ABSTRACT

LOYD, KAYLIE NORA. Encapsulation of Plant Growth Promoting Bacteria in Electrospun Biodegradable Nanofibers (Under the direction of Dr. Michael D. Boyette).

The growing need for agrochemical alternatives has increased research into biofertilizer and biocontrol solutions. Plant associated microorganisms provide a wide range of ecological services for their host plant, including nutrient acquisition and disease protection. The application of these microorganisms to agricultural crops has been shown to increase crops yield and decrease crop damage due to pests. The wide spread application of bioinoculants in agriculture is still limited largely due to inefficient preservation and application techniques. The present research investigates the use of the electrospinning process to encapsulate plant growth promoting bacteria in poly(vinyl alcohol) (PVA) nanofibers. Prior to selecting PVA, guar gum and starch nanofibers were explored. Biopolymers are biodegradable, inexpensive, sustainable, and generally regarded as safe, making them an ideal source for environmentally friendly bioinoculant carriers. This research produced electrospun nanofibers from guar gum and corn starch blended solutions at room temperature for the first time. The effect of amylose content and the addition of glycerol and nanoclay were evaluate using steady state and oscillatory rheological measurements and final fiber morphology imaged using FESEM. Ultrathin, mostly uniform fibers were produced at a total polymer concentration of 4% by weight with high amylose. The addition of glycerol and nanoclay altered the rheological properties of the solutions and resulted in larger nanofibers. However, interaction effects between glycerol and nanoclay prevented nanofiber exfoliation and the large nanoclay particles prevented nanofiber formation. The ultrathin and low-quality nanofibers produced from these biopolymers prevented their use in the second stage of this research.
The encapsulation of bacterial inoculants in electrospun nanofibers provides improved barrier properties against moisture and physical damage and this process has lower temperature and pressure requirements compared to other methods. Additionally, bioactive nanofibers have delayed release which would allow the slow release of bacteria as the seed germinates. This research aims to evaluate the potential of the electrospinning process for the preservation of plant growth promoting microorganism in a nanofibrous seed coat. Three distinct bacterial strains were selected and tested for their survival during storage and their release after resuspension. All the bacterial strains had a significant decrease in viable cells after 5 days of storage with the most significant loss seen within the first 24 hours of storage. The addition of osmoprotectants, including glycerol and trehalose, to the spinning solution had no significant impact on bacterial survival. A release experiment was also performed to determine when the bacteria were released from the fibers. The nanofibers produced in this experiment showed the greatest release after one day and continued release up to 3 days. This process is a promising approach to preserving bioinoculants on the surface of the seed but additional exploration into alternate spinning methods and polymer materials will be necessary to achieve long term survival.
Encapsulation of Plant Growth Promoting Bacteria in Electrospun Biodegradable Nanofibers

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Biological and Agricultural Engineering

Raleigh, North Carolina
2019

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DEDICATION

I would like to dedicate my thesis research to my fiancé, Ben. I could not have completed this project without your endless patience and support. I love you! And to my parents for always believing in me and raising me to be curious.
BIOGRAPHY

I was born in Nashville, Tennessee but was raised in Zebulon, North Carolina. I have always enjoyed being active, growing up that involve track and soccer year round; and most importantly, horseback riding. One advantage of growing up in Zebulon was the endless maze of tobacco and soybean fields that provided excellent riding trails. These fields, many of which have been developed now, were probably the foundation for my interest in sustainability. I graduated from Southeast Raleigh Magnet High School in 2014 as the valedictorian and had just begun to explore the applications of genetic engineering and biotechnology in my senior graduate project. However, I did not know how to bridge my passion for sustainability with my interest in biotechnology until I began my undergraduate career at NC State.

I completed by undergraduate at NC State in the same department, Biological and Agricultural Engineering, with a focus in bioprocessing. I enjoyed taking a wide range of classes from bio-manufacturing to plant biotechnology. For my senior capstone project, a group of fellow seniors and I set out to design our own project. After reaching out to several professors, Dr. Goller in the Biotechnology Department introduced us to *Delftia*, an interesting little soil microbe that secreted gold pellets. Although this was not applicable to biological engineering, we soon discovered that different strains of *Delftia* could fix nitrogen. This project was my introduction into biofertilizers and the use of microorganisms to improve plant health. After researching the effects of *Delftia* on tobacco and tomato growth for my capstone project, I decided to continue the research and pursue a Masters. The following research is the product of an extensive literature review and Dr. Boyette’s faith and interest in this area of study.
ACKNOWLEDGMENTS

I would like to thank the members of my committee for the help and expertise they provided throughout this project. I would especially like to thank Dr. Michael Boyette for everything he has done for me throughout my graduate career and for taking a chance on this project. I would also like to thank Dr. Carlos Goller for allowing me to take over an incubator for several weeks and providing the much need lab space and resources for working with the microbial strains. I hope you never give up on Delftia! I would also like to acknowledge Dr. Chuck Mooney, from the Analytical Instrumentation Facility, for his excellent training and assistance with operating the FESEM and Mr. Chris Pernell, from the Food Rheology Lab, for his training and expertise in rheological measuring methods.

I would also like to thank IngredionTM for generously providing a sample of Hylon VII corn starch for my experiments; and, finally, the College of Agricultural and Life Science for providing funding for this research.
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CHAPTER 1: Background and Literature Review

1.1 Introduction

Current farming practices, especially for field crops such as corn and soybean, rely heavily on synthetic fertilizers, herbicides, and pesticides. The negative environmental and economic impacts associated with their application will only be amplified as soil degradation and pest resistance increases due to climate change. Warmer climates and elevated CO₂ levels have already led to record pest populations and a decrease in the efficacy of current formulations (Matzrafi, 2019).

The introduction of chemical fertilizers, herbicides, and insecticides into large scale agriculture dramatically improved the yield per acre needed to support a growing population and meat consumption. However, these high-energy input and soil amendment techniques have had their consequences. Each year, 500 million tons of nitrogen fertilizer are produced worldwide through the Haber-Bosch process, which accounts for over 1% of the world’s fossil fuel energy consumption (Dent & Cocking, 2017). Yet, nitrogen uptake efficiency in most crops is estimated to be only 30% (Skowrońska & Filipek, 2014). This is partly due to the untimely and overapplication of chemical fertilizers, as well as, the rapid loss through soil leaching and biological activity. Soil microorganisms convert some of this nitrogen back into atmospheric dinitrogen but a considerable amount is converted into nitrous oxide, one of the most harmful greenhouse gasses. Furthermore, the loss of nitrogen and phosphorus from agricultural soils into surface water systems has decreased water quality and negatively impacted aquatic ecosystems (Skowrońska & Filipek, 2014). The agriculture industry is approaching another turning point, spurred by the need for alternative, more sustainable methods for increasing crop yield.
The potential of plant growth promoting microorganisms (PGPM) is one such sustainable alternative. Many of the ecological services performed by the soil are directly related to the biological activity of mycorrhizal fungi and rhizobacteria. Without a healthy symbiome, plants are more susceptible to disease and less resilient to abiotic stresses (Finkel et al., 2017). Until recently the damaged to soil biodiversity due to the application of harsh agrochemicals was not well understood. A comparison between the microbial communities in organically managed fields and conventional farmed fields showed a drastic decline in microbial biomass. Although soil properties can strongly influence these communities, organic farming methods regularly add these microorganisms back to the soil with the application of manure and compost (Hartmann et al., 2015). Furthermore, the application of ammonia and nitrate inhibits biological nitrogen fixation by diazotrophs, nitrogen fixing bacteria. These bacteria are a critical element in the nitrogen cycle and account for over 66% of the atmospheric nitrogen that is fixed every year (Hartmann et al., 2015). Inhibiting this natural biochemical process further increases our reliance on manufactured fertilizers.

Due to the sheltered and nutrient rich environment created by the root system, microbial biomass is much higher within a few millimeters of the root, the rhizosphere, in comparison to the bulk soil. Many PGPM, including arbuscular mycorrhizal fungi (AMF) and rhizobacteria, have been isolated from this zone. Legume symbiosis with rhizobacteria is the most well-known and understood bacterial growth promoting relationship. This complex nodulation pathway is initiated by plant secreted flavonoids and bacterial secreted Nod factors (Cocking, 2003). However, similar chemical communication can be found in non-nodule forming plants that have evolved a variety of mechanisms for interacting with soil microorganisms. The composition of these microbial communities can vary significantly between plant species which means plants have some control over colonization (Finkel et al., 2017). Root-associated and endophytic
symbiotic relationships can be found among all plant taxa. Endophytes represent a small group of bacteria and fungi that can colonize within the root tissue. These microorganisms can fill the spaces between plant cells or enter cell vacuoles without inducing an immune response by the host plant (Carvalho et al., 2014).

1.1.1 Mycorrhizal Fungi

Mycorrhizal fungi are one of the oldest plant symbionts, conserved in over 90% of plant species. Fungal biomass makes up a significant portion the organic biomass in the soil and plays an important role in carbon sequestration (Farrar et al., 2014). For plants, this beneficial relationship is critical for efficient nutrient uptake from the surrounding soil and solubilizing bound phosphorus that would otherwise be unavailable. Beneficial mycorrhizal fungi can also make the plant less susceptible to pathogenic fungi and increases abiotic stress tolerance. Arbuscular mycorrhizal fungi (AMF), or endomycorrhizea, are one category of plant beneficial fungi that live within and between plant cells and send hyphae into the soil, Figure 1-1. Ectomycorrhizea are another class of fungi whose growth is limited to the plant root surface and surrounding soil. In exchange for carbon sources, the fungi provide nutrients absorbed by their hyphae, which act as a second root system. However, due to the long evolutionary history of this relationship, many mycorrhizal species cannot survive or reproduce in the soil without a plant host, making their survival sensitive to seasonal crop rotations and soil disruption (Farrar et al., 2014).
Figure 1-1: Diagram portraying the symbiotic relationships established between plants and PGPM.

1.1.2 Plant Growth Promoting Bacteria

Beneficial bacteria in the rhizosphere have coevolved with plants to provide a diverse range of ecological services. Plant exudates, composed of organic acids, sugars, amino acids, vitamins, and mucilage, provide one of the largest sources of carbon for soil microorganisms. Due to this rich environment the soil surrounding the root structure can contain up to $10^{10}$ bacteria per gram of soil. The plant exudates are key, not only in the maintenance of this biome, but also in the recruitment and selection of specific taxa and this microbial composition can vary significant between plant species and throughout a plant’s life. Plants have the ability to support or reduce this community during extreme stress when the cost-benefit balance changes. Some of these microorganisms are introduced by the seed at the time of germination but a larger number move through the soil in response to plant secreted signals and sugars.
1.2 Modes of Action

Plant growth promoting microorganisms (PGPM) can be divided into two modes of action: a biofertilizer or a biocontrol. Many PGPM also have bioremediation capabilities where specialized enzymes secreted by the microorganism can degrade harmful soil toxins and sequester heavy metals that would otherwise decrease crop yield (Farrar et al., 2014; Mejäre & Bülow, 2001). An overview of these modes of action can be seen in Figure 1-2. The benefits and mechanisms associated with bacterial and fungal colonization are still being investigated. Research into the gene expression between colonized and uncolonized plants have shown a more complex cascade of signaling and pathway regulation than previously predicted (Hardoim et al., 2019).

1.2.1 Biofertilizers

Biofertilizers describe bacteria and fungi that increase the nutrient availability to the plant either directly or indirectly. These microorganisms can directly provide nutrients by fixing atmospheric nitrogen or converting inorganic nutrients, such as phosphorus, potassium, sulfur, and iron to a form plants can absorb. Diazotrophs are believed to make up 15% of all prokaryotic species, spanning up to five phyla and providing a significant portion of the organic nitrogen found on Earth (Dos Santos et al., 2012). Siderophores are a secondary metabolite secreted by any bacteria and act as chelating agents aiding in the uptake of iron. Many plant-associated bacteria also produce phytohormones and phytohormone-like chemicals, such as auxins, cytokines, and ethylene, that stimulates root and shoot growth and increases the root surface area needed for nutrient absorption (Vacheron et al., 2013). Many of these hormones aid in the stimulation or inhibition of key development pathways and alter the root architecture. Other secondary metabolites secreted by microorganisms in the soil have also been shown to increase
the plants innate nutrient uptake abilities and improve plant nutrition. Other microbial enzymes and chemicals can also improve crop performance under abiotic stress; however, specific metabolic mechanisms are not well characterized.

**1.2.2 Biocontrol**

PGPM can also act as biocontrol agents that protect the host plant from external harm. These bacteria can act as biological insecticides, nematicides, and fungicides, as well as, improving the plant's immune response to pathogenic bacteria and viruses (Glare et al., 2012). Biocontrol agents are generally less effective and slower acting than agrochemicals but are more selective in which pest species they kill. Suppressing the pest population and slower kill methods can still result in significant damage to the plant but can alter the pest population over time. Furthermore, they have a lower risk of widespread resistance (Parnell et al., 2016). Perhaps the most well-known example of a biocontrol insecticide is the bacteria, *Bacillus thuringiensis* (Bt). This spore forming bacteria produces a toxin during sporulation that, when ingested by dipterans, attacks the cells lining the gut (Carlton & González, 1986). Although this bacterium was originally used as a pesticide spray, the protein-based toxin has since been genetically inserted into corn, cotton, and soybean for plant produced defense. Aside from direct chemical biocontrol properties, the phytohormones and secondary metabolites described above can also stimulate the plants immune system, induced systemic resistance, and which improves the plants self-defense. Biocontrols are also believed to decrease the risk of infection by outcompeting pathogenic bacteria and fungi.

**1.2.3 Mixed Cultures**

Biodiversity is often related to a community’s resilience to change, and this concept applies when considering the possibility of exploiting these beneficial microorganisms to our advantage. To fully reap the benefits of this technology, the industry needs to develop plant
inoculants that can improve crop yield through a variety of mechanisms. The inoculation of multiple strains of plant growth promoting microorganisms has also been shown to increase the effectiveness of each individual strain due to complex inter-microbial interactions (Parnell et al., 2016). Quickroots®, a product developed by the Monsanto BioAg Alliance, utilizes a combination of the *Bacillus amyloliquefaciens* and, the filamentous fungus, *Trichoderma virens* (Monsanto BioAg Alliance, 2015b). This bacteria and fungi bioinoculant can mechanistically function as a conventional fertilizer by increasing the availability of nitrogen, phosphorus, and potassium in the soil. Field trial data with QuickRoots® applied to corn showed a yield increase ranging from 220 to 500 kg ha\(^{-1}\) (Monsanto BioAg Alliance, 2015a,b). Effective inoculants will need to contain a variety of bacteria and fungi that can colonize the rhizosphere in a variety of soil conditions and provide a range of ecological services. This also calls for application methods and formulations that are compatible with a wide range of microorganisms.
1.3 Current Issues in Application

Although research into PGPM has increased significantly over the last ten years, the widespread acceptance and application of bioinoculants is still relatively low (Finkel et al., 2017). Transitioning these bacteria from the lab scale to the field has produced several complications related to large scale production and maintenance of cell viability. An ideal product should be efficacious, versatile, practical for the farmer, and inexpensive (Parnell et al., 2016). Inoculants should not only be biodegradable, non-toxic, and sustainable, but should be able to preserve bacterial cell counts and promote colonization in the soil. Temperature tolerance, pH sensitivity, sporulation ability, flocculation ability, and additive tolerance has a huge effect on bacterial and fungal survival and proliferation (Swaminathan et al., 2016).

Another limiting factor is PGPM strain selection and efficacy after being applied to the field. With the advent of high throughput sequencing and the growing interest in these complex microbiomes, the list of microbial species that could be harnessed as biofertilizers and biocontrol agents has grown (Finkel et al., 2017). Although these bacteria might perform exceptionally well in the lab and greenhouse environment, they do not always behave as expected in the field environment where soil conditions, competing microbes, and plant interactions inhibit colonization and persistence in the soil. Furthermore, a greater understanding of the core microbiomes or metagenomes among plants would allow microbiome assembly to be more predictable (Busby et al., 2017). Developing a product that can perform reliably and is advantageous to the farmer will require screening for ideal microorganisms as well as an increased understanding of how these microorganisms interact.

Introducing beneficial microbials to the plant can be accomplished using a variety of methods including bio-priming, seedling dip, foliar spray, fruit spray, soil drench, or seed coating (O’Callaghan, 2016). Some methods of application are not practical for large scale farming
without significantly increasing labor but have been shown to be effective in the lab. One of the major considerations when bringing a product to market is the impact on the farmer. The product should have similar time and labor requirements to conventional systems and should utilize existing equipment. Three commonly used and effective methods are described below.

1.3.1 Liquid Inoculants

The use of liquid inoculants is one of the most widely used delivery methods. Most liquid inoculants are microbial suspensions in water, oil, or polymer media (Lee et al., 2016). Although the production and storage of liquid inoculants can be cheaper than solid inoculants, they are more expensive to transport and handle. During extended storage, these inoculants also experience abiotic stress due to nutrient depletion and temperature fluctuations. Additives can significantly increase shelf-life under stress induced conditions. Lee et al. (2016) reported a 50% increase in bacterial survival after one-month storage at 40 °C with glycerol, polyethylene glycol (PEG), glucose, and polyvinylpyrrolidone (PVP). Liquid inoculants can be added to the soil at the time of seeding or sprayed at any stage in the crop’s development. Spraying inoculants can increase the risk of aerosolized bacterial and fungal spores and its effectiveness varies with soil type. Furthermore, only a small percentage of the applied microorganisms successfully colonize the root zone due to a limited range of movement through the soil and competition with native microorganisms. For microbes not acting in the rhizosphere, such as foliar spraying of pesticides, this is a very effective application method.

