2008

Pulp and paper mill bacteria as a potential source of novel encapsulation materials / by Peter R. Godin.

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Pulp and Paper Mill Bacteria as

A Potential Source of Novel Encapsulation Materials

A Thesis

Presented to

The Faculty of Graduate Studies

Of

Lakehead University

By

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In partial fulfillment of requirements

For the degree of

Master of Science

August, 2008

Library and
Archives Canada

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Ottawa ON K1A 0N4
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Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-43401-7
Our file Notre référence
ISBN: 978-0-494-43401-7

Abstract of Thesis

A total of 194 bacteria were isolated from pulp and paper biofilms from a mill in Thunder Bay, Canada. Diversity of the 194 bacteria indicated that the amounts of bacteria found in these biofilms may be influenced by seasonal factors. From this stock of 194 bacteria, 55 isolates were preliminarily screened for their carbohydrate quantity and flocculation ability with hopes of identifying isolates that may be capable of producing an extracellular polymeric substance that could be used as a novel encapsulation material. Four isolates, 1, 2, 8, and 34 were selected from the carbohydrate screening and they were identified as a *Flavobacterium* sp. (isolate 1), *Pseudomonas* sp. (isolate 2), and a *Sphingomonas* sp. (isolate 34). Unfortunately, isolate 8 identity could not be confirmed using 16s rDNA sequencing and biochemical testing. Isolates 1 and 34 carbohydrate was harvested and lyophilized in attempts to create gels. Following gelation experiments using different concentrations of bacterial carbohydrate along with cation addition to the solution, it was observed that isolate 1 carbohydrate produced semi-solid gels at 2.0 and 1.0% w/v concentrations when $FeCl_3$ was added. Both isolate 1 and 34 carbohydrates appeared to enhance gelation of non-gelling concentrations of the known polysaccharides Gellan and Xanthan when mixed gel experiments were conducted.

Acknowledgements

First and foremost I would like to extend my gracious thanks to Dr. Heidi Schraft who advised me during my academic career at Lakehead University. Without her knowledge and guidance this project could not have been completed. I would also like to extend my thanks to my committee members Dr. Kam T. Leung and Dr. Aicheng Chen who's input and criticism was greatly welcomed. I must also humbly thank Dr. Nandakumar Kanavillil who taught me the finer points of research and also helped me start this project.

I've had the pleasure of making many friendships while living in Thunder Bay that will last a lifetime, thanks everyone. Thank you to everyone whom I have lived while here, we had some great times. Most importantly thank you Lakehead Microbiology Laboratory for helping me with this project. Keep up those door posts in the office guys.

Finally, I would like to thank my family for being there for me and supporting my decisions over the years. Your support is always cherished.

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Chapter 1: Literature Review

1.1.1 Biofilm Formation

Biofilms can be defined as communities of microbes associated with a surface, typically encapsulated in an extracellular matrix (Costerton et al, 1987). Microbes attach to abiotic surfaces and begin to secrete extracellular polymeric substance (EPS) creating a biofilm. EPS is mainly composed of polysaccharides, nucleic acids, and proteins and, depending on the species biofilms consist of 10-25% cells and 75-90% EPS matrix (Costerton, 1999). There is debate driving the research as to why bacteria form biofilms, however many speculations have been suggested. For example, Jefferson (2004) proposed the following four reasons for biofilm formation; defense as a stress response, favourable niche formation, community formation of multispecies and division of labour, and finally biofilm formation as a default state for microorganisms.

Under conditions of stress, bacteria may produce a biofilm as a defense mechanism. Once protected by a biofilm, organisms can withstand changes in pH, nutrient deprivation, and even exposure to antimicrobial agents such as antibiotics better than planktonic cells (Jefferson, 2004, Czechowski and Stoodley, 2002, Stewart and Costerton, 2001). Studies have shown that *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* cells in biofilms have a greater survivability than planktonic cells when exposed to the antimicrobial agents rifampin, glutaraldehyde and tobramycin (Zheng and Stewart, 2002, Grobe et al., 2002, Walters et al, 2003). If the bacteria are not destroyed

by antimicrobials, they will continue EPS production and colonization of surfaces. It has also been suggested that some cells in biofilms can sense stress and actively change their physiology in order to cope with this stress (Szomolay et al., 2005). For instance, *P. aeruginosa* biofilm cells actively produced β-lactamase enzyme to deactivate imipenem and could neutralize monochloroamine after multiple exposures to the chemical (Giwercman et al., 1991, Sanderson and Stewart, 1997). It has also been discovered that some β-lactams induce the production of polysaccharide produced by *Escherichia coli* which may also increase resistance to antimicrobials in such a way that the antimicrobial may not diffuse through the entire biofilm (Sailor et al., 2003). These studies all reported increased resistance and defense to antimicrobials when cells are in biofilms as opposed to the planktonic form.

Although the mechanisms behind antimicrobial resistance is not fully understood, five possible principles have been proposed; "(i) depletion of the antimicrobial agent in the bulk fluid bathing the biofilm, (ii) slow penetration of the antimicrobial agent into the biofilm, (iii) an altered chemical microenvironment within the biofilm leading to zones of slow or no growth, (iv) adaptive stress responses, and (v) persister cells" (Ghannoum and O'Tool, 2004).

Biofilms provide a favorable niche for the specific microorganisms present within the protective structure itself and might form as a result of this opportunity for a group of bacteria. A biofilm may consist of aerobic and anaerobic regions that can support the growth of multiple species of bacteria within the single structure (Borenstein, 1994). According to Borenstein, secondary colonizers may also get trapped in the original matrix while passing through the bulk fluid around the biofilm. A particular anaerobic

bacterium in a persister state may not be able to colonize a surface until it has been trapped in a biofilm.

Microorganisms have also shown to evolve adaptations which allow for colonization of a favorable niche. For instance, some opportunistic pathogens including *Staphylococcus aureus* have developed surface binding proteins that have an affinity for host proteins such as fibronectin, fibrinogen, vitronection, and elastin (Patti et al., 1994). Bacteria such as *Vibrio choloerae, E. coli, P. aeruginosa, and Salmonella enterica* have shown to adhere using flagella, pili and fimbriae (Jefferson, 2004). Similar mechanisms may be found in strains of environmental bacteria which allow for attachment to abiotic surfaces.

Another possibility that biofilms are formed may be to develop a communal biofilm with shared labor. Bacteria in nature are generally found in a biofilm state rather than in a planktonic, free-living state (Costerton et al., 1978). Due to the fact that a biofilm is a heterogeneous environment, bacteria must communicate in order for each species in the biofilm to survive. These bacteria communicate with each other via small signaling molecules using a process known as quorum sensing (Ghannoum and O'Tool, 2004). In a study conducted by Nielsen et al. in 2000, a mixed species biofilm composed of *Burkholderia xenovoran* LB400 and *Pseudomonas sp.* B13 displayed commensual behavior. These bacteria together will convert 3-chlorobiophenyl into carbon dioxide and water. *Pseudomonas sp.* B13 will degrade chlorobiophenyl to chlorinated benzoate and *B. xenovoran* LB400 will then utilize this compound to form carbon dioxide and water. However, *Pseudomonas sp.* B13 will not grow on its own and will only grow in the presence of *B. xenovoran* LB400 indicating the dependence of *Pseudomonas sp.* B13 on

the latter. This example illustrates one example of bacterial cooperation by means of providing energy sources. As more species of bacteria are added to a biofilm, each with their own exclusive behavior, the ability for the community to benefit might increase.

Finally, the existence of planktonic cells in nature is not normally found and generally cells are found in a biofilm state. Free-floating cells come to colonize a surface and begin to form a biofilm. In some cases, remaining in planktonic form may provide to be disadvantageous as with the case of oral streptococci which are exclusively found in biofilm form due to the fact that if they were not, they would be washed away or destroyed in their niche (Burne et al., 2003). Environmental biofilms are generally of multispecies composition and pure culture biofilms are mainly found in laboratories.

1.1.2 EPS Composition and Paper Mill Bacteria

Following attachment of a bacterium to a surface, extracellular polymeric substance (EPS) is secreted and a biofilm begins to form. EPS is the key component of any biofilm as it provides structure, a protective covering from antimicrobials and can act as an energy source (Liu and Fang, 2002). Polysaccharides and proteins make up the greatest portion of EPS in a biofilm and there can also be dead cells and nucleic acids present (Sutherland, 2001, Christensen, 1989, Whitchurch et al., 2002). EPS polysaccharides tend to be comprised of larger molecular weight polymers of 10-30 kDa. Most bacterial polysaccharides average 80-100 carbohydrate monomers per molecule (Ghannoum and O'Tool, 2004). They are mainly found in two forms, (i) capsular such as capsular polysaccharides (CPS) and K-antigens associated with cell surfaces and (ii) in

slimy polysaccharides which are superficially bound to the cell surface (Kumar et al., 2007).

Each polysaccharide secreted by an individual bacterium has a unique structure pertaining to that specific bacterium and can in some cases be used to identify the species. Commonly found carbohydrate monomers in EPS include, galactose, glucose, mannose, rhamnose, fructose, glucuronic acid (Ishida et al., 2003, Ratto et al., 2005, Lindberg et al., 2001). There are also discrepancies between the number of glycosidic linkages and the types of functional groups found on a polysaccharide in EPS (Ghannoum and O'Tool, 2004). The many different combinations of these factors allow for bacterial attachment to surfaces by means of van der Waals interactions, hydrogen bonding, and hydrophobic interactions (Ghannoum and O'Tool, 2004).

One such area that has been shown to be a promising environment for biofilms and EPS production is pulp and paper mill machines. The machine's operating temperatures of 30-50°C, white water availability, a pH of 5-8 and the presence of many nutrients all create favorable growth conditions for biofilm producing bacteria (Desjardins and Beaulieu, 2003). A typical paper machine uses 10-100 m^3 of water per tonne of paper produced and there is ample medium for bacteria to rely on for growth and circulation (Vaisanen et al., 1998). Due to the fact that most trees are shipped to mills straight from the forest, the number of microbial species found on machines can vary significantly. Anaerobic and aerobic species such as the *Bacillus, Sphaerotilus, Klebsiella, Achromobacter, Pseudomonas, Paenibacillus, Enterobacter, Cytophaga* and many more have been isolated in paper mill studies (Ratto et al., 2005, Lahtinen et al.,

2006). Some of these bacteria such as *Pseudomonas* sp. are known biofilm producers and have been the focus of many biofilm studies.

Although there are attempts to control the spread of biofilms in pulp and paper mills they have proven futile. The presence of biofilms on machines can contaminate paper, causing product loss, and affect the operation of paper machines themselves (Kolari et al., 2001). Problems that can occur during paper production include plugging of the screens used for primary filtering of pulp, corrosion induced by microbes, additive spoilage, and machine fouling. Problems involving product include holes/spots in paper and sheet breaks caused by biofilm growth on large sheets of paper (Evans, 2000). In order to control this loss of product, biocides are periodically added to the white water with the expectation that the chemicals will aid in the removal of contaminants (Desjardins and Beaulieu, 2003).

Traditionally biofilms are removed from paper machines by mechanical and/or chemical methods. A 'boil-out' may be done where the paper making process is stopped and biofilms are removed by flushing all systems with high alkaline solution. However, this method is expensive as it requires a shutdown of the mill (Evans, 2000). Another method used to remove biofilms is the application of biocides which are added to the white water in the mill. These chemicals can be divided into two groups; oxidizing and non-oxidizing biocides. Oxidizing biocides degrade cell walls and disrupt cell metabolic processes. Commonly used oxidizing biocides are hypohalous acids, chlorine dioxide and peracids (Evans, 2000). Non-oxidizing biocides are believed to be metabolic inhibitors and commonly used chemicals include bromonitropropanediol, carbamates, chlorosulfone, and glutaraldehyde (Evans, 2000). However, biocides have had limited

success in controlling mill slimes (Lassen et al., 2001). No one biocide works best at removing all biofilms because the structure of each biofilm is different. Biofilms continue to grow on paper machines regardless of the amount of control.

1.1.3 Encapsulation of Bacteria

Encapsulation of chemicals is not a new process, however encapsulating bacteria for food purposes and bioremediation is being explored. Encapsulation of probiotics such as *Lactobacillus reuteri* with alginate, κ-carrageenan, gellan and Xanthan has been used to increase survivability in gastrointestinal environments (Muthukumarasamy et al., 2006). *Bifidobacteria* sp. have been encapsulated with alginate to improve their viability in mayonnaise (Khalil and Mansour, 1998). Survival of encapsulated *Bifidobacteria* spp. in milk has also been studied (Hansen et al., 2002). There has also been investigation into the encapsulation of pollution degrading micro-organisms with Gellan gum which has been shown to increase degradation ability of activated sludge compared to that of non-encapsulated activated sludge (Moslemy et al., 2004). Creating a protective coating around a bacterium may create a time-released action and increase the survivability of the bacterium being encapsulated. Commonly used encapsulation materials include Gellan gum derived from *Sphingomonas elodea*, Xanthan gum derived from *Xanthomonas campestris*, alginate derived from brown algae, and agar derived from red algae. These materials are long chained polysaccharides and have been successfully applied for encapsulation studies. The two major areas discussed will be encapsulation of probiotic bacteria and encapsulation of environmental remediation bacteria in different materials.

It is necessary for probiotic bacteria to survive shelf-life storage as well as pass through the stomach in order to reach the intestinal environment where colonization will occur. The acidity level of the stomach is very high with pH levels ranging from 1.0-3.0, depending on the presence of food. The main components of gastric juice which probiotic bacteria must endure are pepsin, rennin, mucus, and hydrochloric acid. One way that probiotics may pass through this climate relatively unharmed is with the aid of an encapsulation material. The bacterium is encapsulated in a material that is not fully digested by the gastric juices of the stomach, thus enabling delivery to the intestine. According to the International Dairy Federation, probiotic bacteria should be present at 10^7 CFU/g minimally in a food product for proper inoculation of an individual (Ouwehand & Salminen, 1998). Rao et al. (1989) concluded that *Bifidobacterium pseudolongum* encapsulated with cellulose acetate phthalate (CAP) increased the survival of bacteria under simulated gastric acid conditions compared to the non-encapsulated bacteria (Sultana et al., 2000). This is just one study that highlights the importance of encapsulation materials for the delivery of probiotic bacteria to the intestine.

Calcium alginate has been shown to be a useful encapsulation material for aiding in the survival of probiotic bacteria. Alginate is a linear copolymer derived from brown algae. When alginate beads are coated with polycations, their chemical and mechanical stability is improved and encapsulation effectiveness increases (Krasaekoopt et al. 2004). This substance has been used due to its non-toxicity to humans and low cost. In Krasaekoopt's study, the rate at which probiotic induced acidification of yogurt occurred was lower when the probiotics were encapsulated with calcium alginate compared to the rate of free, non-encapsulated bacteria incubated at the same temperature. A different

study concluded that it took 6 hours for free, non-encapsulated cells to change a solution of reconstituted skim milk to a pH of 5.0, while it took close to 30 hours for encapsulated cells to change the pH of the solution to 5.0 (Sultana et al., 2000).

It was also concluded that the encapsulated probiotics *Lactobacillus acidophilus*, *B. infantis* and *Lactobacillus casei* had a significant decrease in cell death compared to free cells when exposed to 1% bile after a 2 hour interval. This result depended on the size of the microcapsule (Sultana et al., 2000).

Chitosan-coated alginate microcapsules have also been used to coat probiotic bacteria with hopes of increasing their viability further as it has been demonstrated that alginate alone has limited success due to its poor stability when chelating agents are present (Smidsrod and Skjak-Braek, 1990). Degradation of alginate by chelating agents may increase the release rate of encapsulated materials. Chitosan is a linear polysaccharide derived from chitin. Probiotic bacteria were encapsulated in alginate and then secondarily immersed in a chitosan solution to produce a double encapsulation effect. Microencapsulated cells of *B. bifidum* survived better than free, non-encapsulated cells in yogurt during storage, with an increased survivability of around 1 log cycle when encapsulated with chitosan-coated alginate. There was a decline of about 1 log cycle over a period of 4 weeks for encapsulated cells and 2 log cycles for free, non-encapsulated cells in both yogurts from UHT (milk) and conventionally treated milk (Krasaekoopt et al., 2006). *L. casei* in chitosan-coated alginate capsules showed similar results. Encapsulated cells survived better than free, non-encapsulated cells in both UHT and conventionally prepared yogurt with a difference of 1 log cycle (Krasaekoopt et al., 2006). This method of encapsulation could demonstrate to be an effective means to

extending shelf-life viability of probiotics. However, no simulated gastric juice test was performed to determine whether Chitosan coated alginate microcapsules can indeed resist an acidic environment.

Another study did evaluate the 6.0% bile salt (pH 8.25) tolerance of chitosan coated alginate microcapsules. This study concluded that probiotic bacteria *L. acidophilus*, *B. bifidum*, and *L. casei* were more likely to survive when coated with a chitosan-alginate mixture in a pH level of 8.25 (Krasaekoopt et al., 2004). Once again, survival in low pH environment could not be conclusively proven, and there was no survival of *B. bifidum* in the presence of gastric juice due to its low acid resistance (Krasaekoopt et al., 2004).

Picot and Lacroix (2003) investigated the effects of whey-protein encapsulation on two *Bifidobacteria* species. Whey protein is a globular protein derived from whey. Survival of the bacteria was tested in yogurt and exposed to a gastrointestinal simulation. Encapsulated *Bifidobacterium breve* cells exhibited a better survival rate after a 28 day storage period in low pH yogurt (10^4 cfu/g) compared to non-encapsulated cells (10^3 cfu/g). There was also an increase in viability (+2.6 log cycles) during simulated gastrointestinal conditions when these bacteria were encapsulated with whey protein (Picot and Lacroix, 2003). Whey protein encapsulation could be one method for improving probiotic delivery, however, the recommended dosage of probiotic bacteria is 10^6 cfu/g for therapeutic benefits and further investigation into increasing survivability is needed.

Gum arabic has also been used as an encapsulation material for the probiotic *Bifidobacterium spp.* This material is a mixture of saccharides and glycoprotein derived

from acacia trees. In one study, *B. longum* and *B. infantis* were encapsulated with gum arabic and exposed to simulated gastric juice environments of pH 2.0 for a period of 4 hours. *B. longum* encapsulated with gum arabic remained relatively stable during the entire exposure period. The survival of microencapsulated *B. infantis* was also found to be higher than that of the free, non-encapsulated cells in the presence of the simulated gastric juice (pH 2.0) (Lian et al., 2002). The two bacteria were also exposed to 0.5% and 2.0% bile solutions for 12 hours. Encapsulated *B. longum* or *B. infantis* did not significantly differ from non-encapsulated bacteria with respect to survival after the 12 hour time period in 0.5% bile, but they did show significantly improved survival when exposed to 2.0% bile (Lian et al., 2002). According to this study, gum arabic could prove to be an effective encapsulation material for *Bifidobacterium spp.*

A more recent addition to encapsulation materials is the gelling agent Gellan. The primary structure of Gellan gum has been confirmed as a linear anionic hetero-polysaccharide consisting of D-glucose, D-glucuronic acid, D-glucose and L-rhamnose (Moritaka et al., 2003). Gellan is a linear bacterial exopolysaccharide derived from the Gram-negative bacterium *Sphingomonas elodea*. It forms transparent, elastic-like gels when acetylated. However once de-acetylated with cations such as Ca^{2+}, K^{+}, Na^{+}, a rigid brittle gel is formed. Cation-induced gelation involves the formation of a double helical junction zone followed by aggregation of these double helices to form three dimensional complexes of cation and hydrogen bonds with water (Banik et al., 2000).

Miyazaki et al. (1999) concluded that rheological properties of a Gellan gel changed when calcium chloride (0.016% w/v) was added to different concentrations of Gellan 0.25%, 0.50%, 1.00% (w/v) and allowed to cool at 20°C. As concentration of

Gellan increased, so did the strength of the gel formed (measured in kN/m^2). The same study observed the release time for a given drug (1% w/v theophylline) when encapsulated with different concentrations of Gellan and cation. At simulated gastrointestinal pH of 1.2, the encapsulated material began to release drug at approximately 2.5 hours after "ingestion" and there was no significant difference in release rate between the different concentrations of Gellan.

