particles

5.3.1.1 Material Synthesis of SBA-15

Spherical SBA-15 (Santa Barbara Acid) particles were made using a modified procedure from Schlipf et al, 2013. First, 3.10 grams of Pluronic P123 triblock copolymer (P123; purchased from Sigma Aldrich) was heated in a round bottom flask in a 100°C oven until liquid phase was achieved. Cetyltrimethylammonium bromide (CTAB; 98% pure, obtained from Research Organics, Sigma-Aldrich Corporation, Cleveland, OH) in the amount of 0.465 grams was solubilized in 20 mL of deionized water. This solution

was placed in a 30°C water bath and stirred. To this solution was added 7.8 mL of 200 proof ethanol (purchased from Decon Labs, King of Prussia, PA)and 45.9 mL of 1.5 M HCL. Once the CTAB had completely dissolved in solution, 10 mL of Tetraethyl orthosilicate (TEOS; >98% pure; obtained from Acros Organics, Thermo-Scientific, New Jersey) was added in a drop-wise fashion. The solution was mixed for two hours, then transferred to a Parr 4748 Teflon-lined reactor which had been acclimated to the aging temperature. The solution was held at the desired aging temperature for three days. After the three days, the solution was removed from the reactor and stirred for 10 minutes to homogenize the mixture. The mixture was then filtered and rinsed with 100 mL of deionized water. The washed sample was placed into a single-walled Whatmann cellulose extraction thimble, and the surfactants were removed by washing with 200 mL of 200 proof ethanol for 24 hours.

5.3.1.2 Material synthesis of MCM-48 Silica Particles

Particles with smaller diameter pores (monodispersed spherical mesoporous silica particles with controlled particle size (MCM-48)) were prepared using a modified Stöber particle technique described by Kim et al., 2010. (T.-W. Kim et al., 2010) Cetyl trimethylammonium bromide(CTAB) was used as the templating compound. Tetraethyl orthsilicate (TEOS) and the tri-block copolymer Pluronic F127 were used as the silica source and dispersing agent, respectively. Initially 0.5 g of CTAB and 2.05 g of F127 were dissolved in 96 mL of DIUF water, followed by the addition of 43.1 ml of ethanol and 11.9 ml of NH_4OH solution (29.3 wt%). The solution was stirred until complete dissolution of solutes was achieved. Then, TEOS (1.9 mL) was added to the solution and stirred vigorously for exactly 1 min at room temperature. The solution was then aged at

room temperature for 24 h without any stirring to ensure complete silica condensation. The particles were removed from the solution by ultrahigh speed centrifugation (Beckman-Coulter) at 17,000 rpm and were washed three times with 50mL DIUF water with repeated centrifugation and dried at 80ºC in oven overnight. Finally, template-free silica particles were obtained by washing in 200 ml acidic ethanol (HCl, 1.5 M) for 24 hours followed by repeated centrifugation and washing with DIUF water and ethanol. Template free particles were dried overnight at 84ºC.

5.3.2 Characterization of particles

Particle characteristics were quantified as detailed in Chapter 3.

5.3.3 Loading glucose onto particles

Glucose loading onto particles was detailed in Chapter 3.

5.3.4 Experimental Design

Six treatments were used in the experiment, as described in Table 5-1. The first four treatments serve as controls to separately quantify the treatment effects of the any residual glucose stored in the *S. bovis* (Treatment 1), adsorbed vs soluble glucose (Treatment 2), the microbial effect of the presence of the particles (Treatments 3 and 4), and particle pore size on *S. bovis*' ability to use the adsorbed glucose (Treatments 5 and 6), respectively.

Table 5-1: Experimental Design
Details of the experimental design for the experiment to determine if *S. bovis* can metabolize glucose adsorbed to silica particles.

Treatment	Name	*S. bovis* added	Glucose Concentration (g/L)	Particle Pore Size (nm)
1	Control	X	0	No particles
2	Soluble sugar control	X	4 g/L soluble	No particles
3	Unloaded particles	X	0	2.3
4	Unloaded particles	X	0	7
5	Loaded particles	X	4 g/L	2.3
6	Loaded particles	X	4 g/L	7

5.3.5 Sample Analyses

Fermentation products (ethanol and lactic acid) and residual glucose were quantified by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column with a differential refractive index detector. An aqueous solution of 5 mM H2SO4 was used as the solvent.

5.3.6 Statistics

Data were analyzed by performing an ANOVA to compare treatments (JMP software from SAS®, Research Triangle Park, NC).

5.3.7 Streptococcus bovis glucose uptake screening experiment

5.3.7.1 Microbial Propagation

Streptococcus bovis was obtained from FAPRU, ARS, Lexington, KY and grown in basal medium that contained (per liter): 900mL of deionized water, 40mL of salt A, 40mL of salt B, 0.5g of yeast extract, 1.00g of trypticase, 12.4mL of VFA solution, 0.6g of cysteine hydrochloride, and 1.0mL of resazurin solution. Salt A contained 7.3g/L of

$K_2HPO_4*3H_2O$. Salt B contained 6.0g/L of KH_2PO_4, 12.0g/L of $(NH_4)2SO_4$, 12.0g/L of NaCl, 2.5g/L of $MgSO_4$, and 1.6g/L of $CaCl_2*2H_2O$ (Mantovani & Russell, 2002). The medium pH was adjusted to 6.5 with NaOH and maintained under a 100% carbon dioxide atmosphere. The growth medium was autoclaved at 121 °C for 15 minutes and cooled to room temperature.

