# PRODUCTION POTENTIAL OF CHITOSAN AS A VALUE ADDED COPRODUCT FOR SUGAR BEET PROCESSING FACILITIES

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biosystems Engineering – Master of Science

2019

**ABSTRACT**

PRODUCTION POTENTIAL OF CHITOSAN AS A VALUE ADDED COPRODUCT FOR SUGAR BEET PROCESSING FACILITIES

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Sugar beet pulp residues (SBP) were used as a feedstock for *Rhizopus oryzae* fermentation to produce a value added product – chitosan. Enzymatic conditions for effective liberation of fermentable sugars from SBP were identified. Nitrogen sources, nutritional salts, and plant hormones were investigated as a means to improve the chitosan yield. Optimum conditions identified were applied to a large-scale flask culture. Fungal glucosamine yields reached 3.6% (per gram of SBP input, glucosamine is the monomer unit of chitosan). The economics of a fungal fermentation system for a SBP production of 2,000 metric tons per year was analyzed based on the results achieved in 2L flask culture. Compared to the current application of SBP as animal feed, the high value of the chitosan significantly enhanced the economic performance. A payback period of slightly over 7 months was correspondingly achieved for the studied fungal chitosan production

## ACKNOWLEDGEMENTS

I'd like to thank my committee members, Dr. Bradley Marks, Dr. Randy Beaudry, and a special thanks to Dr. Yan Liu for her expertise and unwavering support; additional thanks to Dr. Linda Hansen and Zhiguo Liu for their help. I'd also like to thank the Michigan Department of Agriculture & Rural Development for supporting this project (#791N4300339), and Michigan Sugar Company for their time, expertise, and resources.

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## KEY TO ABBREVIATIONS

Acid birchwood xylanase unit ..... ABXU

Alkali insoluble material ..... AIM

Analysis of variance ..... ANOVA

Anastomosis group ..... AG

Completely randomized design ..... CRD

Deionized water ..... DI water

Deoxyribonucleic acid ..... DNA

Dinitrosalicylic acid ..... DNS

Filter paper unit ..... FPU

Generally recognized as safe ..... GRAS

Gibberilic acid-3 ..... GA3

High fructose corn syrup ..... HFCS

High performance liquid chromatography ..... HPLC

Least significant difference ..... LSD

Logarithmic acid dissociation constant ..... pKa

Modified accelerated cost recovery system ..... MACRS

Potato dextrose agar ..... PDA

Potato dextrose broth ..... PDB

Randomized complete block design ..... RCBD

Revolutions per minute ..... RPM

Simultaneous saccharification and fermentation ..... SSF

Sugar beet pulp ..... SBP

Total solids ..... TS

# 1. INTRODUCTION

Sugar produced from sugar beets accounts for as much as 70% of the total sucrose consumed in the US every year (James, 2011). Michigan is one of the five most important sugar beet growing states in the U.S., contributing about 12% of the US acreage and produces 4.4 million tons of sugar beets annually (Haley, 2012; USDA, 2015). During the sugar beet processing, about 6.3% of the incoming sugar beet is expelled as sugar beet pulp (SBP) (Donkoh, Degenstein, & Ji, 2012; Spagnuolo, Crecchio, Pizzigallo, & Ruggiero, 1997). SBP currently has a low market value, about $3 per ton (Clark, 2014). Converting SBP into a higher value product or products will provide a significant benefit for the current sugar industry.

One of the major problems facing the sugar industry, and its suppliers, is the threat of pathogenic fungi. Fungal diseases and infections are the leading cause of yield loss for sugar beets, with the pestilent fungus *Rhizoctonia* accounting for an average yield loss of 2% every year; and in particularly humid years, fungal diseases have reduced sugar beet yields by as much as 60% (Neher & Gallian, 2011). Prevention of these fungal infections is costly to farmers, with anti-fungal agents costing as much as $92/acre planted (May, 2001). Cheap and effective alternatives for pesticides synthesis are needed.

One such potential alternate is the natural chemicals, chitin and chitosan. Chitin is the primary structural compound in fungal cell walls; and has been shown to make up as much as 45% of fungal biomass (Dhillon, Kaur, Brar, & Verma, 2013; Wu, Zivanovic, Draughon, Conway, & Sams, 2005). Chitosan is a deacetylated derivative of chitin. Over the last 20 years, Chitosan has generated considerable interest in the food, agriculture, and biomedical fields. As a very common biological molecule, chitosan displays a low toxicity, and is certified as Generally Accepted as Safe (GRAS) by the FDA (Mattia, 2013). Interest in chitosan is due to its spectrum of bioactivity – including antifungal (Anusuya & Sathiyabama, 2014; Aranaz et al., 2009; Ben-Shalom, Ardi, Pinto, Aki, & Fallik, 2003; Devlieghere, Vermeulen, & Debevere, 2004;

El-Diasty, Nesreen, & Hoda, 2012; Eweis, Elkholy, & Elsabee, 2006; Prapagdee, Kotchadat, Kumsopa, & Visarathanonth, 2007; Romanazzi, Feliziani, Santini, & Landi, 2013; Wu et al., 2005), antibacterial (Aranaz et al., 2009; Devlieghere et al., 2004; Wu et al., 2005), antiviral (Chirkov, 2002), and antidesiccation (Han, Zhao, Leonard, & Traber, 2004) properties; additional uses in the biomedical field include antitumoral applications (Aranaz et al., 2009; Tokoro et al., 1988), nutrient immobilization (Aranaz et al., 2009; Han et al., 2004), and applications in tissue engineering (Aranaz et al., 2009; Nwe, Furuike, & Tamura, 2010).

## 1.1 PROBLEM STATEMENT

The low value of SBP residues does not contribute in a significant way to the economic competitiveness of sugar beet processors; meanwhile the high cost of pest prevention can minimize the economic incentive for farmers to plant sugar beets. If there were a way to utilize this low value feedstock to produce a pesticide for use in sugar beet fields, or other higher value applications it would benefit both the sugar producers and the sugar beet farmers in Michigan.

## 1.2 OBJECTIVE

The objective of this project is to convert sugar beet pulp into a value added product – chitosan – and investigate the feasibility of integrating a biological process for this conversion into an existing sugar beet processing facility to improve the economic performance of the sugar industry.

# 2. LITERATURE REVIEW

## 2.1 CHEMISTRY OF CHITIN AND CHITOSAN

As the second most abundant polymer on earth, after cellulose (Peter, 1995), chitin is a linear polymer of 2,000 – 3,000 units of 2-deoxy-2-acetyl amino glucose (N-acetylglucosamine). Structurally, this molecule is very similar to cellulose, the difference being one hydroxyl group is replaced with an acetylamino group (Dhillon et al., 2013; Wu, Zivanovic, Draughon, & Sams, 2004). Chitin is found in insect exoskeletons, making up 20-30% of the shells of crustaceans, and consists of as much as 45% fungal biomass (Cho, No, & Meyers, 1998; Dhillon et al., 2013; Peniston & Johnson, 1972; Wu et al., 2005).

A derivative of chitin, chitosan is a flexible polymer of chitin with various degrees of deacetylation (monomer is N-glucosamine). Typically a chitin molecule must be ≥40% deacetylated before it can be considered chitosan (Nwe et al., 2010; Wu et al., 2005), although industrial chitosan extraction procedures typically produce higher degrees of deacetylation (Dhillon et al., 2013). Chitosan behaves very weakly acidic with a p$K_a$ value of 6.3 (glucosamine monomer p$K_a$=7.47) (Peter, 1995). As a polymer, different sizes and degrees of acetylation produce molecules with different chemical and biological characteristics, and thus different applications.

## 2.2 APPLICATIONS OF CHITIN AND CHITOSAN

One of the largest potential applications of chitin and chitosan polymers is in agriculture. Their myriad of antimicrobial properties makes them ideal compounds for pre-harvest field and soil treatments, as well as post-harvest anti-spoilage and anti-desiccation treatments (Anusuya & Sathiyabama, 2014; Ben-Shalom et al., 2003; Prapagdee et al., 2007; Romanazzi et al., 2013). In addition to chitin's effectiveness as an antimicrobial agent it has the advantage of being a naturally occurring compound, allowing it to be used with organic crops and foods.

In recent years, this market for organic foods has exploded – doubling in sales in less then 10 years since 2005 (Greene, 2014); however, the interest in organic foods has much deeper roots. Since thier introduction, chemically derived pesticides have raised concerns over safety. This has been in regards to human health due to the impacts these chemicals have on the environment, human and animal health with respect to eating crops that have been treated with such agents, and the health of the greater ecosystem and how these chemicals alter it (Dunlap & Beus, 1992; Eskenazi, Bradman, & Castorina, 1999). Additionally, overuse of pesticides can increase resistance in target organisms, causing an escalation in both pesticide usage (as increased dosages are required to produce the same level of inhibition that smaller dosages were able to previously achieve) and resistant strains of pests (Prapagdee et al., 2007). This escalation, combined with the rise in popularity of organic agriculture creates a great opportunity for the development of biologically derived pest control technology for both pre- and post harvest applications (Romanazzi et al., 2013).

### 2.2.1 PRE-HARVEST APPLICATIONS

Chitosan dissolved in dilute organic acids, such as 2% acetic acid (1% chitosan, w/v) has a low viscosity, making it ideal for field applications (Romanazzi et al., 2013); additionally several pathways in which it can act as a biological pest control agent have been demonstrated. With legume plants such as tumeric, and soybeans, as well as non-rhizobial plants like cucumber, applications of chitosan on growing crops were shown to increase the natural defense responses of these plants against a multitude of phytopathogenic fungi (Anusuya & Sathiyabama, 2014; Ben-Shalom et al., 2003; Han et al., 2004; Prapagdee et al., 2007). As was shown with tumeric, the protein content of leaves was increased by 0.1%, and the enzyme activity of two key enzyme complexes that have been linked to the defense of plants from phytopathogenic fungi, chitinase and chitosanase, was also increased. Not only does the increased protein content suggest that these enzymes were present in higher amounts, DNA

analysis of these protein complexes shows an increase in the number of isoforms of each enzyme in plant leaves treated with chitosan (Anusuya & Sathiyabama, 2014). Soybeans demonstrated an increase in chitinase activity of 44% when treated with a 0.3% chitosan solution. Plants treated with chitosan and infected with *Fusarium solani* f. sp. *glycines* fungal species, responsible for soybean sudden death syndrome, showed a significant decrease in the size and number of the necrotic blotches caused by the disease. Additionally, 14 days after inoculation the same chitosan treated plants showed over a 40% increase in dry weight when compared to untreated plants (Prapagdee et al., 2007).

Cucumbers treated with chitosan and then infected with *Botrytis cinerea* fungus resulted in an 82% reduction in disease development when compared to non-chitosan treated cucumbers. These plants also displayed a doubling in chitosanase activity compared to untreated plants. The same study showed that chitin oligomers could increase the activity of the leaf-generated chitosanase 240%. However, the chitin oligomer treated plants did not show any disease development reduction over the control (Ben-Shalom et al., 2003).

A second pathway that chitosan may use in order to inhibit fungal growth could be related to its interactions with cell membranes. It is thought that the positively charged chitosan molecules may bind, or interfere with negatively charged proteins in cell membranes which causes macromolecules and intracellular proteins to be released. This interaction could also interfere with nutrient uptake (Prapagdee et al., 2007; Wu et al., 2005). The membrane interference pathway helps to explain how chitosan can inhibit bacterial and viral pathogenesis, otherwise the chitinase and chitosanase enzymes would not affect these pathogens (Chirkov, 2002; Wu et al., 2005).

### 2.2.2 POST HARVEST APPLICATIONS

Chitosan has shown continued antimicrobial effects, suggesting its use as a natural preservative for extending the shelf life of foods. Short chitosan polymers have been shown to

greatly inhibit the growth of gram(-) bacteria, however these effects varied between different strains of gram(+) bacteria (Devlieghere et al., 2004; Romanazzi et al., 2013). Solutions of 2% acetic acid with 1% (w/v) chitosan applied to strawberries which were stored for 7 days at 1°C followed by 3 days of shelf storage at 20°C (both storage phases at 95-98% humidity) showed a reduction in gray mold McKinney index by 73% and *Rhizopus* rots by 88% over strawberries treated with a 2% acetic acid solution alone. When compared to commercial resistance inducers such as benzothiadiazole, the chitosan solutions showed a 5% decrease in the McKinney index for *Rhizopus* rots (Romanazzi et al., 2013). However, lactic acid forming bacteria were unaffected by the chitosan treatments (Devlieghere et al., 2004). For even longer storage periods, 2% chitosan solutions with 1% acetic acid were shown to reduce the incidence of decay in strawberries 45-60% (14 day storage). In raspberries the 2% chitosan solution prevented any noticeable decay for 21 days. Untreated raspberries were shown to have a 95% incidence of decay after 21 days of shelf storage (Han et al., 2004). Chitosan coatings have been shown to be useful for frozen fruits as well. In frozen strawberries, 2% chitosan coatings were also shown to produce a 24% reduction in drip loss (Han et al., 2004).

Chitosan solutions of 0.1 and 0.2% (in 0.4% acetic acid) have been shown to inhibit the growth of common fruit spoiling fugi: *Penicillium digitatum, Penicillium italicum, Botrydiplodia lecanidion,* and *Botrytis cinerea* as well as, or better than the common post-harvest antifungal agent thiabemdazole (Chien & Chou, 2006). Taken fruits dipped in the previously described solutions displayed 50, 47.5, 32.5, and 32.5% fewer incidents of spoiled fruit vs. treatments in a 0.1% thiabemdazole and 60, 53.7, 37.5, and 35% fewer incidents of spoiled fruit compared to a 0.4% acetic acid control after being inoculated with the fungal pathogens *P. digitatum*, *P. italicum*, *B. lecanidion,* and *B. cinerea*, respectively, and stored for 21 days in spoilage friendly, high humidity conditions (Chien & Chou, 2006).

Fruits dipped in chitosan solutions have also shown reduced dehydration during shelf storage compared to uncoated samples (Devlieghere et al., 2004; Han et al., 2004; Romanazzi

et al., 2013). While coated strawberries had an unpleasant bitter taste at first, this bitterness subsided 48-72 hours after application (Devlieghere et al., 2004). Several studies have suggested that the taste and texture of treated strawberries after 4 days of shelf storage is superior to there untreated counterparts, possibly due to its anti-desiccation effects, resulting in juicier berries (Devlieghere et al., 2004; Romanazzi et al., 2013).

Chitosan's interactions with food components have also been studied. While chitosan is able to severely retard the growth of *C. lambica* at concentrations of 0.005% and inactivate the strain completely at 0.01%, addition of high concentrations of soluble starches (>30% w/v) inhibit this antimicrobial activity. Whey proteins and sodium chloride also displayed this trend at concentrations of 10% and 1% (w/v) respectively. Fats (sunflower oil) did not display any inhibitory effects on the antimicrobial activity of chitosan. The application solution pH can also inhibit chitosan's anti-fungal effects. The previously described test involving *C. lambica* had solutions of pH 4. The same tests with sodium hydroxide adjusted solutions, pH=6, showed no anti-fungal activates (Devlieghere et al., 2004).

### 2.2.3 NUTRACEUTICAL APPLICATIONS

Chitosan can be used to produce glucosamine, the monomer unit of chitosan. Glucosamine is a precursor for glucosaminoglycans – components of the extracellular matric in the joints of humans and other animals. As such, it is thought that glucosamine may aid in the repair and maintenance of joints, specifically individuals with osteoarthritis (Qian et al., 2013).

Europe already recognizes this use and glucosamine is sold as a prescription drug to individuals suffering from osteoarthritis. In the US, glucosamine is not recognized by the FDA as anything more than a nutraceutical supplement (European Medicines Agency, 2006; Mattia, 2013; Qian et al., 2013). Unfortunately, glucosamine is not readily bioavailable, and absorption of the molecule in salt form (the most common from) is limited. However, with highly deactylated chitosan (≈83% deactlyated) having low molecular weights (≤200 kDa) the intestinal

uptake of glucosamine salts was enhanced by as much as 400% when in the presence of chitosan at concentrations of 20 µg/mL in cell culture plates (Qian et al., 2013).

### 2.2.4 OTHER APPLICATIONS

Aside from food, agricultural, and nutraceutical applications, chitosan has shown tremendous promise in the biomedical field. Chitosan has a number of properties that, when taken as a whole, make the molecule ideal for biosensor applications. These properties include non-toxicity, biocompatibility, and many of the anti fungal and anti bacterial (anti-microbial) properties mentioned in the previous section. Chitosan films additionally can be made adhesive and exhibit tremendous mechanical strength (Azmi et al., 2009; Kaur & Dhillon, 2014). Of particular interest to biosensor applications is the ability of these films to be used for protein immobilization, or the immobilization of other charged materials (Kaur & Dhillon, 2014). These attraction and immobilization properties also present the potential use for chitosan as a tool for separations, either at the analytical scale (e.g. HPLC column material) or at the industrial scale as a flocculent (Kaur & Dhillon, 2014; Kumar, 2000).

Chitin and chitosan have also been shown to aid in water treatment and purification. The degree to which the adsorbing molecule is deacetylated is a key factor in it's effectiveness, with chitosan molecules having low degrees of deacetylation being very effective at absorbing dyes, PCBs, and metal ions, and chitin molecules with higher degrees of acetylation being more effective at the adsorption of aromatic hydrocarbon residues from the petroleum processing industry (Aranaz et al., 2009; Crini & Badot, 2008; Dhillon et al., 2013).

## 2.3 CURRENT PRODUCTION TECHNOLOGY

### 2.3.1 SHELL FISH EXTRACTION

Currently, all commercially available chitosan comes from crustacean shells and shell byproducts; the annual production of which is near 2000 tons (Dhillon et al., 2013). As a

byproduct of the fishing industry, this chitosan source is very seasonal. Additionally the chitin content of the shells varies between crustacean species and thus the physiochemical characteristics of chitosan produced from these sources varies greatly depending on the season and the species (Cho et al., 1998; Kaur & Dhillon, 2014; Nwe et al., 2010; Wu et al., 2005; Wu et al., 2004). Chitosan derived from shrimp typically retains significant amounts of shrimp shell antigens which results in a major concern for individuals with shellfish allergies (Kaur & Dhillon, 2014; Nwe et al., 2010)

The current industrial chitin extraction process involves high temperatures, harsh chemical conditions, and high-energy inputs. Hydrochloric acid is first used under high temperatures to remove calcium and other inorganic materials. If the shellfish have a high heavy metal content, additional steps are needed to remove them. In order to remove shell pigments, high temperature chemical baths with potassium permanganate, hydrogen peroxide, or sodium hypochlorite are typically used (Dhillon et al., 2013; Kaur & Dhillon, 2014; Peter, 1995; Wu et al., 2004). The resulting shell mixture is then subjected to dilute alkali conditions and intense heat to remove proteins (Cho et al., 1998; Dhillon et al., 2013; Kaur & Dhillon, 2014; Peniston & Johnson, 1972; Wu et al., 2005; Wu et al., 2004). This alkali solution is removed (containing the dissolved proteins) and the alkali insoluble material (AIM) is placed in organic acids to extract the chitin. Finally this extracted chitin is subject to strong alkali conditions and heat to deacetylate it into chitosan. The concentration of alkali is the primary method of controlling the degree of deactylation with crustacean chitosan; with higher concentrations being used to produce chitosan with a higher degree of deactylation (Dhillon et al., 2013). This final step typically needs to be carried out under anaerobic conditions or in the presences of oxygen scavenging chemicals to prevent the chitosan chains from being aerobically degraded (Cho et al., 1998; Dhillon et al., 2013; Peniston & Johnson, 1972; Wu et al., 2005; Wu et al., 2004).