A mixture of Gellan and drug were combined with sodium citrate (0.175% w/v) and administered orally to rats and rabbits. Sodium citrate prevented gelling from occurring until the Gellan reached an acidic environment as the Ca^{++} ions get bound at high pH and will only release at low pH (Miyazaki et al., 1999). Once the mixture reached the stomach, gelation occurred within 15 minutes, as determined by visually observing the stomach contents of the animals. Sustained release of the drug from Gellan and sodium citrate capsules compared to that of intravenous injection in vivo showed significantly higher levels of drug in the blood stream when encapsulation of drug was used compared to that of the intravenous injection after 6 hours (Miyazaki et al., 1999). This study concluded that an in situ gel could be formed from the encapsulation of drug with Gellan.

Kedzierewicz et al. (1999) discovered that a model drug (Propranolol hydrochloride) could be stored up to 3 weeks in wet or dry conditions with no change in the release of the encapsulated substance when Gellan was used. Calcium chloride was used to induce gelation and form a gel matrix around the proposed drug. The study also found that gelation would be affected by adjusting the pH of the Gellan or by adding calcium chloride to increase the gel strength. By increasing the pH of the Gellan gum to

one unit above the p*K*a of the drug, precipitation of the drug inside the microcapsule increased causing insoluble drug to be entrapped in the gel's matrix (Kedzierewicz et al., 1999). The beads were then freeze-dried or oven-dried for 24 hours at 37°C. The drug was released slower from oven-dried than from wet or freeze-dried gels. The beads were then placed in solutions differing in acidity (pH 5.3 and 12.0) and stored at room temperature for a period of 3 weeks. After the 3 weeks, the beads were submersed in a pH solution of 6.8 to dissolve the gel and no significant difference in drug release was observed in either solution (Kedzierewicz et al., 1999). Once again, Gellan has proven to be a successful encapsulating material by increasing viability of a product.

Gellan has also shown promise when used as an encapsulation material for gasoline-degrading bacteria. Encapsulated cells showed degradation of hydrocarbons immediately after inoculation at different gasoline concentration levels (50, 100, 200, 400, and 600 mg/L) and there also appeared to be a reduced adaptation time required by cells when exposed to gasoline hydrocarbons (Moslemy et al., 2002). Although free, non-encapsulated cells did degrade the hydrocarbons, they did so at a lower rate than the encapsulated bacteria because of the lag period expressed during initial inoculation. The formation of a protective gel matrix around gasoline-degrading bacteria aided tremendously with their ability to perform. Moslemy et al. (2002) concluded that when gasoline degrading microorganisms (2.6-1.0 mg_{cells} per bead) were encapsulated with Gellan gum, it took approximately 5-10 days at 10°C to degrade over 90% of the gasoline (50-600 mg L^{-1}) while degradation of equivalent levels of gasoline by free, non-encapsulated cells required more than 30 days (Moslemy et al., 2002). Again, this indicates that cells may stay viable longer when encapsulated with a polysaccharide.

Another useful encapsulating material which has shown significant value is κ-carrageenan. Fluorescently (green fluorescent protein) labeled *Moraxella* sp. G21 was encapsulated with κ-carrageenan and inoculated into autoclaved and non-autoclaved soil contaminated with *p*-nitophenol (720 μM). Results indicated that after 30 days, encapsulated *Moraxella* sp. G21 had higher survival rates compared to that of non-encapsulated cells in autoclaved soil and non-sterile soil. It was also observed that encapsulated *Moraxella* sp. G21 increased in number in non-sterile soil, suggesting that encapsulation may have provided a protective environment for the bacterium to reproduce (Errampalli et al., 1999).

Many encapsulating materials have been described, and each has benefits depending on the conditions used. Alginate, whey-protein, gum arabic have all shown potential for encapsulating probiotics. Alginate and whey-protein may prove to be successful with extending shelf-life of a product, but the benefits of probiotic encapsulation on survival in high acidic environments has not been fully explored. Alginate has been shown to be useful in encapsulating bacteria however, the best results occurred once a secondary coat of an encapsulation material was applied. On large-scale industrial production, secondarily coating an encapsulation material may increase cost of the final product for the consumer. Although whey has shown success in extending shelf life of food products, it is a milk product and may cause problems for people who are lactose intolerant. Gum Arabic demonstrated potential as an encapsulation material, however mainly studies on the *Bifidobacterium spp.* were found in the literature. Gum Arabic is also mainly used in the food industry as a stabilizer. κ-carrageenan has also shown intriguing ability when used as an encapsulation material. Of the encapsulation

materials discussed, Gellan gum has shown to be most versatile with respect to resisting low pH levels and extending shelf-life of bacteria and drugs. When different cations are added to induce gel formation, Gellan has proven to successfully resist high acidic environments (pH 1.2). Shelf-life has been extended with the use of Gellan as an encapsulation material. Gasoline-degrading bacteria have shown to survive at higher levels when encapsulated with Gellan gum. Gellan gum has shown to increase survivability of encapsulated bacteria in milk products however its application in other food products still needs to be investigated. Viability of product was increased up to 4 weeks when using gellan but most milk-based products can expire long before this. Long term encapsulation studies should be conducted on food products that stay on the shelf for greater than 4 weeks if Gellan is used. The identification of novel encapsulation materials is an ongoing research effort and the need for such materials is even greater to improve food quality and bioremediation efforts. An encapsulation material that can be applied in a single coating and last greater than 4 weeks would benefit the food industry and bioremediation industry.

1.1.4 Study Objectives

This study aims to characterize the extrapolymeric substance from paper mill bacterial isolates in search of isolates capable of producing novel carbohydrate materials for encapsulation of probiotic and pollution degrading microorganisms. Crude bacterial carbohydrates will be tested for their ability to form cation induced gels which may be used for encapsulation purposes.

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Chapter 2: Isolation and Screening of Pulp and Paper Mill Biofilm Isolates

2.1.0 Introduction

Biofilms can develop in a range of different environments as a result of high nutrient availability. As nutrients are depleted, planktonic cells may colonize another abiotic surface starting a new biofilm (O'Toole et al., 2000). Pulp and paper mill machines can offer optimum growth conditions for bacteria and thus biofilms have been reported to form on them. Micro-organisms can colonize paper machines as they are introduced through wood, water, raw materials and chemicals (Vaisanen et al., 1998).

In response to biocides used to control growth and spread of biofilms, the bacteria form resistance to the chemicals and continue to colonize the paper machines (Szomolay et al., 2005). This resistance may be due to the large amount of extracellular polymeric substances found in biofilms which restrict the total exposure to biocides. Approximately 90% of pulp and paper mill exopolymeric substances (EPS) has been found to be polysaccharides (Allison, 1993). Based on this assumption, we hypothesized that pulp and paper mill biofilms would offer bacteria producing polysaccharides useful for novel encapsulation materials.

Polysaccharides consist of monosaccharides connected by glycosidic linkages. The structure of polysaccharides may also include many hydroxyl, hydrogen, and carboxyl substituents which offer regions for bonding with cations, anions and other

polysaccharides. One such polysaccharide, Gellan, derived from the bacterium *Sphingomonas elodea* has been shown to bind with cations (Nickerson et al., 2003). Due to the chemical nature of these bacterial polysaccharides, researchers have conducted experiments regarding "flocculation", a term used to define the aggregation of a material into clumps with or without ion/substituent interactions (Evans, 2000).

Microbial polysaccharides are widely used as stabilizers, suspending agents, dispersants, and thickeners in the food industry (Sandford, 1979). Industrially, microbial polysaccharides are used as detergents, for cosmetics, and pharmaceuticals (Sandford, 1979). Bacterial polysaccharides and other biosynthesized materials have been shown to flocculate in particular materials and precipitate out in solution over time (Kurane et al., 1986, Kurane et al.,1994). Using modified methods developed by Kurane et al. (1986), researchers have used flocculation activity of bacterial polysaccharides in a kaolin clay solution as a means of screening carbohydrates produced by bacteria to predict whether a polysaccharide may have high functional-group composition such as hydroxyl, hydrogen, carboxyl groups, sulphate, phosphoryl, and more complex groups such as carboxylate groups similar to other polysaccharides currently used in the food industry such as Xanthan gum, Gellan gum, alginates, and bacterial cellulose (Prasertsan et al., 2006, Kumar et al., 2004). When purified bacterial polysaccharide is added to a kaolin clay suspension along with cations, the polysaccharide will precipitate out at a particular rate (Kurane et al., 1994). This rate is then used to calculate the polysaccharide's flocculation ability. Polysaccharides are also used in the food industry as 'flocculants' which aid in the removal of microbial cells from fermentation processes (Toeda and Kurane, 1991). Flocculation ability may be an indication as to whether a polysaccharide is useful as a

food additive for emulsification, gel-forming, absorption, film forming, and protection purposes (Kumar et al., 2004). Some identified bacterial polysaccharides used in the food industry include Gellan gum derived from *Sphingomonas elodea* and Xanthan gum derived from *Xanthomonas campestris*.

Our study investigated the flocculation ability and carbohydrate quantity of pulp and paper mill bacterial polysaccharides using methods that have been developed over many years. Investigation of the bacterial diversity found in pulp and paper mill biofilms was also observed based on individual colony morphology. The objective of this portion of the study was to identify microbial isolates from pulp and paper biofilms that produce polysaccharides which may be used as encapsulation materials. This data may be used to further add to the knowledge base of polysaccharide flocculation and serve as a selection method for further isolates of interest.

2.2.0 Methods and materials

2.2.1 Raw Biofilm Sample Collection

Biofilm samples were collected at a pulp and paper mill located in Thunder Bay, Ontario, Canada. Sampling was conducted by mill employees during routine maintenance shut downs of the paper machine on three separate occasions: January 2006, March 2006, and May 2006. Each time, biofilms were sampled at the following four sites on paper machines: inside paper press (PI), outside paper press (PO), inside paper former (FI) and outside paper former (FO). Raw biofilm samples were placed in sterile Fisherbrand 50 ml

disposable plastic screw capped centrifuge tubes and transferred from the paper mill to the Lakehead University Microbiology Laboratory in a cooler box maintained at approximately 4°C using refrigeration ice packs.

2.2.2 Enumeration of Bacteria

Raw biofilms samples were serially diluted by adding 0.5 mL of sample to 0.5 mL sterile distilled water followed by vortexing (Koneman, 1997). Using this initial dilution, a ten-fold serial dilution series was prepared with sterile distilled water and samples were inoculated onto BD Difco™ R2A Agar using triplicate plates. Inoculation was conducted mechanically with a Spiral Biotech Autoplate 4000 Spiral Plater or by spread plating using a sterile glass rod. R2A plates were incubated at 30°C and 50°C for 24 hours and for 48 hours at a 20°C. Total colony numbers were counted and the plates were used to select isolates for further study as described below.

2.2.3 Selection, Characterization, and Purification of Isolates

Once total colony counts on R2A media plates were completed, selection of isolates for screening proceeded. For each sampling date (January, March, May), sample site (PI, PO, FI, FO) and incubation temperature (20°C, 30°C, 50°C), up to eight unknown isolates were chosen resulting in a total of 194 unknown isolates. Isolates were categorized based on colour and size. From this initial selection of 194 bacteria, 55 isolates producing mucoid, ropy colonies were randomly chosen for further study. The

preference for this colony morphology was based on the assumption that these particular isolates would produce large amounts of extracellular polysaccharides (EPS) and thus be good candidates for isolation of novel carbohydrate encapsulation materials.

The 55 isolates selected from R2A media were purified by sub-culturing twice on R2A agar, incubated for 24 hours at 30°C. Pure cultures were inoculated into a 100 mL glass Erlenmeyer flask containing 25 mL of R2A broth and incubated for 24 hours at 30°C while shaking at 30 rpm on a New Brunswick Scientific C1 Platform Shaker. Each culture (0.8 mL) was mixed with 0.8 mL of sterile 50% w/v Fisherbrand glycerol and stored at -80°C.

2.2.4 Inoculum Preparation

A single colony of each purified isolate was inoculated into a 100 mL glass Erlenmeyer flask containing 25 mL of R2A broth and incubated for 24 hours at 30°C while rotating at 30 rpm on a New Brunswick Scientific C1 Platform Shaker. Following incubation, the culture was centrifuged at 3200 x *g* for 10 minutes in Thermo IEC Centra CL3R centrifuge at 4°C. The culture was then washed three times by adding 10 mL of phosphate buffered saline (PBS) and centrifuging for 10 minutes at 3200 x *g* at 4°C. PBS was composed of 0.13M NaCl, 2.7mM KCl, 5mM Na_2HPO_4 and 1.8mM KH_2PO_4 at pH of 7.4.

Following the wash with PBS, the cell density of the culture was adjusted to OD_{600} = 0.06 using a Biochrom Nova Spec Visible Spectrophotometer. Cultures were adjusted to 0.06 because this value was expected to correspond to approximately 10^7

CFU/mL. Cell inoculum quantities were confirmed by drop plating (Hoben, 1982) on R2A media. Then 0.5 mL of density adjusted culture was inoculated into two centrifuge tubes, each containing 225 mL of sterile R2A broth supplemented with dextrose at 10g/L and incubated for 24 hours at 30°C while rotating at 30 rpm. This culture was used to prepare extracellular polysaccharides.

2.2.5 Preparation of Extracellular Polysaccharides (EPS)

EPS was prepared following a protocol adapted from Kurane et al. (1994), Prasertsan et al. (2006), and Kumar et al. (2004). The 24 hour cultures described above were centrifuged initially at 3200 x *g* for 10 minutes at 4°C in order to combine the two 225 mL cultures into one centrifuge tube before washing. The pellets from both tubes were combined and the culture was washed twice by mixing with approximately 10 mL of sterile distilled water and centrifugation at 3200 x *g* for 10 minutes at 4°C. Following the second wash, 10 mL of sterile distilled water was added and the culture was vortexed for 3 minutes to mechanically shear EPS from the cells. The preparation was then centrifuged for 35 minutes at 14,460 x *g* at 4°C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge. Crude EPS was present in the supernatant, and cells that collected in the pellet were discarded (Kurane et al., 1994 and Prasertsan et al., 2006 and Kumar et al., 2004).

2.2.6 Carbohydrate Quantification

Crude EPS was assayed for its carbohydrate content using the Phenol-Sulfuric Acid Assay (Dubois et al., 1956). This assay estimates simple sugars by measuring colour change of furfural and hydroxymethylfurfural compounds produced by sulfuric acid after phenol is added to the sample. EPS supernatant (0.5 mL) was added to 0.5 mL of reagent grade 5% (w/v) Phenol and vortexed in a Fisherbrand Disposable 16x125mm Glass Culture Tube. Then, 2.5 mL of reagent grade concentrated sulfuric acid was added to the solution and vortexed lightly. The mixture was incubated at room temperature for 10 minutes and then incubated for 15 minutes at 25°C in a Fisher Scientific Isotemp 228 water bath. Samples were read against a blank prepared without glucose using a Biochrom Nova Spec Visible Spectrophotometer set at a wavelength of 488 nm. A glucose standard solution was prepared by adding 50 mg of glucose to 50 mL of 0.15% (wt/vol) benzoic acid solution. This solution could be stored at 5°C for several months. To prepare a standard curve, the glucose solution was diluted 1:10 in distilled water resulting in a solution containing 100 μg/ml glucose. Using this stock solution of 100 μg/ml glucose, solutions of 0, 5, 10, 20, 30, 70, and 100 μg/ml were created and assayed as described above. Concentration of carbohydrate in isolate samples could then be calculated by comparing the absorbance of the samples to the absorbance of the plotted glucose standard curve.

2.2.7 Carbohydrate Flocculation

Crude carbohydrate, commercial grade Keltrol® Xanthan (CPKelco) and commercial grade Kelcogel® Gellan gum (CPKelco) were assayed for their ability to flocculate in a kaolin clay solution. It has been reported that there is a correlation between the amount of bacterial carbohydrate and the ability for this carbohydrate to form aggregates in solution (Kurane et al., 1994 and Prasertsan et al., 2006). Carbohydrate will bind to kaolin clay particles and precipitate over a given time interval (Kurane et al., 1994). A suspension of Fisherbrand Kaolin Clay USP (Aluminum silicate, $Al_2Si_2O_5(OH)_4$) was prepared by mixing 5.5g Kaolin with 1000 mL of sterile distilled water. One mL of crude carbohydrate sample and 0.9 mL of 0.5 M $CaCl_2$ were added in order to create a cross-bridging effect between kaolin and carbohydrate molecules. This mixture was vortexed for 1 minute and then left to stand at room temperature. The turbidity of the solution was recorded every 15 minutes for 1 hour using a Biochrom Nova Spec Visible Spectrophotometer set at a wavelength of 550 nm. At each time point (15, 30, 45, and 60 minutes), 1.0 ml of the sample was transferred to a Fisherbrand 1.5 milliliter disposable plastic curvette and absorbance was recorded.

Flocculation ability was calculated using the formula:

$(1/Sample_{OD550}) - (1/Control_{OD550})$

2.3.0 Results

The total number of aerobic bacteria found at each sampling site, namely press outside, press inside, former outside and former inside (PO, PI, FO, and FI), and is shown in Figure 2.1. Overall, January had the lowest number of positive sites, only sites PO, FO, and FI at 30°C yielded countable plates. For several sampling sites only a few colonies grew on the plates incubated at 20°C and 50°C. These were reported as <100 CFU/mL. The highest CFU/mL counts were obtained from plates incubated at 30°C.

As seen in Figure 2.1, for the sampling in March all sites were positive for bacterial growth. Bacteria counts on plates incubated at 20°C and 30°C were approximately 10^4 CFU/mL, while counts on plates incubated at 50 °C were much higher, 10^6 CFU/mL.

The May sampling also was positive for growth at all sites (Figure 2.1). There appeared to be a shift in bacteria contributing to the highest counts during this month, as 30°C plates showed approximately 10^5 CFU/mL and 50°C sites showed only 10^4 CFU/mL. The lowest counts were observed on 20°C plates (10^3 CFU/mL).

The diversity of the 194 isolates was assessed based on colour and size for each sampling month (Figure 2.2). The few colonies collected in January were predominantly coloured. The March sampling produced all three colour types with white colonies predominating mainly on 20°C and 30°C plates. The 50°C plates produced a similar amount of all three colour types during March. In May, coloured colonies predominated on 20°C plates, while white and clear colonies were found in higher numbers on 30°C and

50°C plates. Overall, 19.07% of the isolates collected were clear, 45.36% were white and 35.56% were coloured (Figure 2.4).

With respect to size, colonies were predominantly <1 to 2mm in diameter and very few isolate colonies were >5mm in diameter (Figure 2.3). January and May collection dates provided isolates that were mainly <1 to 2mm with very few isolates >3mm. The March samples were more diverse with respect to size as there was a mixture of isolates ranging from <1 to 2mm, 3 to 5mm and even some 6 to 10 mm (Figure 2.3). The May sampling had the largest colonies, 6 to 10 mm in diameter. Overall, 65.46% of the isolates were <1 to 2mm in diameter, 28.35% were 3 to 5mm, and 6.18% were 6 to 10 mm in diameter (Figure 2.5).

A total of 55 isolates were selected randomly from this 194 isolate stock, but preference was given to isolates that produced mucoid, ropy colonies. Morphology observations based on colony colour and size for these 55 isolates are shown in Figures 2.4 and 2.5. A majority of the 55 isolates were white (43.6%), while clear (30.9%) and coloured (25.4%) were also selected (Figure 2.4). With respect to size, 94.5% of the 55 isolates were <1 to 2mm in diameter, 5.4% were 3 to 5mm, and 0% were 6 to 10 mm in diameter (Figure 2.5).

To assess carbohydrate and flocculation ability, each culture was adjusted to an OD_{600} = 0.06 because it was expected that this value would correspond to approximately 10^7 CFU/mL ($\log_{10}$ 7.00 CFU/mL). This inoculum quantity was verified using drop plating and the range of inocula values (CFU/mL) for the isolates are shown in Figure 2.6. Inocula ranged from $\log_{10}$ 3.75 to 8.16 CFU/mL while the average inoculum for all 55 isolates was $\log_{10}$ 6.34±1.00 CFU/mL.