1.3.2 Dry Inoculants

Another application method involves drying the bacterial culture to produce a highly concentrated powder or impregnating a material with the bacteria. These inoculants can also be applied at the time of seeding or dispersing the powder at different stages in plant development. The drying process decreases the weight and volume but adds additional costs. The most
commonly used drying methods include spray drying and freeze drying which exposes the bacteria to extreme temperature and pressure changes. The effectiveness of these methods are highly dependent on the characteristics of the bacteria. Many legume inoculants such as *Azotobacter spp.* and *Azopirillum spp.* have poor process survival rate and the surviving bacteria have decreased functionality (Campos et al., 2014). Often, these dry products are stored and shipped as dry inoculants and then resuspended before application by the farmer. This adapted method introduces additional cost and education to the farmer and does not eliminate many of the problems associated with the two individual methods.

**1.3.3 Seed Coat Inoculants**

The targeted application of inoculants using a seed coat is the most effective method for giving the bacteria of interest a competitive edge over native microorganisms in the soil (Barret et al., 2015). This is one of the largest factors affecting the reproducibility and effectiveness of this technology in the field. Seed coating can be achieved by using either a dry or liquid initial inoculant. This method is the most targeted method and provides close proximity between the germination plant and the bacteria of interest; however, bacteria application is limited to the time of planting. Seed inoculation is achieved either by the manufacture, by the application of a biofilm or coating, or by the farmer at the time of seeding through a method called bio-priming. The production of pre-inoculated seeds is the most ideal method due to the low impact to the farmer but also complicates inoculant survival during storage and seed costs.

**1.4 Current Formulations**

The practice of applying bioinoculants has been used for decades, although they have not always been referred to by that name. Since the early 1900’s, simple peat carriers were used to prime legumes with nodulating bacteria. Many mineral and organic carriers show a loss of several orders of magnitude in bacterial concentration over storage periods between one to eight
months. A marketed product typically has to be inoculated with two to three times the number of bacteria required by regulatory agencies as the minimum inoculant concentration at the time of application. Even if a product meets minimum requirements, the high loss of bacteria creates uncertainty for the farmer about the contents of the purchased product.

1.4.1 Inorganic Materials

Mineral-based carrier materials such as talc, lignite, vermiculite, perlite, and rockwool have been used as inoculated media that can be dispersed to the field (Nakkeeran et al., 2006; John et al., 2011). The application of high amounts of mineral-based carrier could change the composition of the soil, and material sources are normally regional. Other carriers such as biochar and fly-ash have shown to have a shelf-life of up to eight months. However, an experimental storage assay showed an average decrease of three orders of magnitude in bacterial concentrations and a significant decrease in carrier pH (Kumar et al., 2017).

1.4.2 Organic Materials

More sustainable sources for inoculant carriers include agricultural and industrial wastes. These carriers can vary significantly from source to source and should be sterilized before inoculation. Although, the application of these materials without sterilization can introduce beneficial bacteria to the soil, sterilization would be required to ensure the success of the bacteria of interest. Methods for sterilization, such as autoclaving and gamma-ray irradiation, add additional time, cost and energy consumption to the process (Hong-yuan et al., 2015). Heat sterilization can also release toxic substances or denature beneficial compounds within the organic matter (Nakkeeran et al., 2006). However, organic carriers have better colonization and efficiency compared to mineral and even liquid inoculants. This can be attributed to better porosity, pH buffering, water capacity, and nutrient availability (Shahzad et. al, 2017).
Agricultural wastes such as corn husks and cobs, wheat husks, soybean meal, and manure have shown to be effective carriers as a composted or ground substrate (Rebah et al., 2007; Hongyuan et al., 2015). Similar results were also reported for industrial wastes generated by wastewater treatment, biofuel production, and fermentation operations.

1.4.3 Polymer Formulations

Polymers are a third class of carrier material that includes a variety of natural gums, mucilages, and proteins, as well as, synthetic polymers. The most commonly used synthetic polymers include polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and polyethylene oxide (PEO). These polymers can generally biodegrade and are nontoxic but the application of natural polymers is favored due to their intrinsic properties and sustainable sourcing (Coelho et al., 2016; John et al., 2011).

Exploiting plant derived polymers could provide an inexpensive and multi-functional carrier for bacterial inoculants, Table 1-1. Plants produce a variety of polysaccharides including cellulose, starch, agarose, alginate, gums, and mucilages (Coelho et al., 2016). Gums are generally watersoluble, heterogeneous polysaccharides composed of arabinose, galactose, glucose, mannose, xylose, and/or uronic acids. There are currently no known common functional roles for gums between plant species or even between different tissues in the plant. Plant-based gums such as Guar and Arabic gums are commonly used in industry as gelling agents, stabilizers, and thickeners. Another common plant derived polymer mixture is mucilage. These polysaccharides are compositionally similar to gums but are believed to have physicochemical properties produced by various non-sugar chemical constituents. They also act as key non-starch storage molecules. High concentrations can be found in the roots, seeds, and leaves of many higher plant species. Both gums and mucilages have high water absorption capacity which, when coated on a seed, can increase germination rate (Coelho et al., 2016). Furthermore, these
complex polysaccharides could play a role in signaling and maintaining microbial biofilms (Bashan et al., 2014).

**Table 1-2:** List of common industrial biopolymers. Summarize from Albuquerque et al. (2016)

<table>
<thead>
<tr>
<th>Biopolymer</th>
<th>Polymer Source</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>Seaweed</td>
<td>Mannuronic acid with guluronic acid units</td>
</tr>
<tr>
<td>Xanthan Gum</td>
<td>Microbial</td>
<td>Glucan</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Plant, Microbial</td>
<td>Glucose</td>
</tr>
<tr>
<td>Arabic gum</td>
<td>Plant</td>
<td>Arabinogalactan</td>
</tr>
<tr>
<td>Starch</td>
<td>Plant</td>
<td>Glucan</td>
</tr>
<tr>
<td>Pectin</td>
<td>Plant</td>
<td>Galacturonic acid + arabinose, galactose, xylose, glucose</td>
</tr>
<tr>
<td>Agar</td>
<td>Seaweed</td>
<td>Galactan</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Animal</td>
<td>Protein</td>
</tr>
<tr>
<td>Whey Protein</td>
<td>Animal</td>
<td>Protein</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Animal, Fungi</td>
<td>Glucosamine</td>
</tr>
<tr>
<td>Guar gum</td>
<td>Plant (seed)</td>
<td>Galactomannan</td>
</tr>
<tr>
<td>Carrageenans</td>
<td>Seaweed</td>
<td>Galactan</td>
</tr>
</tbody>
</table>

**1.4.4 Additives**

Another key component of polymeric microbial inoculants is additive selection. A wide variety of chemicals have proven to increase shelf life, as well as, increase bacterial survival during processing. Nanoclay is one way to improve bacterial survival during storage. Nanoclay is normally a silicate-based clay, including montmorillonite, which is able to intercalate into the polymer or organic matrix. Intercalation is a process in which the polymer strands are able to enter the gallery space between the clay sheets. When nanoclay is incorporated within polymeric
products, it decreasing the water absorption rate and limits the available water that can cause contamination or prematurely revive dried inoculants during storage. Furthermore, it has been shown to improve the rheology characteristics of polymer solutions, as well as, increase the mechanical properties.

Osmoprotectants are another additive that can improve survival during processing and storage. These small molecules, typically sugars such as glucose and sucrose, coat and enter the cytosol of microorganisms and improve survival during desiccation. If bacterial cells are dried too quickly, they can experience irreversible protein and cell wall damage. Glycerol is a widely used, inexpensive, organic solvent that can be utilized as a carbon source during large scale microbial fermentation and as a microbial protectant during dissection (Vassilev et al., 2017). Humic acid, skim milk, calcium, and magnesium are also commonly investigated chemical additives (Silva et al., 2007; Young et al., 2006). There is also the potential to incorporate colonization inducing chemicals, such as salicylic acid or lipochitooligosaccharide, that can cause regulation changes in the host plant and improve both endocytic and surface colonization potential (Lebeis et al., 2015; Muñoz et al., 2014).

Another advantage of utilizing glycerol, especially for polymeric formulations, is that it can act as a plasticizer during processing. Plasticizers are used in many polymer systems as a way to increase the flexibility and elongation at break mechanical properties of plastics and fibers. These chemicals typically improve these properties by disrupting the inter-polymer bonds, generally hydrogen bonding, which decreases crystallinity.
1.5 Encapsulation Methods

The encapsulation of PGPM in polymeric matrices is currently the most widely researched alternative over other carrier methods. Encapsulation technology has been widely used in other industries to preserve bioactive compounds but its use has been limited in agriculture. Table 1-2. Encapsulation within a polymer matrix has several advantages over other applications methods including a longer shelf life, higher loading capacity, and more chemically consistent materials (Bashan et al., 2014). Furthermore, microbial release is delayed as the polymer structure degrades and breaks up in the soil. This is ideal for seed coat application, when bacterial colonization and seed germination should coincide. There are a variety of methods for encapsulating the microorganisms depending on the microbial characteristics and the size of the intended product. Some of the most commonly used processes are extrusion, emulsion techniques, solvent evaporation, spray drying, coacervation, droplet freezing, electrospinning, and thermal gelation (John et al., 2011).

1.5.1 Macroencapsulation

Macroencapsulation involves the production of beads ranging from 1 to 4 mm in size. Alginate is the most commonly used polymer for macroencapsulation because it is inexpensive, and its properties are well understood. The macroencapsulation of several rhizobacteria in alginate in combination with humic acid has shown significant survival of the two bacterial strains over a five month storage period at various pH ranges (Young et al., 2006). A long term study has also shown acceptable survival in alginate beads after fourteen years at ambient temperature (Bashan et al., 2014). The long shelf life of this method is promising but the inoculant efficiency in the field environment is still low. The application of macrobeads normally involves an additional step at the time of sowing that would require extra time and possibly equipment. Furthermore, the beads are not a target approach. The microbial inoculants have to
travel through the soil as the polymers decompose which decrease the probability of successful colonization (Bashan et al., 2014).

1.5.2 Microencapsulation

A solution to some of the problems with macroencapsulation can be solved by microencapsulation, where the bead size ranges from 200 to 800 µm (Bashan et al., 2014). The most common method for forming these beads involve extruding the polymer slowly through a needle to form droplets and then solidifying them in CaCl$_2$. This powder-like formulation can easily be coated onto seeds or be applied as a foliar dust depending on the microorganism’s mode of action (Bashan et al., 2014). Pre-coated seeds minimize additional steps during seeding and supply the microorganism directly to the germinating seedlings as they emerge.

Until recently, microencapsulation has not been investigated for agricultural purposes and there is limited data on the success of this technology in the field.

1.5.3 Nanoencapsulation

Nanoencapsulation is another method currently being investigated by other fields for probiotic stabilization and drug delivery (Liu et al., 2017). There are also several precision agriculture applications for targeted and slow release fertilizers (Krishnamoorthy & Rajiv, 2017). The process involves feeding a polymeric solution through a needle and subjecting it to an electric field. The extension of the fiber by electrostatic forces allows for strand entanglement of the polymers. Depending on the processing parameters, the method can produce beads or fibers from 10 to 800 nm in diameter (Liu et al., 2017). The formation of nanofibers using a process called electrospinning can be directly coated onto the seed surface or produce non-woven mats containing the microbe of interest. The mild operating conditions minimizes cell loss from pressure and temperature changes seen in other drying and encapsulation methods (Zussman,
Furthermore, the non-woven textile industry has developed the machinery to mass produce nanofiber material at low cost.

Studies into electrospun seed coats has been limited to only a couple experiments with soybean inoculants using polyvinyl alcohol (PVA), a synthetic polymer. A recent study performed by Gregorio et al. (2017) encapsulated two biofertilizer bacterial strains within nanofibers applied directly to soybean seeds. The process only decreases the bacterial concentration by approximately 20% and a one-month storage assay showed minimal loss in bacterial cell counts. Additionally, the coated seeds had a shorter germinations time in comparison to untreated seeds (Gregorio et al., 2017). Further evaluation is needed to determine reproducibility and versatility of this method although it has the potential to address many of the issues seen with other carriers.

1.6 Future Perspectives

An interdisciplinary approach to product formulation could provide novel solutions to the current issues with inoculant carriers. Research being performed in the medical and food industry could provide additional techniques that can be adapted for agricultural use. The potential of electrospun nanofibers could revolutionize the bioinoculant industry. Future research in the use of biopolymers to produce the fibers could lend additional benefits to the process. A continued understanding of the roles plant-based polymers has on bacterial colonization would allow the production of a multi-function microbial carrier. A formulation containing phytochemicals, such as galactomannan and lipochitooligosaccharide, would effectively preserve the microbial inoculant and induce symbiotic-promoting changes within the plant. Future research using other bacterial and fungal spores to assess the viability and shelf-life of these microorganisms after electrospinning is needed. Testing this technology in multiple field environments is also necessary to determine if this technique will give a competitive advantage to the
Table 1-2: Polymeric encapsulation methods and their effect on microbial survival.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Processing Method</th>
<th>Bacterial Strains</th>
<th>Additives</th>
<th>Storage Conditions</th>
<th>Survival</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA(^1)</td>
<td>Electrospinning</td>
<td><em>Escherichia coli</em>, <em>Staphylococcus albus</em></td>
<td>Glucose, glycerol, sucrose</td>
<td>Temperature: 24°C, 4°C, -20°C, -55°C, Duration: 3 months</td>
<td>No significant change at -20°C and -55°C. Decreased by 10x at 4°C</td>
<td>Salalha et al. 2006</td>
</tr>
<tr>
<td>SDF(^2) and PVA</td>
<td>Electrospinning</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>NA</td>
<td>Temperature: 4°C, Duration: 21 days</td>
<td>Decreased by 10(^2) at 4°C</td>
<td>Fung et al. 2011</td>
</tr>
<tr>
<td>PEO(^3)</td>
<td>Electrospinning</td>
<td><em>E. coli</em> and <em>Micrococcus luteus</em></td>
<td>NA</td>
<td>Temperature: 4°C, 20°C, Duration: 300 hr</td>
<td><em>E. coli</em> not viable after an hour. <em>M. luteus</em>: Decreased by 10x at 4°C, not viable at 20°C after ~100hr</td>
<td>Gensheimer et al., 2007</td>
</tr>
<tr>
<td>PVDF-HFP(^4) and PEG(^5) shell; PVP(^6) core</td>
<td>Co-axial Electrospinning</td>
<td><em>Candida tropicalis</em>, <em>Saccharomyces cerevisiae</em></td>
<td>NA</td>
<td>Temperature: 24°C, Submerged in PBS</td>
<td>No significant change after 17 days</td>
<td>Letnik et al., 2015</td>
</tr>
<tr>
<td>PVA</td>
<td>Electrospinning</td>
<td><em>Pantoea agglomerans</em>, <em>Burkholderia caribensis</em></td>
<td>Glycerol</td>
<td>Temperature: 24°C, Duration: 30 days</td>
<td>Decreased by 10(^2) after 30 days</td>
<td>De Gregorio et al., 2017</td>
</tr>
<tr>
<td>Alginate</td>
<td>Drop Gelation</td>
<td>Azospirillum brasilense, Raoultella terrigena</td>
<td>Starch, clay</td>
<td>Temperature: 4°C Duration: 400 days</td>
<td>A. brasilense had no significant change. R. terrigena decreased by 10³.</td>
<td>Schoebitz et al., 2012</td>
</tr>
<tr>
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<td>---------------------------------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Arabic gum, Maltodextrin, Whey protein</td>
<td>Spray Drying</td>
<td>Lactobacillus acidophilus</td>
<td>NA</td>
<td>Temperature: 20°C Duration: 10 weeks</td>
<td>10x initial decrease, Complete loss of viability after 2 weeks.</td>
<td>Colin-Cruz et al., 2019</td>
</tr>
<tr>
<td>Alginate, chitosan</td>
<td>Electrospraying</td>
<td>Lactobacillus plantarum, Bifidobacterium lactis</td>
<td>NA</td>
<td>Temperature: 25°C, 4°C, -18°C Duration: 90 days</td>
<td>No significant change at 4°C and -18°C. 10² to complete loss at 25°C.</td>
<td>Zaeim et al., 2019</td>
</tr>
<tr>
<td>Alginate, gelatin</td>
<td>Emulsion Gelation</td>
<td>Pseudomonas fluorescens</td>
<td>NA</td>
<td>Release experimentation</td>
<td>NA</td>
<td>Pour et al., 2019</td>
</tr>
<tr>
<td>PVA</td>
<td>Co-axial electrospinning</td>
<td>Bifidobacterium animalis</td>
<td>Skim milk</td>
<td>Temperature: 25°C, 4°C, -20°C Duration: 130 days</td>
<td>Not viable after 40 days at 24°C, no significant decrease at 4°C and -20°C.</td>
<td>López-Rubio et al., 2009</td>
</tr>
</tbody>
</table>

1 Poly(vinyl alcohol), 2 Soluble dietary fiber, 3 Poly(ethylene oxide), 4 Poly(vinylidene fluoride-co-hexafluoropropylene), 5 Poly(vinylpyrrolidone), 6 Poly(ethylene glycol)
target microorganism. An evaluation of the cost for large scale production is also an important indicator of market success because ideally, the inoculant should not dramatically increase seed prices.