In addition to the variation in chitosan's physiochemical characteristics between crustacean species, this chitosan extraction procedure produces inconstant polymers – even

from the same source. Defects like protein contamination, varying degrees of deacatylation and inconsistent polymer chain length and molecular weight are prevalent with this extraction procedure (Dhillon et al., 2013; Kaur & Dhillon, 2014). Disposal of the large quantity of base solution, and heavy metal contaminants also presents environmental concerns (Cho et al., 1998; Dhillon et al., 2013; Kaur & Dhillon, 2014; Nwe et al., 2010; Wu et al., 2005).

### 2.3.2 FUNGAL FERMENTATION

Humans have a long history of utilizing fungi, dating back to the first civilizations. Originally used as purely a foodstuff, alcohol fermentation was the first intentional use of fungi as a food modifier by as early as 10,000 B.C. – this is thought to predate the use of bread (Hanson, 2013). By the 21$^{st}$ century, fungal products are commonplace in industry. Fungi can utilize almost any organic carbon source, fungal fermentations can be carried out very quickly, and as fermentation environments can be precisely controlled, their biological products are consistent and non-seasonal (Dhillon et al., 2013).

With regards to chitosan, fungi have many advantages over crustacean shells. While the basic procedure for chitosan extraction remains the same as for crustacean sources, the degree of acetylation with fungal chitin is typically lower then crustacean sources (Dhillon et al., 2013; Kaur & Dhillon, 2014; Nwe et al., 2010; Wu et al., 2004). As such the concentration and amount of harsh alkali chemicals is significantly lower. As they contain far fewer inorganic compounds and no heavy metals within their biomass, less or no chemical processing is required to remove these compounds (Dhillon et al., 2013; Kaur & Dhillon, 2014; Wu et al., 2005). Chitosan from fungal biomass is far more consistent in its physiochemical properties than chitin from other sources. This is very advantageous for applications that require a high degree of deacetylation, like nutraceuticals, or very low molecular weight, like crop and food applications. As the solubility and viscosity are more consistent, processing of chitosan from fungal sources is easier and more efficient as well (Dhillon et al., 2013; Kaur & Dhillon, 2014; Nwe et al., 2010).

The yield and specific properties of fungal chitosan such as molecular weight and degree of deacetylation can be controlled and augmented by different fermentation conditions. This allows for a high degree of specificity with regards to the physiochemical properties of the produced chitosan. Solid state fermentation causes fungi to produce more mycelia then submerged fermentations, which can result in higher chitosan production from the fungus (Dhillon et al., 2013), however submerged fermentations produced more homogenous reaction environments, which can result in a more efficient, and thus higher chitosan yield per gram of substrate (Nwe & Stevens, 2004). Acidic pH's typically result in chitosan with lower molecular weights than cultures grown under neutral conditions (Nwe et al., 2010). However many strains are known to produce less chitosan under acidic conditions; these factors need to be taken into account when designing chitosan producing fungal fermentations (Dhillon et al., 2013)

The nitrogen content and form of nitrogen being supplied in fungal fermentations is another key factor in maximizing the chitosan yield (Dhillon et al., 2013). In both solid state as well as submerged fermentations, urea was found to be the best nitrogen source to maximize chitosan yield (Nwe et al., 2002; Nwe & Stevens, 2004). However, with *Gongronella butleri*, increasing the nitrogen content of the media with urea resulted in an increase in the molecular weight of the chitosan produced (Nwe & Stevens, 2004). In submerged fermentations, the use of peptone as a nitrogen source has been shown to increase the molecular weight of chitosan as well (Arcidiacono & Kaplan, 1991).

Many fungi in the phylum zygomycota have high chitosan content in their cell walls (in addition to chitin). This is due to these fungi's production of chitin deacetylating enzymes (Dhillon et al., 2013). Several studies have looked at use of these enzymes as an alternative to chemical deacetylation techniques, however significant problems still need to be solved before enzymatic deacetylation is a viable technique (Chatterjee, Chatterjee, Chatterjee, & Guha, 2008; Dhillon et al., 2013).

Studies have also found that chitosan produced by the fungus *Rhizopus oryzae* has both a higher degree of deacetylation than other fungi, along with a higher bioactivity than chitosan produced from crustacean shells (Dhillon et al., 2013). *Mucor rouxii*, while producing less chitosan than *R. oryzae* (only 6-9% of biomass) has shown to peak in biomass production between 48-72h before decreasing slightly. It has been shown that many *Mucor* strains produce respectable quantities of biomass rapidly under submerged fermentation conditions (Arcidiacono & Kaplan, 1991; Dhillon et al., 2013). Acidic pH around 3-3.5 appear to stimulate chitosan production in these strains, which may have to do with the optimum pH of the chitosan deacetylating enzymes *Mucor* strains produce (Dhillon et al., 2013; Rane & Hoover, 1993).

Manganese and ferrous ions were shown to increase the biomass and chitosan yield in *Absidia orchidis,* despite decreasing the specific activity of one the chitosan deacetylase enzyme (Dhillon et al., 2013; Jaworska & Konieczna, 2001). Not surprisingly, multiple studies have shown that culture media containing salts and micronutrient minerals produced fungal cultures with higher biomass and chitosan yields than minimal medias (Chatterjee et al., 2008; Chatterjee, Chatterjee, Chatterjee, & Guha, 2009; Dhillon et al., 2013; Jaworska & Konieczna, 2001; Nwe et al., 2010; Rane & Hoover, 1993; Wu et al., 2005). Byproducts of the fungal fermentation, either metabolites or portions of the biomass have economic potential as valuable coproducts. Such coproducts include β-glucans, enzymes, acetic and lactic acids as well as high crude protein feed for animals (Dhillon et al., 2013).

## 2.4 SUGAR BEETS & SUGAR BEET PULP

Refined sugar consumption in the US is quite high, averaging 45 lbs per person per year in 2010 (Schumacher, Boland, & Brester, 2012). The primary sources of this sugar are refined table sugars and High Fructose Corn Syrup (HFCS). Table sugar, or sucrose, comes from primarily two sources, sugar beets and sugar cane, with a majority of the later being imported from Central and South American countries (Schumacher et al., 2012). The Federal Agriculture

Improvement and Reform Act sets the allotments of sugar to be produced from cane and beet at 45.65 and 54.35% respectively, however sugar from sugar beets has accounted for any where from 57 to 70% of refined sugar in recent years (James, 2011; Schumacher et al., 2012).

Michigan alone produced over 4.4 MM tons of sugar beets from 150 thousand harvested acres in 2014 (USDA, 2015). Each acre harvested can be processed into roughly 4 tons of sugar, making the refined sugar production from Michigan over 600 thousand tons (Schumacher et al., 2012). US refined sugar exports totaled $1.8B in 2011, however in the same year more than $5B worth of sugar was imported into the country, primarily from cane sugar producers, Mexico and Brazil, (Schumacher et al., 2012).

During the sugar beet processing, about 6.3% of the incoming sugar beet is expelled as SBP (Donkoh et al., 2012; Spagnuolo et al., 1997). SBP currently has a low market value, about $3 per ton when it is sold as an animal feed additive (Clark, 2014). However, this SBP is high in cellulose, hemicellulose, and pectin, giving it great potential as a feedstock for fermentation (Donkoh et al., 2012; Mata, Blazquez, Ballester, Gonzalez, & Munoz, 2009; Rombouts & Thibault, 1986; Saulnier & Thibault, 1999; Spagnuolo et al., 1997).

# 3. MATERIALS & METHODS

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich and all experiments were preformed with a minimum of 3 replicates; all standards used were analytical grade.

## 3.1 FUNGAL SEED

*Rhizopus orzyea* NRRL 395 (ATCC 9363), obtained from American Type Culture Collection (Manassas, VA, USA), was cultured on potato glucose agar (lot # BCBG7039V). After 3-4 weeks of culture at 25°C, the hyphal mass was washed and rinsed with autoclaved DI water to remove spores, under aseptic conditions (Y. Liu, Liao, & Chen, 2008). The spore solution was stored at 4°C and used to inoculate seed flasks. A hemocytometer was used to determine the concentration of this spore solution to be $7\times10^7$ spores per mL.

The seed culture medium composed of 24 g/L potato dextrose broth (PDB, lot # SLB7991V) and 3 g/L of calcium carbonate powder (Jade Scientific, Inc. Westland, MI, USA. Lot # G0213). 100µL of spore solution was added into 100 ml of autoclaved seed medium to generate seed inoculum. The seed flasks were cultured for 24 hours in a shaker incubator at 30°C and 180rpm. All fermentations were inoculated with this seed media at 5% (v/v) unless otherwise noted.

## 3.2 SUGAR BEET PULP

Two types of SBP were obtained from Michigan Sugar Company (Bay City, MI, USA). Dry pelletized SBP was used for initial experimentation. After the fall harvest, fresh SBP residues were obtained and used for the rest of the study, as they are more representative of the feedstock that would be used in the proposed implementation. While the switch between SBP was due to availability, it has the added benefit of testing the treatment and fermentation procedure across different seasons and slightly different feedstock compositions.

### 3.2.1 DRY SUGAR BEET PULP

The dry pelletized SBP was ground using a hammer mill (Schutte Buffalo, model: WA-6-H) to a 2.2mm size. The fiber composition of the SBP was analyzed according to the National Renewable Energy Lab (NREL) structural carbohydrate procedure (Sluiter et al., 2008). The pectin content was determined according to the procedure developed by Simpson et al. (Simpson, Egyankor, & Martin, 1984).

Three initial experiments were designed to determine the type of fermentation that would be optimized. The factors that were investigated in these initial tests included the use of an acid pretreatment, preliminary investigation of enzyme complexes for enzymatic hydrolysis and the efficacy of using a simultaneous saccharification and fermentation (SSF) procedure as opposed to a separate hydrolysis and fermentation.

#### 3.2.1.1 ACID PRETREATMENT

Acid pretreatment was preformed by placing dry SBP into 2% (w/w) sulfuric acid (Avantor Performance Materials, Center Valley, PA, USA. Lot # 0000102197) so the final concentration of SBP dry solids was 10% (w/w) (Ruan et al., 2013). The acid pulp slurry was heated to 121°C for 15min (autoclave conditions) and then allowed to cool to room temperature. The hydrolyzed slurry was then adjusted to pH 5 with 1 M sodium hydroxide (Avantor Performance Materials, Center Valley, PA, USA. Lot # 0000106384).

Test treatments included an acid pretreatment only, acid pretreatment followed by enzymatic hydrolysis with a high cellulase loading of 5.69 FPU[1]/g cellulose (Baiyin Sino Bio-Science Co. LTD. Shanghai, China. Lot # SS-12011-CEL), acid pretreatment followed by enzymatic hydrolysis with a high cellulase loading of 5.69 FPU/g cellulose and a high

[1] One Filter Paper Unit (FPU) is the amount of enzyme required to release 1 μmol of reducing sugar from filter paper per milliliter per minute at 50°C, in solution of pH 4.8 (Ghose, 1987).

hemicellulose loading of 520.4 ABXU[2]/g hemicellulose (Genencor, Palo Alto, CA, USA. Lot # 4901131618) and an non-pretreated enzymatic hydrolysis with a cellulase loading of 5.69 FPU/g cellulose and hemicellulose loading of 520.4 ABXU/g hemicellulose (Ruan et al., 2013). All treatments were incubated for 4 days at 50°C, shaking at 180 rpm. Each day, 3 flasks of each treatment were autoclaved (121°C, 15 minutes exposure time; to deactivate enzymes) and centrifuged at 8000 rpm for 10 minutes. The solids were dried to constant weight at 105°C and the percent of the initial solids liquefied during the hydrolysis was calculated.

### 3.2.1.2 ENZYMATIC HYDROLYSIS

The effects and interactions of 2 enzyme complexes were investigated. These enzymatic hydrolysis experiments were conducted with no acid pretreatment. Cellulase was investigated at a high loading for lignocellulosic biomass of 5.69 FPU/g cellulose (Ruan et al., 2013) and an average literature value for high pectin biomass of 1.56 FPU/g cellulose (Grohmann & Baldwin, 1992; Wilkins, Widmer, Grohmann, & Cameron, 2007). Pectinase (Lot # SLBM6360V) was investigated at a low loading for high pectin biomass of 48 U[3]/g pectin (Grohmann & Baldwin, 1992) and an average literature loading of 12,100 U/g pectin (Wilkins et al., 2007). The enzyme treatments were incubated for 4 days at 50°C, shaking at 180 rpm. Each day, 3 flasks of each treatment were autoclaved (121°C, 15 minutes exposure time; to deactivate enzymes) and centrifuged at 8000 rpm for 10 minutes. The reducing sugar concentration of the supernatant was measured via dinitrosalicylic acid method (DNS) (Miller, 1959). The solids were dried to constant weight at 105°C and the percent of the initial solid liquefied during the hydrolysis was calculated.

[2] One Acid Birchwood Xylanase Unit (ABXU) is the amount of enzyme required to generate 1.0 µmol of xylose-reducing sugar per minute at 50°C, in solution of pH 5.3 (Bailey, Biely, & Poutanen, 1992).
[3] One pectinase unit (U) will liberate 1.0 µmole of galacturonic acid from pectin per minute at pH 4.0 and 25°C (Sigma, 1997)

### 3.2.1.3 PRELIMINARY FERMENTATION & SSF

Each of the previously described acid pretreatment and enzymatic hydrolysis conditions was inoculated with 5% (v/v) fungal seed prepared as described in section 3.1. As a control, ground SBP pellets were hydrated with DI water and autoclaved (121°C, 30 minutes exposure time); the final SBP concentration of this media was 10% (w/v). This unpretreated media was inoculated with 5% (v/v) seed. As the pulp is high in pectin and *Rhizopus oryzae* can produce pectinase to hydrolyze pectin as carbon source (Kertes, 1951), tests were conducted to determine ability of the strain to produce pectinase enzymes and quantified by the Sigma Pectinase Activity Assay (Kertes, 1951; Sigma, 1997).

As part of the preliminary fermentation investigation, a solid/liquid separation was explored after the enzymatic hydrolysis to determine if there was any advantage to removing the residual SBP fibers. The high loadings of cellulase and pectinase enzyme (5.69 FPU/g cellulose and 12,100 U/g pectin, respectively) were applied to 2 sets of SBP flasks. After 24 hours of enzymatic hydrolysis, 3 flasks were centrifuged at 8000 rpm for 10 minutes, and the supernatant was autoclaved for fermentation. The other 3 flasks used the whole slurry for fermentation. All flasks had 10 g/L of calcium carbonate added to neutralize the pH. All flasks were then inoculated with seed, as described in section 3.1, and cultured for 4 days to compare the effects of separate fermentation on hydrolysate and fermentation on hydrolyzed pulp with residual fibers present.

Simultaneous saccharification and fermentation (SSF) was explored in this preliminary fermentation experiment as well. SSF cultures were produced by preparing 10% (w/v) SBP media as previously described, no acid pretreatment was conducted and cultures were inoculated with the high loadings of cellulase and pectinase enzymes (5.69 FPU/g cellulose and 12,100 U/g pectin, respectively) and 5% (v/v) seed at the same time.

### 3.2.2 WET SUGAR BEET PULP

Fresh, wet SBP was procured from Michigan Sugar Company. The fiber composition of the SBP was analyzed according to the NREL structural carbohydrate procedure (Sluiter et al., 2008). The pectin content was determined according to the procedure developed by Simpson et al. (Simpson et al., 1984). SBP culture media was made by blending 1 part SBP with 2 parts DI water in a commercial laboratory blender (BlendTec, ES3 EZ Blender) at 90% power for 50 seconds. Samples were taken from the SBP media each time it was made to determine the total solids, as this value varied with the moisture content of the pulp. Typical values were 6.5-7.5% TS (w/w). While the preliminary study on the dry pelletized SBP was used to determine the appropriate fermentation procedure, the enzyme loadings were optimized based on tests preformed on the wet SBP, as this pulp is an accurate representation of the recommended feedstock for this process.

#### 3.2.2.1 ENZYMATIC HYDROLYSIS OPTIMIZATION

Previous analysis indicated that cellulase and pectinase enzymes had the largest contribution to pulp degradation into fermentable sugars (section 3.2.1). The range explored with this preliminary study was very large, so a completely randomized design (CRD) was designed with two factors utilizing the fresh SBP as a substrate. Table 1 shows the range of enzyme loadings used for both pectinase and cellulase.

**Table 1. Enzyme loading ranges used in initial wet pulp hydrolysis study**

| Pectinase loading (U/g pectin) | Cellulase loading (FPU/g cellulose) |
|---|---|
| 48 | 1.00 |
| 500 | 1.56 |
| 1,000 | 2.50 |
| 2,500 | 5.69 |
| 6,000 | - |
| 12,100 | - |

Samples were incubated at 30°C and shaken at 180 rpm for 3 days. Samples were taken every day and reducing sugars were determined as g/L glucose equivalence according to the DNS method (Miller, 1959). After the 3rd day residual fibers were dried to constant weight to determine percent pulp hydrolyzed. As this was a preliminary study on the wet SBP, and the number of treatments was very large, experiments were done in duplicate.

As the cost of enzymes is anticipated to be one of the highest fixed costs of the proposed facility, optimizing the enzyme loading is essential to the economic viability of this process. Based on the preliminary results of the previous enzymatic hydrolysis experiments aCRD study (with 2 enzyme variables) was designed to explore the lower enzyme loadings further. In this study, 3 pectinase loadings were further investigated at the two low cellulase loadings, shown in table 2.