The amount of carbohydrate produced by the 55 isolates was determined by using the Phenol/Sulfuric Acid Assay as described in Section 2.2.6. These initial carbohydrate quantities measured were used as one selection methods of isolates producing potential encapsulation materials. Carbohydrate quantities varied greatly between the isolates with 0.13 μg/ml being the lowest and 2076.87 μg/ml being the highest measured value (Figure 2.7).

Along with carbohydrate quantification, carbohydrate flocculation was evaluated. Carbohydrate produced by the 55 mill isolates was tested for their ability to flocculate in a Kaolin Clay Solution and a wide range of flocculation ability values was calculated (Figures 2.9 & 2.10).

Total average flocculation ability for the 55 isolate carbohydrates increased up to 45 minutes, however began to decrease after this point (Figure 2.8). Total average flocculation ability was low for all the carbohydrates as the highest average flocculation ability was calculated to be 1.25 at 45 minutes. In comparison, flocculation of Xanthan and Gellan in similar concentrations of 45 minutes was 20-60 (Figure 2.13). The variance between flocculation ability at each time interval increased over time for all of the carbohydrates as seen in Figure 2.8. All carbohydrates showed different flocculation abilities from one another at 30 and 45 minutes as seen in Figures 2.9 and 2.10.

Upon completion of carbohydrate quantification and carbohydrate flocculation ability assays, the coefficient of correlation (R^2) was calculated at both 30 and 45 minutes using the line of best fit for the data.

Figure 2.11 depicts no correlation between carbohydrate flocculation ability at 30 minutes and carbohydrate quantity. The correlation of flocculation ability and

carbohydrate quantity (R^2) was calculated to be 0.0027 and increased to 0.1952 once outlier isolates 1 and 34 were removed. All isolates except isolate 34 produced carbohydrate quantity less than 200 μg/ml. All isolates except isolate 1 had a calculated flocculation ability of less than 4. Flocculation abilities ranged from -0.361 to 25.49, while carbohydrate quantities ranged from 0.13 μg/ml to 2076.87 μg/ml.

Figure 2.12 depicts similar results with respect to flocculation ability of carbohydrate at 45 minutes and carbohydrate quantity. The correlation of flocculation ability of carbohydrate and quantity of carbohydrate (R^2) was calculated to be 0.0004 and increased to 0.1867 once outlier isolates 1 and 34 were removed. Approximately 98.18% of the 55 isolates screened had carbohydrate quantities measured of less than 200 μg/ml and 90.90% of isolates screened had flocculation abilities less than 4 after 45 minutes. Isolates 1, 3, 13, 20, and 47 all had flocculation abilities greater than 4 at 45 minutes. Flocculation abilities ranged from -1.87 to 27.84, while carbohydrate quantities ranged from 0.13 μg/ml to 2076.87 μg/ml.

Commercially available Xanthan and Gellan gum were also assessed for their flocculation ability at 250 and 500 μg/mL concentrations (Figure 2.13). Xanthan had higher flocculation ability at 250 μg/mL concentrations while Gellan had higher flocculation at 500 μg/mL concentrations.

2.4.0 Discussion

The total number of aerobic bacteria found at each sampling site, press outside, press inside, former outside, and former inside (PO, PI, FO, and FI), are shown in Figure 2.1. For each sampling date of January, March and May, there was a shift in microbial flora, as illustrated by the shift in highest $\log_{10}$CFU/mL counts obtained, which depended on the incubation temperature of the plates. January was the coldest sampling month while May was the warmest for our study. The seasonal temperature may have had an influence on the counts of bacteria found in each month. Bacteria concentrations found in each site will differ depending on the optimal growth temperature for the organism. As the seasonal temperature increased, one may see an increase or decrease in the number of bacteria per site as it was observed in Figure 2.1. Canada's climate is cooler so it may be expected to find more psychrophile (growth at -10°C-20°C) and mesophile (growth at 10°C-50°C) bacteria in the pulp and paper mills rather than thermophiles (growth at 40°C-70°C) (Pikuta and Hoover, 2007). For our study incubation temperatures of 20, 30 and 50°C were selected which may have been the optimal growth temperatures for some pulp and paper mill bacteria. However, a greater selection of temperatures should be tested for future studies. The optimal temperature for isolates found in January may have been 30°C, March 50°C and 30°C for May as these were the highest CFU/mL count temperatures. The optimal growth temperature may have changed for the microorganisms because the specific species of microorganisms may have changed themselves.

The 194 isolates screened initially were categorized based on colour and size. For the January sampling, several areas had <100 CFU/mL colonies recorded which resulted

in a small number of colonies, which were predominantly coloured. White coloured colonies were present in all three sampling months. White colonies predominated in March and May. Most clear colonies were observed in March.

Most of the isolates collected on all sample dates were <1 to 2 mm in diameter. When colour (Figure 2.2) and size (Figure 2.3) were compared for March and May, it can be seen that sites containing colonies <1 to 2 mm were also sites that contained many colonies that were white and coloured.

The diversity of bacteria found in the mill may be explained as a result of the time of year depending on where the pulp and paper mill received wood from. January and March are colder months compared to May and this may have influenced the types of bacteria found in the soil and on the trees if the bacteria are not psychrophilic. Kubartova et al. (2007) observed a shift in microbial decomposers found around different forest tree stands depending on the season. It was also observed that fungal colonies reached a peak during summer months and rare or infrequent species were also found during this time of the year. Community composition was highly dependent on environmental conditions such as temperature (Kubartova et al., 2007).

Another reason for diversity of bacteria on paper machines may be attributed to the use of river water for paper making processes. Late winter and early spring snow melts can cause flooding of streams and disturbance of benthos materials which may influence bacterial populations. It has been shown that the input of allochothonous cells from the surrounding landscape and the re-suspension of sediment from benthic zones can influence riverine bacterial flora during snow melt periods (Liu and Leff, 2002). Our

study found similar results as we saw an increase in the amount of bacteria during the months of March and May compared to that of January.

From the 194 isolates initially selected, a group of 55 isolates was chosen for screening of carbohydrate. These isolates were selected based on their ability to produce mucoid colonies under the assumption that these particular isolates would produce large amounts of extracellular polysaccharides and thus be good candidates for isolation of novel carbohydrate encapsulation materials. The 55 isolates selected seemed an accurate representation of the colour diversity for the 194 isolates (Figure 2.4). The selection seemed however biased with respect to colony size since most (94.5%) of the 55 isolates formed colonies <1.0 to 2mm in diameter (Figure 2.5). This may not fully represent the colony size of the 194 isolates, where only (65.5%) were <1-2 mm in diameter.

The 55 bacterial isolates were screened for their ability to produce carbohydrate and flocculate in kaolin clay solution. These two approaches were used as a means of selecting isolates capable of producing novel encapsulation materials. Previous studies have investigated wastewater sites (Gao et al., 2006), mudflats (Kumar et al., 2004), seafood sludge from processing plants (Prasertsan et al., 2006) as sources which can offer useful microbial exopolysaccharides. Using flocculation ability and carbohydrate content as screening methods, the above studies identified the bacteria *Vagococcus* sp. W31, *Bacillus* sp. I-450, and *Enterobacter cloacae* WD7 respectively. Based on these previous studies, we considered pulp and paper mill machines may also offer industrially significant polysaccharides as there is significant biofilm growth on paper machines.

Carbohydrate concentrations were measured for all 55 isolates as seen in Figure 2.7. A wide range in carbohydrate production was seen for the 55 isolates which may be a

result of the multiple bacterial species found in pulp and paper biofilms. Selection of these isolates was based on mucoid, ropy colonies, which were thought to be potential sources of high extracellular carbohydrate. However, this assumption was not entirely true as carbohydrate production varied substantially between isolates (Figure 2.7).

Variation in EPS production may be explained by the growth conditions that were applied to the isolates. The 55 unknown isolates were selected for study, but their optimal growth conditions were unknown. An incubation temperature of 30°C was selected and R2A media supplemented with dextrose was used as the growth medium assuming that these conditions would be sufficient enough for all 55 bacteria. As seen in Figure 2.7, for some isolates these growth conditions may be optimal, but for others it was not, as carbohydrate production varied. Other growth temperatures, growth media, and supplements may affect EPS production and should be examined; this could include using growth conditions similar to the conditions found on the collection site of the paper machines where the biofilm samples were taken from.

By calculating how well a polysaccharide flocculates, we could possibly identify novel polysaccharides for encapsulation use because a correlation between extracellular bioflocculant and cell aggregation has been reported (Tenny and Verhoff, 1973). We hoped to use this correlation to identify novel polysaccharides as some microbial polysaccharides such as Gellan may have flocculation abilities due to the molecule's functional group interactions. Our study hypothesizes that some paper mill isolate polysaccharides will have high flocculation ability and we use this to indicate gelation activity of a paper mill polysaccharide.

As seen in Figure 2.8, the average total flocculation for the 55 isolates began to decrease after 45 minutes. It was also observed that as time increased, so did the variance in flocculation abilities between the 55 isolate flocculations at all time intervals. Only measurements at 30 minutes (Figure 2.9) and 45 minutes (Figure 2.10) were graphically shown due to the fact that at these intervals the least amount of variance in flocculation abilities was observed for the triplicate experiments (Figure 2.8). Measurements at 15 minutes were not used because mixtures of kaolin and carbohydrate may not yet have fully settled and this may influence the readings.

Carbohydrate flocculation ability and carbohydrate concentrations did not correlate with most of the 55 bacteria in this study at 30 and 45 minutes. This may be a result of the different species of bacteria that were isolated which in turn produced different types of polysaccharides. A certain bacterium may produce large quantities of a polysaccharide, however, the polysaccharide may not have had the appropriate binding sites for our $CaCl_2$ cation which was used to create cross-bridges with kaolin particles. This may insinuate that the bacteria which produced large amounts of carbohydrate and had low flocculation abilities should have their flocculation tested with other flocculating media and ions.

Although flocculation did not correlate with carbohydrate concentration of the 55 isolates, it did correlate for isolate 1, which was chosen for further study because it had a high carbohydrate concentration (174.07 μg/m) and high flocculation ability at 45 minutes (27.84). Three other isolates (2, 8 and 34) were selected for further study as well. Isolate 2 and 8 were selected because their carbohydrate concentration and flocculation ability were similar to the rest of the 55 isolates as seen in Figures 2.11 and 2.12; they

may thus accurately represent the population of bacteria screened. Isolate 34 was selected because it produced a large amount of carbohydrate (2076.87 μg/mL) but it had a low flocculation ability.

Polysaccharides are complex molecules with many physio-chemical properties. Simple substituents such as hydroxyl, hydrogen, carboxyl groups, sulphate, phosphoryl, and more complex groups such as carboxylate groups which offer binding sites for cations and anions may be present in these bacterial polysaccharides (Sutherland, 1994). Cations such as Na^{+}, K^{+}, Ca^{++}, and Mg^{++} have been shown to bind to polysaccharides such as the Gellan molecule and induce rigid gels (Huang et al, 2004). In order for carbohydrate flocculation to occur in a kaolin clay suspension, a cation must be introduced into the system, similar to the requirement for gelation of certain polysaccharides. Gellan gum, Xanthan gum, chitosan, agarose, and guar gum are different polysaccharides and each has different combinations of functional groups and in different quantities. If the flocculation of these polysaccharides were tested, it may be high as these polysaccharides do form gels. Thus, the flocculation ability of Xanthan and Gellan gum was examined at 250 and 500 μg/mL concentrations (Figure 2.13). At low concentrations Xanthan and Gellan gum had very high flocculation abilities which supported our hypothesis that these known gelling agents should have high flocculation abilities.

Polysaccharides from different pulp and paper mill bacterial isolates may have varying compositions and thus might display different flocculation abilities as shown in our study. Using the carbohydrate quantity and flocculation data, isolates 1, 2, 8 and 34

were selected for further study as they may be producers of a novel encapsulation material.

2.5.0 Figures

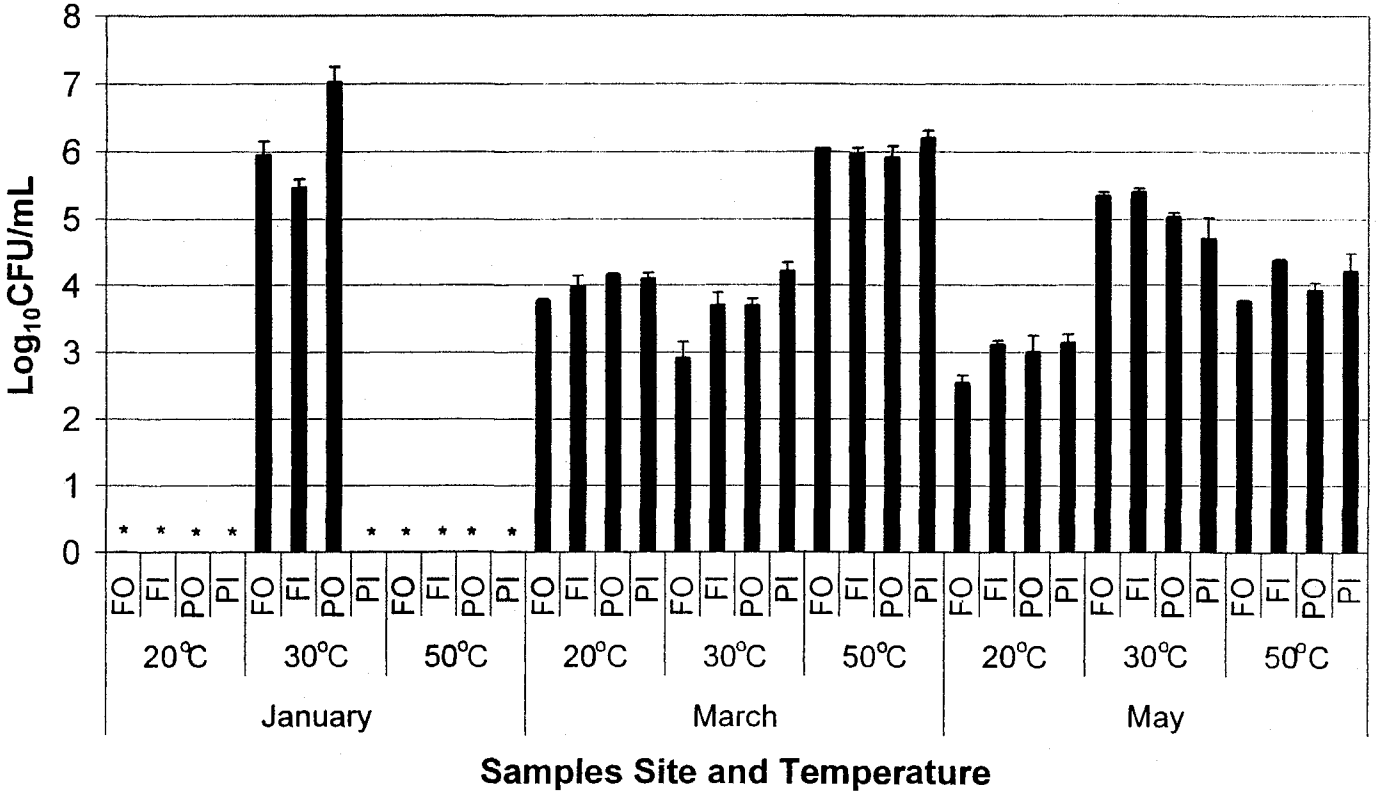

Figure 2.1: Average Log_{10} CFU/mL of aerobic colonies found at each sample site and temperatures for sampling months of January, March, and May 2006.
FO = Former Outside, FI = Former Inside, PO = Press Outside, PI = Press Inside
*For January samples, plates incubated at 20°C and 50°C had <100 CFU/mL at most locations.

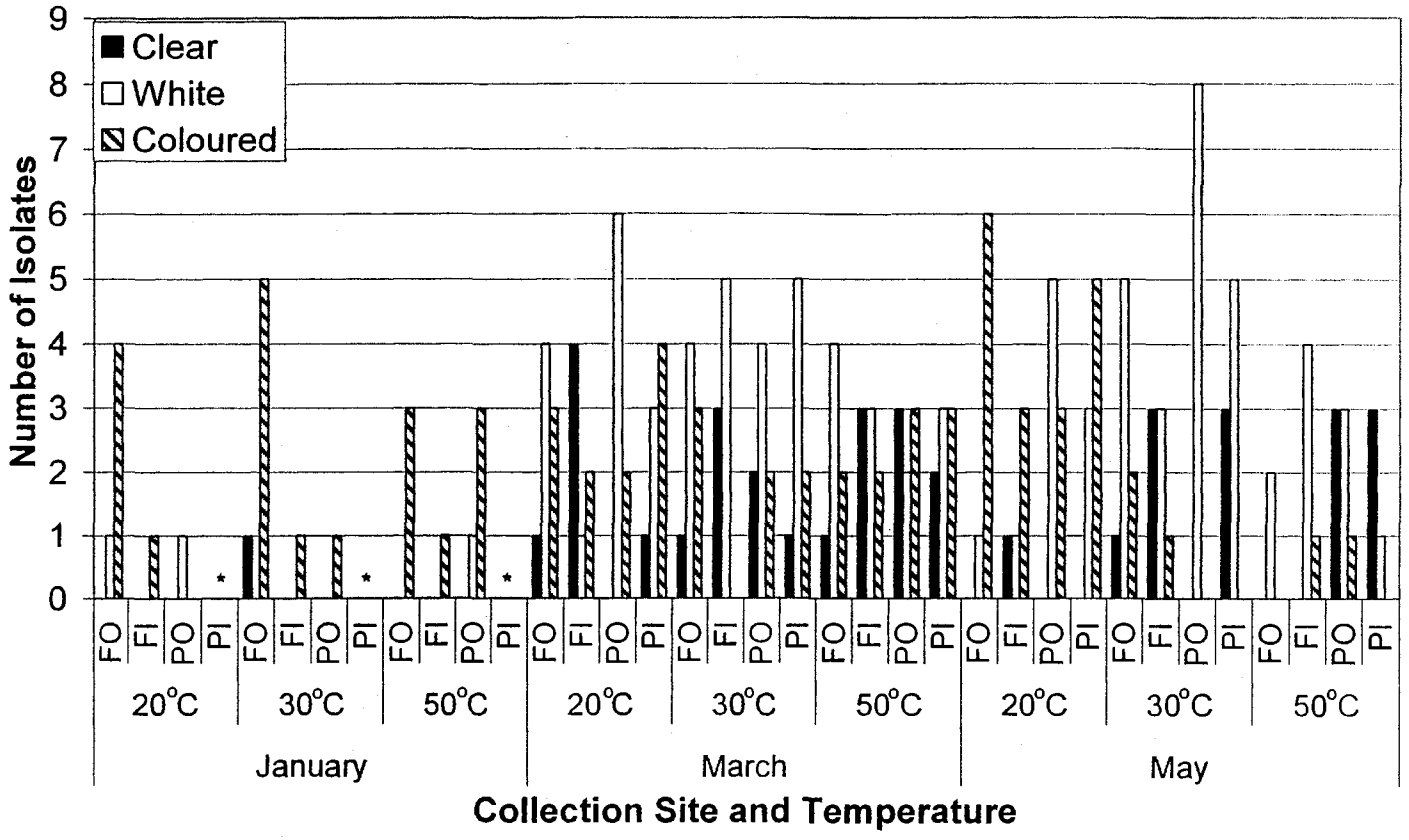

Figure 2.2: Colony diversity of the 194 isolates collected indicate that fewer white colonies were found at 50°C samples sites while 20°C and 30°C samples sites offered more coloured and clear isolate colonies.
FO = Former Outside, FI = Former Inside, PO = Press Outside, PI = Press Inside
*For January, samples collected at the PI site did not yield any isolates.