Inoculum was prepared in 100 mL glass bottles which were placed in a 39 °C water bath for 24 hrs to produce the inoculum used in the subsequent experiment. The inoculum was anaerobically transferred via syringe to Balch tubes (18x150mm and hold 28mL) for centrifugation. After centrifugation, the cell pellet was washed with fresh media, re-centrifuged, and then re-suspended with 50 mL fresh media. This suspension was transferred anaerobically via syringe to Balch tubes containing the treatment.

5.3.8 Fermentations

All samples were fermented in anaerobic conditions. Each sample was housed in a sealed test tube in anaerobic conditions. Test tubes were prepared in an anaerobic glove box environment containing 95% CO_2 and 5% H_2 and sealed with a rubber stopper and metal crimping. Each sample was inoculated with washed pure culture of *S. bovis*. Samples were incubated in a 39°C water bath for 24 hours. After 24 hours, the media was transferred to a micro centrifuge tube, centrifuged, and the supernatant was stored at -30°C for subsequent HPLC analysis.

Glucose was added directly to the fermentation vessels in treatment one and adsorbed onto particles in treatments 5 and 6. In treatments 5 and 6, YSI data were used

to determine the amount of glucose used calculate the weight of particles to be added to the fermentation.

The following example calculation assumes the SBA15 particles are used.

The exact amount of glucose in each can be determined from the total amount of glucose adsorbed:

$$0.720624\frac{g\ glucose}{g\ particle} * 0.2g\ particle = 0.144g\ glucose\ adsorbed\ in\ vessel$$

The amount of glucose needed per vessel is also calculated:

$$\frac{4\ g\ glucose}{L} = \frac{0.016\ g\ glucose}{4mL}$$

Finally, the wet weight of the particles is taken into account because the overall weight of particles is higher due to water adsorption occurring. If the particles now weigh 0.3 g:

$$\frac{0.3\ g\ particles}{0.144g\ glucose} = \frac{0.033\ g\ particles}{0.016\ g\ glucose}$$

In this example, around 0.033g of particles would be weighed into the fermentation vessel to ensure the media contained 4g/L of glucose.

5.4 Results and Discussion

5.4.1 Characterization of particles

Particle physical characteristics are reported here (from Chapter 3) for the convenience of the reader.

Table 5-2: Physical Characteristics of the Two Particles Used.
Physical characteristics of the two particles studied, Table repeated here from Chapter 3 for the reader's convenience.

Characteristic	Particle SBA-15	Particle MCM-48
Diameter	5-15µm	200-230nm
Surface Area	620m^2/g	840m^2/g
Pore Diameter	7nm	2.3nm

5.4.2 Fermentation Products

The mean levels of ethanol, lactate, and glucose are shown below in Table 5-3, Figure 5.1 and Figure 5.2. The level of ethanol in control samples (Treatment 1) was below the reliable detection limit, as expected. However, ethanol was detected in samples with particles and no glucose, which was not expected because the organism did not have a carbon source. The ethanol measured in these treatments was likely due to residual ethanol on the particles after they were synthesized and rinsed with 200-proof ethanol. However, detectable levels of lactate were present only in the soluble glucose and the glucose loaded particle treatments. This indicates that *S. bovis* was able to at least

partially metabolize the glucose adsorbed to the particles, but only for the 7 nm pore size particles.

Table 5-3: Mean Product concentrations in *S. bovis* fermentations. Mean ethanol and lactate concentrations produced by *S. bovis* and residual glucose concentrations in the fermentation broth, averaged over all replicates by treatment.

Treatment	Name	Mean Ethanol (g/L)	Mean Lactate (g/L)	Glucose (g/L)
1	Control	0.02	0	0
2	Soluble sugar control	0.05	0.35	0
3	Unloaded particles – 2.3 nm pores	0.11	0	0
4	Unloaded particles – 7 nm pores.	0.47	0	0
5	Loaded particles – 2.3 nm pores	0.05	0	0
6	Loaded particles – 7 nm pores	0.06	0.08	0.02