A vast array of carriers have been used throughout history to seed soils with beneficial microorganisms but the need for large scale application of bioinoculants requires these carriers to be optimized. Liquid inoculants have acceptable microbial survival during storage but can be difficult to transport and have varying efficiencies in the field environment. Current solid based carriers such as peat and clay like materials have low batch consistency and low microbial loading capabilities. Further exploration of these carriers might marginally improve products; however, a dramatic improvement is needed for microbial inoculants to be realistic. Future research into encapsulated microbial bioinoculants could provide a novel solution to PGPM application at a large scale.

1.7 Objectives

The following research aims to contribute to the growing field of bioactive nanofibers. The overall objective of this research is to develop an effective way to preserve beneficial bacterial inoculants by electrospinning biodegradable polymers directly onto the seed surface. This method aims to increase the shelf life and provide a delayed release of the bacteria after the seeds are planted. Furthermore, this research aims to evaluate the addition of osmoprotectants on bacterial survival after electrospinning and during storage. This research will evaluate several strains of bacteria with a variety of characteristics to determine if this method can be adapted to a wide variety of bioactive formulations for industrial use.

This research was performed in two stages. The first stage will focus on the fiber formation and assessing the processability of mixed biopolymeric solutions. Starch and guar
gum nanofibers will be produced and evaluated for their application as a bacterial carrier. These polymers were selected due to their low cost and innate role in plant development, especially guar gum. Biopolymers can produce much lower quality nanofibers over synthetic polymers; if this stage of the project does not yield viable nanofibers, poly(vinyl alcohol) will be utilized as the encapsulation polymer of the second stage of this research. This will involve electrospinning nanofibers with a high density of bacterial cells with the addition of two osmoprotectants, glycerol and trehalose. Fibers will be coated directly onto the seeds and stored at room temperature, while a second batch of fibers will be evaluated for the release of the bacteria from the polymer matrix over time. The addition of osmoprotectants aims to protect the bacteria during desiccation, as well as, reduce bacterial loss due to humidity during storage in ambient conditions. This research aims to determine if this immobilization process and formulation can maintain bacterial dormancy and improve inoculant efficiency.
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Mejáre, M., & Bülow, L. (2001). *Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals*doi://doi.org/10.1016/S0167-7799(00)01534-1


CHAPTER 2: Rheological Properties of Starch and Guar Gum Nanocomposites and their Impact on Nanofiber Formation

Part 1- Rheological Properties and Nanofiber Formation of Starch and Guar Gum Nanocomposites

2.1 Abstract

Biopolymers are biodegradable, inexpensive, sustainable, and generally regarded as safe making them an ideal source for environmentally friendly packaging, textiles, and nanomaterials. However, the application of these materials with current processing methods can be difficult due to the natural variation in molecular weights, purity, and branching structure. This research aimed to produce electrospun nanofibers from guar gum and corn starch blended solutions at room temperature. Different ratios of unmodified corn starch, approximately 27% amylose, and high amylose corn starch, 70% amylose, were assessed, as well as, the addition of glycerol and nanoclay. The steady state and oscillatory rheological properties of the polymer solution were evaluated and the fibers were imaged using FESEM. Ultrathin fibers with occasional beads, were produced at a total polymer concentration of 4 wt% and high amylose content. These spinning solutions generally had higher viscosities and lower viscoelastic moduli compared to spinning solutions that had unsteady fiber formation. They also demonstrated a greater plasticity over elasticity at higher angular frequencies. The nanoclay and glycerol content in the spinning solution moderately increased the fiber diameter. However, interaction effects between glycerol and nanoclay retarded the exfoliation of the nanoclay into the polymer matrix and the large nanoclay particles prevented nanofiber formation. Guar gum and starch composite nanofibers were produced for the first time at room temperature and demonstrates the emergent properties achieved by mixing biopolymers during electrospinning.

Keywords: Guar gum, corn starch, electrospun nanofibers, viscosity, viscoelastic properties
2.2 Introduction

Biobased polymers are one of the most abundant and chemical diverse natural resources on Earth and they have the potential to reduce our reliance on petroleum-based products. Biobased polymers are a broad class of molecules composed of multiple repeating units of monomers, most commonly nucleic acids, the amino acids, and sugars. Many of these polymers are widely used in food processing as texture and rhetorical modifiers, e.g. gelatin and arabic gum. However, a new focus in sustainable products has driven research into their use in packaging and nanotechnology (Akkurt et al. 2018, Siracusa et al, 2008). Their unique properties, such as the antimicrobial properties of chitosan, could lead to the development of bioactive and multifunctional fabrics, films, and plastic products (Bhardwaj and Kundu, 2010). Furthermore, biobased polymers are sustainable, biodegradable, and nonimmunogenic. However, unlike the well-controlled chemical structure of synthetic polymers, biopolymers can vary considerably in molecular weight, branching pattern, and purity, which has limited their compatibility with current processing methods. Alternative processing methods that utilize chemical altered biopolymers, such as the production of poly(lactic acid) (PLA) from corn starch, or microbial produced biodegradable polymers, such as polyhydroxyalkanoates, have shown continued improvement (Siracusa et al., 2008; Šukytė et al., 2012). However, to utilize the low cost and low environmental impact of naturally occurring polymers, developing processes to handle their inconsistencies and impurities should continue to be explored.

Electrospinning is a process in which a strong electric field is applied to an extruded polymer solution to create micro and nano sized fibers. Electrospinning processing of fibers has been widely used in the fields of nanotechnology and material science sense the 1990’s (Renker & Yarin, 2008). Renewed interest in this processing method aims to exploit the high surface area
to volume ratio and high porosity for the application in filtration and biosensors (Bhardwaj and Kundu, 2010). The mechanisms of this process make it ideal for producing composite fibers due to the rapid evaporation of the solvent preventing phase separation. Another advantage of electrospinning is the simple laboratory set-up and scalability, Figure 2-1 (Bhardwaj and Kundu, 2010). Furthermore, it can be tuned to different specifications by altering the processing conditions, such as the applied voltage, the distance to the collector, and the flow rate of the polymer solution (Deitzel et al., 2001). The fiber diameter can be controlled by the selection of the polymer molecular weight and concentration (Koski et al., 2003).

However, the electrospinning process can be sensitive to many polymer properties and characteristics. Most natural polymers have inconsistence charge distributions and chemical side groups which impact the viscosity. Other properties including the electrical conductivity, viscoelastic properties, interfacial rheology, and surface tension can also impact a solution's ability to form continuous and uniform fibers. The role of these polymer solution properties in

![Figure 2-1: Vertical electrospinning set up. Image borrowed from Senthil et al. (2013).](image-url)
predicting nanofiber formation has been investigated for several different polymers (Rošic et al., 2012, Pelipenko et al., 2012). The bulk and interfacial rheological has shown to have higher correlation with successful fiber initiation and elongation than other polymer characteristics. The bulk rheology includes both the viscosity and viscoelastic characteristics of the polymer in solution. Viscosity is the measure of the flow resistance of a solution and is mainly impacted by particle size and intermolecular interactions (Mezger, 2006). The viscoelastic properties can be further separated into elasticity and plasticity which is represented by the storage (elastic) modulus and the loss (viscous) modulus. These moduli represent energy changes in the polymer during deformation where the storage modulus is stored energy produced when the polymer is stretch within its linear viscoelastic range, material is not destroyed, and the loss modulus is energy lost as heat due to internal friction (Mezger, 2006).

One challenge of working with natural polymers is achieving a viscosity and viscoelastic ratio that is suitable for fiber formation. For biopolymers the rheological properties of the polymer mixture can fluctuate considerably from source to source due to impurities, variation in molecular weights, and branching structure. Currently, many biobased polymer fibers are produced by blending natural polymers with synthetic polymers. However, mixed biopolymer can achieve a similar result by taking advantage of different polymeric structures. The emergent properties of composite mixtures, where the single components alone cannot form fibers, has been shown in the production of chitosan- gelatin, pullulan- pectin, and guar gum- starch nanofibers (Amiri et al., 2018; Liu et al., 2016; Yang et al., 2017).

Another method to alter the rheology and mechanical properties of biopolymer-based nanofibers is the addition of plasticizers and nanoclay. Commonly used plasticizers include glycerol, formamide, and citric acid (Jiugao et al., 2005; Tank et al., 2008). These low molecular
weight molecules alter the interactions and hydrogen bonding between polymer chains which can decrease the viscosity of a solution and increase the elasticity of the final fiber. The addition of nanoclay to polymer products modifies the rheology, increases the mechanical properties, and decrease water vapor permeability (Khodaeimehr et al., 2018). Nanoclay in its native form is composed of layered silicate layers which are held together by exchangeable cations (Tang et al., 2007). When montmorillonite nanoclay is added to a polymer mixture, these layers can become separated during processing and the polymer chains drive the layers farther apart. A true nanocomposite contains completely exfoliated nanoclay where the silicate layers are completely dispersed within the polymer matrix; however, the nanoclay can also be intercalated where there is only partial dispersion (Tang et al., 2007). The addition of nanoclay and glycerol to extruded starch films has been extensively studied (Chiou et al., 2007; Khodaeimehr et al., 2018; Romero-Bastida et al., 2018); however, few studies have analyzed the effects of nanoclay on nanofiber formation (Marras et al., 2007).

This study is a continuation of the work performed by Yang et al. (2017) to produce guar gum and starch nanofibers without the need for additional purification of the component parts. The fabrication of starch and modified starch nanofibers has received considerable attention due to its low cost and abundance (Hemamalini et al., 2017). However, unmodified starch has not been successfully spun into nanofibers using traditional electrospinning techniques. Kong and Ziegler have developed a ‘electro-wet-spinning’ process to form pure starch fibers using dimethyl sulfoxide (DMSO) and a grounded collector in an ethanol bath (2012; 2014; Lancuški et al., 2015). Although, even with this modified system the quality of the fibers is still dependent on the amylose content (Kong and Ziegler, 2012). Natural corn starch is composed of approximately 27% amylose and 73% amylopectin. As shown in Figure 2-2, amylose is
composed of α-(1→4)-glycosidic linkages between glucose molecules which polymerize to form a helical structure. Amylopectin also contains α-(1→4)-glycosidic linkages but has α-(1→6)-glycosidic linkages that produce a highly branched structure. When dispersed in water, amylopectin produces a highly viscous solution due to molecular entanglements between starch molecules. This gelatinization property limits fiber alignment and prevents fiber elongation.

There has been more success in the formation of pure guar gum nanofibers; though additional guar gum purification is needed (Lubambo et al., 2013). Guar gum is extracted from the endosperm of guar beans and is composed of galactomannans, a polysaccharide with a mannose backbone and a single galactose side group placed every other backbone molecule on average (Mudgil et al., 2014). Galactomannans are roughly linear but have high water holding

![Figure 2-2: Physical and chemical structure of (A) amylose, (B) amylopectin, and (C) guar gum.](image-url)
capacity. This highwater retention prevents the denaturation of seed proteins and also aids in stimulating seed germination. Galactomannans are also the main polysaccharide utilized by the sprouting seedling (Srivastava and Kapoor, 2005).

The following research explores the effects of guar gum and starch concentrations on solution rheology and fiber formation at room temperature. Additionally, two forms of corn starch with different amylose content were evaluated. This study also explores the use of glycerol and nanoclay to improve polymer properties and electrospinnability. To evaluate the relationship between bulk solution rheology and fiber morphology, the viscosity and viscoelastic properties of the polymer solutions were evaluated.

2.3 Methods

Reagent grade guar gum (GG), corn starch (St27), and glycerol were purchased from Carolina Biological Supply (Burlington, NC). A high amylose, unmodified corn starch (St70), Hylon® VII, was kindly provided by Ingredion Incorporated (Westchester, Illinois). The reported maximum moisture content was 13.0 percent and the average amylose content was 72 percent. Nanomer® PGV nanoclay was purchased through Sigma-Aldrich (St. Louis, MO). This unmodified, hydrophilic bentonite clay is designed to disperse in water-based polymer mixtures and coatings.

2.3.1 Spinning Solution Preparation

Samples were prepared by dispersing the nanoclay and glycerol, as needed, in deionized water at 100°C followed by the addition of St27, St70, and GG to achieve the required polymer concentrations, Table A1-1. All samples had a constant GG concentration of 2 wt%. Samples were stirred at 350 rpm for 15 minutes and then allowed to sit at room temperature overnight.
The following morning, samples were autoclaved at 121°C for 50 minutes to ensure complete gelatinization of the high amylose corn starch; reported gelatinization temperatures between 154°C and 171°C. Samples were cooled and gently stirred for another hour before electrospinning and rheological analysis to ensure homogeneity.

To evaluate the effects of polymer composition on the rheological properties and fiber formation, samples with varying total concentrations and polymer ratios were assessed. Samples containing 3 wt% and 4 wt% polymer were prepared at ratios of 1:0, 1:1, and 0:1 starch (St27: St70) with a fixed 2 wt% GG. These samples contained no glycerol or nanoclay. Samples created to evaluate the effects of glycerol and nanoclay on the polymer solutions were prepared by adding 0, 2.5, 5 wt% nanoclay, based on polymer weight, and 0, 5, and 10 v/v% glycerol to a polymer solution containing 2wt% GG and 2wt% St70.

2.3.2 Rheological Analysis

Rotational and oscillatory rheological tests were conducted using a rheometer (Anton Paar- Modular Compact Rheometer MCR 302) with a cone and plate configuration (CP50-1/TG, diameter: 50mm, 1° angle) equilibrated to 25°C. Flow curves were generated to evaluate the apparent viscosity over a range of shear rates with a fixed shear stress. Tests were conducted with a logarithmic ramp from 0.01 to 1000 s⁻¹ and data points were collected from 100 to 0.1 seconds to eliminate any transient effects at lower shear rates due to high viscosity. Viscoelastic properties (storage modulus, G’, and loss modulus, G’”) as a function of frequency were generated over a range of 0.01 to 10 Hz with 1% strain. Amplitude sweeps were conducted to ensure the strain fell within the linear viscoelastic range for each sample. All samples were evaluated in triplicate.
2.3.3 Electrospinning Procedure

Nanofibers were produced using a simple electrospinning arrangement comprising of a high voltage power supply (0-30kV), syringe pump, and grounded, stationary collector. The syringe pump was positioned vertically above the collector which was covered in aluminum foil. Before electrospinning, each sample was cooled to room temperature and loaded in a 3mL syringe fitted with a 22g blunt end needle (OD: 0.71mm, ID: 0.41, length: 1in). Each sample was spun for 30 minutes with an applied voltage of 18kV and a working distance of 6 cm. A flow rate of 0.25 mL•hr was used and ensured a stable polymer jet for most samples. The ambient temperature and relative humidity were maintained at 21°C and 20%, respectively, for the duration of fiber formation.

2.3.4 Scanning Electron Microscopy

Field emission scanning electron microscopy was used to visualize the fibers formed for each sample. Fiber samples were collected from the center of the deposition area and mounted on aluminum stubs using adhesive carbon tape. Images were obtained using a FEI Verios 460L FESEM in immersion mode using a through-the-lens detector (TLD). The SEM was operated with a 500V accelerating voltage, 13pA current, and 6mm sample to collector distance. A stage bias of 500V was also applied to eliminate the need for a conductive sample coating.

2.3.5 Statistical Analysis

Analysis of variance (ANOVA) using a general linear model was used to determine the effects of each treatment in the two independent experiments: 1) the effect of polymer concentration and starch ratios and 2) the effect of glycerol and nanoclay. Viscosities at a shear rate of 5.34s\(^{-1}\) and moduli for an angular frequency of 10 Hz were used for analyses.
Furthermore, pairwise comparisons of each factor level were evaluated using Tukey’s test with a significance level of 0.05. ANOVA assumptions were checked using the Shapiro-Wilk test of normality and the Levene’s test for equality of variance. All statistical analyses and data manipulation were performed using R.

2.4 Results and Discussion

2.4.1 Polymer Characterization

Steady state and oscillatory rheological measurements were performed to evaluate the shear rate dependent change in viscosity and a frequency sweep was carried out to determine elastic (storage) and plastic (loss) moduli. Two independent experiments were performed; 1) to evaluate the effect of polymer solution concentration and amylose content on the bulk rheology characteristics and 2) evaluate the effects of glycerol and nanoclay on these characteristics while holding the polymer composition constant. Guar gum in all samples was fixed at 2 wt% based on prior experimentation. A higher guar gum percentage was too viscous for fiber formation and a lower concentration had poor fiber formation and was more sensitive to the addition of starch. Starch ratios of 1:0 St27:ST70 contained approximately 27% amylose, 1:1 with 48.5% amylose, and 0:1 with 70% amylose within the starch portion of the polymer solution were assessed. These ratios were scaled to achieved final fiber concentrations of 3 wt% and 4 wt%.

The highest viscosities were observed when the total polymer concentration was set to 4 wt% and there was a general increase in viscosity as the amylopectin content in the samples increased, Figure 2-3. A more noticeable change in viscosity due to starch content was observed at 4 wt% total polymers. At 3 wt%, samples were clustered together, even at lower shear rates and the flow curves at starch ratios of 1:1 and 0:1 predominately overlapped. As the shear rate increased the apparent viscosity decreased by several orders of magnitude. The variation in
viscosity was less significant at higher shear rates and flow curves begin to converge at about 1000 s\(^{-1}\). The samples exhibited a shear thinning behavior after a shear rate of 0.01s\(^{-1}\), below this shear rate the samples displayed a slight increase in viscosity. Similar peaks have been observed in samples with inadequate time between sampling, transient effects. At low shear rates the polymer solution takes longer to achieve laminar flow and sampling too quickly can result in incorrect viscosity estimates. Additionally, guar gum polymer solutions often show a Newtonian plateau within this range of shear rates which could contribute to the change in shear thinning behavior (Torres et al., 2014).