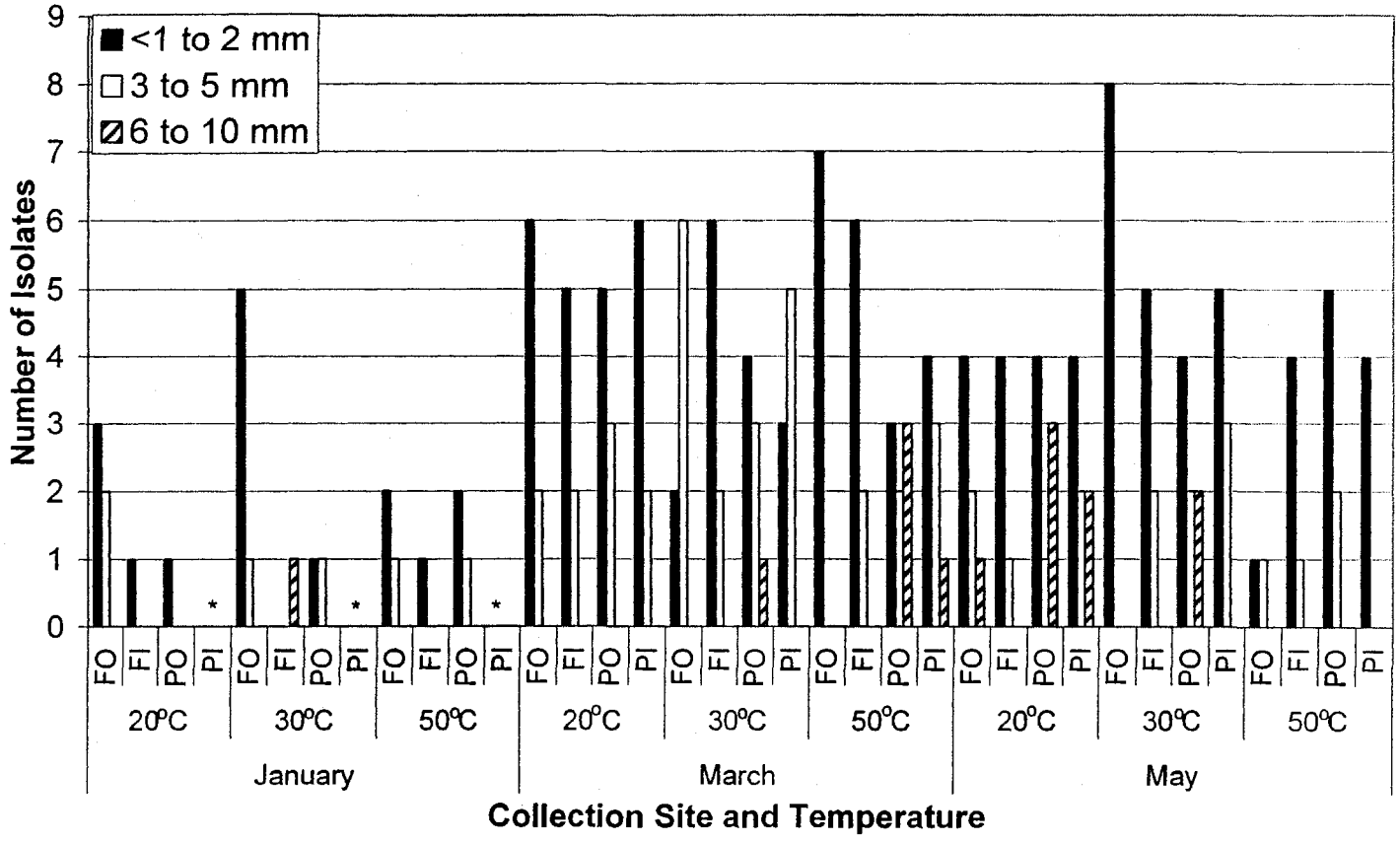

Figure 2.3: Colony diversity based on size of the 194 isolates indicates that many of the isolate colonies were <1 to 2mm in diameter and few were 3 to 5mm in diameter. Very few isolates were greater than 5mm in diameter.
FO = Former Outside, FI = Former Inside, PO = Press Outside, PI = Press Inside
*For January, samples collected at the PI site did not yield any isolates.

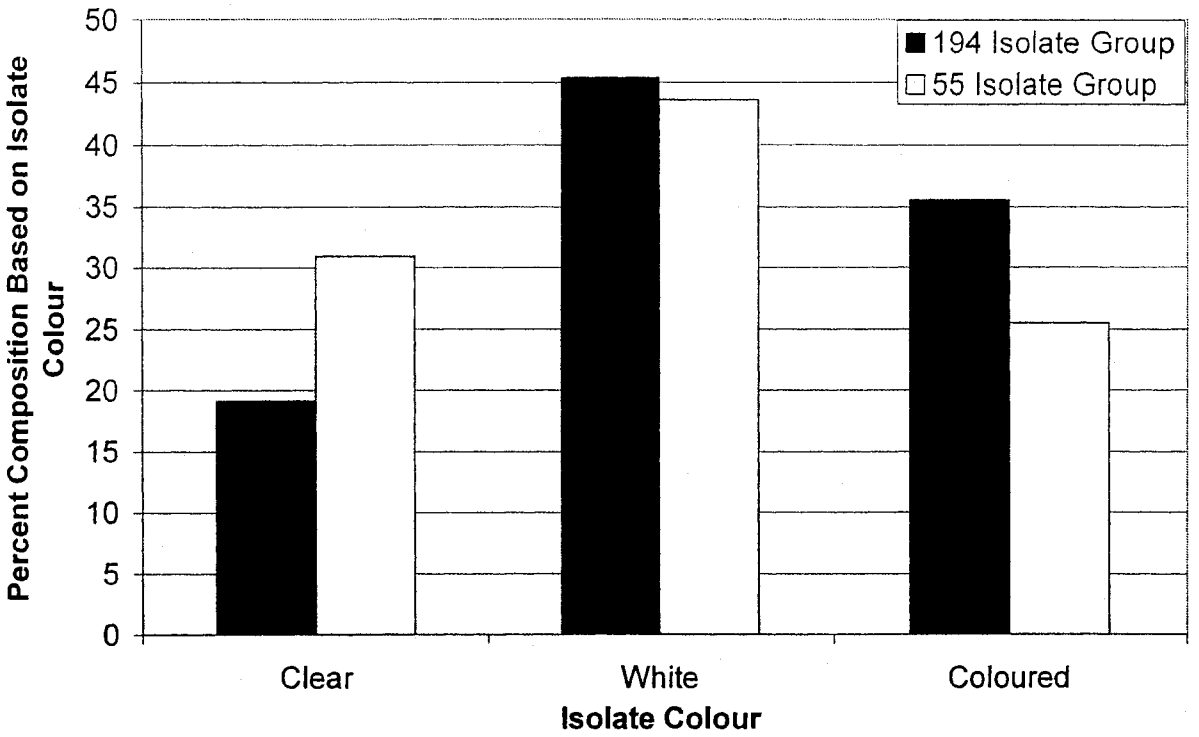

Figure 2.4: A majority of the 55 different isolates screened were white. A similar distribution of colours was selected from the 194 isolate group for further screening.

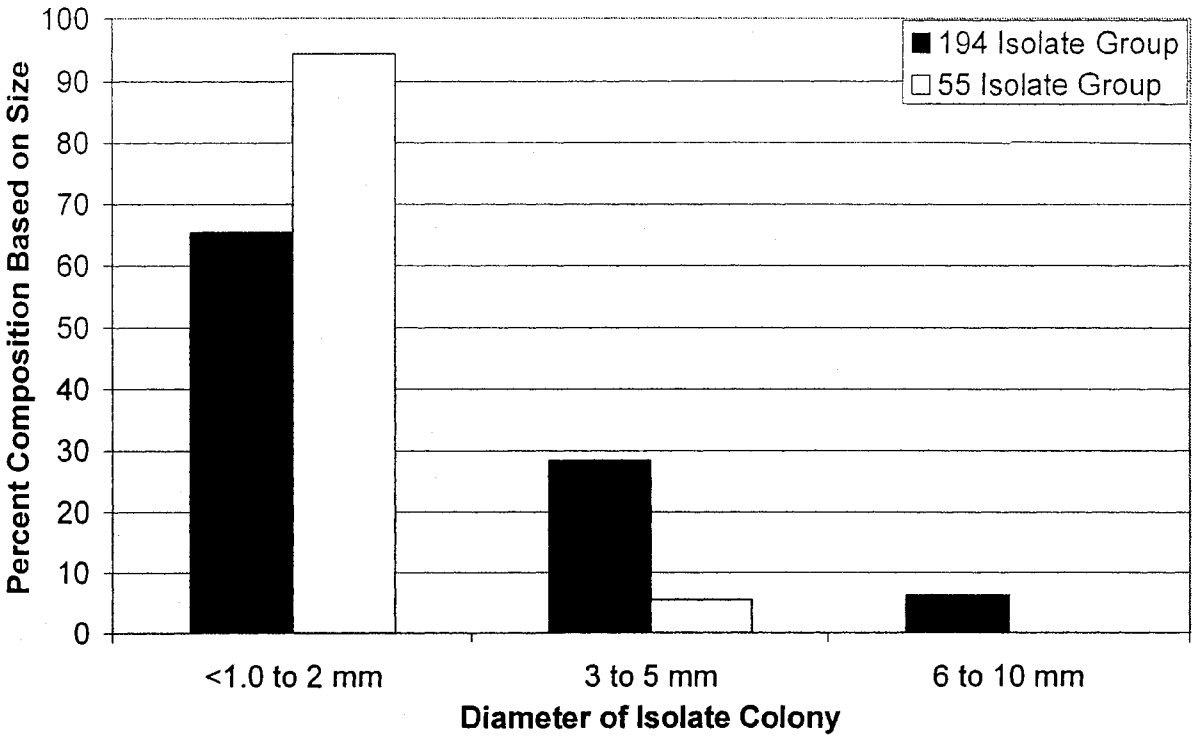

Figure 2.5: Based on colony size, the composition of the 55 isolates screened were mainly <1.0mm in diameter which may not have fully represented the 194 isolate group.

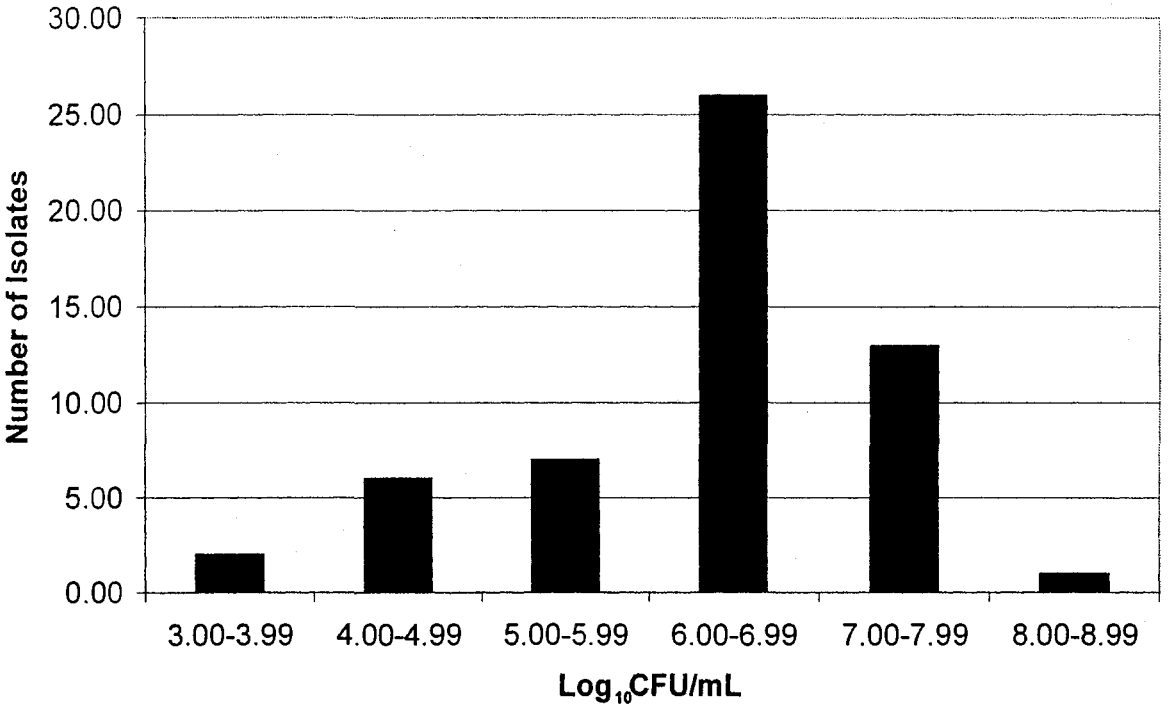

Figure 2.6: Inocula for the 55 isolates varied when cell density was adjusted to an OD_{600}= 0.06. This value was selected because it was expected to correspond to approximately 10^7 CFU/mL; however, this was not always the case.

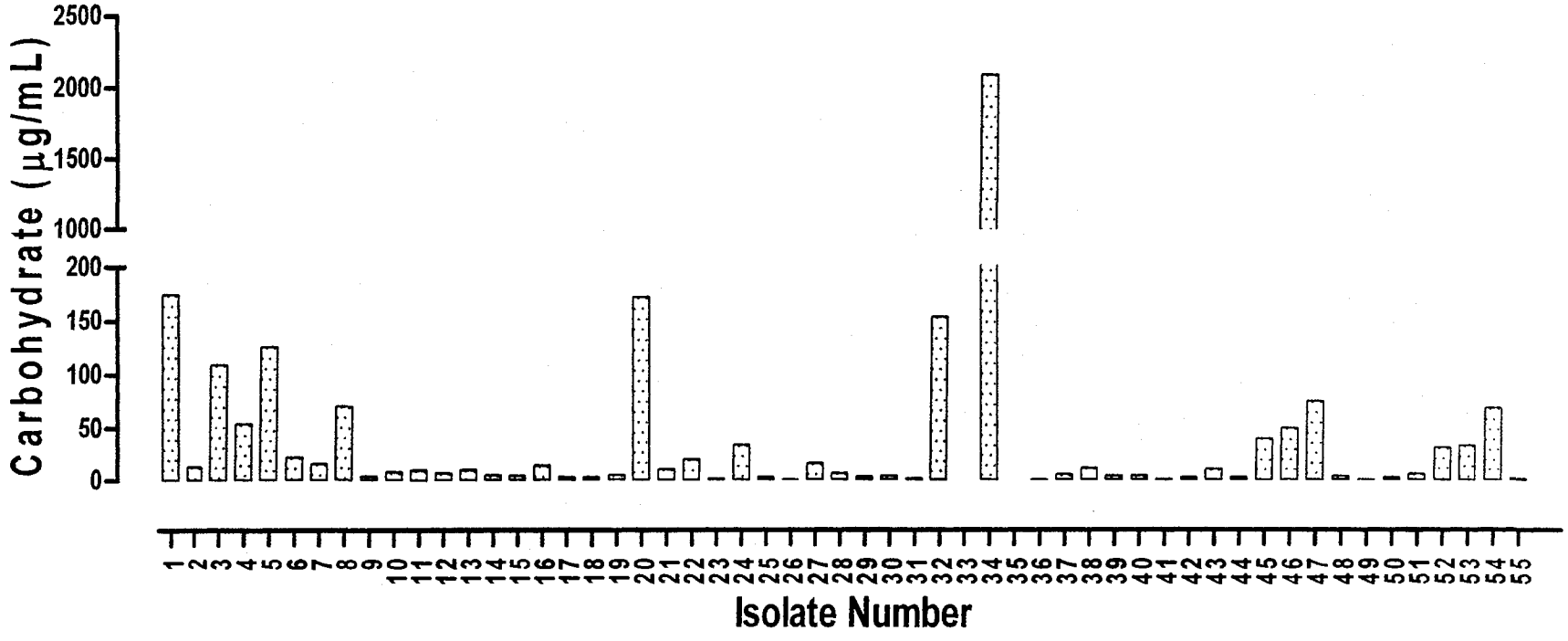

Figure 2.7: Initial paper mill isolate carbohydrate quantities as determined by Phenol/Sulfuric Acid Assay used to select particular isolates for gelation experiments.

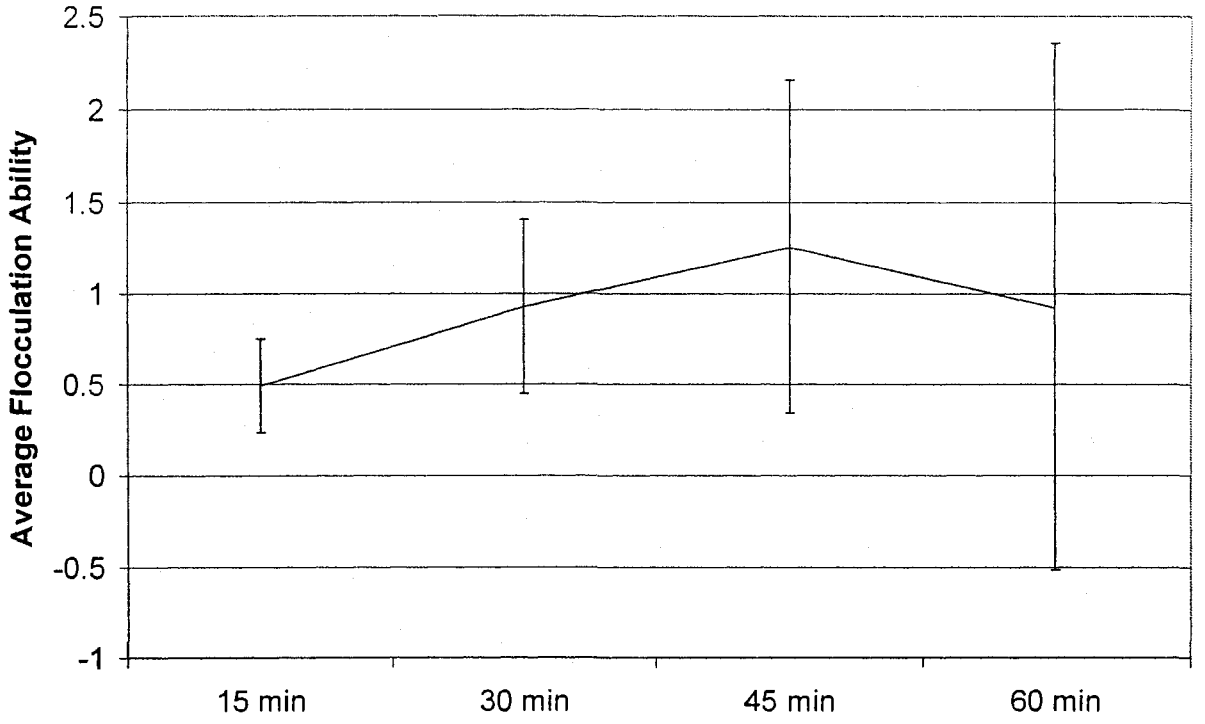

Figure 2.8: Total average flocculation ability of all 55 isolate carbohydrates increased until 45 minutes and then flocculation began to decrease.

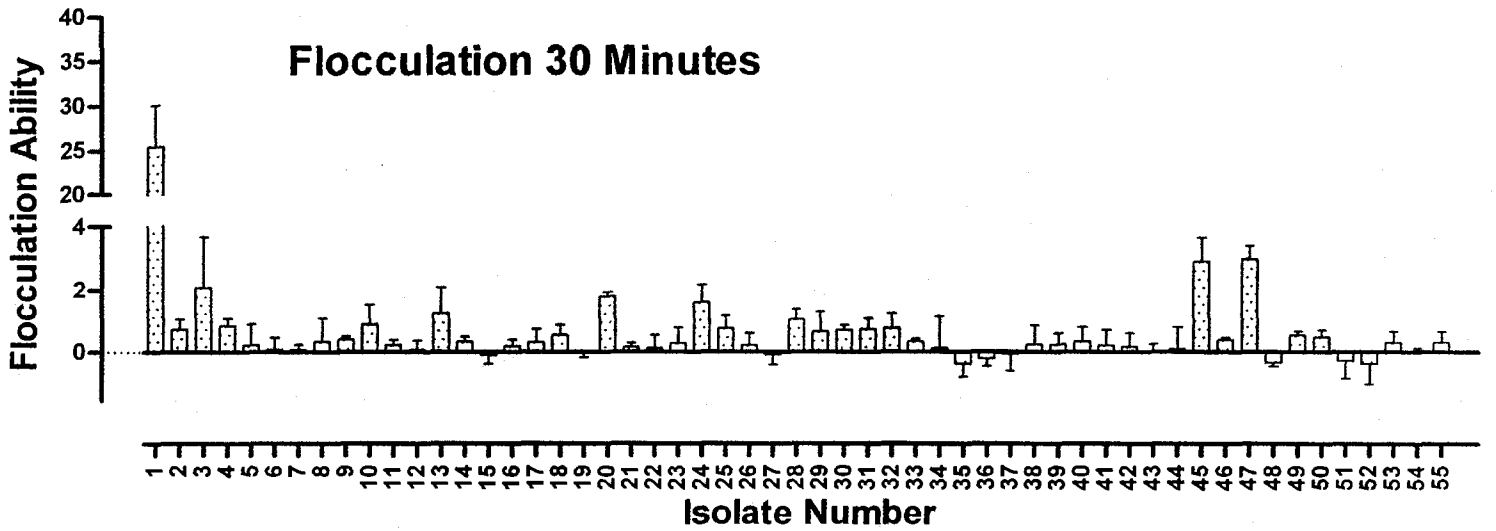

Figure 2.9: Initial paper mill isolate carbohydrate flocculation at 30 minutes as determined by Kaolin Clay Flocculation Assay.