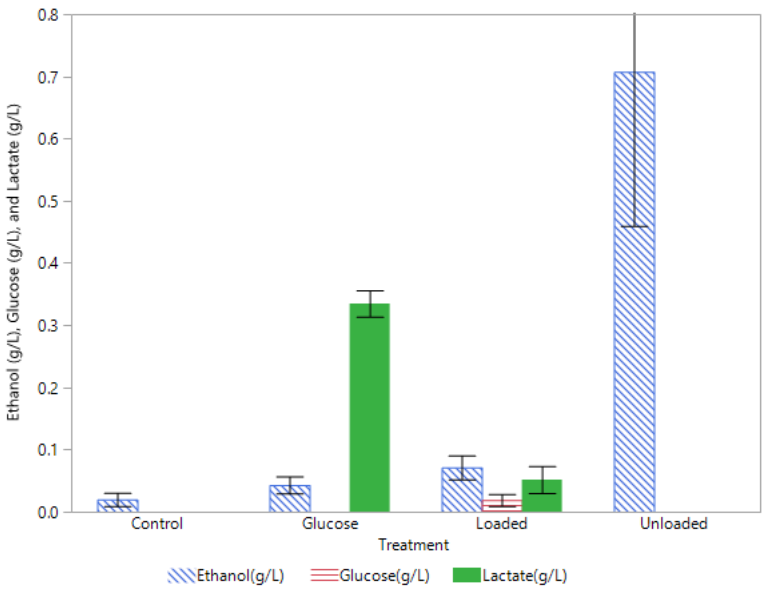

Figure 5.1: Ethanol, glucose and lacate concentrations for the SBA-15 particle treatment.
Concentrations are at harvest in *S. bovis* fermentations using glucose adsorbed to SBA-15 particles as the energy source for the loaded treatments, no energy source for the unloaded treatment. Control and Glucose treatments contained no particles.

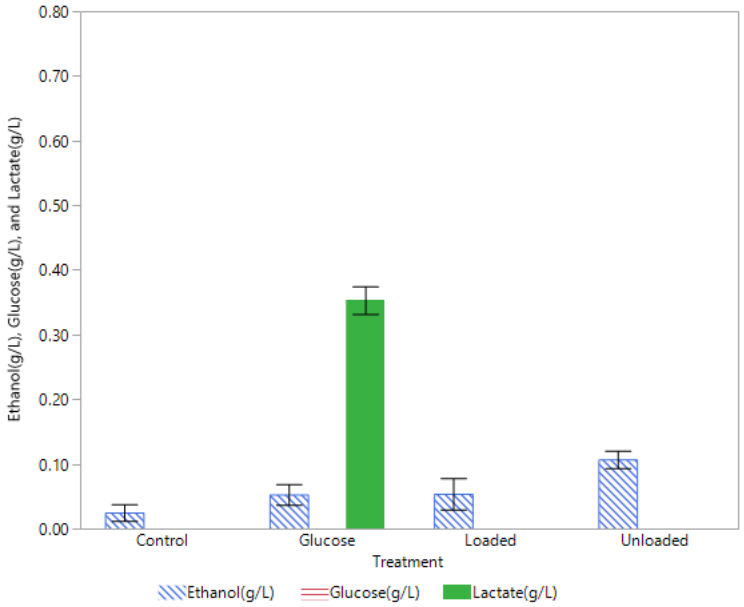

Figure 5.2: Ethanol and Lactate concentrations for MCM-48 Particles
Concentrations are at harvest in *S. bovis* fermentations using glucose adsorbed to MCM-48 particles as the energy source for the loaded treatments, no energy source for the unloaded

treatment. Control and Glucose treatments contained no particles.

Measurable ethanol was present in the unloaded particle treatments indicating that the particles contained residual ethanol from synthesis. However, lactate was present only in the samples with adsorbed glucose (SBA-15) or glucose in solution (both graphs), which indicates that the S. *bovis* was able to at least partially utilize the adsorbed glucose. The product yield from both the adsorbed glucose treatments, and soluble glucose treatments was well below the theoretical yield (approximately 2 g/L ethanol).

Results were similar for both particles, however the 2.3 nm pore size particle fermentations did not generate lactate, whereas the 7 nm pore size particle fermentations did generate lactate, indicating that *S. bovis* was metabolizing the glucose on the 7 nm pore size particle. The loaded and unloaded ethanol in excess of the control and glucose only treatments shown in both graphs indicates that the particles retained residual ethanol from synthesis.

6 Future Work

The synthesis of the particles did not include a sufficient drying time to ensure no residual ethanol remained on the particles from the production process. Residual ethanol was present in the samples even when no glucose was added for the *S. bovis* to metabolize. The amount of ethanol produced by the loaded particles from utilizing adsorbed glucose is therefore unclear. However, lactate present in both treatments with glucose in solution and adsorbed to particles with 7 nm pores, potentially indicating that the *S. bovis* was able to utilize glucose adsorbed to the 7 nm pore particles but not the 2.3 nm pore particles. One suggestion is to try even larger pores; larger than 7 nm and see if the microorganisms can more easily remove the glucose from the particles. More work needs to be conducted to determine if the results are repeatable, and if so, to determine why the larger pores are more desirable.

In the future, some quantification of cell growth should also be recorded. For example, an additional assay which determined initial and final protein content would be informative. Plating to determine the live cell count or a dry cell weight would also be helpful in confirming cell growth vs cell metabolism, and to determine if any cell death occurs over the course of the experiment.

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