**Table 2. Enzyme loading ranges used in second wet pulp hydrolysis study**

| Pectinase loading (U/g pectin) | Cellulase loading (FPU/g cellulose) |
|---|---|
| 50 | 0.50 |
| 100 | 1.00 |
| 250 | - |

Samples were incubated at 30°C and shaken at 180 rpm for 3 days. Samples were taken every day and reducing sugars were determined as g/L glucose equivalence according to the DNS method (Miller, 1959). After the 3rd day residual fibers were dried to constant weight to determine percent pulp hydrolyzed.

## 3.3 CHITOSAN EXTRACTION & GLUCOSAMINE QUANTIFICATION

Chitosan was the primary criteria for quantifying the performance of the different fermentations preformed. Fungal biomass was first suspended in 1 M sodium hydroxide (biomass concentration of 10 g/L) and heated to 121°C for 15 minutes to strip away proteins. This mixture was centrifuged and the supernatant containing the solubilized proteins was

discarded. The biomass was washed with DI water and centrifuged again. Deproteinated biomass was then suspended in 2% acetic acid (Mallinckrodt Baker, NJ, USA, lot # H15A00) solution to hydrolyze and dissolve chitosan polymers. This mixture was incubated at 60°C, shaking at 180 rpm for 24 hours to ensure all soluble chitosan polymers had dissolved.

Samples were then centrifuged and the supernatant (containing the dissolved polymers) was recovered. In order to precipitate the chitosan polymers out of this acetic acid solution, the pH was adjusted to 10 with 1 and 10 M sodium hydroxide and stored at 4°C for ≥12 hours. The precipitated chitosan was centrifuged and washed twice with DI water to remove any salts formed from the extraction. Chitosan was then dried to constant weight and chitosan yield per gram of SBP input was calculated (Cho et al., 1998; Dhillon et al., 2013; Peniston & Johnson, 1972; Wu et al., 2005).

In order to determine the total glucosamine synthesized, a second method was used to hydrolyze and deacetylate the chitin and chitosan molecules. Dried biomass was hydrolyzed with 60% (v/v) sulfuric acid (biomass concentration = 10 g/L) at room temperature for 24 hours. Sulfuric acid concentration was then reduced to 0.5 M by diluting with DI water. Samples were autoclaved at 121°C for 1 hour (exposure time) and neutralized to pH of 7 with 1 M sodium hydroxide. Neutralized samples were diluted with DI water to bring the biomass concentration down to 0.1 g/L and a 0.5 mL sample was taken of each solution. These samples were mixed with 0.5 mL of 5% (w/v) sodium nitrite (lot # MKBH7064V) and 0.5 mL of 5% (w/v) potassium bisulfate (lot # SZBB3500V). Samples were shaken at 250 rpm for 15 minutes, and then centrifuged at 1500 x g for 2 minutes. 0.6 mL of supernatant was mixed with 0.2 mL of 12.5% (w/v) ammonium sulfamate (lot # MKBR8639V) and shaken vigorously for 3 minutes. To each sample 0.2 mL of 0.5% (w/v) methyl-2-benzothiazolinone hydrazone hydrochloride (lot # BCBG8561V) was added and then samples were boiled for 3 minutes and cooled to room temperature. 0.2 mL of 0.5% (w/v) iron(III) chloride hexahydrate (Mallinckrodt Baker, NJ, USA, lot # 851342) was added to each and samples were allowed to stand for 30 minutes before

being measured with a photospectrometer (Shimadzu UV-1800 UV spectrophotometer) at λ=650nm. Fresh methyl-2-benzothiazolinone hydrazone hydrochloride and iron(III) chloride hexahydrate was prepared each time and glucosamine was used to establish a standard curve (Chysirichote, Reiji, Asami, & Ohtaguchi, 2014).

## 3.4 MEDIA OPTIMIZATION

In order to maximize the yield of chitosan per gram of dry SBP the individual and synergistic effects of several important nutritional supplements were investigated. ATCC recommends potato dextrose media as the optimal media for the culturing of *R. oryzea*, therefore media optimization was first done on PDB media consisting of 24 g/L PDB and 5 g/L calcium carbonate (ATCC, 2014).

### 3.4.1 NITROGEN SOURCE

Several nitrogen sources were investigated on PDB media. These included yeast extract, urea, and ammonium sulfate. Previous studies have indicated that 1-2 g/L of yeast extract is sufficient for *R. oryzea* cultured on PDB (Minning et al., 2001; Ruan et al., 2013). These yeast extract concentrations were chosen, as well as a high value of 5 g/L to ensure the strain was not nitrogen limited.

Total nitrogen of the yeast extract used in this study was determined to be 9.86% (w/w) via the persulfate digestion method using a HACH Total Nitrogen kit (HACH, 2014). The three concentrations of yeast extract – 1, 2, & 5 g/L, correspond to 0.10, 0.19, & 0.48 g/L of total nitrogen, respectively. Urea and ammonium sulfate were then added to PDB media in order to produce equivalent total nitrogen concentrations.

### 3.4.2 NUTRITIONAL SUPPLEMNENTS

The addition of mineral salts was investigated as a means of improving the chitosan yield. The final concentration of salts in the culture media was 1 g/L $KH_2PO_4$, 0.5 g/L

$MgCl_2$•$6H_2O$, 1.4 mg/L $ZnSO_4$•$7H_2O$, 1.6 mg/L $MnSO_4$•$H_2O$, 3.6 mg/L $CoCl_2$•$6H_2O$, and 2.75 mg/L $FeSO_4$•$7H_2O$ (Ruan et al., 2013). Yeast extract was added at a concentration of 2 g/L. This test treatment was compared against 2 control treatment media including 24 g/L PDB and 5 g/L calcium carbonate and 24 g/L PDB, 5 g/L calcium carbonate and 2 g/L of yeast extract. Cultures were grown for 24h at 30°C, in a shaking incubator at 180 rpm.

### 3.4.3 ADDITION OF PLANT HORMONES

Several plant hormones have been shown to increase the chitosan content of fungi; of these, Gibberilic acid – 3 (GA3) has shown promising results when added to *R. oryzea* cultures (Chatterjee et al., 2008, 2009). Based on literature, 3 loadings of GA3 were selected: 0.1, 0.2, and 0.5 mg/L. In order to test the effects of this hormone, PDB media was again used, with 5 g/L calcium carbonate. Tests were conducted both with, and without 2 g/L of yeast extract.

## 3.5 PROCESS SCALE UP

Fermentations were scaled up to 2L flasks containing 500 mL of media made with the wet SBP. The results of the enzymatic hydrolysis (section 3.2.2.1) were used to determine the enzyme loading. The results of the media optimization (section 3.4) were used to determine the media components. Cultures were allowed to ferment for 48 and 72h. Yields from this scale up study were used to conduct the economic analysis.

## 3.6 BIOACTIVITY TEST

In order to determine the efficacy of the chitosan produced, a bioactivity assessment was conducted according to the method outlined by Windels & Nabben (Windels & Nabben, 1989). Fungal pathogen *Rhizoctonia solani* anastomosis group – 4 (AG-4), was obtained from Dr. Linda Hanson at Michigan State University. Cultures of this fungus were grown on PDA plates (24 g/L potato dextrose agar) for 6-7 days at 30°C to ensure sufficient time for the culture to cover the entire petri dish. Potting soil was sterilized at 121°C for 30 minutes (exposure time)

and approximately 250 $cm^3$ was placed in plastic cups. One of the *R. solani* covered agar discs was removed from the petri dish and placed on top of this soil so that the fungal mass covered all soil surface area. On top of this fungal mass was placed 50 $cm^3$ of sterilized soil.

USDA certified organic sugar beet and heirloom cucumber seeds were obtained from Mountain Valley Seed Company (Salt Lake City, UT). These seeds were disinfected with a solution of 0.5% (v/v) sodium hypochlorite and 0.1% (v/v) Tween 20. Seeds were suspended 1:2 (v/v) in this solution and shaken at 150 rpm for 5-10 minutes. The disinfectant solution was decanted and the seeds were then washed 3 times with DI water (Windels & Nabben, 1989). In order to accurately capture the effects chitosan had on disease resistance of the seed, 5 treatments were devised and then applied to each seed as follows:

- A seed dipped in sterile DI water (control 1)
- A seed dipped in 2% acetic acid (control 2)
- A seed dipped in a solution of 2% commercial chitosan from shrimp shells, ≥75% deacetylated, polymer size 40-150 KDa (lot # MKBH7256V), dissolved in 2% acetic acid (control 3)
- A seed dipped in a solution of 2% of the produced fungal chitosan (grown on PDB media) dissolved in 2% acetic acid
- A seed dipped in a solution of 2% of the produced fungal chitosan (grown on SBP media) dissolved in 2% acetic acid

These 5 treatments were placed in 2 different sets of soil cups, half had the pathogenic fungi placed in them as described previously (seed was then covered with 50 $cm^3$ of sterilized soil) and half had no pathogen added (negative control treatments, seed was then covered with 50 $cm^3$ of sterilized soil). All treatments were done with 6 replicates and each soil cup was watered every day with 10mL of DI water. Cultures were monitored for 3 weeks; each day the number of plants to sprout were recorded and their heights were measured.

## 3.7 ANALYSIS & DATA INTERPRETATION

All experiments were done using a minimum of 3 replicates unless otherwise noted. The data analysis was preformed with SAS® version 9.4 statistical software. An Analysis of Variance (ANOVA) with pairwise comparisons was conducted for each data set. The significance level used was set at 0.05.

# 4. RESULTS AND DISCUSSION

## 4.1 DRY SUGAR EET PULP

The results of the fiber and pectin analysis on the dry pelletized SBP are shown in Figure 1. This pectin content was slightly lower than values in literature at 9.3%, versus reported values that range from 15-30% (Mata et al., 2009; Rombouts & Thibault, 1986; Saulnier & Thibault, 1999). However, in this study there was considerable variation observed in all fiber components both within a given season's samples and between the previous seasons dry pulp and the current seasons fresh pulp (data can be found in appendix A).

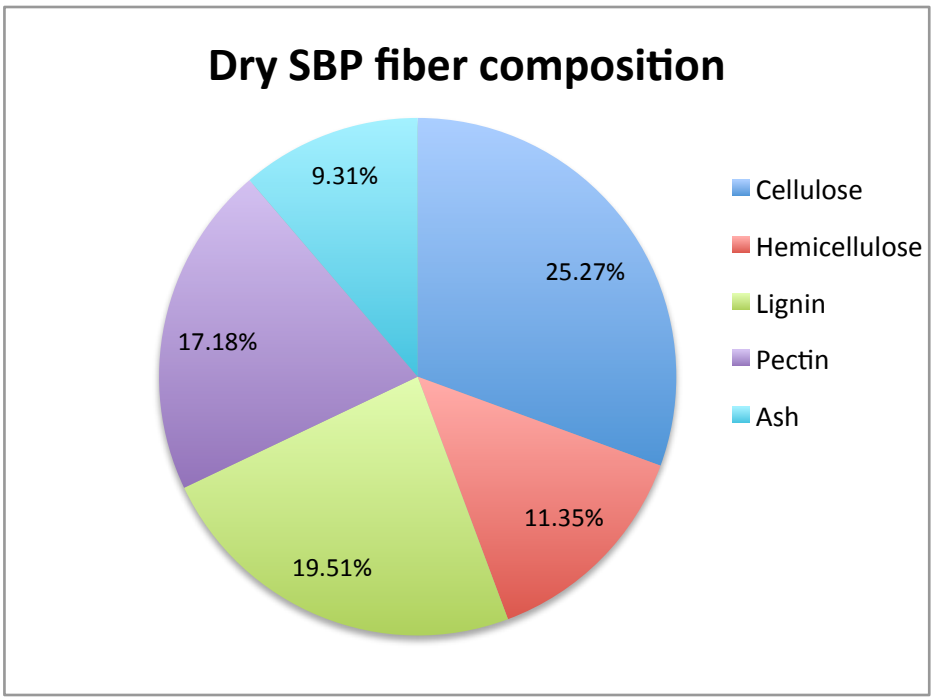


**Figure 1. Dry SBP fiber composition analysis**

### 4.1.1 ACID PRETREATMENT, ENZYMATIC HYDROLYSIS & SSF

Table 3 shows the results of the acid pretreatment and initial enzymatic hydrolysis. The data indicate that acid pretreatment was not able to significantly increase the percent of solids liquefied. This might be related with the composition of SBP, as it has both a lower

hemicellulose and lower lignin content compared with other cellulosic feedstocks. The synergistic effects between the hemicellulase enzyme and the cellulase enzyme did not produce any noticeable benefit over the use of cellulase alone. Table 3 shows data for only 2 replicates of each treatment.

**Table 3. Acid pretreatment and enzymatic hydrolysis**

| Treatment | Solids hydrolyzed |
|---|---|
| No pretreatment | - |
| Acid pretreatment | 6.7% |
| Acid pretreatment + cellulase | 44.9% |
| Acid pretreatment + cellulase & hemicellulase | 41.8% |
| cellulase & hemicellulase | 40.3% |

Table 4 shows whether or not the fungal strain was capable of growing on each of the described SBP treatments. These cultures indicated that *R. oryzea* was capable of growth on untreated pulp. As this was a solid-state fermentation, the observed growth was quite slow. This test did show *R. oryzae* can grow on raw SPB and there were no inhibitors left on the pulp slurry from the sugar extraction process that would prevent fungal growth; it also showed that *R. oryzea* was able to hydrolyze pectin in the pulp residues as the strain produced 3 U/mg solid (58 U/mL in 100 mL of PDB fermentation broth, data shown in appendix A). The fungus didn't grow on acid treated SBP (Table 4). This may be caused by possible inhibitors generated during acid pretreatment, as harsh acid pretreatment can result in the production of some toxic inhibitors such as furfural and phenolic compounds that can inhibit fungal growth (Zha et al., 2014). As such, direct enzymatic hydrolysis and fermentation without pretreatment was selected for further study.

**Table 4. Preliminary fermentation results**

| Treatment | Fermentation |
|---|---|
| No pretreatment | Growth |
| Acid pretreatment | No growth |
| Acid pretreatment + pellulase | No growth |
| Acid pretreatment + cellulase & hemicellulase | No growth |
| Cellulase & hemicellulase | Growth |

The results of the enzymatic hydrolysis test are shown in table 5. This data, along with that shown in table 3 indicate that there is no benefit obtained with the addition of hemicellulase. Therefore, hemicellulase was not used in any of the further studies. The reducing sugars liberated from the SBP over the course of this enzymatic hydrolysis are shown in appendix A.

**Table 5. Dry SBP enzymatic hydrolysis**

| Enzyme combination | | | Solids hydrolyzed (%) | Solids hydrolzed (standard deviation) | DNS sugar concentration (g/L) | DNS sugar concentration (standard deviation) |
|---|---|---|---|---|---|---|
| Cellulase (FPU/g Cellulose) | Pectinase (U/g Pectin) | Hemicellulase (ABXU/g Hemicellulose) | | | | |
| 1.56 | | | 30.03% | 5.06% | 3.90 | 0.2247 |
| 5.69 | | | 51.20% | - | 14.1 | - |
| 5.69 | | 520.4 | 47.10% | - | 15.7 | - |
| | 48 | | 35.90% | 3.79% | 4.05 | 0.3062 |
| | 12,100 | | 69.60% | 2.55% | 50.7 | 4.080 |
| 1.56 | 48 | | 55.44% | 1.84% | 18.5 | 1.673 |
| 1.56 | 12,100 | | 72.83% | 4.28% | 44.7 | 14.569 |
| 5.69 | 48 | | 64.48% | 3.96% | 23.7 | 1.692 |
| 5.69 | 12,100 | | 72.01% | 0.28% | 54.4 | 1.488 |

Figure 2 shows the decrease in reducing activity caused by fungal growth on both the separated hydrolysate supernatant and the hydrolysate with residual fibers. As can be seen, there was no difference between these 2 culture techniques. Figure 2 also shows that there appears to be about 30 g/L of residual reducing activity, or about 56% of the initial reducing activity. The compound producing this reducing activity appears to be unavailable as a carbon source for the fungal strain under the tested fermentation conditions.

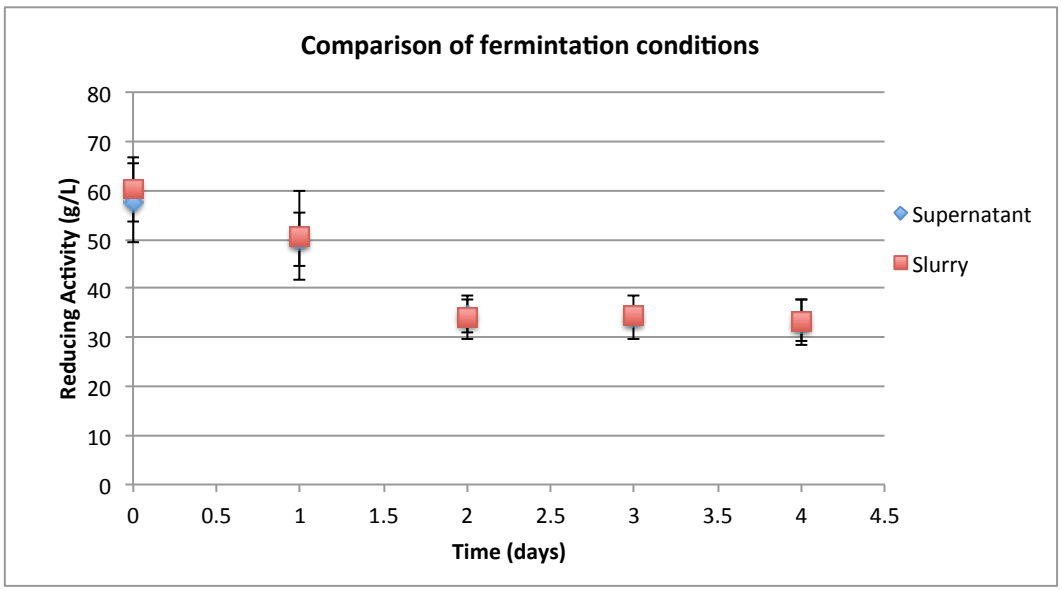


**Figure 2. Reducing sugars liberated from culture on enzymatic hydrolysate and mixed SBP slurry**

The SSF results were similar to the separate enzymatic hydrolysis, with 51% of the initial reducing activity unable to be consumed by the fungus. Chitosan was extracted from the treatments as described and the results of the chitosan extraction are presented in Table 6. Unsurprisingly, with the increase in fermentable sugars released by the enzymatic hydrolysis, more biomass was produced over the 4-day culture resulting in a higher chitosan yield. Although the conditions of the SSF treatment were designed around the needs of the fungal strain, and therefore not optimized for the enzymes, the SSF cultures were able to generate significantly more chitosan per gram of SBP input (Table 6). In addition, SSF simplifies the process by eliminating a liquid solid separation step.