The loss modulus and storage modulus over angular frequencies of 1 rads.s\(^{-1}\) to 100 rads.s\(^{-1}\) are shown in Figure 2-4. Similar to viscosity, the moduli generally increased with the total polymer concentration and amylopectin content. This trend was more apparent when the

![Figure 2 – 3: Flow curve analysis over shear rates of 0.001 to 1000 s\(^{-1}\) for polymer mixtures containing (•) 3 wt% and (▲) 4 wt% total polysaccharide. Starch ratios from low amylose content (lighter blue) to higher amylose content (darker blue) are colored accordingly.](image-url)

- Polymer Concentration
  - 3%
  - 4%
- Starch Ratio [St27:St70]
  - 1:0
  - 1:1
  - 0:1
Figure 2 – 4: Frequency sweep curves over an angular frequency of 1 to 100 rad.s\(^{-1}\) against the (Green) loss modulus and (Orange) storage modulus. Starch ratios of (•) 1:0 St27:St70, (▲) 1:1, and (●) 0:1 are plotted according to polymer concentration; (A) 3 wt% total polymer and (B) 4 wt% total polymer.

The shift in moduli due to the starch composition of the samples demonstrates that the addition of starch can successfully modify the rheological properties of guar gum to values more appropriate for electrospinning. The storage modulus changed more significantly (P < 0.01) with the change in starch ratio compared to the loss modulus (P = 0.83), Table 2-1. The branched structure of amylopectin increased the elastic properties of the polymer solution by increasing polymer concentration was 4 wt% due to the higher overall starch percentage in the polymer solution. Samples exhibited predominately plastic characteristics at low angular frequency. The crossover from plastic to elastic (G’=G’’) shifted according to the amylose content. At 4 wt% polymer and a starch ratio of 0:1 the crossover was approximately 100 rads.s\(^{-1}\); at a starch ratio of 1:0 the cross over point was closer to 20 rads.s\(^{-1}\). No notable change in moduli was observed for 3 wt% samples and the crossover point was at greater than 100 rads.s\(^{-1}\) after which the polymer solution became predominately elastic.
the intermolecular entanglements between polymers and these fix points allow the polymer to return to its previous state after deformation. When the samples contain a higher proportion of amylose, there are few molecular entanglements and the polymers are more likely to travel past each other during deformation. Although amylopectin improves the elastic properties of the solution, its structure also inhibits polymer chain alignment during fiber formation which may impact fiber morphology.

**Table 2-1:** ANOVA results assessing the effects of polymer concentration and starch ratio on the loss modulus, storage modulus, and viscosity.

<table>
<thead>
<tr>
<th>Loss Modulus</th>
<th>Mean Square</th>
<th>F-value</th>
<th>Pr &gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer Concentration</td>
<td>2102</td>
<td>297.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Starch Ratio</td>
<td>0</td>
<td>0.05</td>
<td>0.830</td>
</tr>
<tr>
<td>Polymer Conc * Starch Ratio</td>
<td>108</td>
<td>15.28</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Storage Modulus</th>
<th>Mean Square</th>
<th>F-value</th>
<th>Pr &gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer Concentration</td>
<td>7041</td>
<td>440.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Starch Ratio</td>
<td>511</td>
<td>32.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Polymer Conc * Starch Ratio</td>
<td>1371</td>
<td>85.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Viscosity</th>
<th>Mean Square</th>
<th>F-value</th>
<th>Pr &gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer Concentration</td>
<td>0.0037</td>
<td>3.53</td>
<td>0.081</td>
</tr>
<tr>
<td>Starch Ratio</td>
<td>0.0041</td>
<td>3.91</td>
<td>0.068</td>
</tr>
<tr>
<td>Polymer Conc * Starch Ratio</td>
<td>0.0002</td>
<td>0.15</td>
<td>0.701</td>
</tr>
</tbody>
</table>

The loss modulus significantly changed with an increase in polymer concentration (P < 0.001); however, this effect was dependent on the starch ratio. Although the starch ratio term was not found to be significant, it did significantly change once controlling for the polymer.
concentration. A graph of the interaction terms showed that samples containing only St27 had a greater impact on the loss modulus compared to the other starch ratios, Figure A.2-1. The storage modulus had a similar interaction effect (P < 0.001) and both the polymer concentration (P < 0.001) and starch ratio (P < 0.001) main effects were also found to be significant. Graphically, viscosity appeared dependent of both the polymer concentration and starch ratio but at a shear rate of 1000s⁻¹, no significant difference was found.

2.4.2 Effects of Nanoclay and Glycerol

The mechanical and water vapor permeability effects of nanoclay and glycerol have been studied by Chiou et al. (2007) and Khodaeimehr et al. (2018) for extruded starch nanocomposites but the effect on the solution rheology was not explored. The control samples with no nanoclay or glycerol had the lowest viscosity and corresponded to the flow curve generated in the previous section, Figure 2-5. With the addition of nanoclay, the initial peak observed at 0.01s⁻¹ was less noticeable and could be due the samples reaching laminar flow more quickly or a shift in the Newtonian plateau. Generally, the viscosity increased with the increase in both glycerol and nanoclay. Glycerol significantly impacted the viscosity (P < 0.001) with the greatest difference found between 0% and 5% glycerol content according to a Tukey multiple comparison of the means. Nanoclay also increased the viscosity (P < 0.001) of the samples with each level of nanoclay added.
Figure 2 – 5: Flow curve analysis over shear rates of 0.001 to 1000 s\(^{-1}\) for polymer mixtures containing (▪) 0 wt%, (▲) 2.5 wt%, and (●) 5 wt% nanoclay and glycerol concentrations (Red) 0 v/v%, (Yellow) 5 v/v%, and (Blue) 10 v/v%.

The storage and loss moduli also increased as the nanoclay and glycerol concentrations increased, Figure 2-6. The addition of glycerol increased the storage modulus by approximately three times for all levels of nanoclay. Glycerol alters the hydrogen bonding between polymer chains and thus improves the elasticity of the polymer matrix. An initial increase in the storage modulus was seen between 0% and 5% glycerol content (P < 0.001) but no significant change was found with the additional increase to 10% (P = 0.273). The loss modulus increased slightly due to the addition of glycerol when the nanoclay concentration was low but this effect decreased as nanoclay increased which could be due to an interaction between the nanoclay and glycerol.
Figure 2 – 6: Frequency sweep curves over an angular frequency of 1 to 100 rad.s\(^{-1}\) against the (Green) loss modulus and (Orange) storage modulus. Glycerol concentrations of (•) 0 v/v\%, (▲) 5 v/v\%, and (•) 10 v/v\% are plotted according to nanoclay concentration; (A) 0 wt\%, (B) 2.5 wt\%, and (C) 5 wt\%.

Both the loss (P < 0.001) and storage (P < 0.001) moduli were significantly impacted by the addition of nanoclay. The overall trends in the frequency sweep curves did not change with the addition of nanoclay but the crossover from plastic to elastic behavior shifted by about 40 rad.s\(^{-1}\) for every 2.5 wt\% nanoclay added. This crossover point was further decrease by 90 rad.s\(^{-1}\) with the addition of 5% glycerol but no change was observed between 5% and 10%.

As described previously, there was a significant change in the loss modulus, storage modulus, and viscosity due to both glycerol and nanoclay, but the loss modulus was the only rheological parameter that showed an effect due to an interaction between glycerol and nanoclay. The increase in the mean loss modulus for each increase in treatment was not linear for most nanoclay samples which could signify a chemical interaction between glycerol and nanoclay which impacted their ability to interact with the polymer chains. This effect would not effect would not have impact the loss modulus or viscosity of the solutions.
Table 2-2: ANOVA results assessing the effects of glycerol and nanoclay on the loss modulus, storage modulus, and viscosity.

<table>
<thead>
<tr>
<th></th>
<th>Mean Square</th>
<th>F-value</th>
<th>Pr &gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Loss Modulus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>3285</td>
<td>45.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nanoclay</td>
<td>1146</td>
<td>15.7</td>
<td>0.0007</td>
</tr>
<tr>
<td>Glycerol* Nanoclay</td>
<td>876</td>
<td>12.0</td>
<td>0.0022</td>
</tr>
<tr>
<td><strong>Storage Modulus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>169130</td>
<td>126</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nanoclay</td>
<td>56419</td>
<td>42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glycerol* Nanoclay</td>
<td>0</td>
<td>0</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Viscosity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.011</td>
<td>33.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nanoclay</td>
<td>0.0022</td>
<td>6.71</td>
<td>0.017</td>
</tr>
<tr>
<td>Glycerol* Nanoclay</td>
<td>0.0003</td>
<td>0.94</td>
<td>0.343</td>
</tr>
</tbody>
</table>

2.4.3 Nanofiber Formation

The bulk rheologic properties of a polymer solution play an important role in fiber initiation and elongation during electrospinning. Highly viscous solutions often result in needle clogging after the electric field is applied. The oscillatory rheological properties impact the development of a stable Taylor cone and continuous fiber formation. A balance between elasticity and plasticity generally has better success than polymers with strong plastic or strong elastic properties. The development of a stable Taylor cone at the spinneret is necessary to achieve uniform and continuous fiber formation. If the electrostatic repulsion between the charged particles cannot overcome the surface tension of the solution, Rayleigh instability results in the formation of drops and electrospaying. Higher solution elasticity overcomes the Rayleigh
instability and allows for a continuous fluid jet to form (Yu et al., 2006). As polymer solution exits the Taylor cone as a charged liquid jet it elongates and begins to form a whipping motion due to electrostatic forces. During this process, high polymer solution plasticity is necessary for fiber formation (Rošic et al., 2012). There are conflicting opinions on the role and importance of these two mechanisms (Rošic et al., 2012; Yu et al., 2006). Rošic et al. (2012) found that the ideal polymer solution should have the lowest elasticity necessary for fiber initiation but should have a greater plastic response. They claimed that a high solution elasticity prevented fiber formation and elongation.

FESEM images of the resulting fiber for the two experiments can be seen in Figures 2-7 and 2-8 for the polymer composition experiment and glycerol-nanoclay experiment, respectively. Overall, the fibers formed from both experiments had a beaded fiber morphology with a very small diameter, approximately 50 nm. The polymer concentration seemed to have a greater impact on fiber uniformity and the amylose content reduced the bead size on the fibers. The polymer solutions with only 3 wt% total polymer were not able to achieve stable fiber formation and often produced drops during electrospinning. These fibers contained large beads with inconsistent fiber structures. Solutions with 4 wt% polymer were more stable during electrospinning and produced more uniform fibers. An increase in amylose content seemed to created denser polymer mats which corresponds to an increase in the storage modulus and a decrease in solution viscosity.

Only the glycerol solutions that contained no nanoclay were successful in producing nanofiber mats. The fiber diameter in these mats increased with the addition of glycerol which could be related to either the increase in solution viscosity or the increase in the viscoelastic
**Figure 2 – 7**: FESEM images of guar gum/starch nanofibers deposited on aluminum foil. Guar gum was held constant at 2 wt% across all samples. (A) 1 wt% 1:0 St27:St70, (B) 1 wt% 1:1, (C) 1 wt% 0:1, (D) 2% starch 1:0, (E) 2 wt% 1:1, (F) 2 wt% 0:1. Images were captured at 25kx with a beam energy of 13pA, stage bias of 500 V, and a working distance of approximately 6mm properties. The other samples contained large round structures, most likely nanoclay, with some fibers radiating outwards. These layered nanoclay particles are larger and more frequent in samples with higher glycerol concentrations. Several studies have evaluated the exfoliation of nanoclay in polymer solutions containing glycerol with conflicting results which could be due the nanoclays used (Chiou et al., 2007; Risyon et al., 2016). The inability for the nanoclay to exfoliate in the presence of glycerol agrees with the results published by Risyon et al. (2016). They concluded that the glycerol entered the space between the silicate layers and excluded the polymer chains.
Figure 2 – 8: FESEM images of guar gum/starch nanofibers deposited on aluminum foil. Polymer concentrations were held constant at 2 wt% GG and 2 wt% St70. (A) 0% nanoclay, 0% glycerol; (B) 0% nanoclay, 5% glycerol; (C) 0% nanoclay, 10% glycerol; (D) 2.5% nanoclay, 0% glycerol; (E) 2.5% nanoclay, 5% glycerol; (F) 2.5% nanoclay, 10% glycerol; (G) 5% nanoclay, 0% glycerol; (H) 5% nanoclay, 5% glycerol; (I) 5% nanoclay, 10% glycerol. Images were captured at 25kx with a beam energy of 13pA, stage bias of 500 V, and a working distance of approximately 6mm.

2.5 Conclusions

This research evaluated the effect of polymer solution composition and additives on the final rheological properties and nanofiber formation by electrospinning. Generally, the viscosity and viscoelastic properties of the electrospinning solutions increased with an increase in amylpectin content, nanoclay, and glycerol. Guar gum and starch composite nanofibers were successfully produced at room temperature with the most uniform fibers produced from solutions
with higher amylose corn starch and a total polymer concentration of 4 wt%. Increasing the electrospinning temperature as performed by Yang et al. (2017) could further improve the fiber structure by decreasing the number of beads. Higher nanoclay concentrations resulted in greater fiber diameter but interactions in the presence of glycerol prevent nanoclay exfoliation and inhibited fiber formation. This issue could be resolved by adding the glycerol after autoclaving.

The production of ultrathin electrospun nanofibers from starch and guar gum could have useful applications in products that require high surface area to volume ratios but additional research into the mechanical properties of the final fibers would need to be evaluated.
2.6 Abstract

The electrospinning process is dependent on several solution properties including the viscosity, viscoelasticity, conductivity, and surface tension. Of these properties, the viscosity and viscoelasticity often have the greatest impact of fiber initiation and elongation. Often small changes in the biopolymer composition can significantly affect these properties and the success of biopolymer nanofiber formation is dependent on optimizing these metrics. This study evaluated the ability of several models to predict the viscosity and loss factor of guar gum and starch polymer mixtures. The Scheffé model is the most commonly used model in mixture experiments and can be modified to contain any combination of linear, crossproduct, and exponential terms to fully capture the dynamics of the mixture. This model out-performed the other black box models based on its high model efficiency and robustness to variable variation. The random forest model had greater predictive accuracy than the Scheffé model but it was more sensitive to variable variation. Often overly definite models do not perform well on messy biological data or out-of-samples values. Mixture design experimentation using the Scheffé mixture model is a promising approach to optimizing biopolymer solutions for electrospinning without having to test every possible variable combination.

**Keywords:** Guar gum, corn starch, rheology, Scheffé mixture model, random forest, neural network
2.7 Introduction

The effective incorporation of biopolymers into current polymer processing techniques will require a greater understanding of how these polymers behave and interact in mixtures. As seen in the previous study, the viscosity and viscoelastic properties of guar gum (GG) and starch mixtures had an impact on whether they are suitable for electrospinning. Adjusting the concentration of components of the mixture and additives, such as nanoclay and glycerol, to achieve more desirable polymer solution characteristics would ultimately lead to better biopolymer nanofibers. In reality, a researcher will never be able to test every combination of variables within the sample space which requires more sophisticated sampling methods and modeling. This is particularly useful when optimizing nanofiber formation due to the cost and time required to image the samples created. The following research aims to build off of the work performed in Part 1 by modeling the key rheological properties of the starch and guar gum mixtures as a way to optimize and improve the resulting nanofibers. This study will also evaluate several regression and machine learning models to determine how the predictive qualities compare and if specific models are more sensitive to outlying values.

Two response variables, viscosity and loss factor, are modeled in this analysis. Although the bulk viscosity had little impact the fiber morphology compared to the viscoelastic properties, it should not be overlooked. Higher viscosities often lead to needle clogging and the high-water absorption capacity of guar gum can result in very thick mixtures, even at lower concentrations. Additionally, if the viscosity is not above a critical value, the polymer chains will not interact and entangle within the mixture and eventually form fibers. The impact of the viscoelastic properties on fiber initiation and elongation was extensively covered in the previous section; however, an additional viscoelastic metric is introduced here. The loss factor, Equation (1), is
the proportion between the loss modulus (\(G''\)) and storage modulus (\(G'\)) (Amaratunga et al., 2018; Mezger et al., 2006). The loss factor is also referred to as the damping factor or \(\tan \delta\), where \(\delta\) is the phase shift angle between the preset and resulting oscillatory curves. The loss factor was selected for modeling because of its ability to

\[(1) \quad \tan \delta = \frac{G''}{G'}\]

capture both the elastic and plastic properties of the solution; however, it is not a good measure of magnitudes. A loss factor less than one is predominately elastic and a factor greater than one has more plastic properties (Mezger et al., 2006).

The experimental design was performed in JMP® using a custom mixture design with multiple constraints on polymer composition and the proportion of nanoclay and glycerol to the polymer weight. The constraint proportions were determined by single factor experiments to determine the polymer solution limits. An example of a constrained sampling space can be seen by the tertiary plot in Figure 2-9 for GG, St70, and glycerol. The restricted samples space to known limits improves the optimization of the model to physically achievable and realistic polymer concentrations.