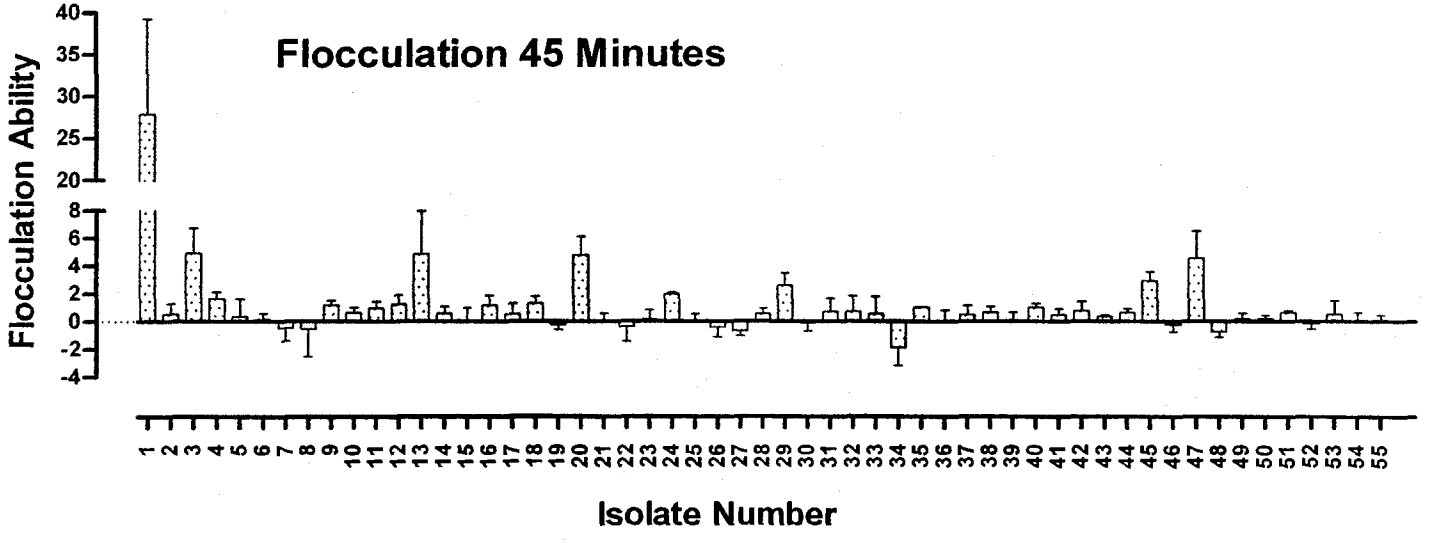

Figure 2.10: Initial paper mill isolate carbohydrate flocculation at 45 minutes as determined by Kaolin Clay Flocculation Assay.

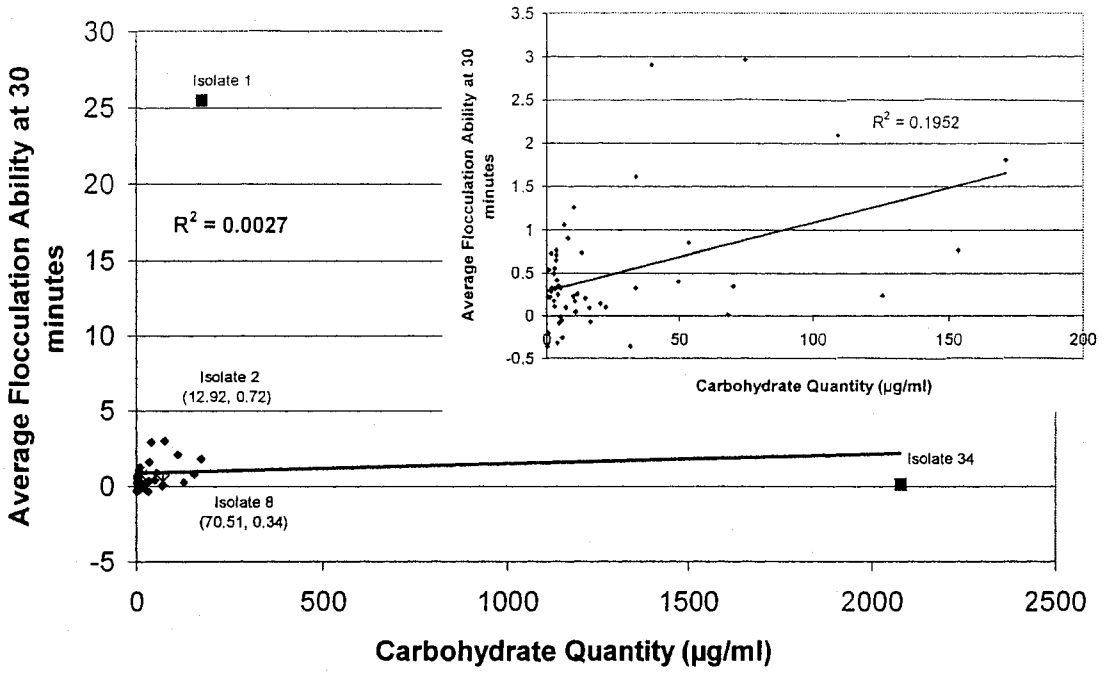

Figure 2.11: Average flocculation ability at 30 minutes of isolate carbohydrate versus isolate carbohydrate quantity displayed no correlation when plotted against one another indicating that carbohydrate quantity may not influence flocculation ability at 30 minutes. Insert Graph: No correlation was observed when outlier isolates 1 and 34 were removed.

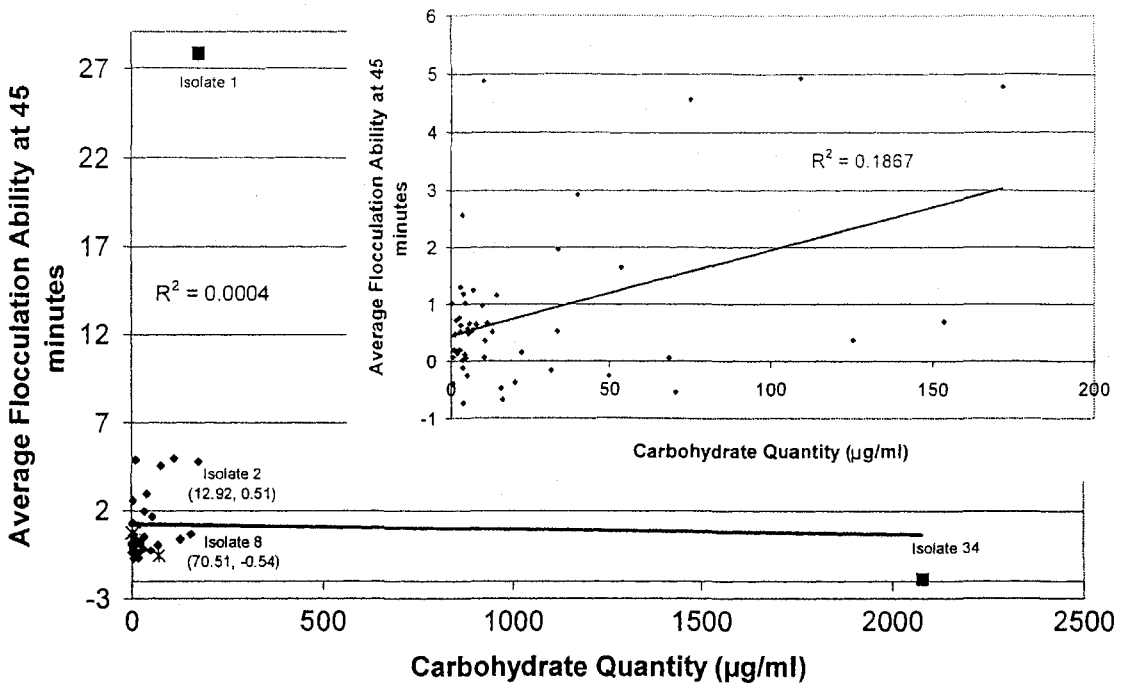

Figure 2.12: Average flocculation ability at 45 minutes of isolate carbohydrate versus isolate carbohydrate quantity displayed no significant correlation when plotted against one another indicating that carbohydrate quantity may not influence flocculation ability at 45 minutes.

Insert Graph: No correlation was observed when outlier isolates 1 and 34 were removed.

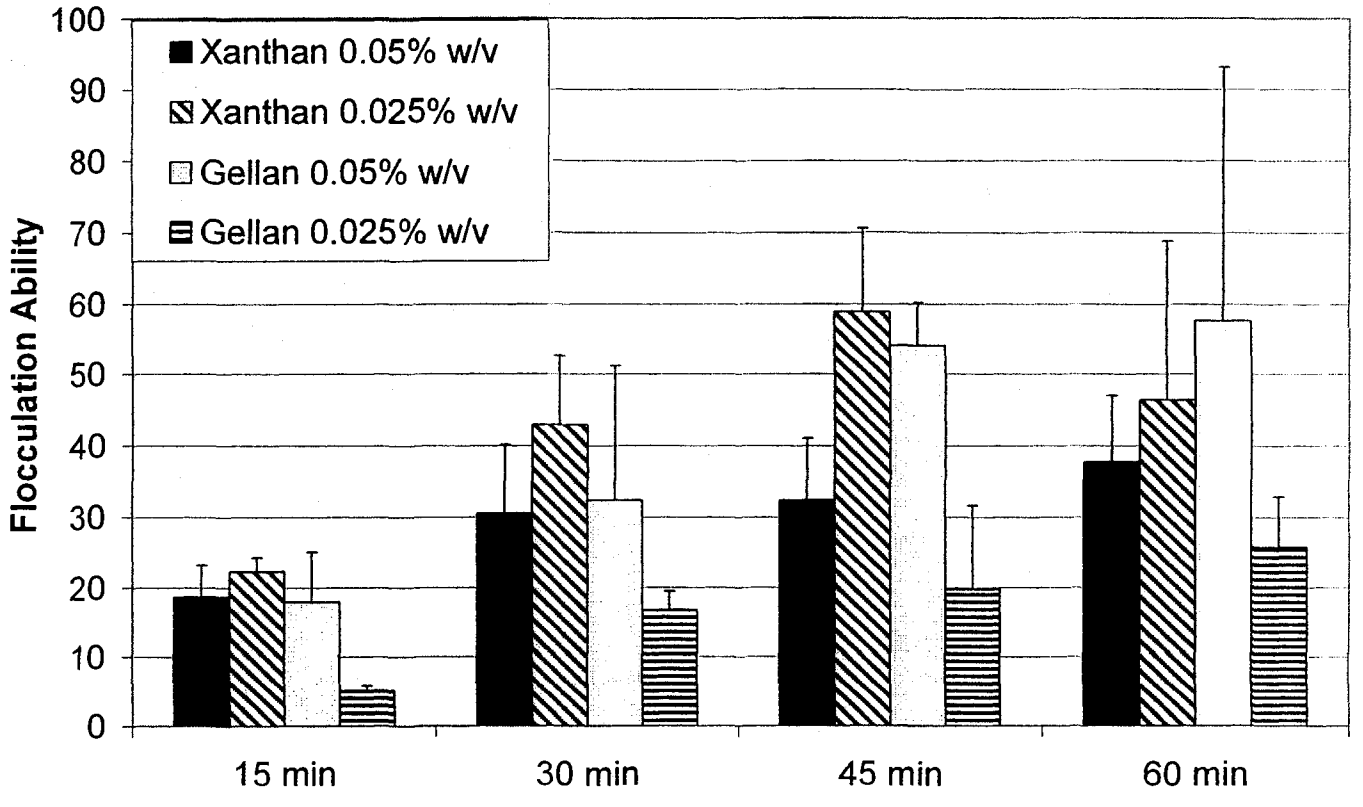

Figure 2.13: Commercially available Xanthan and Gellan gums were tested for their ability to flocculate in Kaolin at 250 and 500 μg/mL concentrations. Both polysaccharides had high flocculation abilities. Xanthan had higher flocculation ability at 250 μg/mL concentrations. Gellan had higher flocculation abilities at 500 μg/mL concentrations.

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Chapter 3: Identification of Carbohydrate Producing Bacteria and Harvesting of EPS

3.0.0 Introduction

Biofilms produced by bacteria are formed in many different environments. Paper machines can offer optimal growth conditions, nutrients, temperatures, and pH for bacteria which can in turn produce biofilms (Desjardin and Beaulieu, 2003 & Ratto et al., 2005). Extracellular polymeric substances (EPS) are the main structural component in a biofilm and aid in the adhesion of bacteria to surfaces (Liu and Fang, 2002). Non-covalent interactions such as London (dispersion) forces, electrostatic interactions and hydrogen bonds all contribute to EPS adhesion forces (Flemming et al., 1998).

Several studies regarding bacterial composition and polysaccharide composition have been conducted using paper mill bacteria because these environments offer optimum growth conditions for specific species of organisms. The operating temperatures of machines ranging from 30-50°C, white water pH 5-8, and the presence of plenty of nutrients create a stable growth region for bacteria (Desjardins and Beaulieu, 2003, Lahtinen et al., 2006, Lindberg et al., 2001, Verhoef et al., 2005).

Identifying bacteria located in paper mills accurately and with confidence has improved with the use of 16S ribosomal DNA sequencing. This portion of the ribosomal subunit is highly conserved between species and can be used to accurately identify prokaryotic organisms (Koneman, 1997). Many studies have successfully identified

aerobic and anaerobic paper mill, sludge, and waste water bacteria using 16S ribosomal sequencing (Yu and Mohn, 2001, Desjardins and Beaulieu, 2003, Chandra et al., 2007, & Roest et al., 2005).

Species diversity is high in mixed culture biofilms formed on pulp and paper machines. Some aerobic and anaerobic species identified in paper mills using 16S ribosomal DNA sequencing include; *Bacillus, Brevundimonas, Cytophaga, Enterobacter, Klebsiella, Paenibacillus, Pseudomonas, Pseudoxanthomanas, and Flavobacters* (Ratto et al, 2005, Desjardin and Beaulieu, 2003). Many of these bacteria are commonly found environmental species and can be opportunistic pathogens to many organisms.

Our study investigated the bacteria found in pulp and paper mill machines in hopes of discovering a novel encapsulation material which can be produced from EPS. As a result of screening described in Chapter 2, four likely isolate candidates were selected for 16S ribosomal DNA identification and carbohydrate analysis.

3.1.0 Methods and materials

3.1.1 Preparation of Pure Culture

Pure cultures of isolates 1, 2, 8 and 34 were inoculated onto R2A agar which was incubated at 30°C for 24 hours. Following this initial incubation period, a single colony of each species was inoculated into 5 mL of R2A broth and incubated at 30°C for 24 hours while shaking at 30 rpm. Pure bacterial isolates (0.8 mL) were mixed with 0.8 mL of 50% w/v Fisherbrand glycerol and stored at -80°C for later use. For each molecular

experiment, biochemical experiment or EPS batching experiment, a new freezer culture was used.

3.1.2 Chromosome Extraction

Using the procedure for isolating Gram-negative genomic DNA outlined in the Promega Wizard Genomic DNA Purification Kit, genomic DNA was extracted from bacterial isolates 1, 2, 8 and 34. Following this procedure, DNA extracts were electrophoresed in tris-borate buffer in a 1.0% w/v agarose gel, containing ethidium bromide (10 mg/mL) used to fluoresce the DNA. Fermentas Lambda HindIII molecular ladder (5μg/μL) was used as a standard. Gels were visualized with UV light using Syngene Chemi Genius Bio Imaging System illuminator and Genesnap© version 7.01 software.

3.1.3 Primer Sets 1.5 kb and 600 bp

Once chromosome extraction was successful and verified by electrophoresis, 16s rDNA was amplified using polymerase chain reaction. Two DNA fragment sizes were desired, a larger 1.5 kb fragment and a smaller 600 base pair fragment as seen in Figure 3.1. The primers 1492-R and 27-F were used for the 1.5 kb reaction as they would yield the maximum amount of sequence information for analysis, however larger fragments are harder to amplify because yields are lower (Trevors and van Elsas, 1995). The 16s universal primers 907–R and 341-F were used for the 600 bp reaction (Muyze et al.,

1998). The two reactions were used in order to completely sequence the 1.5kb DNA fragment as using the 1.5 kb reaction alone might not produce the best quality of data for sequence analysis. Including the 600 bp sequence produced by 16s universal primers 907-R and 341-F for sequencing created a more robust 1.5kb DNA sequence (Figure 3.1).

3.1.4 Calculating Annealing Temperatures for Primers (Farell, 2005)

Using the equation,

$T_{anneal} = 4$ (Guanine + Cyosine) + 2 (Adenine + Thymine) **(Equation 3.1)**

annealing temperature for 1492-R was calculated as 64°C and for 27-F, it was 62°C. To find the Total Annealing Temperature (T_{an}) for the two primers Equation 3.2 was used.

T_{an} $[(T_{anneal}\ 1 + T_{anneal}\ 2)/2] - 5$ **(Equation 3.2)**

Where,

T_{anneal} 1 represents the temperature calculated for primer 1

T_{anneal} 2 represents temperature calculated for primer 2

The Total Annealing Temperature for 1.5kb primer set was calculated to be T_{an} (64°C + 62°C /2) – 5 = 58°C.

Using Equation 3.1 annealing temperature for 16s 907-R was calculated as 56°C and 16s 341-F, it was 58°C. To find the Total Annealing Temperature (T_{an}) for the two primers Equation 3.2 was used. The Total Annealing Temperature for 600bp primer set was calculated to be T_{an} (56°C + 58°C /2) – 5 = 52°C.

3.1.5 Polymerase Chain Reaction Amplification

Chromosomal DNA that was highly concentrated after extraction, was diluted 10x in sterile ddH_2O. PCR reactions were performed in a total volume of 50 µl containing 1x PCR buffer without $MgCl_2$, 2.5mM $MgCl_2$, 1 µM of each PCR primer, 0.2mM dNTPs, 1U Taq DNA polymerase and 1 µl of isolate genomic DNA. PCR reagents were purchased from Fermentas, Burlington, Ontario. PCR conditions were set as follows, after initial denaturation for 5 minutes at 94°C, 30 cycles of amplification consisting of denaturation for 1.5 minutes at 94°C, annealing for 1 minute at 55°C, and extension for 1.5 minutes at 72°C were performed. The procedure was then followed by a final extension for 10 minutes at 72°C and then a holding temperature of 4°C completed the program. Following amplification, 5 uL of PCR products were electrophoresed in a 0.8% agarose gel containing ethidium bromide (10mg/mL) and eletrophoresed at 120 volts for 30 minutes. Fermentas GeneRuler™ 1kb DNA Ladder Plus (0.5µg/µl) was used as a standard. Gels were visualized with UV light using Syngene Chemi Genius Bio Imaging System illuminator and Genesnap© version 7.01 software.