**Table 6. Initial chitosan extraction**

| Treatment | Chitosan yield | Standard deviation |
|---|---|---|
| Unhydrolyzed SBP | 0.20% | - |
| Hydrolyzed SBP | 0.62% | 0.14% |
| SSF | 1.53% | 0.83% |

## 4.2 WET SUGAR BEET PULP

The results of the fiber and pectin analysis on the fresh wet SBP are shown in Figure 3. These values deviated slightly from the dry SBP, showing some of the seasonal variability

present in agricultural residues. Aside from the reduction in pectin present, there were considerable changes in the cellulose and ash contents. A large part of this variation is due to the change in ash content. It is believed that the ash content of the dry pulp was high, partially due to coming from the dry pellets at the end of the season. As the tail end of the pulp these pellets are far more likely to have excess sand and dirt picked up from the bottoms of containers or piles.

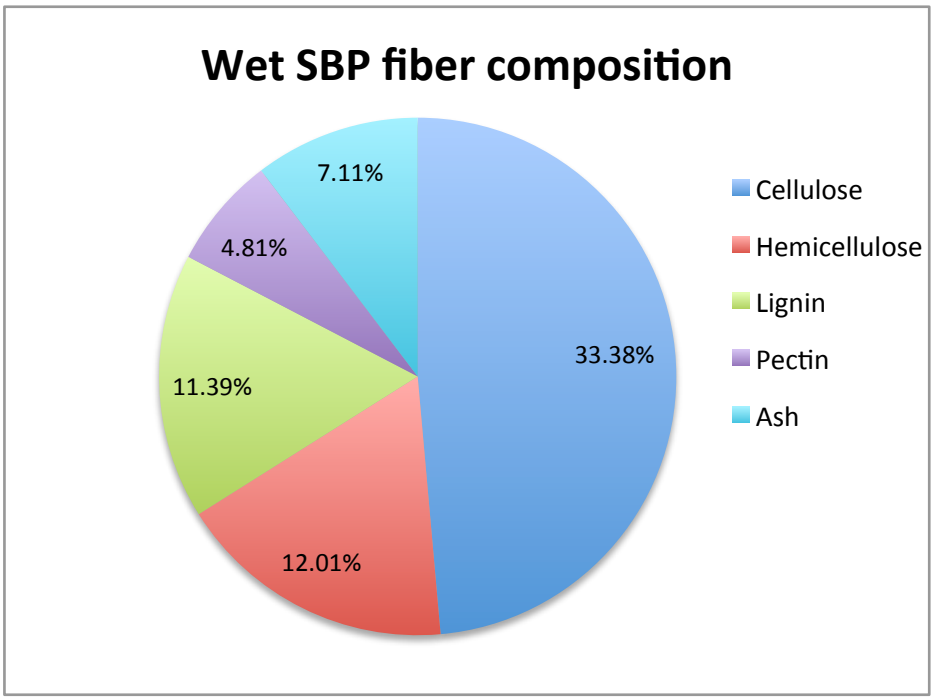


**Figure 3. Wet SBP fiber composition analysis**

## 4.3 ENZYMATIC HYDROLYSIS OPTIMIZATION

Table 7 shows the results of the enzymatic hydrolysis experiments preformed on the wet pulp. For the total amount of solids hydrolyzed, the interaction between enzymes was not found to be statistically significant. There was no statistically significant difference between any of the treatments in the 1$^{st}$ test group. The lowest loading in this 1$^{st}$ group (pectinase=500 U/g pectin, cellulase=1.0 FPU/g cellulose) was significantly better than all of the lower loadings explored in test group 2. The percent of total solids hydrolyzed was found to be sensitive primarily to the

pectinase loading, while the cellulase loading produced no statistically significant changes in hydrolysis performance. The full statistical analysis can be found in appendix B.

For the DNS sugar concentrations (reported as g/L glucose equivalence) the interaction between the enzymes was found to be statistically significant. The second highest loading explored in the 1st group (pectinase=2,500 U/g pectin, cellulase=2.5 FPU/g cellulose) was significantly better then almost all of the other treatments. Reducing sugars liberated was found to be sensitive to both pectinase and cellulase loadings. The full statistical results can be found in appendix B.

**Table 7. Results of the enzymatic hydrolysis optimization on wet SBP and Kg of reducing sugars released per dollar input on enzymes**

| Test group | Enzyme combination | | Solids hydrolyzed (%) | DNS sugar concentration (g/L) | Kilograms sugar released per dollar input on enzymes |
|---|---|---|---|---|---|
| | Cellulase (FPU/g Cellulose) | Pectinase (U/g Pectin) | | | |
| 1 | 2.5 | 6,000 | 86.02 | 58.29 | 725 |
| 1 | 2.5 | 2,500 | 84.41 | 65.18 | 1502 |
| 1 | 2.5 | 1,000 | 85.01 | 60.83 | 2211 |
| 1 | 2.5 | 500 | 82.12 | 58.71 | 2642 |
| 1 | 1.0 | 6,000 | 80.55 | 50.49 | 719 |
| 1 | 1.0 | 2,500 | 80.84 | 59.47 | 1790 |
| 1 | 1.0 | 1,000 | 81.22 | 62.91 | 3625 |
| ***1*** | ***1.0*** | ***500*** | ***74.07*** | ***43.24*** | ***3584*** |
| 2 | 1.0 | 100 | 39.10 | 22.14 | 2827 |
| 2 | 1.0 | 50 | 46.81 | 24.50 | 3355 |
| 2 | 0.5 | 250 | 46.92 | 18.03 | 2989 |
| 2 | 0.5 | 100 | 47.29 | 26.67 | 6002 |
| 2 | 0.5 | 50 | 52.97 | 21.59 | 5516 |

As the reducing sugars liberated was far more sensitive to the enzyme loading than pulp hydrolysis the loadings that produced a significant increase in sugars liberated were broken down by cost. With the anticipated costs of pectinase at $80,000 per ton (A. Liu, 2015) and cellulase at $6,270 per ton (G. Liu, Zhang, & Bao, 2015), the grams of reducing sugar liberated per dollar input are shown in the last column of Table 7.

These values were derived by multiplying the anticipated enzyme cost and units used by the total substrates present in one year of operation (9,620 metric tons of pectin, 66,760 metric tons of cellulose). The product of the hydrolysis performance for each enzyme combination (in grams of reducing sugar released per gram SBP input) and the total SBP hydrolyzed per year (200,000 tons) was then divided by the total cost for one years worth of the selected enzyme loading.

As is shown by Table 7, a low loading of 0.5 FPU/g cellulose and 100 U/g pectin produced the highest grams of reducing sugars per dollar spent on enzymes. However, the results of an ANOVA pairwise comparison (located in appendix B) show there was no statistical difference between any of the bottom 5 treatments in Table 7. The loading of 500 U/g pectin and 1.0 FPU/g cellulose produced the highest amount of grams reducing sugar per dollar at a statistically significant level (there was no statistically significant difference between the 500 U/g pectin and 1 FPU/g cellulose treatment and the 1000 U/g pectin and 1 FPU/g cellulose treatment). For this reason the loadings of pectinase and cellulase of 500 U/g pectin and 1.0 FPU/g cellulose, respectively, were selected (italicized treatment in Table 7).

## 4.4 MEDIA OPTIMIZATION

The 3 nitrogen sources investigated were: yeast extract, urea, and ammonium sulfate. These sources were explored on a nitrogen equivalence basis; N concentrations in PDB media were 0.097, 0.194, and 0.484 g/L. Table 8 shows the results of the nitrogen source study. While the biomass yield from the lowest loading of yeast extract was not found to be statistically different from the control, it was found to be better than any of the ammonium sulfate treatments, and the low urea treatment. There was no statistically significant difference between the low yeast extract treatment and the higher 2 urea treatments. The mid level and high-level loading of yeast extract were both considerably better than any of the other nitrogen sources used. The

chitosan yield for each treatment produced the same statistical results and significances as the biomass yield.

**Table 8. Biomass and glucosamine yield (per gram sugar input) from various nitrogen sources on PDB media**

| Treatment | Biomass yield | Biomass standard deviation | Glucosamine yield | Glucosamine standard deviation |
|---|---|---|---|---|
| Control | 11.70% | 1.35% | 1.222% | 0.25% |
| Yeast extract (0.097 g/L N) | 15.21% | 2.86% | 1.995% | 0.62% |
| Yeast extract (0.194 g/L N) | 30.91% | 7.93% | 4.105% | 0.93% |
| Yeast extract (0.484 g/L N) | 42.52% | 6.18% | 5.293% | 0.90% |
| Urea (0.097 g/L N) | 8.09% | 0.18% | 1.961% | 0.09% |
| Urea (0.194 g/L N) | 11.42% | 1.95% | 1.807% | 0.75% |
| Urea (0.484 g/L N) | 14.32% | 1.25% | 2.478% | 0.63% |
| Ammonium sulfate (0.097 g/L N) | 6.62% | 0.57% | 1.227% | 0.17% |
| Ammonium sulfate (0.194 g/L N) | 7.48% | 0.61% | 1.236% | 0.18% |
| Ammonium sulfate (0.484 g/L N) | 6.51% | 0.96% | 1.255% | 0.15% |

The results of the addition of nutritional supplements are shown in Table 9. The addition of nutritional salts produced no statistically significant difference in biomass yield than the control media composed of only PDB. The yeast extract only control produced the highest biomass yield almost 3 times as much as other treatments.

**Table 9. Effects of nutritional salts on the yield of biomass and glucosamine on PDB media**

| Treatment | Biomass yield | Biomass standard deviation | Glucosamine yield | Glucosamine standard deviation |
|---|---|---|---|---|
| Control | 11.70% | 1.35% | 1.222% | 0.25% |
| Yeast extract (0.194 g/L N) | 30.91% | 7.93% | 4.105% | 0.93% |
| Yeast extract + nutritional salts | 10.96% | 0.11% | 2.456% | 0.16% |

Figure 4 shows the nutritional salts results alongside the results of the GA3 study. GA3 produced a reduction in biomass at all levels of its application. Despite the reduction in biomass, the cultures grown on GA3 media, specifically at the 0.2 mg/L loading, displayed a very slight increase in chitosan yield. However none of these differences were found to be of statistical significance (shown in appendix C). This, combined with the high cost of GA3, roughly $700,000/metric ton (Rarexoticsseeds, 2015), precluded investigating GA3 any further for beneficial effects.

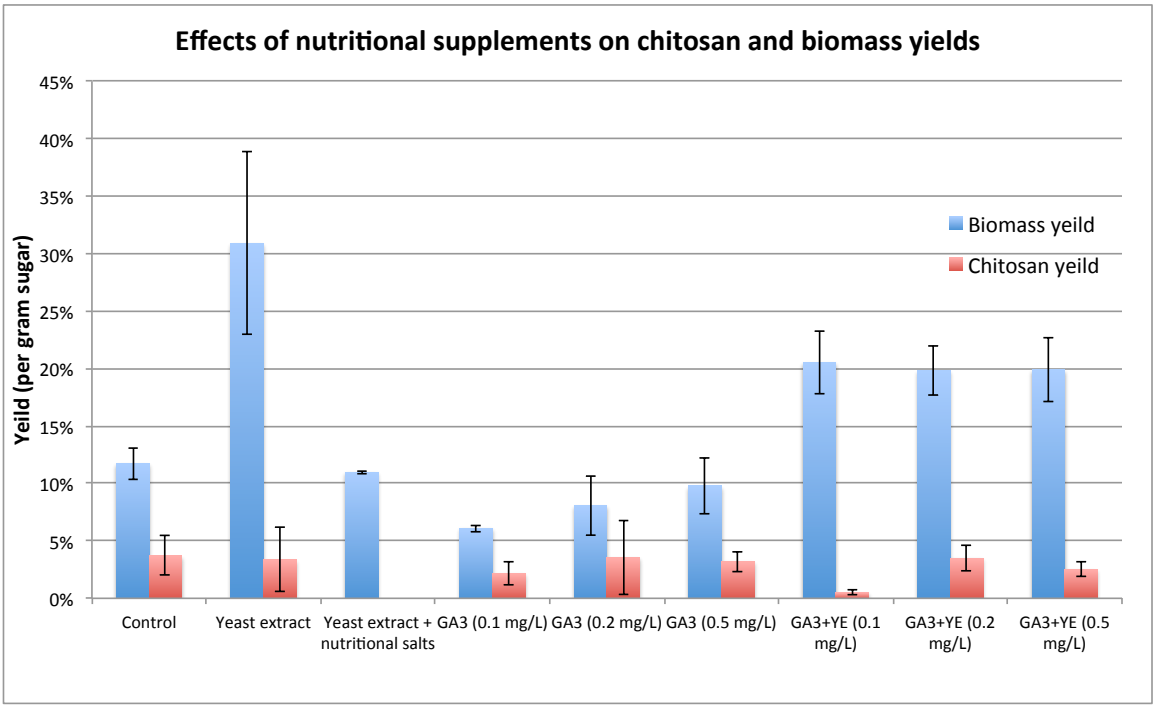


**Figure 4. Biomass and chitosan yields of PDB media with yeast extract and nutritional supplements**

## 4.4.1 SCALE UP

The 2 L flasks cultures were stopped after 48h and 72h. Figure 5 shows the performance of scale up cultures in glucosamine and chitosan production. Other fungal metabolites have potential value as industrial coproducts; the production of one such metabolite, lactic acid, was quantified in the scale up study. Lactic acid production is given as yield (grams per gram SBP consumed).

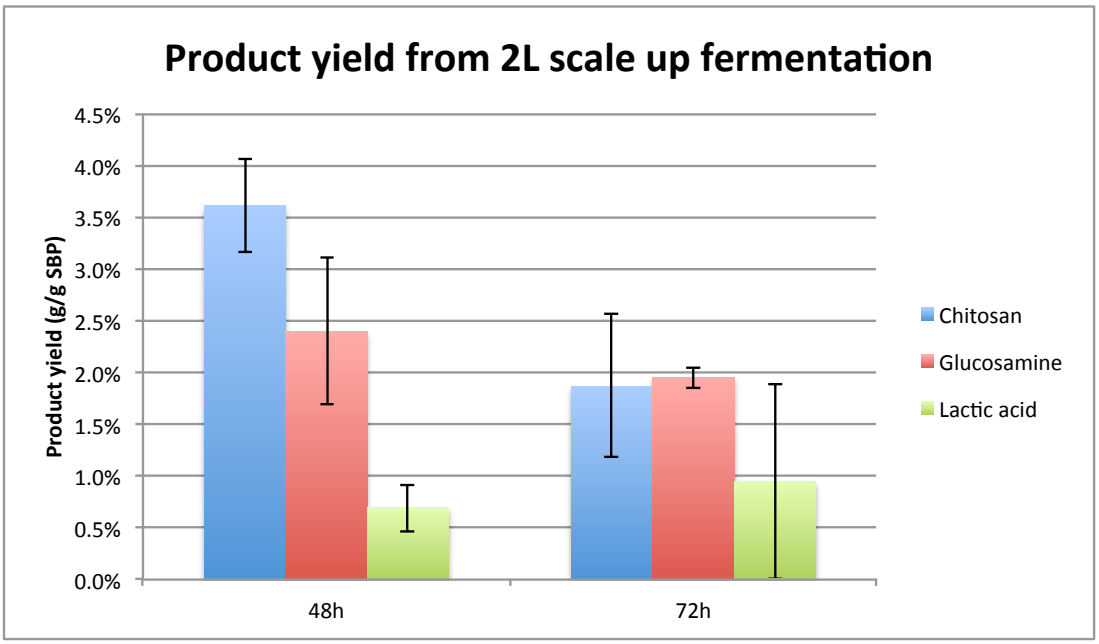


**Figure 5. Yields of various products from the 2L scale up fermentation**

The differences in chitosan and glucosamine production between the 48h cultures and the 72h cultures were found to be statistically significant. As figure 5 shows, the 48h culture generated better results in all metrics. This is to be expected as previous studies have shown that the all of the available sugars present in the SBP cultures are consumed within the first 48 hours.

Figure 6 shows the comparison between the 48h scale up culture and the 100mL flask cultures in chitosan and glucosamine yields. The differences observed between the two cultures were found to be statistically significant for both chitosan and glucosamine. One possible reason for the lower yields observed in the scale up compared to the 100mL flasks might be the low oxygen level when scaled up to 2L flasks. More experiments should be conducted using bioreactors to generate more information for process scale up and commercialization.

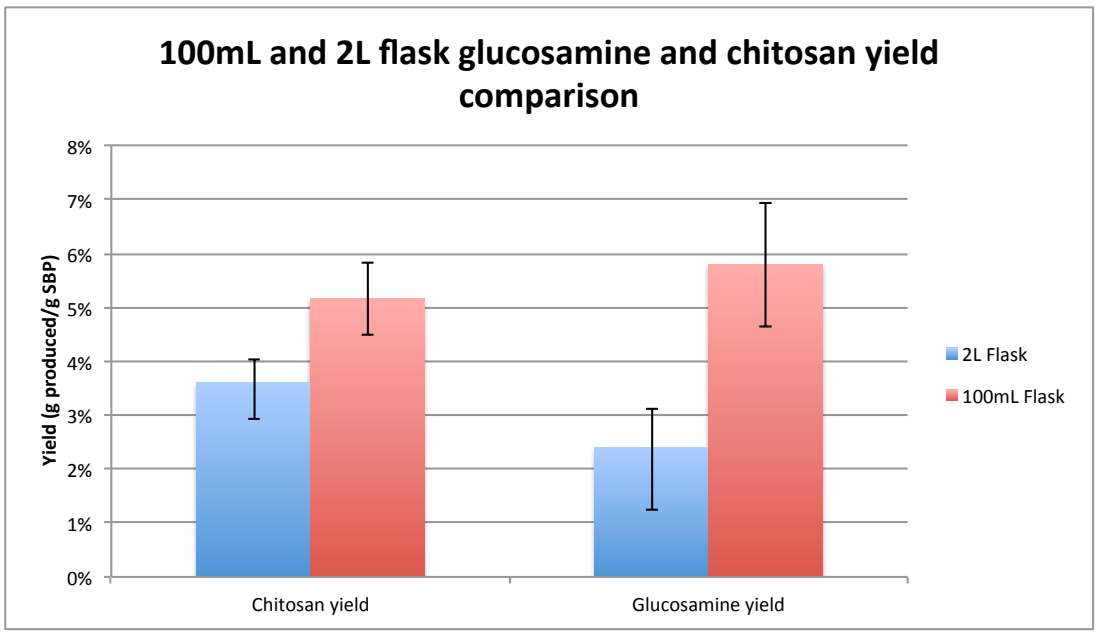


**Figure 6. Yield comparison between 2L scale up flask and previous 100mL cultures in chitosan and glucosamine yield**

## 4.5 BIOACTIVITY TEST

Figure 7 shows the average heights of the cucumbers for each treatment. Seed treatments include:

- A seed dipped in sterile DI water (control 1)
- A seed dipped in 2% acetic acid (control 2)
- A seed dipped in a solution of 2% commercial chitosan from shrimp shells, ≥75% deacetylated, polymer size 40-150 KDa (lot # MKBH7256V), dissolved in 2% acetic acid (control 3)
- A seed dipped in a solution of 2% of the produced fungal chitosan (grown on PDB media) dissolved in 2% acetic acid
- A seed dipped in a solution of 2% of the produced fungal chitosan (grown on SBP media) dissolved in 2% acetic acid

While the soil only control (control 1 -) had the highest average plant height after 14 days, each of the test treatments inoculated with pathogenic fungi preformed better than the positive

soil only control (control 1 +). However, no treatment inoculated with the pathogen preformed better than the simple application of acetic acid.