Several models were evaluated for their predictive accuracy including the Scheffé mixture model and two machine learning models, random forest and neural network. The Scheffé mixture model is the most commonly used model found in food and material sciences. It was developed to provide more interpretable results from constrained mixture experiments and can take multiple forms depending on the complexity of polymer interactions (Piepel et al., 2002). The linear and quadratic forms used in this study are shown in Equation (2) and (3), respectively. These equations include an intercept term, \(a_0\), as an additional equation for comparison but this constant term
Figure 2-9: Constrained sample space for three variable dimensions used during the mixture design.

often creates errors in the analysis and model fit and can lead to a better model fit than described by the system (Cornell, 2002). The typical Scheffé-type models would not include this term. Additional Scheffé mixture model forms include full quadratic, with both crossproduct and squared terms, and reduced quadratic models. Reduced quadratic models only contain significant quadratic terms typically determined using stepwise regression for the addition or removal of terms based on importance (Amaratunga et al., 2018). These models were explored but did not perform as well as the models evaluated in this study.

\[
(2) \ E(y) = \alpha_o + \sum_{i=1}^{q} \alpha_i x_i \\
(3) \ E(y) = \alpha_o + \sum_{i=1}^{q} \alpha_i x_i + \sum_{i<}^{q} \sum_{j}^{q} \alpha_{ij} x_i x_j
\]
A growing trend in predictive modeling is the use of machine learning models. These models can offer much better predictive qualities compared to typical regression models and are typically less sensitive to outlying values, although that depends on the model used. However, these models are also commonly referred to as black box models because only the inputs and outputs are visible to the modeler. These models provide limited mechanistic or relational information about the model parameters and variables. Two machine learning models were select to compare to the Scheffé models, the random forest model and the neural network models. The application of neural network models has begun to permeate into the literature for optimizing mixture components. Yusoff et al. (2019) utilize a multilayer perceptron neural network model to evaluate the rheological response to nanosilica and polymer-modified bitumen compositions. Random forest models have not been as widely utilized for the evaluation of mixture models but an advantage of this model is the calculation of variable importance. In this study, the permutation method was used to determine which variables had the greatest impact on the predictive quality of the model. This method iteratively removes variables from the model and calculates the decrease in skill score associated with its removal.

The objectives of this model evaluation are to evaluate the predictive performance of several regression-based models and determine their sensitivity to outliers. The viscosity and loss factor response to guar gum and starch nanocomposites are evaluated as a promising method to optimize the properties effecting nanofiber formation from these biopolymers.

2.8 Methods

Reagent grade guar gum (GG), corn starch (St27), and glycerol were purchased from Carolina Biological Supply (Burlington, NC). A high amylose, unmodified corn starch (St70), Hylon® VII, was kindly provided by Ingredion Incorporated (Westchester, Illinois). The
reported maximum moisture content was 13.0 percent and the average amylose content was 72 percent. Hydrophilic bentonite clay was purchased through Sigma-Aldrich (St. Louis, MO). The nanoclay was untreated and had a particle size less than 25 µm.

2.8.1 Experimental Design

Sample concentrations for modeling were generated using a custom mixture design in JMP®, Table B.1-2. The mixture design was constrained to a range of total starch concentration between 0.5 wt% and 3.0 wt% and the total polymer concentration between 3.0 wt% and 5.0 wt%. The starch concentrations were determined by experimentation to determine the maximum concentration before complete gelation. Similarly, the total polymer maximum was set to ensure a polymer solution and the total polymer minimum was approaching the critical entanglement concentration need to for nanofiber formation (Yang et al., 2017). Additionally, the glycerol and nanoclay proportions, in relation to the polymer mixture, was set to be no larger than 67% of the polymer concentration for each sample. The mixture design samples were randomized within blocks and five samples were created and tested within each block. Samples were prepared and tested as described in the previous section. All rheological measurements were performed in triplicate.

2.8.2 Scheffé and Regression Modeling

A single point from the flow curve and frequency sweep analyses was selected and used for model training. The apparent viscosity at a shear rate of 1000s⁻¹ provided an approximation of the viscosity experienced during the electrospinning process (Han et al., 2008). A loss factor for an angular frequency of 100 rads.s⁻¹ ensured that all samples were above the cross-over point (G’=G’’) and samples became predominantly elastic, a loss factor less that one. Data was partitioned using a 75:25 training to testing data split and model performance metrics were
calculated using the testing data. All models were trained using the caret package in R with tuning parameters optimized using 5-fold cross validation. Variable importance for the random forest model was estimated using the permutation method.

2.8.3 Model Performance

Model performance was evaluated using two approaches to determine the predictive accuracy and the sensitivity in model performance to training data variation. The predictive accuracy for each model was determined using the root mean squared error (RMSE) and the model efficiency (EF) with the naïve predictor set as the mean. To evaluate the impact of outliers and extreme values on the model coefficients and predictive capabilities, data was partitioned again using 5-fold cross validation and the mean and standard deviation of the RMSE and EF metrics across the folds were calculated.

2.9 Results and Discussion

2.9.1 Scheffé Mixture Model

Both linear and partial quadric Scheffé mixture models with and without intercept terms were constructed for both response variables. This resulted in a total of four models for each response which were compared using the root mean squared error and model efficiency of the model based on the testing data, data no used in the model training. Between both response variables, the partial quadratic model performed better than the linear model, which was predicted due to the interaction effects observed in the previous section, Table 2-3. The models with intercepts performer comparably to the models without but the parameter estimates differed greatly. Although the models with intercepts have acceptable predictive accuracy, the parameters cannot be interpreted in any meaningful way. The high number of parameters tuned in the
Table 2-3: RMSE and EF for the linear and partial quadratic Scheffé models for viscosity and loss factor.

<table>
<thead>
<tr>
<th></th>
<th>Viscosity</th>
<th></th>
<th>Loss Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Linear</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Intercept</td>
<td>0.0718</td>
<td>0.847</td>
<td>0.0964</td>
</tr>
<tr>
<td>With Intercept</td>
<td>0.0678</td>
<td>0.863</td>
<td>0.0964</td>
</tr>
<tr>
<td><strong>Partial Quadratic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Intercept</td>
<td>0.0589</td>
<td>0.897</td>
<td>0.0669</td>
</tr>
<tr>
<td>With Intercept</td>
<td>0.0593</td>
<td>0.896</td>
<td>0.649</td>
</tr>
</tbody>
</table>

quadratic models and the high model efficiency does add concern for overparameterization which might reduce the performance of these models when applied to other data.

The parameter estimates and significance levels for the model with the best predictive accuracy for each response are shown in Table 2-4. In agreement with the previous results, the polymer composition had a significant impact on the solution viscosity and the interaction between nanoclay and glycerol had not impact on the viscosity. Of the polymers added to the solution, the addition of guar gum had the greatest impact on the viscosity which is typical of its high-water holding capacity. Every term in this quadratic model of the viscosity response was significant (P < 0.05) except the glycerol term and the glycerol: nanoclay interaction term. No significant change in viscosity due to glycerol conflicts with the previous findings; however, the glycerol interaction terms with the polymers were significant. The loss factor had slightly lower predictive accuracy (EF = 0.8) which could be due to the low number of significant terms in the quadratic model, although it still out performed the linear model (EF = 0.58). The polymer main effects and interaction terms seemed to have a more significant (P < 0.05) impact on the loss.
Table 2-4: Estimated parameters, t statistic, and P-value for the quadratic Scheffé models for viscosity and loss factor, without an intercept.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Viscosity</th>
<th></th>
<th></th>
<th>Loss Factor</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>t- value</td>
<td>Pr &gt; t</td>
<td>Estimate</td>
<td>t- value</td>
<td>Pr &gt; t</td>
</tr>
<tr>
<td>GG</td>
<td>1499.009</td>
<td>6.839</td>
<td>&lt;0.0001</td>
<td>-628.849</td>
<td>-2.086</td>
<td>0.041</td>
</tr>
<tr>
<td>St27</td>
<td>700.465</td>
<td>3.313</td>
<td>0.002</td>
<td>-629.939</td>
<td>-2.007</td>
<td>0.049</td>
</tr>
<tr>
<td>St70</td>
<td>382.382</td>
<td>3.913</td>
<td>&lt;0.0001</td>
<td>-31.483</td>
<td>-0.232</td>
<td>0.817</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-5.885</td>
<td>-1.928</td>
<td>0.059</td>
<td>4.197</td>
<td>0.909</td>
<td>0.367</td>
</tr>
<tr>
<td>Nanoclay</td>
<td>18.725</td>
<td>2.643</td>
<td>0.010</td>
<td>10.926</td>
<td>1.113</td>
<td>0.270</td>
</tr>
<tr>
<td>Solvent</td>
<td>0.351</td>
<td>2.177</td>
<td>0.033</td>
<td>0.513</td>
<td>2.406</td>
<td>0.019</td>
</tr>
<tr>
<td>GG: St27</td>
<td>-1855.446</td>
<td>-6.784</td>
<td>&lt;0.0001</td>
<td>1269.499</td>
<td>3.190</td>
<td>0.002</td>
</tr>
<tr>
<td>GG: St70</td>
<td>-1268.903</td>
<td>-6.868</td>
<td>&lt;0.0001</td>
<td>573.870</td>
<td>2.116</td>
<td>0.038</td>
</tr>
<tr>
<td>GG: Glycerol</td>
<td>-1509.060</td>
<td>-6.670</td>
<td>&lt;0.0001</td>
<td>626.881</td>
<td>2.011</td>
<td>0.049</td>
</tr>
<tr>
<td>GG: Nanoclay</td>
<td>-1513.362</td>
<td>-6.407</td>
<td>&lt;0.0001</td>
<td>555.485</td>
<td>1.732</td>
<td>0.088</td>
</tr>
<tr>
<td>GG: Solvent</td>
<td>-1530.362</td>
<td>-6.725</td>
<td>&lt;0.0001</td>
<td>658.067</td>
<td>2.107</td>
<td>0.039</td>
</tr>
<tr>
<td>St27:St70</td>
<td>-571.010</td>
<td>-3.130</td>
<td>0.003</td>
<td>631.469</td>
<td>2.315</td>
<td>0.024</td>
</tr>
<tr>
<td>St27: Glycerol</td>
<td>-729.166</td>
<td>-3.352</td>
<td>0.001</td>
<td>635.647</td>
<td>1.956</td>
<td>0.055</td>
</tr>
<tr>
<td>St27: Nanoclay</td>
<td>-702.603</td>
<td>-3.103</td>
<td>0.003</td>
<td>658.281</td>
<td>1.987</td>
<td>0.052</td>
</tr>
<tr>
<td>St27: Solvent</td>
<td>-723.995</td>
<td>-3.295</td>
<td>0.002</td>
<td>640.422</td>
<td>1.968</td>
<td>0.054</td>
</tr>
<tr>
<td>St70: Glycerol</td>
<td>-405.410</td>
<td>-3.893</td>
<td>0.000</td>
<td>55.223</td>
<td>0.382</td>
<td>0.704</td>
</tr>
<tr>
<td>St70: Nanoclay</td>
<td>-374.544</td>
<td>-3.445</td>
<td>0.001</td>
<td>52.939</td>
<td>0.353</td>
<td>0.726</td>
</tr>
<tr>
<td>St70: Solvent</td>
<td>-406.642</td>
<td>-3.861</td>
<td>0.000</td>
<td>26.751</td>
<td>0.183</td>
<td>0.855</td>
</tr>
<tr>
<td>Glycerol: Nanoclay</td>
<td>-12.999</td>
<td>-1.732</td>
<td>0.088</td>
<td>-6.518</td>
<td>-0.609</td>
<td>0.545</td>
</tr>
<tr>
<td>Glycerol: Solvent</td>
<td>7.681</td>
<td>2.092</td>
<td>0.041</td>
<td>-6.555</td>
<td>-1.183</td>
<td>0.242</td>
</tr>
<tr>
<td>Nanoclay: Solvent</td>
<td>-20.372</td>
<td>-2.656</td>
<td>0.010</td>
<td>-15.469</td>
<td>-1.444</td>
<td>0.154</td>
</tr>
</tbody>
</table>

factor compared to the glycerol and nanoclay. Only one of these terms, the guar gum: glycerol interaction term, was found to be significant (P = 0.049). The parameter estimates show guar gum and starch both decreased the loss factor while the additive and interaction term increased
the loss factor. The opposite trend was seen for viscosity which was increased by the addition of all the polymers.

Both the training and testing data was predicted using the trained and tuned models and the predicted versus actual values can be seen in Figure 2-10. The quadratic models for both response variables showed strong clustering to the 1:1 line which is indicative of a strong model fit. The error for the viscosity model seemed to increase at higher viscosities shown by the increase in spread. The loss factor data clustered between a loss factor of 0.2 and 0.6 with several outliers that might have impact the model and variable significance.

![Figure 2-10: Predicted values vs observed values for the quadratic Scheffé models for (A) viscosity, and (B) loss factor. Both training and testing data sets included.](image)

2.9.2 Alternate Regression Models

Two alternate regression models were also explored to determine if the commonly used Scheffé mixture model could perform as well as two machine learning models. The ransom forest and neural network models for viscosity performed well with only a marginal increase in accuracy for the random forest model, Table 2-5. The models for loss factor, however, performed poorly. Both models had model efficiency of less than zero which signifies that the
model performed worse than the naïve model which was the sample mean value. However, the simulated versus observed plot showed that the model predictions for the random forest model clustered around the identity line, Figure 2-11. The poor model performance could be due to low variability in the response or could signify additional pre-processing steps are needed. Typical pre-processing steps including removing near zero variables and variable scaling seemed to have no impact on the model performance.

Table 2-5: RMSE and EF for the random forest and neural network models of viscosity and loss factor.

<table>
<thead>
<tr>
<th></th>
<th>Viscosity</th>
<th>Loss Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RMSE</td>
<td>EF</td>
</tr>
<tr>
<td>Random Forest</td>
<td>0.0615</td>
<td>0.889</td>
</tr>
<tr>
<td>Neural Network</td>
<td>0.0694</td>
<td>0.857</td>
</tr>
</tbody>
</table>

Figure 2-11: Predicted values vs observed values for random forest models for (A) viscosity and (B) loss factor with an intercept. Both training and testing data sets included.
An advantage of the random forest model is the ability to calculated variable importance during the model training. The variable importance was calculated using the permutation method for both response models, **Table 2-6**. These values represent the degree of error introduced by removing that variable from the model. The guar gum and nanoclay terms had the largest impact on the model accuracy for both response variables, followed by the solvent or water term. Although these values cannot be directly compared to the significance levels calculated for the Scheffé mixture models, they can still provide a glimpse inside the black box of these models. However, the random forest model does not directly consider term interactions which limits the interpretation of these importance values to gain insight into the mixture dynamics.

**Table 2-6**: Variable importance calculated using the permutation method for the random forest models of viscosity and loss factor.

<table>
<thead>
<tr>
<th></th>
<th>GG</th>
<th>St27</th>
<th>St70</th>
<th>Glycerol</th>
<th>Nanoclay</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viscosity</strong></td>
<td>0.0402</td>
<td>0.00371</td>
<td>0.00228</td>
<td>0.00106</td>
<td>0.0110</td>
<td>0.00441</td>
</tr>
<tr>
<td><strong>Loss Factor</strong></td>
<td>0.0246</td>
<td>0.00145</td>
<td>0.00372</td>
<td>0.00356</td>
<td>0.0176</td>
<td>0.0117</td>
</tr>
</tbody>
</table>

**2.9.3 Model Comparison**

In addition of the model accuracy calculations for the final models, RMSE and EF, the quadratic and random forest models for both responses were evaluated for their sensitivity to training data variation. This was achieved by using a second 5-fold data split that was iterated over as the training and testing data. From these folds, 5 separate RMSE and EF values were generated and the mean and standard deviation of these values were calculated. For viscosity, the random forest model had a higher model efficiency (EF = 0.91) and lower model error (RMSE = 0.05) but the standard deviation across the folds was greater, **Table 2-7**. This indicates that this model was more sensitive to data variation and outliers. For the loss factor random forest model,
the model efficiency was also higher (EF = 0.08) and the model error was lower (RMSE= 0.06). However, neither the quadractic or random forest model displayed any clear advantage over the other based on the standard deviations.

Table 2-7: K-fold cross validation of selected model. The mean and standard deviation for each performance metric was calculated across the folds.