3.1.6 Purification of PCR Products

To purify PCR products, 45 μL of product was electrophoresed in a 1.0% w/v agarose gel containing ethidium bromide (10mg/mL). Fermentas GeneRuler™ 1kb DNA Ladder Plus molecular ladder (0.5μg/μl) was used as a standard. The products were run at 110 volts for 40 minutes to ensure greater separation of bands. Once separated, DNA bands of the desired size were manually cut from the agarose gel and purified using Wizard SV Gel and PCR Clean-Up System. The concentration of DNA was measured using a UV spectrophotometer by comparing the ratios of absorbance at OD_{260}:OD_{280} of the purified product (Sambrook & Russell, 2001). Purified DNA was stored at -20°C until shipped for sequencing by Mobix Laboratory at McMaster University.

3.1.7 Sequence Analysis

Once complete, the DNA sequences for both the 1.5kb fragment and 600bp fragment were combined using DNAMan for Windows (Lynnon Corporation, Quebec, Canada). The complete sequence of the 1.5kb fragment was matched using the Basic Local Alignment Search Tool (BLAST) available online at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

3.1.8 Biochemical Tests

All isolates were characterized with Gram stain, oxidase and catalase tests. Glucose fermentation and ONGP tests were conducted on isolate 1 to further aid in identification while nitrogen gas production, hydrogen sulfide production, ornithine decarboxylase, and urea hydrolysis were conducted to identify isolate 8. Overnight cultures taken from R2A agar were used for all biochemical testing.

3.1.9 Carbohydrate Lyophilization

Isolates 1, 2, 8, 34 were selected for further analysis and carbohydrate lyophilization. Isolate 1 was selected because it had high carbohydrate production and high flocculation, isolates 2 and 8 because they had similar carbohydrate quantities and flocculation abilities to the entire 55 isolate group, and isolate 34 was selected because it produced a large amount of carbohydrate. Several batches of each isolate culture was grown and prepared in order to produce enough carbohydrate for gelation tests. Isolates were inoculated onto R2A media and incubated overnight at 30°C. A single colony was then inoculated into 500 mL of R2A broth and incubated for 24 hours at 30°C while rotated at 30 rpm. Carbohydrate was prepared as described in Chapter 2: Sections 2.2.4 and 2.2.5. Initial inocula were adjusted to OD_{600} = 0.06 using a Biochrom Nova Spec Visible Spectrophotometer. Some carbohydrate batches were mechanically filtered using a Fisherbrand 0.22 μm plastic disposable filter and BD Brand Disposable Syringe to remove cells, while other batches were not filtered (Ishida et al., 2003, Hernandez-Mena

and Friend, 1993). For each batch of carbohydrate preparation, 1 mL of sample was put aside and assayed using the Phenol-Sulfuric Acid Assay to confirm carbohydrate quantity. The remaining 9 mL of carbohydrate was then placed into two 10 milliliter glass bottles and sealed with a plastic stopper and placed in a -80°C freezer over night before lyophilization to ensure homogeneous freezing of the carbohydrate. Substances to be lyophilized must be frozen to eutectic temperature before proper freeze-drying can occur. Frozen samples were placed in Labconco Freezezone 12 Vacuum Freeze Drying System set to a temperature of -30°C. Lyophilization occurred over a 3-5 day period until samples were dry. Lyophilized samples were stored in a dessicator at 4°C to prevent re-hydration due to atmospheric moisture.

3.2.0 Results and Discussion

Studies have been conducted in Europe to characterize the types of bacteria found on pulp and paper machines (Lahtinen et al, 2006 & Linberg et al, 2001). However, to our knowledge, only two previous studies (Desjardins and Beaulieu, 2003, Evenleigh and Brewer, 1964) have attempted to characterize aerobic bacteria on Canadian pulp and paper machines. It has also been established that bacterial species isolated are highly dependant on the paper machine environment (Harju-Jeanty and Vaatanen, 1984, Hughes-van, 1988, Oppong et al, 2000, Vaisanen et al, 1998). Many different genera, such as *Bacillus, Brevundimonas, Cytophaga, Enterobacter, Klebsiella, Paenibacillus, Pseudomonas, Pseudoxanthomanas, and Flavobacters* have been isolated from pulp and paper machines around the world (Ratto et al, 2005, Desjardin and Beaulieu, 2003). This

study isolated bacteria from a Canadian pulp and paper mill in Thunder Bay, Ontario and identified four isolates of interest with respect to production of EPS.

3.2.1 Identification of Isolates 1, 2, 8 & 34 using Molecular Techniques and Biochemical Tests

As seen in Figure 3.2, DNA bands were visible at the 1500bp marker region using primers 27-F and 1492-R for all four isolates. In addition, bands were visible at the 600bp region after gel electrophoreses verification of PCR products obtained with primers 341-F and 907-R was conducted. Once sequences were obtained and combined, a nucleotide analysis was conducted using the Basic Local Alignment Search Tool (BLAST). Total results for all isolate sequences are outlined in Table 3.2. The maximum identity for Isolate 1 was *Epilithonimonas tenax* (98% identification), isolate 2 as *Pseudomonas* sp. (99% identification), isolate 8 as Bacterium N25 (99% identity), and isolate 34 as *Sphingomonas* sp. (99% identity) based on a maximum identity score calculated by the BLAST search engine that incorporates the percent of the query length that is included in the aligned sequences. Only organisms with at least 96% maximum identification were considered and the five most similar identifications arranged in decreasing maximum identity scores are listed in Table 3.2.

In addition to molecular techniques, biochemical tests were conducted on each isolate. Following an incubation period for each isolate on R2A media, Gram staining, oxidase, and catalase tests were conducted on fresh isolates. The biochemical test results for isolates 1, 2, 8, and 34 are listed in Table 3.3 and reported biochemical reactions for

the closest related genera of bacteria are listed in Table 3.4. All isolates were Gram negative. Isolate 1 was oxidase positive, catalase positive, ONPG negative, and did not produce acid from glucose utilization. Isolate 2 was oxidase positive and catalase positive. Isolate 8 was oxidase negative, catalase negative, did not produce N_2 gas, did not produce hydrogen sulfide, and did not hydrolyze ONPG or urea. Isolate 34 was oxidase positive and catalase negative (Table 3.3).

Isolate 1 was identified as *Epilithonimonas tenax* (98%), *Flavobacteriaceae bacteria* (98% identification), or *Chryseobacterium lactis* (97% identification). *Epilithonimonas tenax* is a newly described bacterium isolated from river water that is found in the Flavobacteriaceae family. However, very little information has been published about it (O'Sullivan et al., 2006). The Chryseobacteria are a recent addition to the Flavobacteria genus as Vanamme et al. (1994) reported that some of the Flavobacteria were not related to *F. aquatile* and should be a separate grouping. Generally *Chryseobacterium* spp. are ONPG positive and do produce acid from glucose utilization while *Flavobacteria* spp. are ONPG negative and do not produce acid from glucose nor was a colony bright orange. *Epilithonimonas tenax* has been described to be cytochrome oxidase positive, catalase positive and bright orange. Acid is not produced from glucose (O'Sullivan et al., 2006). Both *Epilithonimonas tenax* and *Chryseobacterium lactis* can be found in the Flavobacteriaceae family so that may be why these species were suggested by the 16s rDNA sequences. Using these biochemical tests along with 16s rDNA results, isolate 1 can be successfully identified as a *Flavobacterium* sp.

Isolate 2 was identified as a *Pseudomonas* sp. clone (99%) and *Pseudomonas mendocina* (99%) using 16s rDNA sequencing results (Table 3.2). The oxidase positive

and catalase positive results (Table 3.3) along with the molecular identification support the identification that isolate 2 is a *Pseudomonas* spp.

Isolate 8 was identified as Bacterium N25 (99%), *Mangroveibacter plantisponsor* (99%), *Salmonella enterica* (96%), or *Enterobacter* sp. (96%). A sequence of 96% or less similarity may not be high enough to be considered a match for species identification. Table 3.3 indicates that isolate 8 was oxidase negative, catalase negative, did not produce hydrogen sulfide, did not produce nitrogen gas, did not decarboxylate ornithine, nor hydrolyze urea. Bacterium N25 was described by a Chinese group (Zhang et al., Diversity of bacteria isolated from mangrove system, Feb 2007, unpublished); however, no other data have been published on the bacterium aside from its identity (NCBI, 2008). Very few data were found on *Mangroveibacter plantisponsor*, however the species has been placed in the order Enterobacteriales (NCBI, 2008). Not producing H_2S also rules out *Salmonella enterica* as this species produces hydrogen sulfide (Table 3.4). *Enterobacter* spp. are oxidase negative, catalase positive, do produce nitrogen gas, do decarboxylate ornithine, and do hydrolyze urea. Isolate 8 was found to be catalase negative, did not produce nitrogen gas, and did not decarboxylate ornithine or hydrolyze urea. None of the biochemical tests support the 16s rDNA results and identification of isolate 8 is inconclusive based on our data. However *Mangroveibacter plantisponsor*, *Salmonella enterica*, *Enterobacter* sp., are all found in the same order of Enterobacteriales which may explain the variance between the 16s rDNA results.

Isolate 34 was identified as *Sphingomonas* sp. ATCC 53159 (99%) and *Sphingomonas elodea* (99%) (Table 3.2). The isolate was oxidase positive and catalase negative. Generally *Sphingomonas* spp. are catalase positive. This discrepancy may be

explained by the fact that species name was not identified and not every species found in a particular genus or species may abide by biochemical classification. There is also the possibility that isolate 34 was weakly catalase positive, although it was not observed macroscopically when the test was done. Isolate 34 was previously identified using 16s rDNA as a *Sphingomonas* sp. (data not shown) and this identification was confirmed in this study.

The isolates reflect the types of bacteria found in other pulp and paper mills in Canada and around the world (Ratto et al, 2005, Desjardin and Beaulieu, 2003). Desjardins and Beaulieu (2003) reported large amounts of *Pseudomonas*, but they did not detect any Flavobacteria. *Sphingomonas* spp. have also been identified in paper mill effluent systems (Mohn and Stewart, 1997). Bacterium N25 and *Mangroveibacter plantisponsor* are two bacteria that have been isolated from environmental water areas and are placed in the same *Flavobacteriaceae* family. There is a possibility that these bacteria may have been positively identified in our study, but this could not be confirmed as very little data has been published about them.

Sphingomonads, Pseudomonads and Flavobacters are all environmental bacteria found in soils and water as Bacterium N25 and *Mangroveibacter plantisponsor* have been located in as well. Our study did positively identified Pseudomonads, Sphingomonads and reported a *Flavobacterium* spp. and an unknown isolate and these particular bacteria are regularly found in freshwater. The pulp and paper mill biofilm samples were collected from a mill located on the Kaministiquia River in Thunder Bay and it may use river water to process its materials. Thus, these bacteria may have been introduced into the mill from the river water.

3.2.2 Carbohydrate Analysis

As a result of carbohydrate screening in Chapter 2, isolates 1, 2, 8, and 34 were selected for carbohydrate harvesting. Inocula preparation for EPS lyophilization was conducted as outlined in Chapter 2 section 2.2.4 and 2.2.5. Average inocula for isolates 1, 2, 8, and 34 are outlined in Table 3.5 for unfiltered and filtered batching after cell density of the culture was adjusted to $OD_{600} = 0.06$ using Biochrom Nova Spec Visible Spectrophotometer. Cultures were adjusted to 0.06 because this value was expected to correspond to approximately 10^7 CFU/mL. Adjusting initial inocula to $OD_{600} = 0.06$ did not appear to result in 10^7 CFU/mL for each isolate (Table. 3.4). Isolates 1 and 34 had lower CFU/mL inocula than expected, while isolates 2 and 8 had expected inoculum concentration of 10^7 CFU/mL. The variance in inoculum values may be explained by clumping of bacteria during the washing process which would have an influence on the calculated CFU/mL. Also, if the bacteria are not fully separated from EPS, the optical density of the culture would be affected.

The carbohydrate from each isolate was harvested in several batches and carbohydrate yield was recorded for each batching session. The data show that isolates 1, 2, and 8 produced consistent amounts of carbohydrate with very little variance between the different batches of carbohydrate preparation (Figure 3.3). Since additional experiments would be required to determine growth conditions resulting in higher EPS yields, isolates 2 and 8 were not further investigated. Interestingly, for isolate 34 a large variation of the average amounts of carbohydrate produced was observed.

Carbohydrate quantity for the batches that were filtered and those that were not filtered were measured before lyophilization. Filtering the carbohydrate preparations was assumed to aid in the removal of any bacterial cells that remained suspended following high speed centrifugation (Ishida et al., 2003). As most carbohydrates are soluble in water, the 0.22 μm filter should have let carbohydrate pass and restrict bacteria. Although no direct comparison of the filtration effect was made, it appeared that filtering carbohydrate to remove cells prior to lyophilization may not have had an effect on the concentration of the carbohydrate for isolates 1, 2 and 8 (Figure 3.3). For isolate 34 however, the carbohydrate yield for filtered batches seemed to be higher than the yield for unfiltered batches, although the difference was not statistically significant by the variation of the inoculum used for the different batches.

When average unfiltered carbohydrate yields and inoculum concentration were compared, it was determined that a relationship between the two existed ($R^2 = 0.89$) for all isolates. A similar relationship was seen when filtered carbohydrate was plotted against inoculum concentration ($R^2 = 0.90$). As inoculum concentration decreased, carbohydrate concentration increased for all isolates whether filtered or not. This relationship may be explained by nutrient availability. As fewer cells are present in the culture, there are more nutrients for those cells to produce more EPS where increased competition for nutrients may occur with increased cells present in a culture.

The inocula for carbohydrate batching was compared using a T-test and it was concluded that there was no significant difference between the unfiltered and filtered inoculums with respect to carbohydrate production ($p = 0.38$).

The average inoculum concentration for isolate 1 was lower than isolates 2 and 8, yet higher than that of isolate 34 and it produced the second highest amount of carbohydrate (Figure 3.3). Average inocula for isolates 2 and 8 were approximately $\log_{10}$ 7.00 CFU/mL, the highest inoculum concentrations, and these bacteria produced the least amount of carbohydrate. The variance in the inoculum concentration for isolate 34 was large as seen in Table 3.5. For the unfiltered trials with isolate 34, the average inoculum was higher ($\log_{10}$ 5.81 ± 0.64 CFU/mL) than the average inoculum for filtered trials ($\log_{10}$ 5.29 ± 0.58 CFU/mL) and largest quantity of carbohydrate was recorded for the filtered trials (Table 3.4). This variance may be explained by the nature of the bacterium itself as it may have clumped when optical density was adjusted causing a miss calculation in the actual inoculum value. If inoculum concentration was reduced or increased from the expected 10^7 CFU/mL, this may have caused the increase or decrease in carbohydrate production. This might be explained by considering that isolate 34 was a slow growing bacterium. If carbohydrate was harvested before 24 hours, reduced carbohydrate concentrations would be recorded. It might also be a possibility that isolate 34 does not store well in freezer cultures at -80°C and revival might have been affected. The bacteria may have become lab adapted and less carbohydrate may have been produced.

Isolates 1, 2, 8 and 34 were selected based on screening results from Chapter 2 to be identified using 16s rDNA sequencing and to have their carbohydrate batched for gelation experiments. Isolates 1, 2, and 34 were successfully identified as a *Flavobacterium* sp., *Pseudomonas* spp., and *Sphingomonas* sp. respectively. Isolate 8 was identified as Bacterium N25 or *Mangroveibacter plantisponsor* however, true identity

could not be confirmed as molecular sequencing and biochemical tests could not be found for these bacteria. These bacteria are all found in the same order of Enterobacteriales which may explain the variance between the 16s rDNA results.

The carbohydrate from each of the isolates was harvested in several batches. Isolates 1 and 34 carbohydrates were mainly investigated as isolates 2 and 8 would require additional experiments in order to optimize growth conditions for EPS yield. Adjusting initial inocula to $OD_{600} = 0.06$ did not appear to result in 10^7 CFU/mL for each isolate. Isolates 1, 2, and 8 produced consistent amounts of carbohydrate even though inocula varied while isolate 34 had a large variance in the amount of carbohydrate it produced. A relationship was observed between inoculum concentration and carbohydrate production. As inocula concentration decreased, carbohydrate concentration increased for all isolates whether filtered or not. Isolates 1 and 34 may be successful in produced a novel encapsulation material as *Sphingomonas elodea* is currently used to produce Gellan gum. Further investigation into the gel producing ability for *Flavobacterium* sp. and *Sphingomonas sp.* will be examined in the following chapter.

3.5.0 Tables and Figures

Table 3.1: Primer Sets used for Amplification of Each Isolates 1.5 kb Sequence

Primer	Primer Sequence 5'-3'	Primer Length	Position on *E. coli* 16s rDNA	Reference
1492-R	CACGGATCCTACGGGTACCTTG TTACGACTT	31-mer	1492	(Trevors and Elsas, 1995)
27-F	GTGCTGCAGAGAGTTTGATCCT GGCTCAG	29-mer	27	
16s-907R	CCGTCAATTCCTTTGAGTTT	20-mer	907	(Muyzer et al., 1998)
16s-341F	CCTACGGGAGGCAGCAG	17-mer	341	

Table 3.2: BLAST Results for Mill Isolates

Isolate 1

BLAST Description	Max Identity
Epilithonimonas tenax strain EP105 16S ribosomal RNA gene, partial sequence	98%
Flavobacteriaceae bacterium TSBY-57 16S ribosomal RNA gene, partial sequence	98%
Flavobacteriaceae bacterium TSBY-39 16S ribosomal RNA gene, partial sequence	98%
Unidentified bacterium clone MEB004 16S ribosomal RNA gene, partial sequence	98%
Chryseobacterium lactis isolate H1 16S ribosomal RNA gene, partial sequence	97%

Isolate 2

BLAST Description	Max Identity
Uncultured *Pseudomonas* sp. clone DGG18 16S ribosomal RNA gene, partial sequence	99%
Pseudomonas mendocina ymp, complete genome	99%
Pseudomonas sp. SMCC B0259 16S ribosomal RNA gene, partial sequence	99%
Pseudomonas sp. SMCC B0310 16S ribosomal RNA gene, partial sequence	99%
Pseudomonas sp. SMCC B0280 16S ribosomal RNA gene, partial sequence	99%

Table 3.2 Continued: BLAST Results for Mill Isolates

Isolate 8

BLAST Description	Max Identity
Bacterium N25 16S ribosomal RNA gene, partial sequence	99%
Mangroveibacter plantisponsor strain MSSRF40 16S ribosomal RNA gene, partial sequence	99%
Salmonella enterica subsp. salamae strain DSM 9220 16S ribosomal RNA gene, partial sequence	96%
Enterobacter sp. mcp11b 16S ribosomal RNA gene, partial sequence	96%
Enterobacter sp. WAB1938 partial 16S rRNA gene, strain WAB1938	96%

Isolate 34

BLAST Description	Max Identity
Sphingomonas sp. ATCC 53159 16S ribosomal RNA gene, partial sequence	99%
Sphingomonas sp. ATCC 31853 16S ribosomal RNA gene, partial sequence	99%
Sphingomonas sp. ATCC 31554 16S ribosomal RNA gene, partial sequence	99%
Sphingomonas elodea 16S ribosomal RNA gene, partial sequence	99%
Sphingomonas sp. BR12199 16S ribosomal RNA gene, partial sequence	99%

Table 3.3: Biochemical Test Results of Isolate 1, 2, 8, and 34

Isolate	Oxidase	Catalase	ONPG	Acid from Glucose	H_2S	Gas Production (N_2)	Ornithine Decarboxylase	Urea Hydrolysis
1	(+)	(+)	(-)	(-)	ND	ND	ND	ND
2	(+)	(+)	ND	ND	ND	ND	ND	ND
8	(-)	(-)	ND	ND	(-)	(-)	(-)	(-)
34	(+)	(-)	ND	ND	ND	ND	ND	ND

(+) = positive result, (-) = negative result, (ND) = test Not Done

Table 3.4: Reported Biochemical Reactions for the Closest Related Genera of the Four Isolates (Koneman, 1997)

Bacterium	Oxidase	Catalase	ONPG	Acid from Glucose	H_2S	Gas Production (N_2)	Ornithine Decarboxylase	Urea Hydrolysis
Flavobacteriaceae	(+)	(+)	(-)	(-)	ND	ND	ND	ND
Chryseobacterium spp.	(+)	(+)	(+)	(+)	ND	ND	ND	ND
Pseudomonas spp.	(+)	(+)	(+/-)	(+/-)	(-)	(-)	ND	ND
Salmonella spp.	(-)	(+)	(-)	ND	(+)	(+)	(+)	(-)
Enterobacter spp.	(-)	(+)	(+)	ND	(-)	(+)	(+/-)	(+)
Sphingomonas spp.	(+)	(+)	(+/-)	(+)	(-)	ND	ND	(+)