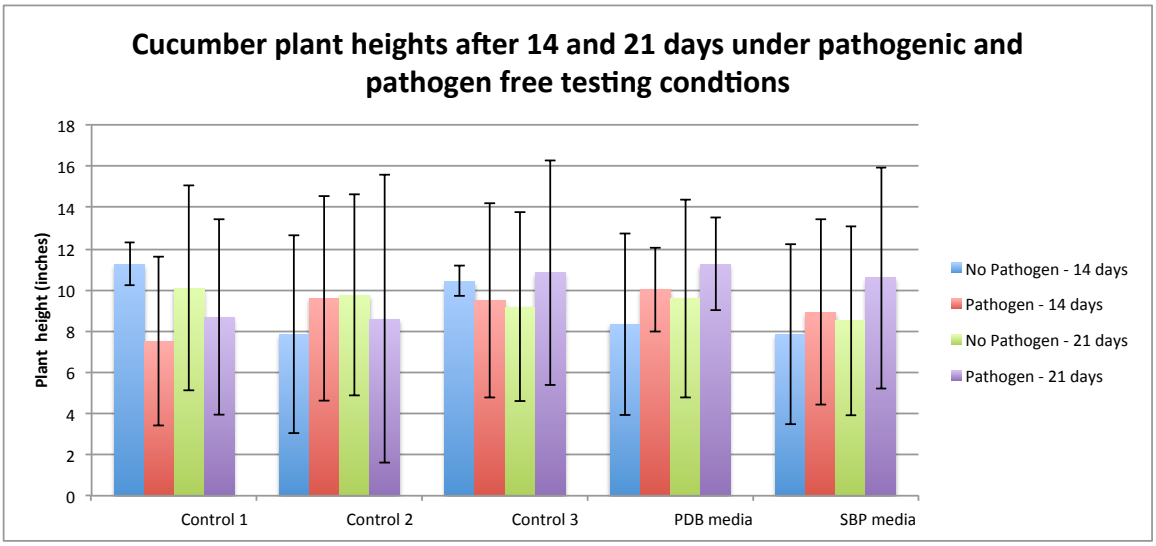


**Figure 7. Height of cucumber plants after 2 and 3 weeks of the bioactivity assessment**

After 21 days of growth, all of the positive treatments with chitosan applied were taller then both the soil only and acetic acid controls (control 1, and control 2). This seems to indicate that the application of chitosan produces some benefit above the application of acetic acid alone, however more research is needed on this topic as none of the observed differences in the cucumber plants at either 14, or 21 days was found to be statistically significant.

Figure 8 shows the average heights of the sugar beet plants for each treatment at 14 and 21 days. Again the soil only control (control 1 -) grew the best over the first 14 days, with the acetic acid positive control outperforming the soil only positive control slightly. On the negative treatments, the addition of chitosan from either the PDB or SBP media improved the growth slightly over the acetic acid control, however this relationship was not statistically significant.

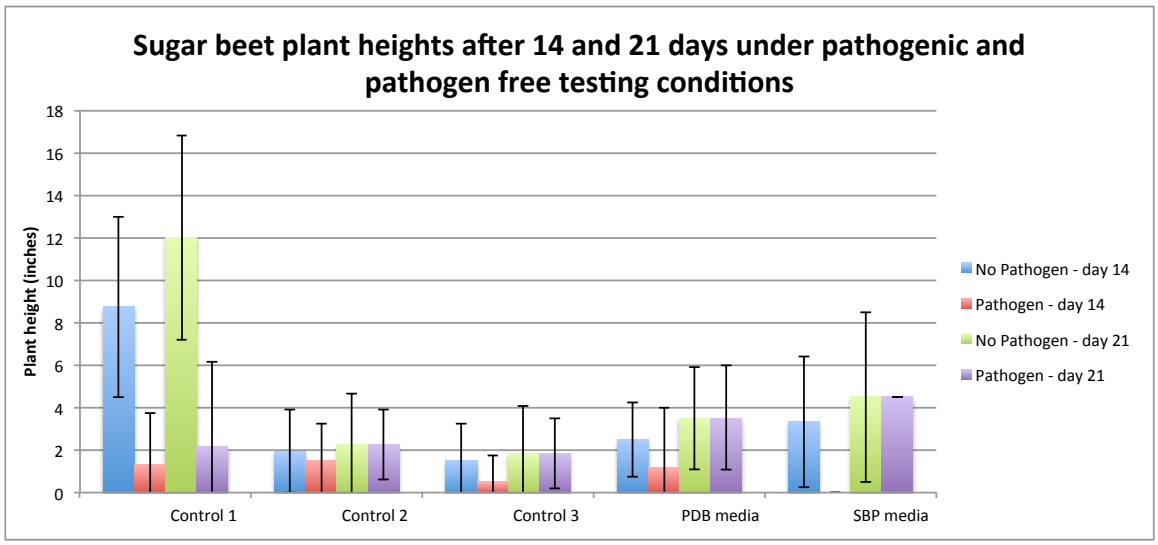


**Figure 8 Height of sugar beet plants after 2 and 3 weeks of the bioactivity assessment**

After the 21 day period, both of the fungal chitosan positive treatments preformed better than all of the controls, as with the cucumber this suggests that the addition of chitosan may benefit the plant in some way, when in the presence of the tested phytopathogen (*R. solani* AG-4). Before such a conclusion can be drawn, more research must be conducted, as the previously described observations were not found to be statistically significant. Pictures were taken during the growth period and the full statistical results for both cucumbers and sugar beets can be found in appendix D.

## 4.6 ECONOMIC ANALYSIS

### 4.6.1 PARAMETERS

Economic feasibility was done using the production of 200,000 dry tons of SBP residues per year (Donkoh et al., 2012). It is assumed the proposed plant would be built on site at an existing sugar beet processing facility, so the purchase of land was omitted from the economic analysis. The full set of parameters used for the economic analysis are described in Table 10. These parameters were based on the 2L flask experiments, and scaled up to the operational production of the proposed plant.

**Table 10. Economic analysis parameters and descriptions**

| System components | |
|---|---|
| **Feedstock** | 200,000 metric ton per year |
| Type | Food wastes |
| Availability | Half year |
| **Fermentation Unit** | |
| Fermentation | Batch |
| TS in the fermentation broth (%) | 10 |
| Fermentation time (day) | 2 |
| Fermentation temperature (°C) | 30 |
| Fermenter size ($m^3$) | 100 |
| Fermenter number | 240 |
| **Post-treatment** | |
| TS in the fermented broth (%) | 10 |
| Alkali tank ($m^3$) | 100 |
| Alkali tank number | 120 |
| Alkali treatment temperature (°C) | 60 |
| Alkali treatment time (hours) | 8 |
| Tank occupation (batch/day) | 2 |
| Weight ratio of NaOH to dry solid residue | 1:2.5 |
| NaOH recycle time | 10 |
| NaOH amount (metric ton per year) | 8,000 |
| | |
| Alkali/solid separation[a] | centrifuge |
| | |
| Wet biomass amount (metric ton per year) | 933,333 |
| TS in the solid after separation (%) | 15 |
| Acid tank ($m^3$) | 100 |
| Acid tank number | 40 |
| Acid treatment temperature (°C) | 60 |
| Acid treatment time (hours) | 24 |
| Tank occupation (batch/day) | 1 |
| Ratio of acid to dry separated solid residue | 1:5 |
| Acetic acid amount (metric ton per year) | 28,000 |
| | |
| NaOH needed to neutralize the acetic acid | The spent NaOH in alkali treatment will be used here |
| **Products** | |
| Chitosan (metric ton/year) | 16,060 |
| Lactic acid (metric ton/year) | 1,387 |
| **Labor cost** | |
| Operator | 30 |
| **Other expense** | |
| Maintenance | 5% of the operation cost |
| **Financial analysis** | |
| Inflation rate | 4% |
| Depreciation | MACRS[b] |

a. 50% of the biomass was removed by alkali treatment.

b. The depreciation period is set as 10 years. The annual depreciation rates from MACRS (Modified Accelerated Cost Recovery System) for the next 10 years are: 0.100, 0.188, 0.144, 0.115, 0.092, 0.074, 0.066, 0.065, 0.065, 0.033, 0.033.

## 4.6.2 COST ANALYSIS

Parameters used in the analysis of costs and revenues are shown in Table 11. Capital costs are a single investment, revenue and operational costs area all reported as a cost per annum. As was anticipated, the cost of enzymes is the largest operating costs of the system.

**Table 11. Cost parameters**

| System component | |
|---|---|
| **Unit capital costs**[a] | |
| Fermentation unit | $22,000,000 |
| Alkali post-treatment unit | $11,000,000 |
| Centrifuge | $300,000 |
| Acid post-treatment unit | $5,500,000 |
| Dryer | $200,000 |
| Accessories[b] | $3,900,000 |
| Control and automation[c] | $1,950,000 |
| Wastewater treatment | $3,500,000 |
| **Total capital cost** | **$22,770,000** |
| **Revenue (yearly)** | |
| Chitosan[d] | $240,900,000.00 |
| Lactic acid[e] | $2,010,740.76 |
| **Total expenses on the system (yearly)** | |
| Enzymes[f] | $43,862,659 |
| Acetic acid | $28,000,000 |
| NaOH[g] | $2,320,000 |
| Maintenance[h] | $4,584,356 |
| Net energy cost[i] | $23,825,006 |
| Labor (30 full time employees) | $3,000,000 |
| | |
| **Total operating costs (yearly)** | $127,610,572 |
| **Revenue (yearly)** | $242,910,740.76 |
| **Net Revenue (yearly)** | **$115,300,168.76** |

a. The capital costs do not include design and engineering fees.

b. The accessories is 10% of the total equipment cost of fermentation unit, alkali post-treatment unit, centreifuge, acid post-treatment, and dryer.

c. The cost for the control and automation is 5% of the total equipment cost.

d. The price of chitosan is $15,000 per ton (Ensymm, 2008).

e. The price of lactic acid is $1,450 per ton (Higson, 2011)

f. Enzymes include 3,927 ton per year cellulase and 241 ton per year pectinase. The cost for pectinase and cellulase are $6,270 per ton and $80,000 per ton (A. Liu, 2015; G. Liu et al., 2015).

g. NaOH is $290 per ton (A. Liu, 2015; Wang, 2016).

h. The maintenance cost is 10% of the total energy cost.

i. The total energy cost for the system is $45,843,577/year, the current cost of drying and peletizing the SBP is $23,825,006/year. The net energy cost is difference between these two and represents the additional operational costs, above what a the sugar beet plant already spends.

### 4.6.3 CASH FLOW AND ECONOMIC SUMMARY

Chitosan production from SBP can turn a low value product stream into a high value product stream, with significant economic benefits for sugar processors. The production of chitosan greatly improves the utilization efficiency of SBP, and reduces the residues from the sugar industry. Tables 13 and 14 clearly show the economic potential of the proposed chitosan production system. At the current rate of SBP production, 200,000 dry ton per year, the added chitosan production can generate a net revenue, on average, of $115,220,168 per year, which is approximately 6 times higher than current revenue – based on selling sugar beet pulp as an animal feed. The economic advantage of taking a waste product and utilizing it for the production of a value added material is, in this scenario immense. Figure 9 shows the cash flow of the proposed system over the first 5 years. With a payback period of a little over 7 months the system is economically favorable.


**Figure 9. Cash flow diagram of the chitosan production facility for the first 5 years**

# 5. SUMMARY & CONCLUSION

As this study has shown, resource recovery operations have tremendous potential. By optimizing the enzymatic hydrolysis for wet SBP, pretreatments were able to achieve 74% liquefaction and liberate 60 g/L of sugars based on a 10% SBP solids loading. Nutritional elements were evaluated to increase the production of chitosan and an optimal loading for yeast extract was found to generate the most chitosan per gram of SBP input, producing 3.6 grams of chitosan for every 100g of SBP input. With the value of both the produced chitosan and other cellular metabolites, the economic feasibility analysis showed a favorable return, with a payback period of just over 7 months.

# APPENDICES

# APPENDIX A: DRY PULP DATA

Table A1 shows the data used for the determination of the dry pulp fiber analysis.

**Table A1. Dry pulp fiber analysis data**

| Sample Weight (g) | Crucible + Dry (g) | Crucible + Ash (g) | HPLC Glucose (g/L) | HPLC Xylose (g/L) | Absorbance (λ=320nm) | Soluble | Insoluble | Cellulose Content | Xylan Content | Lignin Content | Ash Content |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.3000 | 45.3582 | 45.3471 | 1.0040 | 0.4591 | 0.855 | 2.48% | 3.70% | 26.11% | 11.72% | 6.18% | 3.70% |
| 0.3200 | 45.0021 | 44.9040 | 0.9982 | 0.4586 | 0.801 | 2.18% | 30.66% | 24.42% | 10.97% | 32.83% | 30.66% |
| 0.3300 | 45.2676 | 45.1884 | 0.9963 | 0.4726 | 0.867 | 2.29% | 24.00% | 23.64% | 10.96% | 26.29% | 24.00% |
| *Averages* | | | | | | | | *24.72%* | *11.22%* | *21.77%* | *19.45%* |
| *Standard deviation* | | | | | | | | *1.26%* | *0.44%* | *13.89%* | *14.04%* |

Table A2 shows the data from the initial acid pretreatment and enzymatic hydrolysis study. Acid conditions consisted of 2% w/w sulfuric acid (SBP concentration was 100 g/L), and heated to 121°C for 1 hour (autoclave conditions) before being neutralized to pH 7 with sodium hydroxide. Cellulase (5.69 FPU/g cellulose) and hemicellulose (520.4 ABXU/g hemicellulose) enzymes were then added to the neutralized samples and incubated at 50°C for 96 hours in a shaking incubator at 180rpm.

**Table A2. Acid pretreatment & enzymatic hydrolysis data on dry SBP**

| Sample | Residual Solids (g) | Initial Solids (g) | Percent hydrolyzed | Avg. hydrolyzed | Standard deviation |
|---|---|---|---|---|---|
| Acid Pretreatment | 4.8055 | 5 | 3.89% | | |
| Acid Pretreatment | 4.5260 | 5 | 9.48% | 6.69% | - |
| Acid Pretreatment | - | 5 | - | | |
| Acid pretreatment + cellulase | 2.2853 | 5 | 54.29% | | |
| Acid pretreatment + cellulase | 2.0568 | 5 | 58.86% | 44.93% | 20.31% |
| Acid pretreatment + cellulase | 3.9189 | 5 | 21.62% | | |
| Acid pretreatment + cellulase & hemicellulase | 3.0042 | 5 | 39.92% | | |
| Acid pretreatment + cellulase & hemicellulase | 2.1920 | 5 | 56.16% | 41.76% | 13.57% |
| Acid pretreatment + cellulase & hemicellulase | 3.5398 | 5 | 29.20% | | |
| cellulase & hemicellulase | 2.6239 | 5 | 47.52% | | |
| cellulase & hemicellulase | 3.4132 | 5 | 31.74% | 40.29% | 7.98% |
| cellulase & hemicellulase | 2.9189 | 5 | 41.62% | | |

Table A3 shows the data used in the determination of the pectinase enzyme activity produced by *R. oryzae* when grown on 24 g/L of PDB media. Equitation A1 shows the calculation used in analyzing this data (Sigma, 1997).

**Table A3. Pectinase enzyme activity production from *R. oryzae* when grown on PDB media**

| Sample | Volume of $Na_2S_2O_3$ reagent used in titration | Time of reaction (min) | Volume of enzyme solution used (mL) | Dilution factor | Units/mL of enzyme |
|---|---|---|---|---|---|
| Blank | 5.25 | 5 | 0.1 | 1 | 0 |
| Test | 5.22 | 5 | 0.1 | 1 | 3 |
| Pectinase std | 5.13 | 5 | 0.1 | 1 | 12 |

Eq. A1 $$\frac{Units}{mL\ of\ enzyme} = \frac{(1)\times(100)\times(mL\ of\ Reagent\ used\ in\ blank - mL\ of\ Reagent\ used\ in\ Test)\times(dilution\ factor)}{(Reaction\ time\ in\ mins)\times(Volume of\ enzyme\ solution\ used\ (mL))\times(2)}$$

$(1) = One\ \mu mole\ glacturonic\ acid\ is\ oxidized\ by\ 1\ microequivalent\ of\ I_2$
$(100) = microequivalents\ of\ S_2O_3\ per\ mL\ of\ Na_2S_2O_3\ reagent$
$(Reagent) = Na_2S_2O_3\ reagent$
$(2) = microequivalents\ of\ S_2O_3\ oxidized\ per\ microequivalent\ of\ I_2\ reduced$

Figure A1 shows the reducing sugars liberated over the course of the initial enzymatic hydrolysis with cellulase (5.69 FPU/g cellulose) and hemicellulose (520.4 ABXU/g hemicellulose) enzymes.