<table>
<thead>
<tr>
<th></th>
<th>Reduced Quadratic Model</th>
<th>Random Forest</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viscosity</strong></td>
<td><strong>Mean</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td>RMSE</td>
<td>0.0602</td>
<td>0.0121</td>
</tr>
<tr>
<td>EF</td>
<td>0.884</td>
<td>0.0396</td>
</tr>
<tr>
<td><strong>Loss Factor</strong></td>
<td><strong>Mean</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td>RMSE</td>
<td>0.0787</td>
<td>0.0198</td>
</tr>
<tr>
<td>EF</td>
<td>0.654</td>
<td>0.278</td>
</tr>
</tbody>
</table>

**2.10 Conclusions**

The viscosity and loss factor for starch and guar gum polymer mixtures were modeled using the prevalent Scheffé mixture model and two machine learning models; random forest model and neural network model. Taking into account all the terms used to compare the models, the partial quadratic Scheffé model was the most robust and demonstrated good predictive capabilities. The random forest model also showed promise as a method for predicting mixture experiment response but it showed a greater sensitivity to sample variation. Neither the random forest model or the neural network model performed well for the loss factor response. These results suggest the Scheffé mixture model is still the best performing model for mixture response prediction and optimization. Furthermore, it provides important information about the variable interactions which are not available for black box models. A modeling approach to optimize the
formation of electrospun nanofibers has the potential to be applied to other biopolymers and would decrease the sampling space needed to achieve the desired result.
2.11 Implications and Future Work

The research presented in the previous sections attempts to achieve a better understanding of the mixture dynamics and how they impact the fibers formed using the electrospinning process. By understanding how components of the mixture impact the final rheological properties, this research offers a method for optimizing the electrospinning process to those rheological parameters to improve fiber formation. The fiber formed from this experimentation exhibited a bead-on-fiber morphology which seemed to be improved by increasing the elasticity of the sample while maintaining a viscosity around 0.2 Pa.s. Several samples created in Part 1 were also found in the mixture design in Part 2. A sample containing 2 wt% GG and 1 wt% St27 had a predicted viscosity of 0.26 Pa.s and an observed viscosity of 0.22 Pa.s. The predicted and observed loss factors were found to be approximately 1.0. This polymer mixture formed low quality nanofibers but when the loss factor was gradually decreased without changing the viscosity, as seen in a sample containing 2 wt% GG and 2 wt% St70, the fiber structure improved. The addition of 5% glycerol improved the elasticity, loss factor equal to 0.5, and also increased the viscosity to 0.3 Pa.s. This mixture produced thicker fibers but were difficult to image, so the overall fiber structure could not be determined. A lower concentration of glycerol, such that the elasticity is improved without compromising the balance between the two viscoelastic properties, would produce the most uniform nanofibers. It was difficult to evaluate how both the addition of nanoclay and glycerol impacted fiber morphology due to the incomplete exfoliation of those samples; however, the addition of nanoclay seemed to increase the fiber diameter. A guar gum and starch mixture with high amylose content and low concentrations of glycerol and nanoclay would be the best combination to created uniform fibers with a slightly larger diameter.
This research produced guar gum and starch nanofibers for the first time at room
temperature. However, the ultrathin and low-quality fibers achieved in the previous experiments
made them unsuitable for the next stage in the research. The ideal fiber diameter for the
encapsulation experiment should be between 100 and 900 nm. Therefore, the next stage of
research will use poly(vinyl alcohol) (PVA) fibers as the encapsulation material for the bacterial
inoculants. This polymer is biodegradable and non-toxic. Although the biopolymer fibers could
not be evaluated in the encapsulation research, continued improvement in quality and fiber size
of biopolymeric fibers could make them an attractive source of material in the future.
References


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Rošic, R., Pelipenko, J., Kocbek, P., Baumgartner, S., Bešter-Rogač, M., & Kristl, J. (2012). The role of rheology of polymer solutions in predicting nanofiber formation by electrospinning. doi://doi.org/10.1016/j.eurpolymj.2012.05.001


CHAPTER 3: Survival of Plant Growth Promoting Bacteria in an Electrospun Biodegradable Seed Coating

3.1 Abstract

The encapsulation of bacterial and fungal inoculants in biopolymeric structures have several advantages over other bioinoculant formulations, including improved barrier properties against moisture and physical damage, as well as, lower microbial stress during processing. This research aims to evaluate the potential of the electrospinning process for the preservation of plant growth promoting microorganism in a nanofibrous seed coat. Three distinct bacterial strains were selected and tested for their survival during storage and their release after resuspension. *B. amyloliquefaciens* showed the highest survival rate after electrospinning and *B. amyloliquefaciens* and *D. acidovorans* had better survival during storage. However, all the bacterial strains, including *P. putida*, had a significant decrease in viable cells after 5 days of storage. A significant loss in viability was seen within the first 24 hours of storage and after 3 days this rate began to slow. Additionally, several osmoprotectants were added to the spinning solution but they showed no significant impact on bacterial survival. A release experiment was also performed to determine when the bacteria were released from the fibers. For seed coat inoculants the release of the bacteria should coincide with seed germination. The nanofibers produced in this experiment showed the greatest release after one day and continued release up to 3 days. This process is a promising approach to preserving bioinoculants on the surface of the seed. Exploration into alternate spinning methods and polymer materials will be necessary to achieve long term survival.

**Keywords:** PGPM, seed coat, bioencapsulation, PVA, electrospinning
3.2 Introduction

Plant growth promoting microorganisms (PGPM) are beneficial bacteria and fungi that colonize the rhizosphere and intercellular spaces within root hairs of plants. Perhaps the most well understood symbiotic colonizers are the nitrogen fixing bacteria responsible for nodulation in legumes (Carvalho et al. 2014; Campoos et al., 2014). Although not all plant-bacteria symbiotic relationships are this coordinated, PGPM have been isolated from the rhizosphere of a wide range of plants and assist in nutrient acquisition and disease resistance (Farrar et al., 2014). Some of the most well understood processes are the conversion of insoluble phosphorous and sulfur to plant-available forms, as well as, the production of siderophores, phytohormones, and biocides that target a broad spectrum of pests and pathogens (O’Calloghan, 2016; Dent and Cocking, 2016). Siderophores are microbial secreted chelating molecules for the uptake of iron. Phytohormones, such as indole-3-acetic acid (IAA), can stimulate plant growth and also increase the plants natural defense against harmful pathogens.

As our understanding increases and the discovery of new PGPM grows, an opportunity arises to harness these organisms as bioinoculants to decrease our reliance on conventional fertilizers and pesticides. The use of the Bt toxin, a protein produced by bacterium Bacillus thuringiensis when they sporulate, has been used widely in organic farming as a pesticide (Carlton and González, 1986). Furthermore, wide selection of biofertilizers and biocontrol agents are already on the market including, but not limited to, Novozymes® microbial fungicide Actinovate®, biofertilizers BioRise™2, JumpStart®, and QuickRoots®, as well as, Syngenta®’s Afla-Guard® and Taegro® fungicides (Novozymes, 2019; Syngenta, 2019). Although these products provide a sustainable and environmentally friendly alternative to chemical fertilizers
and crop protectants, the effectiveness and reliability of these products are still limited by low microbial survival during processing and after being applied to the field (Busby et al., 2017).

Bioinoculants can be applied as a seed coat, foliar spray, or soil drench depending on the need and product formulation Nakkeeran et al., 2005). Currently liquid bioinoculants have proved to have an enhanced self-life but are less efficient at suppling the bacteria or fungi to the root zone. Seed coats are believed to be the most effective and economic approach to guarantee the bacteria colonize the root zone during early plant development and to ensure the beneficial bacteria are not outcompeted by native soil microorganisms (John et al., 2011). However, the short shelf-life of bioinoculants adhered to the seed coat produces a need for improved PBPM seed carrier formulations (Bashan et al., 2014).

A variety of organic and inorganic carrier materials have been studies over the years but recent research has begun to favor polymeric formulations (Shahzad et al., 2017; Wang et al., 2015). Biopolymers and biodegradable, synthetic polymers provide a cost-effective method to ensure high cell densities, improved survival, and allow for the gradual release of these microorganisms after being introduced to the soil environment. Furthermore, the addition of osmoprotectants within the polymer matrix or during bacterial culture, has also shown to increase bacterial survival during processing and storage (Vassiley et al., 2017; Young et al., 2016; Streeter, 2003). Small, neutral molecules, mainly sugars, at high concentrations can improve survival under extreme osmotic stress and, in the case of desiccation, can stabilize proteins and cell membranes (Berninger et al. 2018; Boumahdi et al., 1999).

The versatility of polymer processing also provides methods for the encapsulation and desiccation microorganism without extreme conditions as seen in lyophilization and vacuum drying (Caiyuan et al., 2010). Electrospinning is one such method that utilized a strong electric
field to extend and entangle polymers dissolved in solution to form nanosized fibers. This process allows for the simultaneous encapsulation and desiccation of bacteria within the fiber structure. The polymeric shell then provides a barrier to humidity, light, and physical damage to the bacterial inoculants. Several studies have investigated the encapsulation of several species of plant growth promoting bacteria (PGPB), as well as, probiotics, in electrospun nanofibers with varying level of success (Fung et al., 2011; De Gregorio, 2017; Zussman, 2011).

The purpose of this research was to investigate the survival and release of bacteria from electrospun PVA nanofibers to test variability between bacterial species. Two gram-negative bacteria, *Delftia acidovorans* and *Pseudomonas putida*, and one gram-positive bacteria, *Bacillus amyloliquefaciens*, were selected for comparison. *D. acidovorans* is a known sulfur oxidizer, which makes it more chemically available to plants, and is believed to improve plant root growth. It is currently an active ingredient in BrettYoung’s BioBoost®+ for soybean and canola (BrettYoung, 2019). *P. putida* has been found to decrease the effects of some causes of wilt and root rot and it has also been utilized in bioremediation (Loeschcke and Thies, 2015). Strains of *B. amyloliquefaciens* are currently being used in several fungicide products including Syngenta’s Taegro® (Syngenta, 2019). The effect of osmoprotectants were also investigated by comparing the effect of glycerol and trehalose on bacterial survival when it was added to the spinning solution. The bacterial inoculated polymer solutions were directly electrospun on to soybean seeds as a model for on-seed survival. The electrospinning set-up, as seen in Figure 3-1, was adapted from the protocols described by Damasceno et al. (2013) and De Gregorio et al. (2017).
3.3 Methods

Poly(vinyl alcohol), 98% hydrolyzed and with an average molecular weight between 13,000 and 23,000 was purchased from Sigma-Aldrich (Milwaukee, WI). Trehalose dihydrate from starch with greater than 99% purity also purchased through Sigma-Aldrich. Reagent grade glycerol was purchased from Carolina Biological Supply (Burlington, NC).

3.3.1 Microorganisms and Culture Conditions

Bacterial cultures were obtained from the USDA Agricultural Research Services (ARS), the bacteria and strains are as follows: Delftia acidovorans (B-783), Bacillus amyloliquefaciens (B-14393), and Pseudomonas putida (B-8). Strains were received in a dormant state and were revived as using the procedure provided by the USDA ARS facility. Bacterial suspensions were then plated and overnight cultures of selected colonies were stored in 50% glycerol stocks at -
80°C. Tryptone-yeast extract- glucose agar (TGY) was the recommended growth medium and was prepared by dissolving 5g tryptone, 5g yeast extract, 1g K$_2$HPO$_4$, 1g glucose in 1 liter deionized water with the addition of 15g agar if the media was prepared for plating. The pH was adjusted to 7.0 before autoclaving for 50 minutes at 121°C.

Every two weeks for the duration of the experiments, agar plates were prepared from the glycerol stocks and stored at -4°C. Bacterial samples for electrospinning were prepared by selecting a single colony from these plates to be cultured in 2.5mL TGY broth for 12 hours before being transferred to a 250 mL flask containing 50mL TGY media. These cultures were placed in an orbital shaker set to 250 rpm and 28°C overnight. The following morning, cultures were centrifuged at 3000 rpm for 5 minutes and resuspended in 5mL 1x phosphate buffered saline (PBS). A subset of the resuspended bacteria was normalized in a spectrophotometer (600nm wavelength) to an OD of 1. The bacteria concentration (CFU.mL$^{-1}$) of the overnight cultures were then calculated using:

\[
\begin{align*}
(1) \text{ Dilution Factor (DF)} &= \frac{V_{\text{culture}}}{V_{\text{total}}} \\
(2) C &= \frac{X_{\text{norm}}}{DF} \left\{ \begin{array}{l}
X_{\text{norm}} (D. acidovorans, P. putida) = 3 \times 10^8 \text{ CFU} \\
X_{\text{norm}} (B. amyloliquefaciens) = 8 \times 10^6 \text{ CFU}
\end{array} \right.
\end{align*}
\]

(1) Where $V_{\text{culture}}$ is the volume of bacteria culture added to the cuvette and $V_{\text{total}}$ is the total volume of bacteria and PBS added to obtain an OD of 1.

(2) Where $C$ is the culture concentration (CFU.mL$^{-1}$) and $X_{\text{norm}}$ is the number of bacteria per milliter at OD 1 determined through a series of normalization and dilution trials.

The re-suspended cultures were then diluted with PBS to $1 \times 10^9 \text{ CFU.mL}^{-1}$ for $D. acidovorans$ and $P. putida$ and $1 \times 10^8 \text{ CFU.mL}^{-1}$ for $B. amyloliquefaciens$. 
3.3.2 Electrospinning

The inoculated polymer solution was electrospun directly onto sterilized soybean seeds. The seed were surface sterilized the day before using a bleach solution of 30% commercial bleach and 0.02% Tween20 (Sigma-Aldrich, Milwaukee IL). Fifty seeds were soaked in the solution for 15 minutes and then rinsed 3 times with sterile, deionized water (Schiefelbein, 2019). Seeds were allowed to dry in sterile petri dishes and then stored at -4°C until use. Three groups of 50 seeds were set aside as a control for surface sterilization.

3.3.2.1 Polymer Solution Preparation

The spinning solutions were prepared by dissolving 20wt% PVA in deionized water heated to 100°C until there were no visible granules (Koski et al., 2004). The polymer solution was autoclaved at 121°C for 50 minutes and allowed to cool. Before electrospinning the solution was stirred at 350 rpm for 30 minutes before the bacteria culture was added. An hour before electrospinning 0.5mL sugar solution containing either 0.1M glycerol or trehalose was added to 0.5mL of bacteria culture; PBS was added for the control. The bacteria and sugar solution was then added to the polymer solution and gently stirred for 30 minutes before being added to a 3mL syringe affixed with a 22g blunt end needle (OD: 0.71mm, ID: 0.41, length: 1in). The final bacteria concentration in each sample was approximately 5x10⁸ CFU.g⁻¹ for *D. acidovorans* and *P. putida* and 5x10⁷ CFU.g⁻¹ for *B. amyloliquefaciens*.

3.3.2.2 Electrospinning Procedure

Nanofibers were produced using a simple electrospinning arrangement comprising of a high voltage power supply (0-30kV), syringe pump, and grounded collector. The syringe pump was positioned vertically above the collector as seen in Figure 3-1. For the on-seed storage
experiment a vortex containing a sterile, aluminum foil-lined petri dish with 50 soybean seeds was used to collect the nanofibers. The vortex was set to gently shake the seeds to evenly distribute the fibers over the seeds. For nanofiber samples used for the release experiment, the collector was a stationary platform covered in aluminum foil. Each sample was spun for 20 minutes with an applied voltage of 18kV and a working distance of 6 cm. A flow rate of 0.25 mL•hr was used and ensured a stable polymer jet. The ambient temperature and relative humidity were maintained at 21°C and 20%, respectively, for the duration of fiber formation. Each sample treatment was created in triplicate with run order randomized by bacteria and then by sugar treatment. Polymer solutions were serially diluted and plated to determine the initial loading concentration.

### 3.3.3 Fiber Evaluation

#### 3.3.3.1 On Seed Survival

Seeds were stored under dark conditions with ambient temperature and humidity. The on-seed bacterial survival was determined using a serial dilution method. Five seeds were randomly selected from each treated sample and the seed controls, 0, 1, 3, and 5 days after treatment. The seeds were placed in 10mL PBS buffer and vortexed for 20 minutes. Plates were incubated at 28°C for 48 hours before colonies were recorded.

#### 3.3.3.2 Release

Release fiber samples were deposited on a stationary, aluminum foil covered collector. The foil was removed after electrospinning and a 2cm x 2cm section of foil form the center of the deposition area was cut and weighed. The fiber sample was then submerged in a small, sterile petri dish with 10mL of PBS. Samples were then stored under dark condition with occasional
shaking. A sample of the PBS was collected every 1 hour, 1, 2, 3, and 5 days and bacterial release was determined using the serial dilution method. After 5 days the foil was removed from the buffer, rinsed, dried, and weighed. A standard for bacterial survival in PBS buffer was created by adding a known concentration of bacteria to 10mL PBS buffer and samples as before. The loss of viable bacteria over time was recorded and used to adjust the number of bacteria released over time.

3.3.3.3 Scanning Electron Microscopy

Field emission scanning electron microscopy was used to visualize the fibers formed for select samples produced with the stationary collector. Fiber samples were collected from the center of the deposition area and mounted on aluminum stubs using adhesive carbon tape. Images were obtained using a FEI Verios 460L FESEM in immersion mode using a through-the-lens detector (TLD). The SEM was operated with a 500V accelerating voltage, 13pA current, and 6mm sample to collector distance. A stage bias of 500V was also applied to eliminate the need for a conductive sample coating.

3.3.4 Statistical Analysis

Due to violation to the analysis of variance (ANOVA) assumption of normality, the Kruskal-Wallis test was performed to evaluate the difference between treatment groups: a) variability between bacteria strains and b) effect of osmoprotectants on survival and release for the two experiments, 1) the on-seed survival after normalization to account for variable initial concentrations and 2) the release over time. Furthermore, pairwise comparisons of each factor level were evaluated using Dunn’s test for multiple comparisons with a significance level of 0.05. ANOVA assumptions were checked using the Shapiro-Wilk test of normality and the
Levene’s test for equality of variance. All statistical analyses and data manipulation were
performed using R.

3.4 Results and Discussion

3.4.1 On-Seed Survival

Seeds were stored in dark, ambient conditions to test the bacterial survival in inoculated
nanofibrous seed coats as a simulated storage experiment. After the electrospinning procedure,
the soybean seeds had a noticeable white coating, appearing to be evenly distributed around the
seed and over all seeds in each sample. Controls for the seed surface sterilization process and for
the uninoculated polymer coating had no growth for the duration of the storage experiment.