(+) = positive result, (-) = negative result, (ND) = test Not Done

Table 3.5: Inoculum Quantities of Isolates 1, 2, 8 and 34 Used for EPS Batching

Isolate	Average Cell Counts of Inoculum (Log_{10}CFU/mL)	
	Unfiltered Carbohydrate Production	Filtered Carbohydrate Production
1	6.5 ± 0.35	6.45 ± 0.61
2	7.31 ± 0.04	7.15 ± 0.11
8	7.06 ± 0.28	7.11 ± 0.36
34	5.81 ± 0.64	5.29 ± 0.58

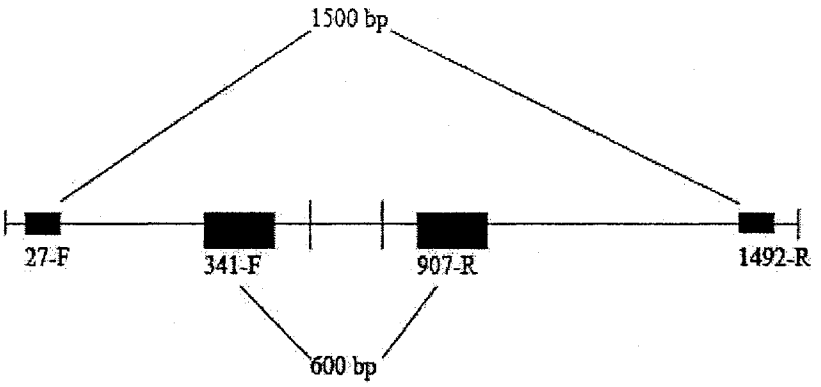

Figure 3.1: Schematic of the 16s rDNA gene from *Escherichia coil* and the position of PCR primers used in this study. Two DNA fragments were necessary in order to completely sequence the 16s rDNA 1.5 kb fragment. Using only 1492-R and 27-F primers for sequencing may result in poor sequencing data for the middle section of the 1.5kb fragment, as a standard sequencing reaction normally covers approximately 700 bp

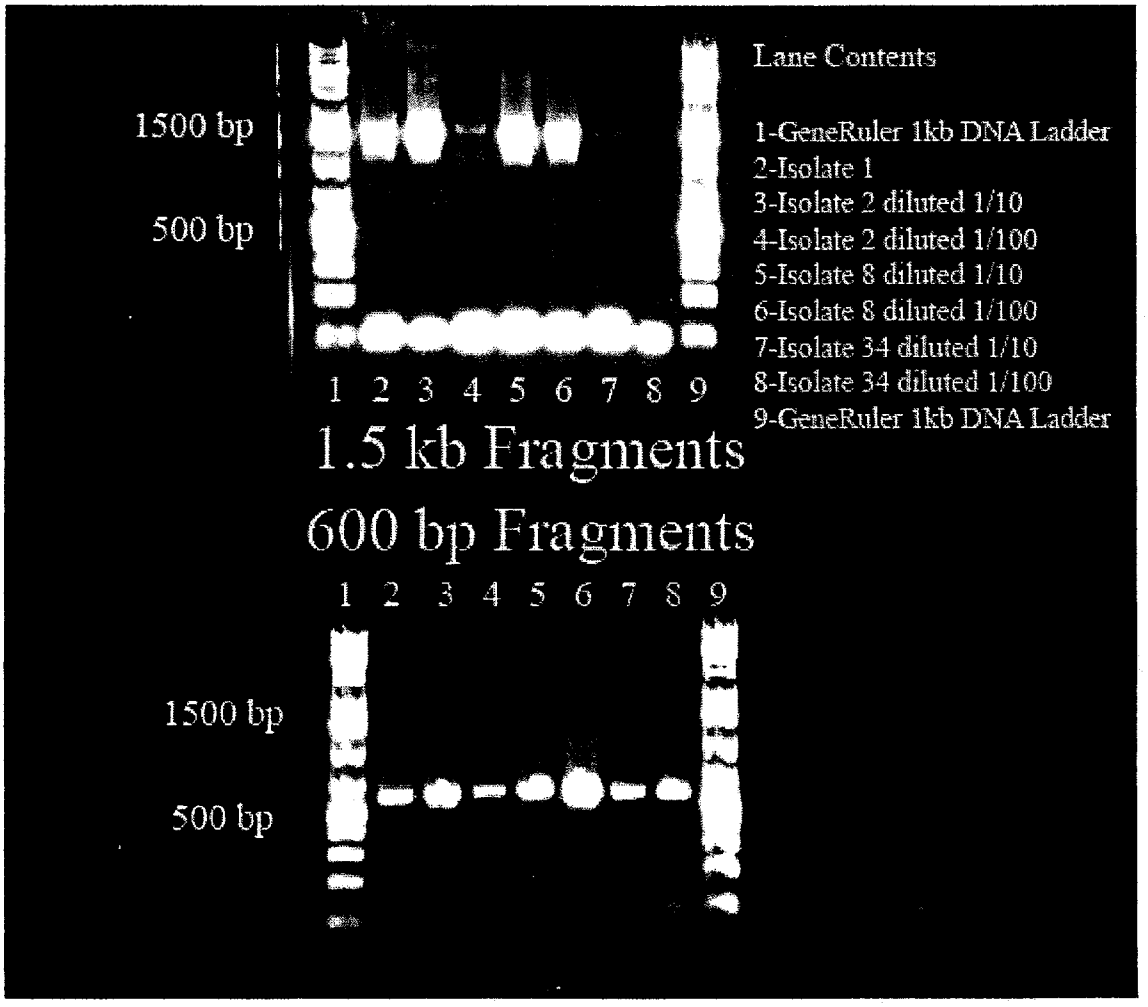

Figure 3.2: 1.5kb and 600bp Isolate PCR products obtained for isolates 1, 2, 8, and 34, eletrophoresed on agarose gels to verify amplification.

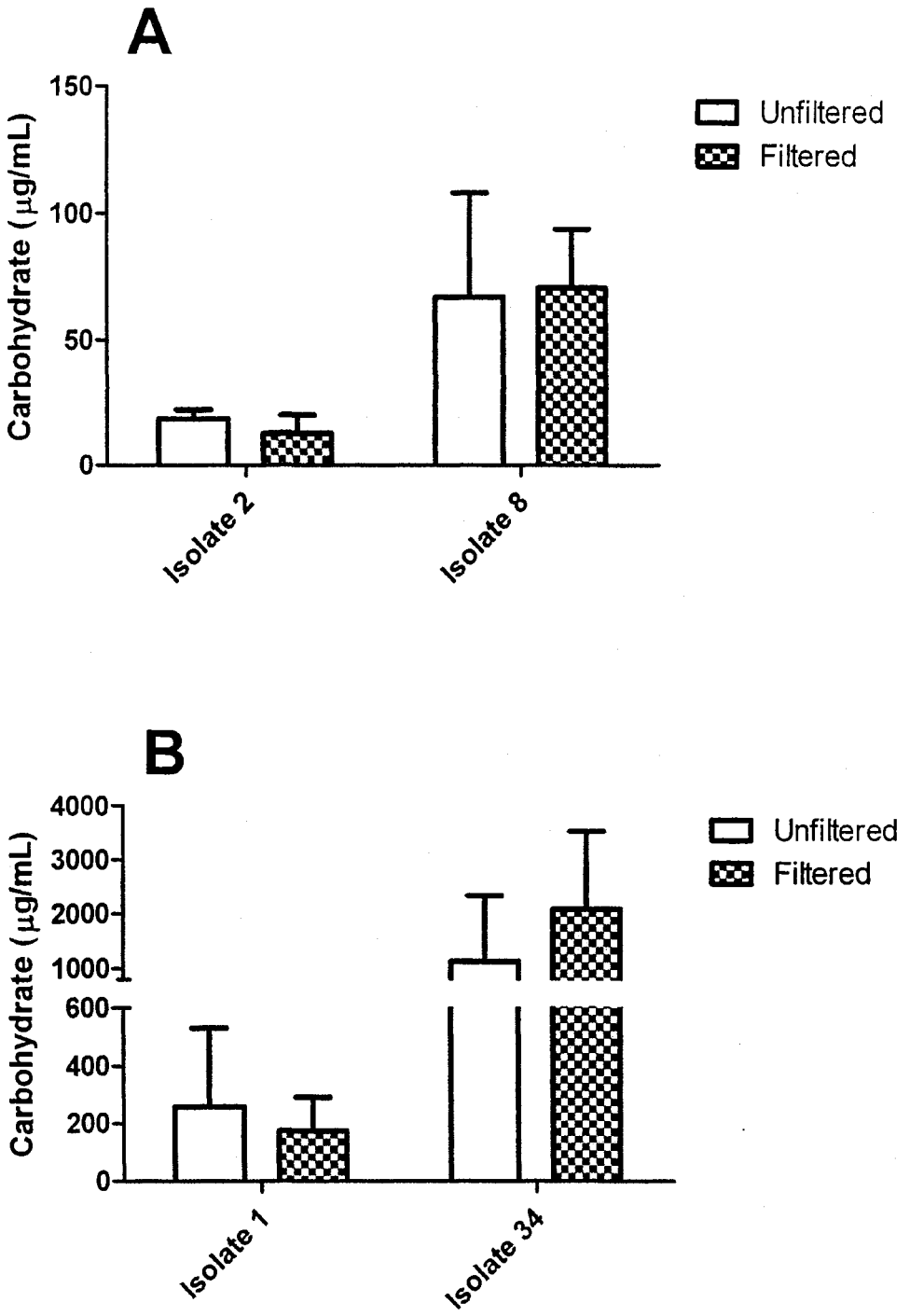

Figure 3.3: Carbohydrate concentrations in filtered and unfiltered batches of the four pulp and paper mill isolates.

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Chapter 4: Gelation of Mill Isolate Carbohydrate

4.0.0 Introduction

Some bacteria produce polysaccharides as a strategy to enhance adhesion to abiotic surfaces and over time this will create a biofilm. Pulp and paper machines act as sites where multi-species biofilms are produced. These biofilms contain extracellular polymeric substances (EPS) which are composed of polysaccharides, living and dead cells, nucleic acids, and proteins. Polysaccharides are long monosaccharides joined together by glycosidic linkages. They also have many different functional groups which may include hydroxyl, hydrogen, and carboxyl groups. These functional groups offer sites where substances can attach to the existing polysaccharide matrix and create physio-chemical reactions.

The food industry currently uses polysaccharides for encapsulation purposes, as thickeners, gelling agents, and stabilizers (Khan et al., 2007). Encapsulation of such materials as probiotics, vitamins, fatty acids, and antioxidants has improved the delivery of bioactive compounds into food for consumption (Champagne and Fustier, 2007). Polysaccharides are also used as encapsulation materials for degradative microorganisms for degradation of pollutants such as gasoline (Moslemy et al., 2004). Encapsulation with polysaccharides has been shown to increase the effectiveness of microbial survival and tolerance to environmental stresses (Goel et al., 2006, Zache and Rehm, 1989, Moslemy et al., 2004). Identifying novel polysaccharides that can be applied industrially can dramatically improve the quality of foods and bioremediation causes.

This study used two bacteria from pulp and paper machines as possible candidates for novel encapsulation materials. For each isolate carbohydrate was harvested and lyophilized as described in Chapter 3. Carbohydrate solutions were prepared and tested for their ability to gelate using $CaCl_2$ and $FeCl_3$ at different concentrations. The objective of this portion of the study was to identify a suitable concentration of carbohydrate and cation mixture that can successfully induce gelation of novel carbohydrates.

4.1.0 Methods and materials

4.1.1 Polysaccharides

Commercial grade Keltrol® Xanthan (CPKelco) and Kelcogel® Gellan gum (CPKelco) were used for preliminary cation gelation, water content, PCR tube, and glass microscope slide experiments. For gelation experiments with isolate 1 and 34 carbohydrate only filtered preparations were used.

4.1.2 Preparation of Polysaccharide and Cation Solutions

All carbohydrate solutions were prepared using percent weight per volume (grams/100 ml) calculations. Solutions were made with sterile ddH_2O. A 2.0% w/v stock of Gellan, Xanthan, isolate 1 carbohydrate and isolate 34 carbohydrate was prepared and diluted to concentrations required for the experiments (0.025% - 2.0%).

Cation stock solutions were prepared in ddH_2O and appropriate amounts were used to achieve final experimental concentrations of 5.4 mM, 10 mM, 15 mM, 20 mM calcium chloride ($CaCl_2$), 8.04 mM potassium chloride (KCl), 10.2 mM sodium chloride (NaCl), 14.1 mM lithium chloride (LiCl), and 0.25M iron(III)chloride ($FeCl_3$).

4.1.3 Preliminary Gelation Experiments

Using commercially available CPKelcogel ®CG Gellan Gum, preliminary gelation experiments were conducted. The effect of four cations ($CaCl_2$, KCl, NaCl and LiCl) on gel firmness was tested. Gellan stock solutions were diluted to 1.0% (w/v), 0.75% (w/v), 0.50% (w/v) and 0.25% (w/v) and heated to approximately 100°C while on a stir plate in order to completely dissolve it in water. Once completely dissolved, 1.0 mL of Gellan at a desired concentration was dispensed into 1.5 mL plastic centrifuge tubes and 5 µl of the desired cation solution was added. Final cation concentrations were 5.4 mM $CaCl_2$, 8.0 mM KCl, 10.2 mM NaCl, and 14.1 mM LiCl. These concentrations of cations were based on 0.06% w/v cation solutions which were then converted to molar concentrations. Gellan mixed with cation solution was vortexed for 30 seconds and then allowed to cool on ice at 4°C for 15 minutes to induce gelation (Figure 4.1).

Using commercially available CPKelcogel ®CG Gellan Gum, preliminary water content experiments were conducted to further quantify gel firmness. Gellan solutions (1.0 and 0.75% w/v) along with 5.4 mM $CaCl_2$ and 8.0 mM KCl were used because these cations produced firm Gellan gels. The gel solutions were prepared as described above and cooled either on ice (4°C) or in a freezer (-20°C) for 15 minutes. Tubes were then

placed in a Fisher Scientific Isotemp Incubator at 50°C with their lids open for 2-3 days to evaporate moisture. The gels were weighed daily until a plateau was reached. Weights on day 0 and after a plateau was reached were used to calculate the percent water for each gel.

4.1.4 Mill Isolate Carbohydrate Gelation with $CaCl_2$ in PCR Tubes

Stock solutions of isolate 1 and 34 carbohydrates were diluted to 2.0, 1.5, and 1.0% w/v final concentrations and mixed with $CaCl_2$ to final concentration of 5.4 mM, 10 mM, 15 mM, and 20 mM (Huang et al., 2004) by mixing 0.1 mL of carbohydrate with 0.005 mL of $CaCl_2$ at the appropriate concentration. Before mixing, carbohydrate and $CaCl_2$ solutions were heated separately to 98.0-99.9°C in a Hybaid PCR Sprint Thermal Cycler for 15 minutes. The mixture was kept at room temperature for 30 minutes to induce gelation. After placing the solutions at 4°C for 24 hours, the mixtures were probed with a pipette tip and rated as (-) = no gel, (+) = viscous, (++) = semi solid, and (+++) solid gel formation.

4.1.5 Gelation Using on Glass Microscope Slides

Isolate 1 and 34 carbohydrate along with commercial grade Gellan and Xanthan gum were mixed with $CaCl_2$ and $FeCl_3$ and tested for their ability to form gels on glass microscope slides. Stock solutions of Gellan and Xanthan were diluted to final concentrations of 2.0, 1.5, 1.0, 0.5, 0.25, 0.10, 0.05, or 0.025% w/v and cations were

added to final concentration of 5.4 mM $CaCl_2$ and 0.25 M $FeCl_3$. Carbohydrate and cation solutions were heated to 90°C before carbohydrate (0.1 mL) and cation (0.01 mL) were mixed by pipetting on a glass microscope slide. The slides with the gel solution were incubated at 40°C for 30 minutes to induce gelation (Iijima et al., 2007). After holding at 4°C for 24 hours, the gels were rated using the scale described above.

4.1.5.1 Effect of Mixed Cation Species on Gelation

Solutions of commercial grade Gellan and Xanthan as well as isolate 1 and 34 carbohydrates were prepared as described above and mixed with cations. Instead of using a single cation, the two cations were combined to achieve final concentrations of 0.25 M $FeCl_3$ and 5.4 mM $CaCl_2$. This cation combination was then added to carbohydrate solutions and gelation experiments were performed on glass microscope slides as described above.

4.1.5.2 Mixed Carbohydrate Gels

Upon determining concentrations of carbohydrate that did not form gels using Gellan Gum (0.05%) and Xanthan Gum (0.025%), mixed carbohydrate gels were created to evaluate whether gelation may be enhanced in mixed gels. The addition of known gelling agents at non-gelling concentrations has been shown to enhance gelation when mixed together (Pongjanuakul and Puttipipatakhachorn, 2007). Stock solutions of Gellan and Xanthan were diluted such that when mixed with isolate 1 or 34 carbohydrate (2.0,

1.0, or 0.5% w/v) or Xanthan (2.0% to 0.025%), the final concentrations would be 0.05% w/v Gellan and 0.025% w/v Xanthan. Gelation experiments for these mixtures were performed on glass microscope slides as describe above.

4.2.0 Results & Discussion

Bacterial polysaccharides have demonstrated rudimentary gelation after cation introduction (Huang et al., 2004 & Pongjanyakul and Puttipipatakhachorn, 2007, Sandolo et al., 2007). Some of the more common bacterial polysaccharides currently used in the food industry include Gellan derived from *Sphingomonas elodea* and Xanthan derived from *Xanthomonas campestris*. This study isolated and identified two pulp and paper mill bacteria which produce carbohydrates that may have potential for use as encapsulation or thickening agents.

Gelation experiments were conducted with Gellan, a common additive used in food products as a thickener, emulsifier or stabilizer. It was observed that no gelation occurred at carbohydrate concentrations less than 0.50% w/v and that $CaCl_2$ produced the most rigid gel with 1.0% w/v Gellan although KCl and NaCl did produce semi-solid gels with 0.75% w/v Gellan as seen in Figure 4.1.

The strength of gels formed by Gellan was estimated by determining the water content of gels. Two cations, $CaCl_2$ (5.4 mM) and KCl (8.0 mM) were added to 1.0 or 0.75% w/v Gellan because these cations performed best at producing rigid gels (Figure 4.1). The gels were cooled at 4°C (on ice) or at -20°C (freezer) to observe if cooling temperature would affect gelation. There was no significant difference in gelation

depending on which cation was used, however there was a difference between the concentrations of carbohydrate used. Figure 4.2 indicates that 1.0% w/v Gellan produced the most rigid gel with $CaCl_2$ based on the fact that these gels contained the least water. The gelation temperature (4°C or -20°C) did not appear to affect gelation.

In agreement with other studies, $CaCl_2$ produced the best results as seen in the literature when tested for its ability to increase the viscosity of Gellan. These studies also reported that $CaCl_2$ contributes to increasing gelling temperature of gellan, and increasing the critical shear value of gellan (Nickerson and Paulson, 2003, Nickerson et al., 2004 & Huang et al., 2004). A semi-solid gel was produced when 0.75% w/v Gellan along with $CaCl_2$, NaCl, KCl, or LiCl was used. Thus, the valency (monovalent or divalent) of the cation did not appear to affect gelation at this concentration. Huang et al. (2007) also observed that divalent cations and monovalent cations did not have a different effect on the gelation of Gellan.

Once this data was gathered, the quantity of carbohydrate was reduced to mimic the amount of carbohydrate that could be obtained with the mill isolates. Experiments were conducted in 0.2 mL PCR tubes. Gellan appeared to produce semi-solid gels at 1.0% w/v concentration when no cation was present, however no gelation occurred at lower concentrations (Table 4.1). Earlier Gellan results were confirmed and 1.0% Gellan with 5.4 mM $CaCl_2$ produced the best gelation as a solid gel was formed. Also, Gellan appeared to increase in viscosity at 0.5% w/v, even without cation (Table 4.1). No solid gel was produced using any of the other cations (KCl, NaCl, or LiCl).