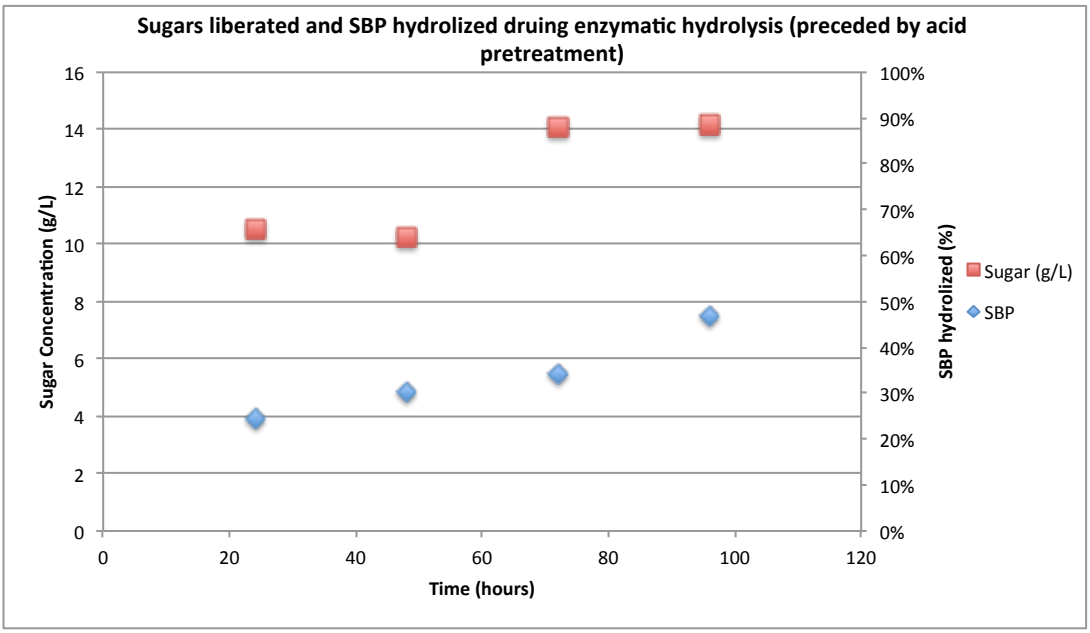


**Figure A1. Enzymatic hydrolysis performance on SBP pretreated with $H_2SO_4$**

Figure A2 shows the reducing sugars liberated over the course of the 4 day enzymatic hydrolysis with the combinations of pectinase and cellulase. The low and high values of pectinase were 48 and 12,100 U/g pectin respectively; the low and high values for cellulase were 1.56 and 5.69 FPU/g cellulose respectively.

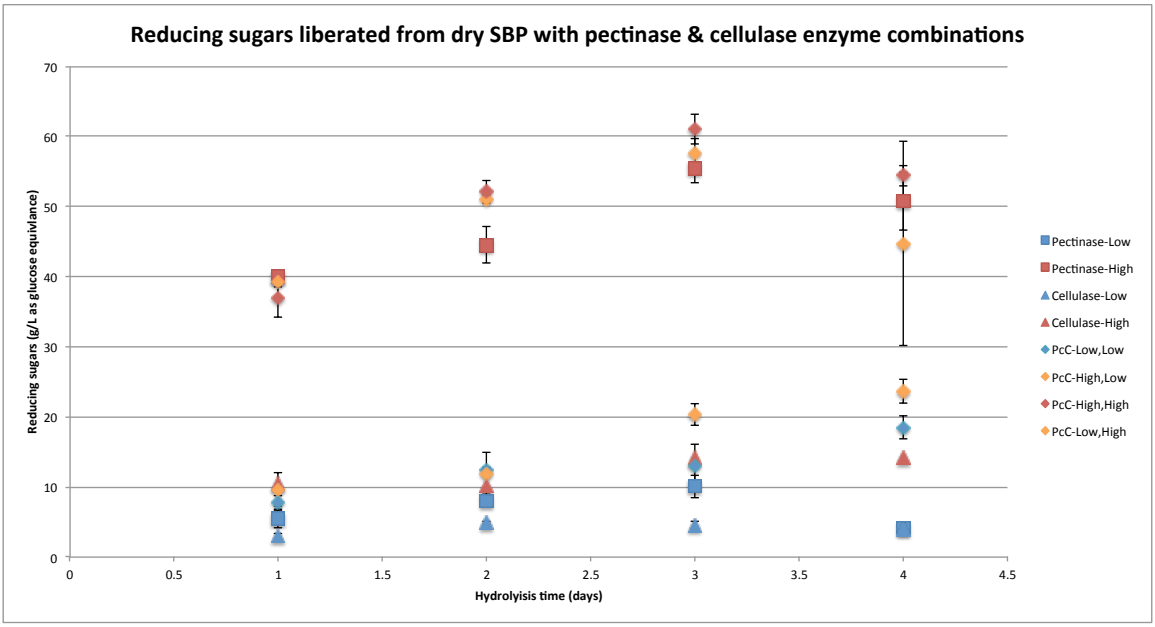


**Figure A2. Reducing sugars liberated from dry SBP over a 4 day enzymatic hydrolysis**

# APPENDIX B: WET PULP DATA

Table B1 shows the data used for the determination of the wet pulp fiber analysis.

**Table B1. Wet pulp fiber analysis data**

| Sample Weight (g) | Crucible + Dry (g) | Crucible + Ash (g) | HPLC Glucose (g/L) | HPLC Xylose (g/L) | Absorbance (λ=320nm) | Soluble | Insoluble | Cellulose Content | Xylan Content | Lignin Content | Ash |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.1500 | 44.9944 | 44.9820 | 0.6606 | 0.2295 | 0.724 | 4.20% | 8.27% | 34.48% | 11.72% | 12.47% | 8.27% |
| 0.1500 | 43.5757 | 43.5620 | 0.7300 | 0.2563 | 0.788 | 4.57% | 9.13% | 38.11% | 13.08% | 13.70% | 9.13% |
| 0.1500 | 43.4249 | 43.4190 | 0.5276 | 0.2200 | 0.701 | 4.07% | 3.93% | 27.54% | 11.23% | 8.00% | 3.93% |
| *Averages* | | | | | | | | *33.38%* | *12.01%* | *11.39%* | *7.11%* |
| *Standard deviation* | | | | | | | | *5.37%* | *0.96%* | *3.00%* | *2.79%* |

Table B2 shows the data from the two CRD enzymatic hydrolysis experiments

**Table B2. Data from wet pulp enzymatic hydrolysis**

| Enzyme combination | | Solids unhydrolyzed (g) | Initial solids (g) | Solids unhydrolyzed (%) | Reducing sugars (g/L) |
|---|---|---|---|---|---|
| Cellulase (FPU/g Cellulose) | Pectinase (U/g Pectin) | | | | |
| 0.50 | 50 | 0.2910 | 0.579 | 50.26% | 26.6 |
| 0.50 | 50 | 0.2871 | 0.579 | 49.59% | 14.2 |
| 0.50 | 50 | 0.3416 | 0.579 | 59.00% | 24.0 |
| 1.00 | 50 | 0.2546 | 0.579 | 43.97% | 24.2 |
| 1.00 | 50 | 0.3135 | 0.579 | 54.15% | 19.1 |
| 1.00 | 50 | 0.2446 | 0.579 | 42.25% | 30.2 |
| 0.50 | 100 | 0.2525 | 0.579 | 43.61% | 30.0 |
| 0.50 | 100 | 0.0901 | 0.579 | 15.56% | 23.8 |
| 0.50 | 100 | 0.2949 | 0.579 | 50.93% | 26.2 |
| 1.00 | 100 | 0.2939 | 0.579 | 50.76% | 18.2 |
| 1.00 | 100 | 0.2237 | 0.579 | 38.64% | 24.7 |
| 1.00 | 100 | 0.2688 | 0.579 | 46.42% | 23.5 |
| 0.50 | 250 | 0.2325 | 0.579 | 40.16% | 19.1 |
| 0.50 | 250 | 0.3016 | 0.579 | 52.09% | 22.6 |
| 0.50 | 250 | 0.2806 | 0.579 | 48.46% | 12.4 |
| 1.00 | 100 | 0.0551 | 0.273 | 20.18% | 65.9 |
| 1.00 | 100 | 0.0475 | 0.273 | 17.40% | 67.5 |
| 1.00 | 500 | 0.0884 | 0.273 | 32.38% | 44.5 |
| 1.00 | 500 | 0.0533 | 0.273 | 19.52% | 42.0 |
| 2.50 | 500 | 0.0497 | 0.273 | 18.21% | 54.8 |
| 2.50 | 500 | 0.0480 | 0.273 | 17.58% | 55.4 |
| 2.50 | 100 | 0.0426 | 0.273 | 15.60% | 58.3 |
| 2.50 | 100 | 0.0393 | 0.273 | 14.40% | 68.1 |
| 1.00 | 2500 | 0.0495 | 0.273 | 18.13% | 53.6 |
| 1.00 | 2500 | 0.0552 | 0.273 | 20.22% | 39.2 |
| 2.50 | 2500 | 0.0407 | 0.273 | 14.91% | 79.7 |
| 2.50 | 2500 | 0.0445 | 0.273 | 16.30% | 64.8 |
| 1.00 | 6000 | 0.0518 | 0.273 | 18.97% | 65.5 |
| 1.00 | 6000 | 0.0545 | 0.273 | 19.96% | 48.3 |
| 2.50 | 6000 | 0.0306 | 0.273 | 11.21% | 52.7 |
| 2.50 | 6000 | 0.0458 | 0.273 | 16.78% | 63.9 |

Table B3 shows the information used to derive the cost per grams of reducing sugar produced per year based on the enzyme loading.

**Table B3. Data used in the calculation of the grams of reducing sugars released per dollar input on enzymes over the course of 1 year**

| Pectinase (U/g pectin) | Cellulase (FPU/g cellulose) | Total units of pectinase used (per year) | Total units of cellulase used (per year) | Cost of pectinase (per year) | Cost of cellulase (per year) | Total cost (per year) | Sugars released (per year, based on 200,000 tonnes of SBP) | Grams of sugar released per dollars spent on enzymes (per year) |
|---|---|---|---|---|---|---|---|---|
| 500 | 1 | 4.81E+12 | 6.68E+10 | $19,240,000 | $24,622,659 | $43,862,659 | 1.57E+14 | 3584362 |
| 500 | 2.5 | 4.81E+12 | 1.67E+11 | $19,240,000 | $61,556,647 | $80,796,647 | 2.13E+14 | 2642376 |
| 1000 | 1 | 9.62E+12 | 6.68E+10 | $38,480,000 | $24,622,659 | $63,102,659 | 2.29E+14 | 3625410 |
| 1000 | 2.5 | 9.62E+12 | 1.67E+11 | $38,480,000 | $61,556,647 | $100,036,647 | 2.21E+14 | 2211080 |
| 2500 | 1 | 2.41E+13 | 6.68E+10 | $96,200,000 | $24,622,659 | $120,822,659 | 2.16E+14 | 1789755 |
| 2500 | 2.5 | 2.41E+13 | 1.67E+11 | $96,200,000 | $61,556,647 | $157,756,647 | 2.37E+14 | 1502419 |
| 6000 | 1 | 5.77E+13 | 6.68E+10 | $230,880,000 | $24,622,659 | $255,502,659 | 1.84E+14 | 718579 |
| 6000 | 2.5 | 5.77E+13 | 1.67E+11 | $230,880,000 | $61,556,647 | $292,436,647 | 2.12E+14 | 724794 |
| 250 | 0.5 | 2.41E+12 | 3.34E+10 | $9,620,000 | $12,311,329 | $21,931,329 | 6.56E+13 | 2988973 |
| 100 | 1 | 9.62E+11 | 6.68E+10 | $3,848,000 | $24,622,659 | $28,470,659 | 8.05E+13 | 2827484 |
| 100 | 0.5 | 9.62E+11 | 3.34E+10 | $3,848,000 | $12,311,329 | $16,159,329 | 9.70E+13 | 6001937 |
| 50 | 1 | 4.81E+11 | 6.68E+10 | $1,924,000 | $24,622,659 | $26,546,659 | 8.91E+13 | 3355358 |
| 50 | 0.5 | 4.81E+11 | 3.34E+10 | $1,924,000 | $12,311,329 | $14,235,329 | 7.85E+13 | 5515987 |

The full statistical breakdown of the enzymatic hydrolysis optimization preformed on wet SBP is shown in figure B4

**Table B4. Statistical analysis of different enzyme loadings with response variable of reducing sugars liberated**

| i/j | Pectinase 50 U/g pectin, Cellulase 0.5 FPU/g cellulose | Pectinase 50 U/g pectin, Cellulase 1 FPU/g cellulose | Pectinase 100 U/g pectin, Cellulase 0.5 FPU/g cellulose | Pectinase 100 U/g pectin, Cellulase 1 FPU/g cellulose | Pectinase 250 U/g pectin, Cellulase 0.5 FPU/g cellulose | Pectinase 500 U/g pectin, Cellulase 1 FPU/g cellulose | Pectinase 500 U/g pectin, Cellulase 2.5 FPU/g cellulose | Pectinase 1000 U/g pectin, Cellulase 1 FPU/g cellulose | Pectinase 1000 U/g pectin, Cellulase 2.5 FPU/g cellulose | Pectinase 2500 U/g pectin, Cellulase 1 FPU/g cellulose | Pectinase 2500 U/g pectin, Cellulase 2.5 FPU/g cellulose | Pectinase 6000 U/g pectin, Cellulase 1 FPU/g cellulose | Pectinase 6000 U/g pectin, Cellulase 2.5 FPU/g cellulose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pectinase 50 U/g pectin, Cellulase 0.5 FPU/g cellulose | | 0.5812 | 0.3386 | 0.9173 | 0.4987 | 0.0015 | <.0001 | <.0001 | <.0001 | 0.0004 | <.0001 | <.0001 | <.0001 |
| Pectinase 50 U/g pectin, Cellulase 1 FPU/g cellulose | 0.5812 | | 0.6785 | 0.6535 | 0.2265 | 0.0045 | <.0001 | <.0001 | <.0001 | 0.0013 | <.0001 | <.0001 | <.0001 |
| Pectinase 100 U/g pectin, Cellulase 0.5 FPU/g cellulose | 0.3386 | 0.6785 | | 0.3916 | 0.1115 | 0.0102 | 0.0001 | <.0001 | <.0001 | 0.0031 | <.0001 | <.0001 | <.0001 |
| Pectinase 100 U/g pectin, Cellulase 1 FPU/g cellulose | 0.9173 | 0.6535 | 0.3916 | | 0.4365 | 0.0018 | <.0001 | <.0001 | <.0001 | 0.0005 | <.0001 | <.0001 | <.0001 |
| Pectinase 250 U/g pectin, Cellulase 0.5 FPU/g cellulose | 0.4987 | 0.2265 | 0.1115 | 0.4365 | | 0.0004 | <.0001 | <.0001 | <.0001 | 0.0001 | <.0001 | <.0001 | <.0001 |
| Pectinase 500 U/g pectin, Cellulase 1 FPU/g cellulose | 0.0015 | 0.0045 | 0.0102 | 0.0018 | 0.0004 | | 0.0767 | 0.0016 | 0.0055 | 0.622 | 0.0002 | 0.0441 | 0.0286 |
| Pectinase 500 U/g pectin, Cellulase 2.5 FPU/g cellulose | <.0001 | <.0001 | 0.0001 | <.0001 | <.0001 | 0.0767 | | 0.0831 | 0.2183 | 0.1857 | 0.0144 | 0.7776 | 0.622 |
| Pectinase 1000 U/g pectin, Cellulase 1 FPU/g cellulose | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0016 | 0.0831 | | 0.583 | 0.0048 | 0.3934 | 0.139 | 0.1991 |
| Pectinase 1000 U/g pectin, Cellulase 2.5 FPU/g cellulose | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0055 | 0.2183 | 0.583 | | 0.0162 | 0.1689 | 0.3357 | 0.4489 |
| Pectinase 2500 U/g pectin, Cellulase 1 FPU/g cellulose | 0.0004 | 0.0013 | 0.0031 | 0.0005 | 0.0001 | 0.622 | 0.1857 | 0.0048 | 0.0162 | | 0.0007 | 0.1137 | 0.0767 |
| Pectinase 2500 U/g pectin, Cellulase 2.5 FPU/g cellulose | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0002 | 0.0144 | 0.3934 | 0.1689 | 0.0007 | | 0.0262 | 0.0405 |
| Pectinase 6000 U/g pectin, Cellulase 1 FPU/g cellulose | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0441 | 0.7776 | 0.139 | 0.3357 | 0.1137 | 0.0262 | | 0.8322 |
| Pectinase 6000 U/g pectin, Cellulase 2.5 FPU/g cellulose | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0286 | 0.622 | 0.1991 | 0.4489 | 0.0767 | 0.0405 | 0.8322 | |

# APPENDIX C: MEDIA OPTIMIZATION

Figure C1 shows the results of the statistical analysis on the GA3 media study. Response variable was chitosan yield.

**Table C1. GA3 study statistical analysis**

| i/j | GA3 - 0.1 mg/L | GA3 - 0.1 mg/L + Y.E. | GA3 - 0.2 mg/L | GA3 - 0.2 mg/L + Y.E. | GA3 - 0.5 mg/L | GA3 - 0.5 mg/L + Y.E. | PDB only | PDB + Y.E. |
|---|---|---|---|---|---|---|---|---|
| GA3 - 0.1 mg/L | | 0.2597 | 0.3597 | 0.3731 | 0.4975 | 0.8173 | 0.3068 | 0.4322 |
| GA3 - 0.1 mg/L + Y.E. | 0.2597 | | 0.0508 | 0.0535 | 0.0810 | 0.1796 | 0.0409 | 0.0659 |
| GA3 - 0.2 mg/L | 0.3597 | 0.0508 | | 0.9790 | 0.8067 | 0.4891 | 0.9116 | 0.8926 |
| GA3 - 0.2 mg/L + Y.E. | 0.3731 | 0.0535 | 0.9790 | | 0.8271 | 0.5054 | 0.8908 | 0.9134 |
| GA3 - 0.5 mg/L | 0.4975 | 0.0810 | 0.8067 | 0.8271 | | 0.6522 | 0.7224 | 0.9125 |
| GA3 - 0.5 mg/L + Y.E. | 0.8173 | 0.1796 | 0.4891 | 0.5054 | 0.6522 | | 0.4238 | 0.5760 |
| PDB only | 0.3068 | 0.0409 | 0.9116 | 0.8908 | 0.7224 | 0.4238 | | 0.8058 |
| PDB + Y.E. | 0.4322 | 0.0659 | 0.8926 | 0.9134 | 0.9125 | 0.5760 | 0.8058 | |

Table C2 shows the results of the nitrogen source media optimization with the response variable of biomass yield. Table C3 shows the same study with the response variable of glucosamine yield.