The storage experiment was carried out of the duration of 5 days with sampling
immediately, 1, 3, and 5 days after electrospinning. A steady decrease in bacterial counts was
observed with the most significant decrease in viable cells between the day of sample creation
and the first day of sampling, where the number decreased between one and two orders of
magnitude, Figure 3-2. The bacterial loss began to level off after 3 days for most samples with
no significant decrease between days 3 and 5. However, the number of viable cells decreased
three-fold for most samples, with some having no viable cells by the end of the storage
experiment.
Figure 3-2: On-Seed bacterial survival over a storage period of 5 days. Survival is plotted as the average log$_{10}$ CFU per seed over time for (A) *B. amyloliquefaciens*, (B) *D. acidovorans*, and (C) *P. putida*. The osmoprotectant treatment is differentiated by (Green) control group, (Orange) glycerol, and (Blue) trehalose.

To adjust for the variability in the initial inoculation concentration the CFU per seed counts were normalized within each sample. Changes in bacterial counts was due the length of storage in PBS before the sample was run and the addition of sugar to the PBS before electrospinning. These normalized counts were used for all statistical analyses to evaluate differences between treatment groups. As seen in Table 3-1, there was a significant difference in between bacterial strains in their ability to withstand storage; however, the addition of osmoprotectants had not significant impact on survival. It was expected that the bacterial strain and characteristics would have an impact on how well it was able to survive the high electric field and stresses associated with the encapsulation and storage. Gram-positive, *B. amyloliquefaciens* deviated the most from the other bacteria and showed the highest initial counts immediately after electrospinning even with a lower inoculation concentration. This implies a greater percent of the bacteria were able to withstand the electrospinning procedure. Due to the nature of the fibers, an accurate survival rate due to the electrospinning process itself
could not be determined. There was no significant difference between the *Delftia* and *Pseudomonas* strains. These strains are both gram-negative, non-sporulating bacteria isolated from the soil environment.

The addition of osmoprotectants to the spinning solution did not have a significant impact on the bacterial survival. Other studies have shown that the addition of sugars and other small molecules has significantly impact the survival of bacterial cells during desiccation. The results found in this experiment could signify that another processing condition other than desiccation resulted in higher bacterial stress or the concentration of the osmoprotectant was too low in the cytosol of the bacteria. Increasing the osmoprotectant loading concentration or culturing the bacteria with the protectant, as described by Gregorio et al. (2017) could increase the transport of the molecules across the cell membrane.

The considerable decrease in bacterial viability over this short storage experiment conflict with the work performed by Gregorio et al. (2017) but similar results were reported for several probiotic bacteria. Modifications to the processing conditions and system could result in better storage survival. Gregorio et al. (2017) determined that the applied voltage the system had no impact on the survival rate of the bacteria but nanofibers used in this experiment had a larger diameter which might have provided more protection against storage conditions such as humidity. Furthermore, electrospinning systems, such as coaxial electrospinning, has proven successful with several probiotic bacterial strains.

### 3.4.2 Release

One of the advantages of encapsulating bioactive compounds in electrospun nanofibers is the controlled release of the substance over time. This would result in the beneficial bacteria being released as the plant is sprouting to ensure the early colonization of the root zone. Studies
Table 3-1: Kruskal-Wallis Test significance levels and Dunn’s Test for the pairwise difference between bacterial strains.

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<th>P-value</th>
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<td><strong>Dunn’s Test [Bacteria]</strong></td>
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<td>P. unadj</td>
<td>P. adj</td>
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<td><em>Bacillus- Deftia</em></td>
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<td>0.051</td>
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<td><em>Bucillus- Pseudomonas</em></td>
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<td>0.020</td>
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<td><em>Delfia- Pseudomonas</em></td>
<td>-0.5</td>
<td>0.635</td>
<td>0.635</td>
</tr>
</tbody>
</table>

have shown that the early biome of the root structure has a lasting impact on the plant’s health and growth even if this biome changes. To test the release of the bacteria from the polymer matrix, a sample of the inoculated fibers were submerged in PBS and gently shaken for 5 days. The PBS solution was then sampled and plated to determine the number of CFUs released over time. These CFU counts were then adjusted to account for the bacteria that survival from the previous sample.

Both the actual CFUs per gram and adjusted counts are shown over time in Figure 3-3. The greatest release of bacteria was observed within 1 hour and 1 day after the fiber mat was placed in the PBS buffer. A few samples, mainly the osmoprotectant controls, had another increase in viable cells in the solution after 2 and 3 days; however, it was not as significant as the initial release and this trend was less apparent after the counts were adjusted.
Figure 3-3: Bacterial release into PBS buffer over a period of 5 days. Release is plotted as the average CFU per gram over time for (A, D) B. amyloliquefaciens, (B, E) D. acidovorans, and (C, F) P. putida. (Top) actual bacterial counts observed, (Bottom) adjusted counts based on bacterial survival in PBS. The osmoprotectant treatment is differentiated by (Green) control group, (Orange) glycerol, and (Blue) trehalose.

The Kruskal-Wallis test showed a significant difference for both treatment groups in this experiment: 1) bacterial strain, and 2) the osmoprotectant, Table 3-2. The difference between bacterial strain release was similar to the significant levels found for the storage experiment. There was no significant difference between D. acidovorans and P. putida, however there was a significant difference between P. putida and B. amyloliquefaciens. Furthermore, the trehalose
treatment was significantly different from the other two treatments and no difference was seen between the osmoprotectant control and the addition of glycerol. This difference in bacteria counts with the addition of trehalose could be due to the bacterial growth on the trehalose released to the PBS buffer.

Table 3-2: Kruskal-Wallis Test significance levels and Dunn’s Test for the pairwise difference between bacterial strains and osmoprotectant treatment.

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<tr>
<th></th>
<th>DF</th>
<th>Chi-squared</th>
<th>P-value</th>
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</thead>
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<td><strong>Osmoprotectant</strong></td>
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<td>P. adj</td>
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<tr>
<td>Bacillus-Deftia</td>
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<tr>
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<td>Delftia-Pseudomonas</td>
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</tr>
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<td><strong>Dunn’s Test [Osmoprotectant]</strong></td>
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<td>P. unadj</td>
<td>P. adj</td>
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<td>Control-Glycerol</td>
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<td>Control-Trehalose</td>
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<td>Glycerol-Trehalose</td>
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</table>

3.4.3 Fiber Characterization

The bacteria species added to the spinning solution seemed to have a considerable impact on the fiber characteristics which could have some effect on survival during the process and during storage. The fibers containing *P. putida* and *D. acidovorans* appeared generally uniform with frequent beads along the fiber, Figure 3-4. These beads ranged in size from approximately 0.5µm to 2 µm in size. The addition of trehalose and glycerol seemed to have little effect on the final fiber size or uniformity. Glycerol is commonly added to the polymer solution as a way to modify the final fiber characteristics but the concentration added to the spinning solution was too low to impact the interpolymeric interactions. Preliminary spinning trials and sample controls
showed smooth and consistent fibers so any variation in fiber structure should be due to experimental treatments. The fibers produced from the spinning solutions containing *B. amyloliquefaciens* deviated considerably from the other bacteria strains. Fibers were not continuous and contained many round beads of polymer with a similar size distribution as the other samples. These beads are commonly caused by Rayleigh instabilities during jet initial. The extensive bead formation seen in these samples could be the result of the larger bacterial size interfering with the surface tension required for stable fiber elongation. *P. putida* and *D. acidovorans* are small relative to the gram-positive *B. amyloliquefaciens* and would not inhibit fiber formation. However, these large beads might be beneficial and could be an improved barrier to external conditions as seed in the increased survival of *B. amyloliquefaciens* in storage. This premise would need additional trials to determine if this property does have an effect on survival.
Figure 3-4: FESEM images of bacteria inoculated PVA nanofibers produced by electrospinning. Fibers contain (A-C) *P. putida*, (D-F) *D. acidovorans*, and (G-I) *B. amyloliquefaciens*. Osmoprotectants added to the spinning solution are (A, D, G) control, (B, E, H) trehalose, and (C, F, I) glycerol.
3.5 Conclusions

Developing more efficient and cost-effective bacterial inoculants for commercially important crops could help decrease our reliance on chemical fertilizers and pesticides, as well as, decrease the environmental impacts caused by runoff and drifting. The application of these beneficial microorganisms as a seed coat is the most promising application method but faces many challenges. The use of electrospinning to produce bacterial inoculated nanofibers would be a scalable and adaptable method for applying a coating direct to the seed. However, the survival of the bacteria during processing and storage varied considerably between bacterial species and the storage survival was only 5 days. The addition of osmoprotectants has been proven to increase survival; however, the conditions used in this research had no significant effect.

Exploring other electrospinning processing methods, such as coaxial electrospinning, could significantly extend the viable cell counts after storage. Coaxial electrospinning involves spinning the polymer molecules around a liquid core solution. In this process, a bacterial solution is extruded through the center of the polymer spinneret. This process would provide improved barrier properties and would decrease stresses to the bacterial cultures from desiccation. Electrospinning is a promising process for the storage of beneficial bacteria but additional research into alternate methods and process optimization is needed.
References


for potential application as soybean seed bioinoculants. *Plos One, 12*(5), e0176930. doi:10.1371/journal.pone.0176930


Appendix A. Results and Statistical Analyses Accompanying Chapter 2- Part 1

Appendix A.1. Experimental Set-up and Consolidated Rheological Results

Table A.1-1: Sample names and concentrations for the two independent experiments outlined in Chapter 2-Part 1. JMP samples were used to evaluate polymer concentration effects and JGN samples contained nanoclay and glycerol to test their effects on rheology and fiber formation.

<table>
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<tr>
<th>Sample</th>
<th>GG</th>
<th>St27</th>
<th>St70</th>
<th>Nanoclay</th>
<th>Glycerol</th>
<th>Solvent</th>
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<td>JMP01</td>
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Table A.1-2: Consolidated rheological measurements used for statistical analyses.

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<td>0.26856</td>
<td>0.27321</td>
</tr>
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</table>
Appendix A.2 ANOVA Output Generated in R

** ANOVA formulas

lossmodulus ~ poly_conc + starch_ratio + poly_conc:starch_ratio

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poly_conc ***  
starch_ratio  
poly_conc:starch_ratio **  
Residuals

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

$`factor(poly_conc)`
  diff lwr  upr  p  adj
   4-3  21.614 18.0154 25.2126 0

$`factor(starch_ratio)`
  diff  lwr  upr  p  adj
0.5-0  -1.7856667 -20.35113 16.77980 0.9662550
1-0   -0.3366667 -18.90213 18.22880 0.9987776
1-0.5  1.4490000  -17.11646 20.01446 0.9776315

** storagemodulus ~ poly_conc + starch_ratio + poly_conc + poly_conc:starch_ratio

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poly_conc ***  
starch_ratio ***  
poly_conc:starch_ratio ***  
Residuals

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

$`factor(poly_conc)`
  diff  lwr  upr  p  adj
   4-3  39.55522 28.08961 51.02084 0.00000017
** viscosity ~ poly_conc + starch_ratio + poly_conc:starch_ratio

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<td>0.000161</td>
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Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

** factor(poly_conc)

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** factor(starch_ratio)

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<th>p adj</th>
</tr>
</thead>
<tbody>
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<td>-0.08972661</td>
<td>0.01578661</td>
</tr>
<tr>
<td>1-0.5</td>
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<td>-0.07059494</td>
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Figure A2-1: Interaction graphs produced during ANOVA analysis. (A) loss modulus ~ polymer concentration: starch ratio (B) storage modulus~ polymer concentration: starch ratio
** lossmodulus ~ glycerol + nanoclay + glycerol:nanoclay

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<tr>
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<td>0.000000959 ***</td>
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<tr>
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<td>1146</td>
<td>1146</td>
<td>15.70</td>
<td>0.000661 ***</td>
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<tr>
<td>glycerol:nanoclay</td>
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<td>876</td>
<td>876</td>
<td>12.01</td>
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Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

$\text{factor(glycerol)}$
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<th>p adj</th>
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$\text{factor(nanoclay)}$
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<td>0.002-0.001</td>
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** storagemodulus ~ glycerol + nanoclay + glycerol:nanoclay

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<td>0.000001622647 ***</td>
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<td>0</td>
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<td>0.991</td>
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Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

$\text{factor(glycerol)}$
<table>
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<tr>
<td>0.05-0</td>
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<tr>
<td>0.1-0</td>
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<tr>
<td>0.1-0.05</td>
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<td>-22.79724</td>
<td>101.2528</td>
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$\text{factor(nanoclay)}$
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<th>upr</th>
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<td>169.6004</td>
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<tr>
<td>0.002-0</td>
<td>102.97639</td>
<td>-13.35982</td>
<td>219.3126</td>
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<tr>
<td>0.002-0.001</td>
<td>49.71222</td>
<td>-63.15048</td>
<td>162.5749</td>
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** viscosity ~ glycerol + nanoclay + glycerol:nanoclay

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<td>33.198</td>
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<td>nanoclay</td>
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<td>0.002213</td>
<td>6.708</td>
<td>0.0167 *</td>
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<td>0.000310</td>
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Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘’ 1
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

```
$`factor(glycerol)`
diff  lwr  upr  p  adj
0.05-0  0.04122292  0.01800837  0.06443746  0.0005227
0.1-0  0.05145625  0.02824171  0.07467079  0.0000347
0.1-0.05  0.01023333 -0.01228808  0.03275475  0.5011897
```

```
$`factor(nanoclay)`
diff  lwr  upr  p  adj
0.001-0  -0.00004569444 -0.03459336  0.03450197  0.9999940
0.002-0  0.01929652778 -0.01525113  0.05384419  0.3580242
0.002-0.001  0.01934222222 -0.01417393  0.05285838  0.3351832
```

Figure A2-2: Interaction graphs produced during ANOVA analysis. Loss modulus ~ nanoclay: glycerol
Appendix B. Results and Additional Model Outputs from Chapter 2- Part 2

Appendix B.1. JMP® Experimental Design Constrains and Sample Generation

Table B.1-1: JMP® mixture model constraints.

<table>
<thead>
<tr>
<th></th>
<th>Starch max (%)</th>
<th>Starch min (%)</th>
<th>Polymer max (%)</th>
<th>Polymer min (%)</th>
<th>Ratio max (%)</th>
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<tr>
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<td>1</td>
<td>1</td>
<td>-1.5</td>
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<tr>
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<tr>
<td>Glycerol</td>
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<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
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<td>0.05</td>
<td>0.03</td>
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</table>
Table B.1-2: Sample concentrations determined using a JMP® mixture design with the previous constrains.

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<th>St70</th>
<th>Glycerol</th>
<th>Nanoclay</th>
<th>Solvent</th>
<th>Block</th>
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<tr>
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**Appendix B.2. Consolidated Rheological Results**

**Table B.2-1:** Viscosity and loss factor values used for modeling. Apparent viscosity was recorded at a shear rate of 1000s\(^{-1}\) and the loss factor at an angular frequency of 100 rads.s\(^{-1}\).