**Table C2. Nitrogen source optimization with biomass yield as response variable**

| i/j | Ammonium sulfate (0.097 g/L N) | Ammonium sulfate (0.194 g/L N) | Ammonium sulfate (0.484 g/L N) | Control | Urea (0.097 g/L N) | Urea (0.194 g/L N) | Urea (0.484 g/L N) | Yeast extract (0.097 g/L N) | Yeast extract (0.194 g/L N) | Yeast extract (0.484 g/L N) |
|---|---|---|---|---|---|---|---|---|---|---|
| Ammonium sulfate (0.097 | | 0.7619 | 0.9691 | 0.0853 | 0.6053 | 0.1022 | 0.0125 | 0.0062 | <.0001 | <.0001 |
| Ammonium sulfate (0.194 | 0.7619 | | 0.7327 | 0.1485 | 0.8296 | 0.1752 | 0.0243 | 0.0122 | <.0001 | <.0001 |
| Ammonium sulfate (0.484 | 0.9691 | 0.7327 | | 0.0792 | 0.5788 | 0.0951 | 0.0115 | 0.0056 | <.0001 | <.0001 |
| Control | 0.0853 | 0.1485 | 0.0792 | | 0.2135 | 0.9234 | 0.3612 | 0.2253 | <.0001 | <.0001 |
| Urea (0.097 g/L N) | 0.6053 | 0.8296 | 0.5788 | 0.2135 | | 0.2489 | 0.0382 | 0.0197 | <.0001 | <.0001 |
| Urea (0.194 g/L N) | 0.1022 | 0.1752 | 0.0951 | 0.9234 | 0.2489 | | 0.3144 | 0.1926 | <.0001 | <.0001 |
| Urea (0.484 g/L N) | 0.0125 | 0.0243 | 0.0115 | 0.3612 | 0.0382 | 0.3144 | | 0.7548 | <.0001 | <.0001 |
| Yeast extract (0.097 g/L N) | 0.0062 | 0.0122 | 0.0056 | 0.2253 | 0.0197 | 0.1926 | 0.7548 | | <.0001 | <.0001 |
| Yeast extract (0.194 g/L N) | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | | 0.0005 |
| Yeast extract (0.484 g/L N) | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0005 | |

**Table C3. Nitrogen source optimization with glucosamine yield as response variable**

| i/j | Ammonium sulfate (0.097 g/L N) | Ammonium sulfate (0.194 g/L N) | Ammonium sulfate (0.484 g/L N) | Control | Urea (0.097 g/L N) | Urea (0.194 g/L N) | Urea (0.484 g/L N) | Yeast extract (0.097 g/L N) | Yeast extract (0.194 g/L N) | Yeast extract (0.484 g/L N) |
|---|---|---|---|---|---|---|---|---|---|---|
| Ammonium sulfate (0.097 | | 0.9846 | 0.9528 | 0.9905 | 0.1253 | 0.2209 | 0.0130 | 0.1101 | <.0001 | <.0001 |
| Ammonium sulfate (0.194 | 0.9846 | | 0.9682 | 0.9750 | 0.1298 | 0.2279 | 0.0136 | 0.1141 | <.0001 | <.0001 |
| Ammonium sulfate (0.484 | 0.9528 | 0.9682 | | 0.9433 | 0.1393 | 0.2428 | 0.0149 | 0.1226 | <.0001 | <.0001 |
| Control | 0.9905 | 0.9750 | 0.9433 | | 0.1227 | 0.2167 | 0.0127 | 0.1077 | <.0001 | <.0001 |
| Urea (0.097 g/L N) | 0.1253 | 0.1298 | 0.1393 | 0.1227 | | 0.7403 | 0.2739 | 0.9432 | 0.0001 | <.0001 |
| Urea (0.194 g/L N) | 0.2209 | 0.2279 | 0.2428 | 0.2167 | 0.7403 | | 0.1595 | 0.6874 | <.0001 | <.0001 |
| Urea (0.484 g/L N) | 0.0130 | 0.0136 | 0.0149 | 0.0127 | 0.2739 | 0.1595 | | 0.3049 | 0.0020 | <.0001 |
| Yeast extract (0.097 g/L N) | 0.1101 | 0.1141 | 0.1226 | 0.1077 | 0.9432 | 0.6874 | 0.3049 | | 0.0002 | <.0001 |
| Yeast extract (0.194 g/L N) | <.0001 | <.0001 | <.0001 | <.0001 | 0.0001 | <.0001 | 0.0020 | 0.0002 | | 0.0176 |
| Yeast extract (0.484 g/L N) | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0176 | |

# APPENDIX D: BIOACTIVITY

Figure D1 shows the control cucumber bioactivity results at day 14. These controls were not inoculated with pathogenic fungi.

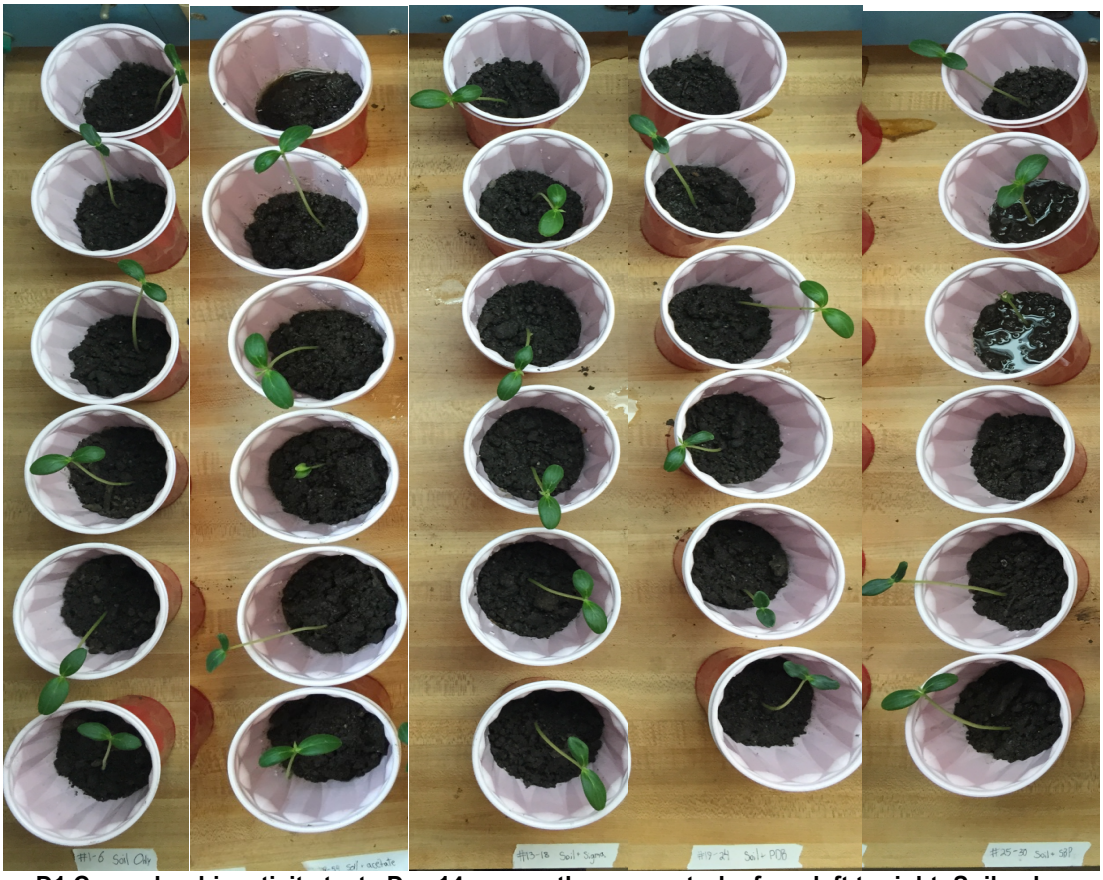

**Figure D1 Cucumber bioactivity test. Day 14, non-pathogen controls; from left to right: Soil only control, seed dipped in 2% acetic acid solution, seed dipped in 2% commercial chitosan, seed dipped in 2% chitosan produced from PDB media, seed dipped in 2% chitosan produced from SBP media**

Figure D2 shows the cucumber test treatments at 14 days. These cultures were inculcated with pathogenic fungi as described in section 3.6.

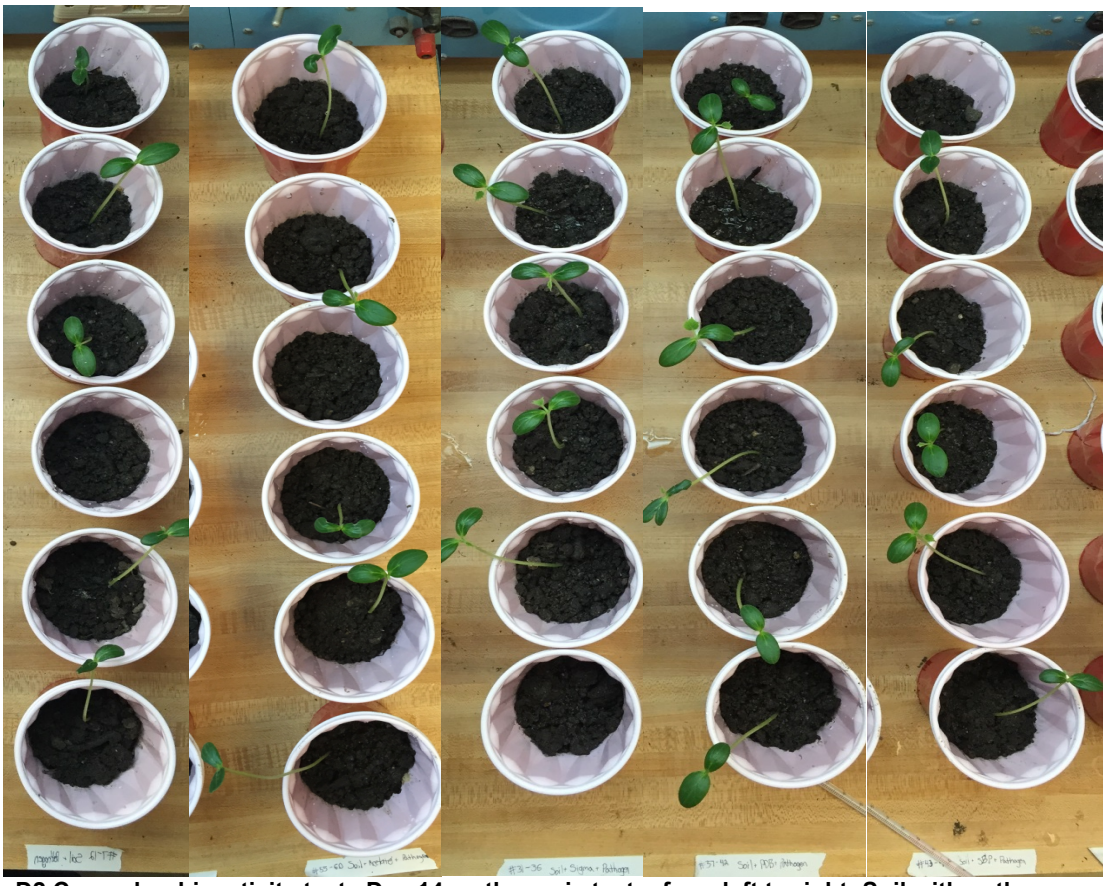

**Figure D2 Cucumber bioactivity test. Day 14, pathogenic tests; from left to right: Soil with pathogen control, seed dipped in 2% acetic acid solution control, seed dipped in 2% commercial chitosan, seed dipped in 2% chitosan produced from PDB media, seed dipped in 2% chitosan produced from SBP media**

Table D1 shows the height of the individual cucumber plants at 14 days (in centimeters); table D2 shows the height of the individual cucumber plants at 21 days (in centimeters).

**Table D1. Cucumber plant height, in centimeters, measured at 14 days**

| Cucumber plant heights at day 14 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
| 10.5 | 10.0 | 10.5 | 14.0 | 9.5 | 0.0 | 10.0 | 11.5 | 10.5 | 10.0 |
| 11.0 | 10.0 | 9.0 | 11.5 | 9.5 | 11.0 | 7.0 | 10.0 | 11.5 | 11.5 |
| 11.5 | 0.0 | 4.0 | 11.0 | 11.0 | 11.5 | 11.0 | 11.0 | 0.0 | 12.5 |
| 11.5 | 8.0 | 11.5 | 0.0 | 10.5 | 13.0 | 10.0 | 9.0 | 8.0 | 10.0 |
| 10.0 | 11.0 | 12.0 | 12.0 | 11.0 | 10.5 | 12.0 | 12.0 | 6.0 | 9.5 |
| 13.0 | 6.0 | 0.0 | 9.0 | 11.0 | 11.0 | 0.0 | 6.5 | 11.0 | 0.0 |

**Table D2. Cucumber plant height, in centimeters, measured at 21 days**

| Height at day 21 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
| 11.5 | 12.0 | 12.0 | 16.5 | 10.0 | 0.0 | 13.0 | 12.0 | 12.0 | 11.0 |
| 11.0 | 11.5 | 12.0 | 0.0 | 10.0 | 12.0 | 9.5 | 10.5 | 12.0 | 13.5 |
| 13.0 | 0.0 | 10.0 | 12.0 | 11.0 | 14.0 | 11.5 | 13.0 | 0.0 | 15.0 |
| 12.5 | 9.0 | 11.5 | 0.0 | 11.5 | 15.0 | 11.5 | 12.0 | 10.0 | 12.5 |
| 12.5 | 12.5 | 13.0 | 13.0 | 0.0 | 12.0 | 0.0 | 13.0 | 6.5 | 11.5 |
| 0.0 | 7.0 | 0.0 | 10.0 | 12.5 | 12.0 | 12.0 | 7.0 | 10.5 | 0.0 |

Table D3 shows the height of the individual sugar beet plants at 14 days (in centimeters); table D4 shows the height of the individual sugar beet plants at 21 days (in centimeters).

**Table D3. Sugar beet height, in centimeters, at 14 days**

| Height at day 14 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
| 11.0 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 |
| 9.0 | 0.0 | 1.0 | 0.0 | 2.0 | 0.0 | 2.0 | 0.0 | 8.0 | 0.0 |
| 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 3.0 | 0.0 |
| 11.0 | 2.0 | 0.0 | 3.0 | 3.0 | 3.0 | 4.0 | 7.0 | 4.0 | 0.0 |
| 6.0 | 6.0 | 5.0 | 2.0 | 4.0 | 0.0 | 2.0 | 0.0 | 0.0 | 0.0 |
| 13.5 | 0.0 | 3.0 | 4.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.0 | 0.0 |

**Table D4. Sugar beet height, in centimeters, at 21 days**

| Height at day 21 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
| 13.5 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.0 | 0.0 |
| 12.0 | 0.0 | 2.0 | 0.0 | 2.0 | 0.0 | 4.5 | 0.0 | 10.5 | 0.0 |
| 3.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.0 | 0.0 | 5.5 | 0.0 |
| 14.0 | 3.0 | 0.0 | 4.0 | 4.0 | 4.0 | 6.0 | 6.0 | 5.0 | 0.0 |
| 11.0 | 10.0 | 6.5 | 0.0 | 5.0 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 |
| 18.0 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.0 | 0.0 |

Table D5 shows the statistical results of the interaction between the seed treatment and the presence of the fungal pathogen for the cucumber bioactivity study at 14 days.

**Table D5. Comparison of means at 14 days for different seed treatments on the height of cucumber plants in the presence or absence of pathogenic fungi**

| i/j | Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
|---|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | | 0.1013 | 0.1346 | 0.4616 | 0.7122 | 0.4397 | 0.2001 | 0.5804 | 0.1346 | 0.304 |
| Control 1 Pathogen | 0.1013 | | 0.8826 | 0.3582 | 0.2001 | 0.3776 | 0.7122 | 0.2711 | 0.8826 | 0.5312 |
| Control 2 No Pathogen | 0.1346 | 0.8826 | | 0.4397 | 0.2556 | 0.4616 | 0.8248 | 0.3395 | 1 | 0.6317 |
| Control 2 Pathogen | 0.4616 | 0.3582 | 0.4397 | | 0.7122 | 0.9706 | 0.5804 | 0.8536 | 0.4397 | 0.7679 |
| Control 3 No Pathogen | 0.7122 | 0.2001 | 0.2556 | 0.7122 | | 0.685 | 0.3582 | 0.8536 | 0.2556 | 0.5074 |
| Control 3 Pathogen | 0.4397 | 0.3776 | 0.4616 | 0.9706 | 0.685 | | 0.6058 | 0.8248 | 0.4616 | 0.7962 |
| PDB No Pathogen | 0.2001 | 0.7122 | 0.8248 | 0.5804 | 0.3582 | 0.6058 | | 0.4616 | 0.8248 | 0.7962 |
| PDB Pathogen | 0.5804 | 0.2711 | 0.3395 | 0.8536 | 0.8536 | 0.8248 | 0.4616 | | 0.3395 | 0.6317 |
| SBP No Pathogen | 0.1346 | 0.8826 | 1 | 0.4397 | 0.2556 | 0.4616 | 0.8248 | 0.3395 | | 0.6317 |
| SBP Pathogen | 0.304 | 0.5312 | 0.6317 | 0.7679 | 0.5074 | 0.7962 | 0.7962 | 0.6317 | 0.6317 | |

Table D6 shows the statistical results of the interaction between the seed treatment and the presence of the fungal pathogen for the cucumber bioactivity study at 21 days.

**Table D6. Comparison of means at 21 days for different seed treatments on the height of cucumber plants in the presence or absence of pathogenic fungi**

| i/j | Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
|---|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | | 0.6253 | 0.9084 | 0.6052 | 0.7519 | 0.7958 | 0.8630 | 0.6875 | 0.5854 | 0.8630 |
| Control 1 Pathogen | 0.6253 | | 0.7087 | 0.9771 | 0.8630 | 0.4559 | 0.7519 | 0.3746 | 0.9541 | 0.5093 |
| Control 2 No Pathogen | 0.9084 | 0.7087 | | 0.6875 | 0.8405 | 0.7087 | 0.9541 | 0.6052 | 0.6665 | 0.7738 |
| Control 2 Pathogen | 0.6052 | 0.9771 | 0.6875 | | 0.8405 | 0.4389 | 0.7302 | 0.3595 | 0.9771 | 0.4911 |
| Control 3 No Pathogen | 0.7519 | 0.8630 | 0.8405 | 0.8405 | | 0.5658 | 0.8857 | 0.4733 | 0.8181 | 0.6253 |
| Control 3 Pathogen | 0.7958 | 0.4559 | 0.7087 | 0.4389 | 0.5658 | | 0.6665 | 0.8857 | 0.4222 | 0.9313 |
| PDB No Pathogen | 0.8630 | 0.7519 | 0.9541 | 0.7302 | 0.8857 | 0.6665 | | 0.5658 | 0.7087 | 0.7302 |
| PDB Pathogen | 0.6875 | 0.3746 | 0.6052 | 0.3595 | 0.4733 | 0.8857 | 0.5658 | | 0.3448 | 0.8181 |
| SBP No Pathogen | 0.5854 | 0.9541 | 0.6665 | 0.9771 | 0.8181 | 0.4222 | 0.7087 | 0.3448 | | 0.4733 |
| SBP Pathogen | 0.8630 | 0.5093 | 0.7738 | 0.4911 | 0.6253 | 0.9313 | 0.7302 | 0.8181 | 0.4733 | |

Table D7 shows the statistical results of the interaction between the seed treatment and the presence of the fungal pathogen for the sugar beet bioactivity study at 14 days.