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<td>3</td>
<td>0.224</td>
<td>0.253</td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>
Appendix B.3. Scheffé Mixture Models: R Outputs

** Trained model formula

\[ \text{Viscosity} = \text{GG} + \text{ST27} + \text{ST70} + \text{Glycerol} + \text{Nanoclay} + \text{Solvent} \]

Residuals:

<table>
<thead>
<tr>
<th>Min</th>
<th>1Q</th>
<th>Median</th>
<th>3Q</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.150446</td>
<td>-0.055771</td>
<td>-0.001446</td>
<td>0.048427</td>
<td>0.282734</td>
</tr>
</tbody>
</table>

Coefficients:

| Estimate | Std. Error | t value | Pr(>|t|) |
|----------|------------|---------|----------|
| guar_gum | 20.66639   | 1.31744 | 15.687   | < 2e-16 *** |
| starch27 | 7.76947    | 1.39330 | 5.576    | 3.70e-07 *** |
| starch70 | 6.90199    | 1.00333 | 6.879    | 1.56e-09 *** |
| glycerol | -0.03759   | 0.13147 | -0.286   | 0.776 |
| nanoclay | 1.36500    | 0.20510 | 6.655    | 4.08e-09 *** |
| solvent  | -0.27449   | 0.04486 | -6.119   | 3.95e-08 *** |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.0829 on 75 degrees of freedom
Multiple R-squared: 0.9338, Adjusted R-squared: 0.9285
F-statistic: 176.2 on 6 and 75 DF, p-value: < 2.2e-16

** Viscosity= GG + ST27 + ST70 + Glycerol + Nanoclay + Solvent + GG*St27 + GG*St70 + GG*Glycerol + GG*Nanoclay + GG*Solvent + St27*St70 + St27*Glycerol + St27*Nanoclay + St27*Solvent + St70*Glycerol + St70*Nanoclay + St70*Solvent + Glycerol*Nanoclay + Glycerol*Solvent + Nanoclay*Solvent

Residuals:

<table>
<thead>
<tr>
<th>Min</th>
<th>1Q</th>
<th>Median</th>
<th>3Q</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.180855</td>
<td>-0.019171</td>
<td>-0.000025</td>
<td>0.023932</td>
<td>0.153986</td>
</tr>
</tbody>
</table>

Coefficients:

| Estimate   | Std. Error | t value | Pr(>|t|) |
|------------|------------|---------|----------|
| guar_gum   | 1464.3233  | 225.3038 | 6.499   | 1.78e-08 *** |
| starch27   | 527.3664   | 216.3592 | 2.437   | 0.017772 * |
| starch70   | 387.7976   | 100.4050 | 3.862   | 0.000278 *** |
| glycerol   | -4.1417    | 3.0850   | -1.343  | 0.184481 |
| nanoclay   | 18.7661    | 7.0264   | 2.671   | 0.009725 ** |
| solvent    | 0.3403     | 0.1662   | 2.048   | 0.044991 * |
| `guar_gum:starch27` | -1558.6930 | 271.1046 | -5.749  | 3.21e-07 *** |
| `guar_gum:starch70` | -1232.2288 | 194.9991 | -6.319  | 3.58e-08 *** |
| `guar_gum:glycerol` | -1480.0944 | 233.1588 | -6.348  | 3.20e-08 *** |
| `guar_gum:nanoclay` | -1472.5667 | 242.6772 | -6.068  | 9.46e-08 *** |
| `guar_gum:solvent` | -1495.9218 | 233.7041 | -6.401  | 2.60e-08 *** |
| `starch27:starch70` | -390.9353 | 177.3096 | -2.205  | 0.031312 * |
| `starch27:glycerol` | -567.8897 | 222.1683 | -2.556  | 0.013135 * |
| Model                      | Estimate | Std. Error | t value | Pr(>|t|) |
|---------------------------|----------|------------|---------|---------|
| guar_gum                  |  7.55884 |  1.32918   |  5.687  | 2.36e-07 *** |
| starch27                  | -0.23434 |  1.36069   | -0.172  |  0.864  |
| starch70                  |  1.06827 |  0.606     |  1.73   |  0.189  |
| glycerol                  |  0.07233 |  0.13925   |  0.53   |  0.603  |
| nanoclay                  | -1.27968 |  0.20955   | -6.107  | 4.15e-08 *** |
| solvent                   |  0.34564 |  0.04733   |  7.303  | 2.50e-10 *** |

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.086 on 75 degrees of freedom
Multiple R-squared: 0.9268,  Adjusted R-squared: 0.9209
F-statistic: 158.2 on 6 and 75 DF,  p-value: < 2.2e-16

**Loss Factor = GG + ST27 + ST70 + Glycerol + Nanoclay + Solvent + GG*ST27 + GG*ST70 + GG*Glycerol + GG*Nanoclay + GG*Solvent + St27*St70 + St27*Glycerol + St27*Nanoclay + St27*SorventSt70*Glycerol + St70*Nanoclay + St70*Sorvent + Glycerol*Nanoclay + Glycerol*Sorvent + Nanoclay*Sorvent

| Model                      | Estimate | Std. Error | t value | Pr(>|t|) |
|---------------------------|----------|------------|---------|---------|
| guar_gum                  | -530.1499|  272.7593  | -1.944  |  0.05663 .|
| starch27                  | -75.9147 |  274.2638  | -0.277  |  0.78289 |
| starch70                  |  83.3923 |  114.0530  |  0.731  |  0.46752 |
| glycerol                  | -0.1203  |  4.1311    | -0.029  |  0.97686 |
| nanoclay                  |  17.6646 |   8.1035   |  2.180  |  0.03320 * |
| Component          | Estimate  | Std. Error | t value | Pr(>|t|) |
|--------------------|-----------|------------|---------|---------|
| (Intercept)        | 11.135    | 6.189      | 1.799   | 0.0761  |
| guar_gum           | 9.185     | 6.512      | 1.410   | 0.1626  |
| starch27           | -3.753    | 6.550      | -0.573  | 0.5684  |
| starch70           | -4.218    | 6.259      | -0.674  | 0.5025  |
| glycerol           | -11.165   | 6.186      | -1.805  | 0.0752  |
| nanoclay           | -9.691    | 6.148      | -1.576  | 0.1192  |
| solvent            | -11.402   | 6.185      | -1.844  | 0.0693  |

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 1

Residual standard error: 0.08169 on 74 degrees of freedom
Multiple R-squared:  0.8269,  Adjusted R-squared:  0.8128
F-statistic:  58.9 on 6 and 74 DF,  p-value: < 2.2e-16

**Viscosity= GG + ST27 + ST70 + Glycerol + Nanoclay + Solvent + Intercept**

**Viscosity= GG + ST27 + ST70 + Glycerol + Nanoclay + Solvent +**

**Viscosity= GG*St27 + GG*St70 + GG*Glycerol + GG*Nanoclay +**

**Viscosity= GG*Solvent + St27*St70 + St27*Glycerol + St70*Nanoclay +**

**Viscosity= GG*SolventSt70*Glycerol + St70*Nanoclay +**

**Viscosity= St70*Solvent + Glycerol*Nanoclay + Glycerol*Solvent +**
Nanoclay*Solvent + Intercept

Residuals:

<table>
<thead>
<tr>
<th></th>
<th>Min</th>
<th>1Q</th>
<th>Median</th>
<th>3Q</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.180782</td>
<td>-0.019064</td>
<td>0.002468</td>
<td>0.023266</td>
<td>0.154005</td>
</tr>
</tbody>
</table>

Coefficients:

| (Intercept) | Estimate  | Std. Error | t value | Pr(>|t|) |
|-------------|-----------|------------|---------|---------|
| guar_gum    | 1480.087  | 251.861    | 5.877   | 2.07e-07 *** |
| starch27    | 545.684   | 252.117    | 2.164   | 0.03449 *   |
| starch70    | 409.008   | 177.953    | 2.298   | 0.02510 *   |
| glycerol    | -2.499    | 11.751     | -0.213  | 0.83230 .   |
| nanoclay    | 20.090    | 11.562     | 1.738   | 0.08750 .   |
| solvent     | 1.823     | 10.231     | 0.178   | 0.85919 .   |
| guar_gum:starch27 | -1551.309 | 278.051 | -5.579 | 6.38e-07 *** |
| guar_gum:starch70 | -1226.905 | 200.012 | -6.134 | 7.4e-08 *** |
| guar_gum:glycerol | -1496.146 | 259.868 | -5.757 | 3.26e-07 *** |
| guar_gum:nanoclay | -1487.859 | 266.464 | -5.584 | 6.27e-07 *** |
| guar_gum:solvent | -1511.797 | 259.850 | -5.818 | 2.59e-07 *** |
| starch27:starch70 | -387.598 | 180.251 | -2.150 | 0.03564 *   |
| starch27:glycerol | -586.644 | 258.693 | -2.268 | 0.02702 *   |
| starch27:nanoclay | -527.516 | 265.525 | -1.987 | 0.05161 .   |
| starch27:solvent | -566.056 | 260.504 | -2.173 | 0.03381 *   |
| starch70:glycerol | -445.675 | 184.113 | -2.421 | 0.01859 *   |
| starch70:nanoclay | -392.834 | 183.935 | -2.354 | 0.02194 *   |
| starch70:solvent | -432.929 | 183.935 | -2.354 | 0.02194 *   |
| glycerol:nanoclay | -14.516 | 8.041 | -1.805 | 0.07612 .   |
| glycerol:solvent | 5.231 | 3.950 | 1.350 | 0.1860 .   |
| nanoclay:solvent | -20.817 | 7.762 | -2.682 | 0.00948 **  |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.05569 on 59 degrees of freedom
Multiple R-squared: 0.9358, Adjusted R-squared: 0.913
F-statistic: 40.98 on 21 and 59 DF, p-value: < 2.2e-16

**Loss Factor = GG + ST27 + ST70 + Glycerol + Nanoclay + Solvent + Intercept

Residuals:

<table>
<thead>
<tr>
<th></th>
<th>Min</th>
<th>1Q</th>
<th>Median</th>
<th>3Q</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.23197</td>
<td>-0.04214</td>
<td>-0.00631</td>
<td>0.02751</td>
<td>0.34049</td>
</tr>
</tbody>
</table>

Coefficients:

| (Intercept) | Estimate  | Std. Error | t value | Pr(>|t|) |
|-------------|-----------|------------|---------|---------|
| guar_gum    | 12.011    | 5.926      | 2.027   | 0.0463 *   |
| starch27    | 4.210     | 5.923      | 0.711   | 0.4794 .   |
| starch70    | 3.560     | 5.560      | 0.640   | 0.5240 .   |
| glycerol    | 4.217     | 5.563      | 0.758   | 0.4509 .   |
| nanoclay    | 2.977     | 5.524      | 0.539   | 0.5916 .   |
| solvent     | 4.647     | 5.578      | 0.833   | 0.4075 .   |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 0.08623 on 74 degrees of freedom
Multiple R-squared: 0.5901,  Adjusted R-squared: 0.5569
F-statistic: 17.76 on 6 and 74 DF,  p-value: 1.245e-12

**Viscosity= GG + ST27 + ST70 + Glycerol + Nanoclay + Solvent +
GG*St27 + GG*St70 + GG*Glycerol + GG*Nanoclay +
GG*Solvent + St27*St70 + St27*Glycerol + St27*Nanoclay +
St27*SolventSt70*Glycerol + St70*Nanoclay +
St70*Solvent + Glycerol*Nanoclay + Glycerol*Solvent +
Nanoclay*Solvent + Intercept

Residuals:
     Min  1Q Median  3Q Max
-0.15726 -0.02022 0.00264 0.02227 0.34330

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) -11.801     12.282   -0.961     0.3405
 guar_gum  -373.047     318.160  -1.173     0.2457
 starch27   74.711      316.055   0.236     0.8139
 starch70  271.579     226.678   1.198     0.2357
 glycerol  13.314      14.580   0.913     0.3648
 nanoclay  29.374      14.637   2.007     0.0494
 solvent  12.616      12.594   1.002     0.3206
 guar_gum:starch27 904.578     361.516   2.502     0.0151
 guar_gum:starch70 655.105     244.099   2.684     0.0094
 guar_gum:glycerol 366.632     328.168   1.117     0.2684
 guar_gum:nanoclay 276.048     331.907   0.832     0.4089
 guar_gum:solvent 389.111     327.541   1.188     0.2396
 starch27:starch70 166.034     248.039   0.669     0.5086
 starch27:glycerol  -97.683     326.447  -0.299     0.7658
 starch27:nanoclay   -78.146     332.601  -0.235     0.8150
 starch27:solvent   -80.859     325.230  -0.249     0.8045
 starch70:glycerol  -274.525     233.861  -1.174     0.2451
 starch70:nanoclay  -280.595     233.269  -1.203     0.2383
 starch70:solvent   -284.278     234.142  -1.214     0.2295
 glycerol:nanoclay  -11.405      9.235  -1.235     0.2217
 glycerol:solvent   -1.907       5.300  -0.360     0.7203
 nanoclay:solvent  -21.357       8.884  -2.404     0.0194

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.06727 on 59 degrees of freedom
Multiple R-squared: 0.8011,  Adjusted R-squared: 0.7304
F-statistic: 11.32 on 21 and 59 DF,  p-value: 9.979e-14
Table B.3-1: Root mean squared error and model efficiency calculated using K-fold cross validation for the LMM viscosity model without an intercept.

<table>
<thead>
<tr>
<th>RMSE</th>
<th>EF</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0637</td>
<td>0.887</td>
<td></td>
</tr>
<tr>
<td>0.0492</td>
<td>0.923</td>
<td></td>
</tr>
<tr>
<td>0.0674</td>
<td>0.788</td>
<td></td>
</tr>
<tr>
<td>0.0596</td>
<td>0.914</td>
<td></td>
</tr>
<tr>
<td>0.0480</td>
<td>0.933</td>
<td></td>
</tr>
</tbody>
</table>

Table B.3-2: Root mean squared error and model efficiency calculated using K-fold cross validation for the LMM loss factor model with an intercept.

<table>
<thead>
<tr>
<th>RMSE</th>
<th>EF</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0612</td>
<td>0.913</td>
<td></td>
</tr>
<tr>
<td>0.0476</td>
<td>0.860</td>
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</tr>
<tr>
<td>0.122</td>
<td>0.313</td>
<td></td>
</tr>
<tr>
<td>0.0654</td>
<td>0.838</td>
<td></td>
</tr>
<tr>
<td>0.110</td>
<td>0.650</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B.4. Alternate Regression Models: R Outputs

Random Forest: Viscosity

Type: Regression
Number of trees: 500
Sample size: 81
Number of independent variables: 6
Mtry: 4
Target node size: 5
Variable importance mode: permutation
Splitrule: variance
OOB prediction error (MSE): 0.003991428
R squared (OOB): 0.8880527

Random Forest: Loss Factor

Type: Regression
Number of trees: 500
Sample size: 81
Number of independent variables: 6
Mtry: 6
Target node size: 5
Variable importance mode: permutation
Splitrule: extratrees
OOB prediction error (MSE): 0.005021544
R squared (OOB): 0.7007648

Table B.4-1: Root mean squared error and model efficiency calculated using K-fold cross validation for the random forest model of viscosity.

<table>
<thead>
<tr>
<th>RMSE</th>
<th>EF</th>
<th>EF</th>
<th>R Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0637</td>
<td>0.887</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0492</td>
<td>0.923</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0674</td>
<td>0.788</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0596</td>
<td>0.914</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0480</td>
<td>0.933</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table B.4-2: Root mean squared error and model efficiency calculated using K-fold cross validation for the random forest model of loss factor.

<table>
<thead>
<tr>
<th>RMSE</th>
<th>EF</th>
<th>EF</th>
<th>R Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0613</td>
<td>0.896</td>
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</tr>
<tr>
<td>0.0322</td>
<td>0.967</td>
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</tr>
<tr>
<td>0.0675</td>
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</tr>
<tr>
<td>0.0624</td>
<td>0.906</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0304</td>
<td>0.973</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix C. Preliminary Growth Curve Experimentation and Statistical Analyses for Chapter 3

Appendix C.1. Growth Curve Analysis

Figure C.1-1: Growth curve analysis in 50mL TGY medium for the three bacterial strains. Optical densities (OD600) were recorded every 0, 1, 2, 4, 8, 12, and 24 hours after inoculation. The final optical densities are adjusted for dilutions.
**Figure C.1-2:** Growth curve analysis in modified TGY medium for the three bacterial strains. Medium contained 5.6 mM either (Red) glucose, (Green) glycerol, and (Blue) trehalose. OD600 measurements were taken every 20 minutes for two hours in a 96 well plate reader spectrophotometer. Run program was set to a temperature of 28°C and 15 seconds of shaking before each read.

**Appendix C.2. Additional SEM Images**

**Figure C. 2- 1:** FESEM images of PVA nanofiber controls generated in two independent experiments. (A) 5,000x magnification (B) 25,000x magnification
Appendix C.3. Statistical Outputs Generated in R

On Seed Storage- Kruskal-Wallis rank sum test

data: norm by factor(bacteria)
Kruskal-Wallis chi-squared = 8.3733, df = 2, p-value = 0.0152

data: norm by factor(sugar)
Kruskal-Wallis chi-squared = 1.0827, df = 2, p-value = 0.582

data: norm by factor(sample)
Kruskal-Wallis chi-squared = 61.888, df = 3, p-value = 0.000000000002321

Dunn (1964) Kruskal-Wallis multiple comparison
p-values adjusted with the Holm method.

Comparison Z  P.unadj  P.adj
1 amylo - deftia -2.2350686 0.025412843 0.05082569
2 amylo - putida -2.7091741 0.006745093 0.02023528
3 deftia - putida -0.4741055 0.635424699 0.63542470

Release- Kruskal-Wallis rank sum test

data: value by factor(sample)
Kruskal-Wallis chi-squared = 29.123, df = 4, p-value = 0.000007382

data: value by factor(bacteria)
Kruskal-Wallis chi-squared = 6.6912, df = 2, p-value = 0.03524

data: value by factor(sugar)
Kruskal-Wallis chi-squared = 7.3431, df = 2, p-value = 0.02544
Dunn (1964) Kruskal-Wallis multiple comparison
p-values adjusted with the Holm method.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Z</th>
<th>P.unadj</th>
<th>P.adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 R0 - R1</td>
<td>-2.34901336</td>
<td>0.01882323139316</td>
<td>0.0941161569658</td>
</tr>
<tr>
<td>2 R0 - R2</td>
<td>0.75859006</td>
<td>0.44809782022919</td>
<td>1.00000000000000</td>
</tr>
<tr>
<td>3 R1 - R2</td>
<td>3.10760342</td>
<td>0.00188610979347</td>
<td>0.0169749881412</td>
</tr>
<tr>
<td>4 R0 - R3</td>
<td>0.09765757</td>
<td>0.92220420786513</td>
<td>0.92220420786513</td>
</tr>
<tr>
<td>5 R1 - R3</td>
<td>2.44667093</td>
<td>0.01441824116113</td>
<td>0.0865094469668</td>
</tr>
<tr>
<td>6 R2 - R3</td>
<td>-0.66093249</td>
<td>0.50865560919523</td>
<td>1.00000000000000</td>
</tr>
<tr>
<td>7 R0 - R5</td>
<td>2.98378757</td>
<td>0.00284704357244</td>
<td>0.0227763485796</td>
</tr>
<tr>
<td>8 R1 - R5</td>
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<td>0.00000009670931</td>
<td>0.00000009670931</td>
</tr>
<tr>
<td>9 R2 - R5</td>
<td>2.22519751</td>
<td>0.02606798185929</td>
<td>0.1042719274372</td>
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<tr>
<td>10 R3 - R5</td>
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<td>0.00390011023546</td>
<td>0.0273007716482</td>
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</table>

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Z</th>
<th>P.unadj</th>
<th>P.adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 amylo - defolia</td>
<td>0.8712709</td>
<td>0.38360623</td>
<td>0.38360623</td>
</tr>
<tr>
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