**Table D7. Comparison of means at 14 days for different seed treatments on the height of sugar beet plants in the presence or absence of pathogenic fungi**

| i/j | Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
|---|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0002 | <.0001 |
| Control 1 Pathogen | <.0001 | | 0.6693 | 0.9028 | 0.9028 | 0.5422 | 0.3943 | 0.9028 | 0.1471 | 0.3309 |
| Control 2 No Pathogen | <.0001 | 0.6693 | | 0.7602 | 0.7602 | 0.3018 | 0.6693 | 0.5832 | 0.3018 | 0.1643 |
| Control 2 Pathogen | <.0001 | 0.9028 | 0.7602 | | 1.0000 | 0.4649 | 0.4649 | 0.8071 | 0.1831 | 0.2746 |
| Control 3 No Pathogen | <.0001 | 0.9028 | 0.7602 | 1.0000 | | 0.4649 | 0.4649 | 0.8071 | 0.1831 | 0.2746 |
| Control 3 Pathogen | <.0001 | 0.5422 | 0.3018 | 0.4649 | 0.4649 | | 0.1471 | 0.6256 | 0.0420 | 0.7143 |
| PDB No Pathogen | <.0001 | 0.3943 | 0.6693 | 0.4649 | 0.4649 | 0.1471 | | 0.3309 | 0.5422 | 0.0715 |
| PDB Pathogen | <.0001 | 0.9028 | 0.5832 | 0.8071 | 0.8071 | 0.6256 | 0.3309 | | 0.1169 | 0.3943 |
| SBP No Pathogen | 0.0002 | 0.1471 | 0.3018 | 0.1831 | 0.1831 | 0.0420 | 0.5422 | 0.1169 | | 0.0176 |
| SBP Pathogen | <.0001 | 0.3309 | 0.1643 | 0.2746 | 0.2746 | 0.7143 | 0.0715 | 0.3943 | 0.0176 | |

Table D8 shows the statistical results of the interaction between the seed treatment and the presence of the fungal pathogen for the sugar beet bioactivity study at 21 days.

**Table D8. Comparison of means at 21 days for different seed treatments on the height of sugar beet plants in the presence or absence of pathogenic fungi**

| i/j | Control 1 No Pathogen | Control 1 Pathogen | Control 2 No Pathogen | Control 2 Pathogen | Control 3 No Pathogen | Control 3 Pathogen | PDB No Pathogen | PDB Pathogen | SBP No Pathogen | SBP Pathogen |
|---|---|---|---|---|---|---|---|---|---|---|
| Control 1 No Pathogen | | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0002 | 0.0002 |
| Control 1 Pathogen | <.0001 | | 0.9645 | 0.9645 | 0.8587 | 0.8587 | 0.4776 | 0.4776 | 0.2163 | 0.2163 |
| Control 2 No Pathogen | <.0001 | 0.9645 | | 1.0000 | 0.8240 | 0.8240 | 0.5054 | 0.5054 | 0.2329 | 0.2329 |
| Control 2 Pathogen | <.0001 | 0.9645 | 1.0000 | | 0.8240 | 0.8240 | 0.5054 | 0.5054 | 0.2329 | 0.2329 |
| Control 3 No Pathogen | <.0001 | 0.8587 | 0.8240 | 0.8240 | | 1.0000 | 0.3753 | 0.3753 | 0.1586 | 0.1586 |
| Control 3 Pathogen | <.0001 | 0.8587 | 0.8240 | 0.8240 | 1.0000 | | 0.3753 | 0.3753 | 0.1586 | 0.1586 |
| PDB No Pathogen | <.0001 | 0.4776 | 0.5054 | 0.5054 | 0.3753 | 0.3753 | | 1.0000 | 0.5939 | 0.5939 |
| PDB Pathogen | <.0001 | 0.4776 | 0.5054 | 0.5054 | 0.3753 | 0.3753 | 1.0000 | | 0.5939 | 0.5939 |
| SBP No Pathogen | 0.0002 | 0.2163 | 0.2329 | 0.2329 | 0.1586 | 0.1586 | 0.5939 | 0.5939 | | 1.0000 |
| SBP Pathogen | 0.0002 | 0.2163 | 0.2329 | 0.2329 | 0.1586 | 0.1586 | 0.5939 | 0.5939 | 1.0000 | |

# REFERENCES

# REFRENCES


Anusuya, S., & Sathiyabama, M. (2014). Effect of Chitosan on Rhizome Rot Disease of Turmeric Caused by Pythium aphanidermatum. *ISRN Biotechnology, 2014*, 5. doi: 10.1155/2014/305349µ

Aranaz, I., Mengibar, M., Harris, R., Panos, I., Miralles, B., Acosta, N., . . . Heras, A. (2009). Functional Characterization of Chitin and Chitosan. *Current Chemical Biology, 3*(2), 203-230.

Arcidiacono, S., & Kaplan, D. L. (1991). Molecular Weight Distribution of Chitosan Isolated from Mucor rouxii under Different Culture and Processing Conditions. *Biotechnology and Bioengineering, 39*(3), 281-286.

ATCC. (2014). Culture Method. In A. T. C. Collections (Ed.), *Rhizopus oryzae Went et Preinsen Geerlings, teleomorph (ATCC 9363)*: ATCC.

Azmi, N. E., Ahmad, M., Abdullah, J., Sidek, H., Heng, L. Y., & Karuppiah, N. (2009). Biosensor based on glutamate dehydrogenase immobilized in chitosan for the determination of ammonium in water samples. *Analytical Biochemistry, 388*, 28-32. doi: 10.1016/j.ab.2009.02.005

Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology, 23*, 257-270.

Ben-Shalom, N., Ardi, R., Pinto, R., Aki, C., & Fallik, E. (2003). Controlling gray mould caused by *Botrytis cinerea* in cucumber plants by means of chitosan. *Crop Protection, 22*, 285-290.

Chatterjee, S., Chatterjee, S., Chatterjee, B. P., & Guha, A. K. (2008). Enhancement of growth and chitosan production by Rhizopus oryzae in whey medium by plant growth hormones. *Int J Biol Macromol, 42*(2), 120-126. doi: 10.1016/j.ijbiomac.2007.10.006

Chatterjee, S., Chatterjee, S., Chatterjee, B. P., & Guha, A. K. (2009). Influence of plant growth hormones on the growth of Mucor rouxii and chitosan production. *Microbiol Res, 164*(3), 347-351. doi: 10.1016/j.micres.2007.05.003

Chien, P.-J., & Chou, C.-C. (2006). Antifungal activity of chitosan and its application to control post-harvest quality and fungal rotting of Tankan citrus fruit (*Citrus tankan Hayata*). *Journal of Science of Food and Agriculture, 86*, 1964-1969. doi: 10.1002/jsfa

Chirkov, S. N. (2002). The Antiviral Activity of Chitosan (Review). *Applied Biochemistry and Micobiology, 38*(1), 5-13.

Cho, Y., No, H. K., & Meyers, S. P. (1998). Physicochemical Characteristics and Functional Properties of Various Comercial Chitin and Chitosan Products. *Journal of Agricultural Food Chemistry, 46*, 3839-3843.

Chysirichote, T., Reiji, T., Asami, K., & Ohtaguchi, K. (2014). Quantification of the glucosamine content in the filamentous fungus *Monascus ruber* cultured on solid surfaces. *Journal of Basic Microbiology, 54*, 350-357. doi: 10.1002/jobm.201200350

Clark, G. (2014). Sale price of beet pulp residues.

Crini, G., & Badot, P.-M. (2008). Application of chitosan, a natural aminopolysaccharide, for dye removal from aqueous solutions by adsorption processes using batch studies: A review of recent literature. *Progress in Polymer Science, 33*(4), 399-447.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antimicrobial activity, interactions with food components and applicability as a coatin gon fruit and vegetables. *Food Microbiology, 21*, 703-714.

Dhillon, G. S., Kaur, S., Brar, S. K., & Verma, M. (2013). Green synthesis approach: extraction of chitosan from fungus mycelia. *Critical Reviews in Biotechnology, 33*(4), 379-403.

Donkoh, E., Degenstein, J., & Ji, Y. (2012). Process integration and economics evaluation of sugar beet pulp conversion into enthanol. *Int J Agric & Biol Eng, 5*(2), 52-61.

Dunlap, R. E., & Beus, C. E. (1992). Understanding Public Concerns about Pesticides: An Empirical Examination. *Journal of Consumer Affairs, 26*(2), 418-461.

El-Diasty, E. M., Nesreen, Z., & Hoda, A. (2012). Using of Chitosan as Antifungal Agent in Kariesh Cheese. *New York Science Journal, 5*(9), 5-10.

Ensymm (Producer). (2008). Abstract For Chitosan Production. [Abstract]

Eskenazi, B., Bradman, A., & Castorina, R. (1999). Exposures of Children to Organophosphate Pesticides and Their Potential Adverse health Effects. *Envrionmental Health Perspectives, 107*, 409-419.

European Medicines Agency. (2006). *Committee for medicinal products for human use (CHMP) opinion following an article 29(4) referral for Glucomed and associated names International Non-Proprietary Name (INN): glucosamine hydrochloride*. London.

Eweis, M., Elkholy, S. S., & Elsabee, M. Z. (2006). Antifungal efficacy of chitosan and its thiourea derivatives upon the growth of some sugar-beet pathogens. *Int J Biol Macromol, 38*(1), 1-8. doi: 10.1016/j.ijbiomac.2005.12.009

Ghose, T. K. (1987). Meaurment of cellulase activities. *Pure & Applied Chemistry, 59*(2), 257-268.

Greene, C. (2014). Organic Market Overview *Economic Research Service - Organic Agriculture*: USDA.

Grohmann, K., & Baldwin, E. (1992). Hydrolysis of orange peel with pectinase and cellulase enzymes. *Biotechnology Letters, 14*(12), 1169-1174.

HACH. (2014). Nitrogen, Total, Persulfate Digestion TNT Method *HACH Water Analysis Handbook* (pp. 1-8): HACH, GmbH.

Haley, S. (2012). *Sugar and Sweeteners Outlook*.

Han, C., Zhao, Y., Leonard, S. W., & Traber, M. G. (2004). Edible coatings to improve storability and enhance nutritional value of fresh and frozen strawberries and raspberries. *Postharvest Biology and Technology, 33*, 67-78.

Hanson, D. J. (2013). Health Background. *Alcohol: Problems and Solutions.*

Higson, A. (2011). Lactic Acid (Vol. Platform Chemicals: Renewable Chemicals Factsheet): The National Non-Food Crops Centre.

James, C. (2011). ISAAA Brief: Global Status of Commercialized Biotech/GM Crops. (43).

Jaworska, M. M., & Konieczna, E. (2001). The influence of supplemental components in nutrient medium on chitosan formation by the fungus Absidia orchidis. *Applied Microbiology and Biotechnology, 56*(1-2), 220-224.

Kaur, S., & Dhillon, G. S. (2014). The versatile biopolymer chitosan: potential sources, evaluation of extraction methods and applications. *Critical Reviews in Microbiology, 40*(2), 155-175. doi: 10.3109/1040841X.2013.770385

Kertes, K. (1951). Pectic Enzymes *Enzymes of Carbohydrate Metabolism*.

Kumar, M. N. V. (2000). A review of Chitin and chitosan applications. *Reactive & Functional Polymers, 46*, 1-27.

Liu, A. (2015). High enzyme activity pectinase enzyme powder: Alibaba.

Liu, G., Zhang, J., & Bao, J. (2015). Cost evaluation of cellulase enzymes for industrial-scale cellulosic ethanol production based on rigorous Aspen Plus modeling. *Bioprocess and Biosystems Engineering*. doi: 10.1007/s00449-015-1497-1

Liu, Y., Liao, W., & Chen, S. (2008). Study of pellet formation of filamentous fungi Rhizopus oryzae using a multiple logistic regression model. *Biotechnology and Bioengineering, 99*(1), 117-128.

Mata, Y., Blazquez, M., Ballester, A., Gonzalez, F., & Munoz, J. (2009). Sugar-beet pulp pectin gels as biosorbent for heavy metals: Preparation and determination of biosorption and desorption characteristics. *Chemical Engineering Journal, 150*, 289-301. doi: 10.1016/j.cej.2009.01.001

Mattia, A. (2013). *Agency Response Letter GRAS Notice No. GRN 000443*. Office of Food Additive Safety.

May, M. (2001). Pesticide Outlook - Crop Protection in Sugar Beet. *The Royal Society of Chemistry*, 188-191. doi: 10.1039/b108605g

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry, 31*(3), 426-428.

Minning, S., Serrano, A., Ferrer, P., Solá, C., Schmid, R. D., & Valero, F. (2001). Optimization of the high-level production of Rhizopus oryzae lipase in Pichia pastoris. *Journal of Biotechnology, 86*(1), 59-70. doi: http://dx.doi.org/10.1016/S0168-1656(00)00402-8

Neher, O. T., & Gallian, J. J. (2011). Rhizocotonia on Sugar Beet. *Pacific Northwest Extension*.

Nwe, N., Chandrkrachang, S., Stevens, W. F., Maw, T., Tan, T. K., Khor, E., & Wong, S. M. (2002). Production of fungal chitosan by solid state and submerged fermentation. *Carbohydrate Polymers, 49*, 235-237.

Nwe, N., Furuike, T., & Tamura, H. (2010). Production of Fungal Chitosan by Enzymatic Method and Applications in Plant Tissue Culture and Tissue Engineering: 11 Years of Our Progress, Present Situation and Future Prospects. *Biopolymers*, 135-162.

Nwe, N., & Stevens, W. F. (2004). Effect of urea on fungal chitosan production in solid substrate fermentation. *Process Biochemistry, 39*, 1639-1642.

Peniston, Q. P., & Johnson, E. L. (1972). United States Patent No. 3852122. United States Patent.

Peter, M. G. (1995). Applications and Envrionmental Aspects of Chitin and Chitosan. *Journal of Macromolecular Science, Part A: Pure and Applied Chemistry, 32*(4), 629-640.

Prapagdee, B., Kotchadat, K., Kumsopa, A., & Visarathanonth, N. (2007). The role of chitosan in protection of soybean from sudden death syndrome caused by *Fusarium solani* f. sp. *glycines*. *Bioresource Technology, 98*, 1353-1358.

Qian, S., Zhang, Q., Wang, Y., Lee, B., Betageri, G. V., Chow, M. S. S., . . . Zuo, Z. (2013). Bioavalibility enhancement of glucosamine hydrochloride by chitosan. *International Journal of Parmaceutics, 455*, 365-373.

Rane, K. D., & Hoover, D. G. (1993). An Evaluation of ALkali and Acid Treatments for Chitosan Extraction from Fungi. *Process Biochemistry, 28*, 115-118.

Rarexoticsseeds. (2015). Gibberellic Acid Powder (90%).

Romanazzi, G., Feliziani, E., Santini, M., & Landi, L. (2013). Effectiveness of postharvest treatment with chitosan and other resistance inducers in the control of storage decay of strawberry. *Postharvest Biology and Technology, 75*, 25-27. doi: 10.1016/j.postharvbio.2012.07.007

Rombouts, F., & Thibault, J.-F. (1986). Feruloylated pectic substances from sugar-beet pulp. *Carbohydrate Reaserch, 154*, 177-187.

Ruan, Z., Zanotti, M., Zhong, Y., Liao, W., Ducey, C., & Liu, Y. (2013). Co-hydrolysis of lignocellulosic biomass for microbial lipid accumulation. *Biotechnology and Bioengineering, 110*(4), 1039-1049.

Saulnier, L., & Thibault, J.-F. (1999). Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *Journal of the Science of Food and Agriculture, 79*, 396-402.

Schumacher, S., Boland, M., & Brester, G. (2012). Sugarbeet Profile. In D. Huntrods (Ed.). Agricultural Marketing Resource Center: Iowa State University, Kansas State University, Montana State University.

Sigma. (1997). Enzymatic Assay of Pectinase *Quality Control Test Procedure*: Sigma-Aldrich.

Simpson, B., Egyankor, K., & Martin, A. (1984). Extraction, purification and determination of pectin in tropical fruits. *Journal of Food Processing and Preservation, 2*, 63-72.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). Determination of structural carbohydrates and lignin in biomass. *Laboratory analytical procedure*.

Spagnuolo, M., Crecchio, C., Pizzigallo, M., & Ruggiero, P. (1997). Synergistic Effects of Cellulolytic and Pectinolytic Enzymes in Degrading Sugar Beet Pulp. *Bioresource Technology, 60*, 215-222.

Tokoro, A., Tatewaki, N., Suzuki, K., Mikami, T., Suzuki, S., & Suzuki, M. (1988). Growth-Inhibitory Effect of Hexa-N-acetylchitohexaose and Chitohexaose against Meth-A Solid Tumor. *Chemical and Pharmaceutical Bulletin, 36*, 784-790.

USDA. (2015). *2014 State Agriculture Overview*.

Wang, A. (2016). Price for industrial sodium hydroxide: Alibaba.

Wilkins, M. R., Widmer, W. W., Grohmann, K., & Cameron, R. G. (2007). Hydrolysis of grapefruit peel waste with cellulase and pectinase enzymes. *Bioresource Technology, 98*(8), 1596-1601. doi: 10.1016/j.biortech.2006.06.022

Windels, C. E., & Nabben, D. J. (1989). Characterization and Pathogenicity of Anastomosis Groups of *Rhizoctonia solani* Isolated from *Beta vulgaris*. *Ecology and Epidemiology, 79*(1), 83-88.

Wu, T., Zivanovic, S., Draughon, F., Conway, W., & Sams, C. (2005). Physicochmeical Properties and Bioactivity of Fungal Chitin and Chitosan. *J Agric Food Chem, 53*, 388-3894.

Wu, T., Zivanovic, S., Draughon, F., & Sams, C. (2004). Chitin and Chitosan - Value-Added Products from Mushroom Waste. *Journal of Agricultural Food Chemistry, 52*, 7905-7910. doi: 10.1021/jf0492565

Zha, Y., Westerhuis, J. A., Muilwijk, B., Overkamp, K. M., Nijmeijer, B. M., Coulier, L., . . . Punt, P. J. (2014). *Identifying inhibitory compounds in lignocellulosic biomass hydrolysates using an exometabolomics approach* (Vol. 14).