Carbohydrate from isolates 1 and 34 was tested for gelation in a PCR tube using 10 mM, 15 mM and 20 mM $CaCl_2$ as divalent cations at 2-80 mM have been shown in

previous studies to create gels (Huang et al., 2004). As seen in Table 4.1, no concentration of carbohydrate or $CaCl_2$ produced a solid gel with either isolate carbohydrates, however viscosity of isolate 1 carbohydrate at 2.0% did increase when $CaCl_2$ was present in concentrations of 10 mM or higher.

Following these experiments, there was a need to further reduce the volume of carbohydrate mixtures as future experiments were conducted on glass microscope slides. Glass microscope slides were used for subsequent experiments because they were easier to manage and use at the 40°C incubation temperature. This temperature was selected as gels would have to be cool enough as to not kill any bacteria once culture was added and because cooling temperatures from 40-45°C have shown to produce the most rigid gels when using Xanthan (Pongjanuakul and Puttipipatakhachorn, 2007).

Using then known carbohydrates Gellan and Xanthan as models, a series of gelation experiments were designed. Gellan gum and Xanthan gum are anionic polysaccharides that contain many carboxyl groups which can interact with cations added to induce gelation (Kani et al., 2005, Mohammed et al., 2007). $FeCl_3$ was selected to use because Fe^{3+} has shown to produce solid gels with Xanthan (Mohammed et al., 2007, Muthukumarasamy et al., 2006, Ma and Barbosa-Canovas, 1997). Trivalent cations such as the ones found in $FeCl_3$, $AlCl_3$ and $CrCl_3$ have shown best results forming Xanthan gels. However, $FeCl_3$ was chosen because Cr^{3+} and Al^{3+} are not fit for human consumption (Ma and Barbosa-Canovas, 1997, Gioia and Ciriello, 2006, Marudova-Zsivanovits et al., 2006). The concentration at which Xanthan and Gellan form gels and with which cations was determined. In addition, cation concentrations required for

gelation were evaluated using mixtures of $CaCl_2$ and $FeCl_3$. Xanthan and Gellan acted as controls and bases for comparison to our unknown carbohydrates.

Gellan did not have any solid gel production when no cation was present and solid gel production did not occur with individual cation introduction at carbohydrate concentrations of 0.75% or less (Table 4.2). Gellan produced solid gels at 1.0% w/v after $CaCl_2$ or $FeCl_3$ was added. Only $FeCl_3$, but not $CaCl_2$, produced semi-solid gels at 0.50 and 0.25% w/v concentrations indicating that $FeCl_3$ may be the better cation to induce gelation at lower carbohydrate concentrations. Once mixed cation solutions were used, Gellan formed a solid gel at concentrations of 0.5% w/v or higher (Table 4.2). Gelation activity also increased in all carbohydrate categories except at 0.025% w/v concentration when a combination of both cations was used. This amplifying effect may be explained by the fact that Gellan gum produces a gel matrix once a divalent cation is added to it and further adding $FeCl_3$, a trivalent cation, may increase the gel matrix binding.

Xanthan did not form solid or semi-solid gels when cations were absent (Table 4.2) nor with $CaCl_2$. Xanthan did not produce gels at any concentration tested; yet, it produced solid gels at concentrations greater than 0.5% w/v when $FeCl_3$ was introduced. Viscosity of Xanthan solutions was increased at 0.25% w/v and 0.1% w/v. Enhanced gelation was observed when a mixed cation solution containing $FeCl_3$ plus $CaCl_2$ was used. A semi-solid gel formed at carbohydrate concentrations at 0.25% w/v, and between 0.025% to 0.1% w/v the viscosity of the solution did increase slightly. This may indicate that a combination of cations amplifies gelation activity better than using $FeCl_3$ alone. Again, this could be a result of introducing more binding sites for negatively charged carbohydrates to form gel matrices.

The effect of mixed carbohydrate solutions was evaluated by varying the concentration of Xanthan and keeping the Gellan concentration at the non-gelling level of 0.05% w/v. No solid gels were produced when only $CaCl_2$ was added. Solid gel formation occurred with Xanthan concentrations of 0.5% w/v and higher when $FeCl_3$ was used. Interestingly, no gelation activity was found at 0.1 and 0.05% w/v concentrations when $FeCl_3$ was used, but viscosity was increased at 0.25% and 0.025% w/v. This may indicate that $FeCl_3$ has an antagonistic affect on mixed gel solutions at lower carbohydrate concentrations. It is also possible that a homogeneous mixture of the carbohydrates was not present as such small quantities of carbohydrate were used. If this was the case, gelation activity would be influenced. This might also explain why at 0.025% w/v viscosity of the mixture increased, while at 0.1 and 0.05% w/v concentration gelation activity was absent (Table 4.2).

When a mixture of both cations was used with mixed carbohydrate solutions, solid gel formation occurred at concentrations of 0.25% w/v and above (Table 4.2). An increase in gelation activity was observed at 0.1% w/v as a semi-solid gel was produced. Using mixed cation solutions may increase the production of solid gels as more potential cation binding sites were present in solution. There may have been a new compound formed when Xanthan and Gellan were combined, and using mixed cation solutions could aid in binding and this may explain why a solid gel was formed at the 0.25% w/v Xanthan concentration (Table 4.2).

To allow for comparisons, the same conditions used with Gellan and Xanthan were applied to the mill isolate carbohydrates at 2.0, 1.0, or 0.5% w/v concentrations. With isolate 1 carbohydrate, no gel production was observed when $CaCl_2$ was added

although isolate 1 carbohydrate was viscous when no cation was present (Table 4.3). This may indicate that $CaCl_2$ reduces the thickening ability of isolate 1 carbohydrate and is not the optimum cation of choice. Semi-solid gel production was observed at carbohydrate concentrations of 1.0 and 2.0% w/v when 0.25 M $FeCl_3$ was added suggesting that $FeCl_3$ is the cation of choice to induce gelation of isolate 1 carbohydrate. No gelation occurred with $FeCl_3$ at 0.5% w/v concentration. This suggests that gelation of isolate 1 carbohydrate may be dependent on cation species and carbohydrate concentration. It may also indicate that the structure of isolate 1 carbohydrate is more similar to Xanthan than to Gellan, as Xanthan gelation was also enhanced by $FeCl_3$.

When a mixture of both cations was added to isolate 1 carbohydrate, the viscosity of the solution did increase; however, the gelling activity was not as strong as when $FeCl_3$ was used alone. In fact, it appeared that gelation activity was similar to not using any cation. Compared to adding $CaCl_2$ alone, the gel activity was increased when a mixture of cations ($CaCl_2$ and $FeCl_3$) was used. This may indicate that $CaCl_2$ may be a poor cation choice for gelation of isolate 1 carbohydrate. Further gelation activity of isolate 1 carbohydrate should be investigated using higher concentrations, as 5.4 mM $CaCl_2$ was the used cation concentration. Gel activity may possibly increase at higher $CaCl_2$ concentrations similar to affect that was observed when 0.25 M $FeCl_3$ was used.

Mixed carbohydrate and mixed cation solution experiments were conducted with isolate 1 carbohydrate. Non-gelling concentrations of 0.025% w/v Xanthan and 0.05% w/v Gellan were added to isolate 1 carbohydrate along with mixed cation solutions. When either Xanthan or Gellan along with mixed cation species were added to 2.0% and 1.0% w/v solutions of isolate 1 carbohydrate, gelation activity was increased and a semi-

solid gel was formed (Table 4.3). A viscosity increase was observed when 0.5% w/v carbohydrate solutions of isolate 1 was mixed with 0.025% w/v Xanthan or 0.05% w/v Gellan when both cations were present. However, at these concentrations Xanthan and Gellan alone showed an increase in viscosity (Table 4.2). This suggests that the viscosity increase with 0.5% w/v isolate 1 carbohydrate was due to Xanthan and Gellan alone and not to isolate 1 carbohydrate. A semi-solid gel was formed with isolate 1 carbohydrate at 0.5% w/v when both Xanthan and Gellan along with mixed cation species were present (Table 4.3). This increase in gelation indicates that gelation was enhanced and a new carbohydrate structure may have formed when all three carbohydrates were combined.

The same experiments were conducted with carbohydrate of isolate 34. No gelation activity was recorded with isolate 34 carbohydrates at the concentrations tested (Table 4.3). This indicates that isolate 34 carbohydrate does not gel on its own, or in the presence of $CaCl_2$ and/or $FeCl_3$.

Isolate 34 carbohydrate was then mixed with non-gelling concentrations of Xanthan and/or Gellan along with a mixed cation solution. As seen in Table 4.3, gelation activity was increased slightly when 0.025% w/v Xanthan was added to 2.0% and 1.0% w/v solutions of isolate 34 carbohydrate, but no gel activity was recorded for 0.5% w/v solution. Xanthan on its own at 0.025% w/v had increased viscosity when both cations were present (Table 4.2) and this may explain the viscosity increase of isolate 34 carbohydrate at 2.0 and 1.0% w/v. This trend should have continued at the 0.5% w/v concentration if Xanthan alone influenced the gelation. Since no viscosity change was observed at the lowest concentrations, it is possible that isolate 34 carbohydrate at 0.5% w/v inhibited gel activity. When isolate 34 carbohydrate was mixed with 0.05% w/v

Gellan, a semi-solid gel was formed at 2.0 and 1.0% w/v concentrations (Table 4.3). Gellan alone at this concentration did not produce semi-solid gels when both cations were present (Table 4.2), suggesting that a new compound may have been produced when Gellan was added to isolate 34 carbohydrate and a mixture of cations was needed to induce gelation (Table 4.3).

Mixed carbohydrate and mixed cation solution experiments were conducted with isolate 34 carbohydrate. Non-gelling concentrations of 0.025% w/v Xanthan and 0.05% w/v Gellan were added to isolate 34 carbohydrate along with mixed cation solutions. Semi-solid gels were produced at all isolate 34 carbohydrate concentrations (Table 4.3). This increase in gel activity suggests that gelation was amplified when non-gelling concentrations of Xanthan and Gellan were added to isolate 34 carbohydrate. As seen in Table 4.3, gelation of a 0.5% w/v solution of isolate 34 carbohydrate increased as the carbohydrate solution became more complex starting a no gelation, increasing viscosity to semi-solid gel production.

The addition of cation is necessary for rigid rudimentary gel formation of polysaccharides as it has been demonstrated in other studies (Mohammed et al., 2007, Prasertsan et al., 2006, Kani et al., 2005). Cations are also a necessary component when forming gels with isolate 1 and isolate 34 carbohydrates

The data collected suggest that the structure of gels formed by isolate 1 and 34 carbohydrate became more complex as more carbohydrates were present in solution. There may also be the possibility that isolate 1 and 34 carbohydrates enhanced gelation of non-gelling concentrations of the known carbohydrates Gellan and Xanthan. The structure of Xanthan is based on a β-1,4-D-glucan(cellulose) backbone, substituted on O-

3 with alternate backbone residues and side chains of β-D-Man ρ-1,4- β-D-GlcAρ-1,2-α-D-Man-ρ to create a branched pentasaccharide (Jansson et al., 1975, Melon et al., 1976). Similar to Xanthan, isolate 1 carbohydrate showed gelation with iron suggesting that its structure may be more similar to Xanthan than Gellan. The structure of Gellan is [3)- β-D-Glucose-(14)- β-D-Glucuronic acid-(14)- β-D-Glucose-(14)- α-L-Rhamnose-(1]$_n$ and Gellan gelates with $CaCl_2$ (Nickerson et al., 2003), while isolate 1 carbohydrate did not gelate with $CaCl_2$.

In this study, isolate 1 and isolate 34 carbohydrates were tested for their ability to form gels alone, with addition of cations, and as mixed gel systems. Amplification of gelation for non-gelling concentrations of isolate 1 and 34 carbohydrates, Xanthan and Gellan gum did occur once both $FeCl_3$ and $CaCl_2$ were added to the solution (Table 4.3). Isolate 1 carbohydrate may be suitable for encapsulation as it has shown gelation activity using $FeCl_3$. However, further investigation into its gelation properties should be done. Isolate 34 may also be suitable to amplify gelling activity of other non-gelling carbohydrates.

4.4.0 Tables and Figures

Table 4.1: Preliminary Gelation Experiments Conducted in 0.2 mL PCR Tubes

					Polysaccharide Concentration (% w/v)					
Polysaccharide Type	**Cation Concentration**	**Container**	**Initial Temp**	**Gelation Temp (°C)**	**2**	**1.5**	**1**	**0.75**	**0.5**	**0.25**
Gellan	none	PCR tube	90	4	ND	ND	++	+	+	-
Gellan	5.4 mM $CaCl_2$	PCR tube	90	4	ND	ND	+++	++	++	+
Gellan	8.04 mM KCl	PCR tube	90	4	ND	ND	++	++	+	-
Gellan	10.2 mM NaCl	PCR tube	90	4	ND	ND	++	++	+	-
Gellan	14.1 mM LiCl	PCR tube	90	4	ND	ND	+	+	+	-
Isolate 1	5.4mM $CaCl_2$	PCR tube	90	4	-	-	-	ND	ND	ND
Isolate 1	10mM $CaCl_2$	PCR tube	90	4	+	-	-	ND	ND	ND
Isolate 1	15mM $CaCl_2$	PCR tube	90	4	+	-	-	ND	ND	ND
Isolate 1	20 mM $CaCl_2$	PCR tube	90	4	+	-	-	ND	ND	ND
Isolate 34	5.4mM $CaCl_2$	PCR tube	90	4	-	-	-	ND	ND	ND
Isolate 34	10mM $CaCl_2$	PCR tube	90	4	-	-	-	ND	ND	ND
Isolate 34	15mM $CaCl_2$	PCR tube	90	4	-	-	-	ND	ND	ND
Isolate 34	20 mM $CaCl_2$	PCR tube	90	4	-	-	-	ND	ND	ND

ND = Not Done, (-) = no gel, (+) = viscous, (++) = semi-solid, (+++) = solid

Table 4.2: Xanthan and Gellan Gelation Conducted on Glass Microscope Slides

					Polysaccharide Concentration (% w/v)								
Polysaccharide Type	Cation Concentration	Container	Initial Temp	Gelation Temp (°C)	2	1.5	1	0.75	0.5	0.25	0.1	0.05	0.025
Gellan	none	Glass Slide	90	40	++	++	++	+	+	-	-	-	-
Gellan	0.25 M $FeCl_3$	Glass Slide	90	40	+++	+++	+++	++	++	++	-	-	-
Gellan	5.4mM $CaCl_2$	Glass Slide	90	40	+++	+++	+++	++	+	-	-	-	-
Gellan	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	+++	+++	+++	+++	+++	++	+	+	-
Xanthan	none	Glass Slide	90	40	+	+	+	-	-	-	-	-	-
Xanthan	0.25 M $FeCl_3$	Glass Slide	90	40	+++	+++	+++	+++	+++	+	+	-	-
Xanthan	5.4mM $CaCl_2$	Glass Slide	90	40	+	+	+	-	-	-	-	-	-
Xanthan	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	+++	+++	+++	+++	+++	++	+	+	+
Xanthan + Gellan 0.05%	5.4mM $CaCl_2$	Glass Slide	90	40	++	++	++	++	++	++	+	++	++
Xanthan + Gellan 0.05%	0.25 M $FeCl_3$	Glass Slide	90	40	+++	+++	+++	+++	+++	+	-	-	+
Xanthan + Gellan 0.05%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	+++	+++	+++	+++	+++	+++	++	+	+

ND = Not Done, (-) = no gel, (+) = viscous, (++) = semi-solid, (+++) = solid

Table 4.3: Gelation of Polysaccharide from Isolate 1 and Isolate 34 Examined on Glass Microscope Slides

Polysaccharide Type	Cation Type	Container	Initial Temp	Gelation Temp (°C)	Polysaccharide Concentration (% w/v) 2	1	0.5
Isolate 1	none	Glass Slide	90	40	+	+	+
Isolate 1	0.25 M $FeCl_3$	Glass Slide	90	40	++	++	-
Isolate 1	5.4mM $CaCl_2$	Glass Slide	90	40	-	-	-
Isolate 1	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	+	+	+
Isolate 1 + Xanthan 0.025%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	++	++	+
Isolate 1 + Gellan 0.05%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	++	++	+
Isolate 1 + Xanthan 0.025% + Gellan 0.05%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	++	++	++
Isolate 34	none	Glass Slide	90	40	-	-	-
Isolate 34	0.25 M $FeCl_3$	Glass Slide	90	40	-	-	-
Isolate 34	5.4mM $CaCl_2$	Glass Slide	90	40	-	-	-
Isolate 34	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	-	-	-
Isolate 34 + Xanthan 0.025%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	+	+	-
Isolate 34 + Gellan 0.05%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	++	++	+
Isolate 34 + Xanthan 0.025% + Gellan 0.05%	0.25 M $FeCl_3$ & 5.4mM $CaCl_2$	Glass Slide	90	40	++	++	++

ND = Not Done, (-) = no gel, (+) = viscous, (++) = semi-solid, (+++) = solid

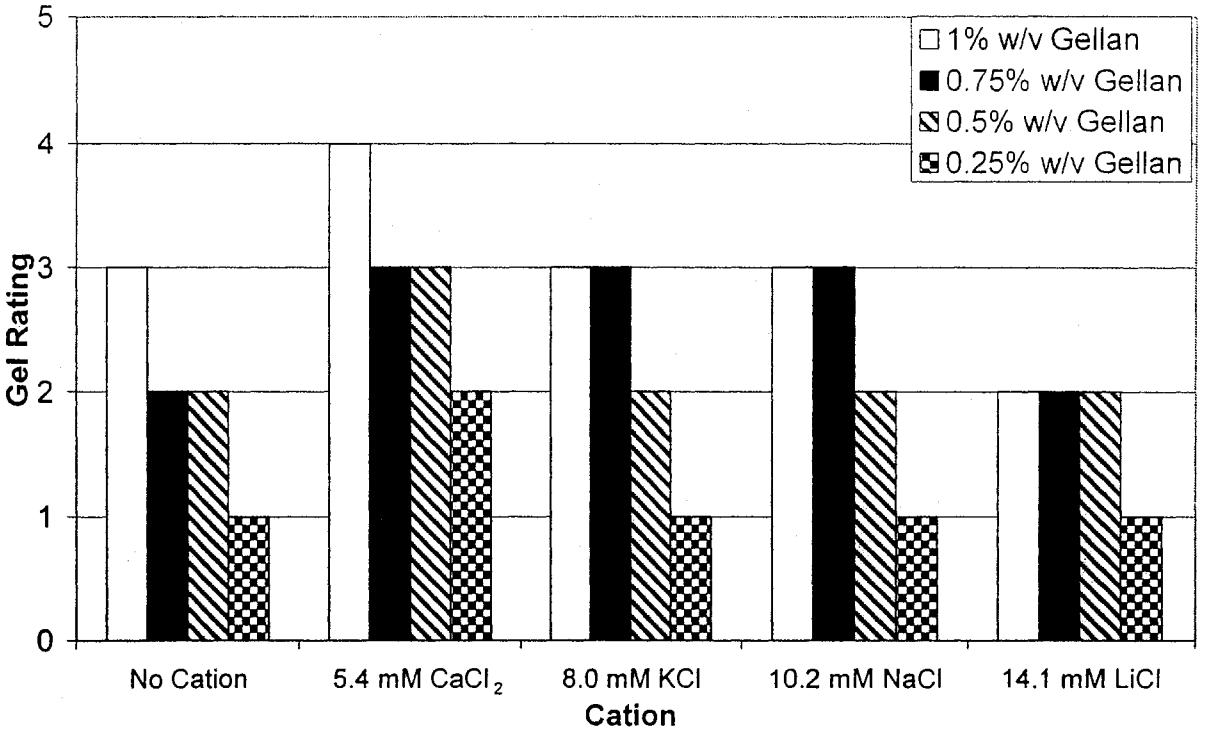

Figure 4.1: Preliminary experiments conducted with Kelcogel® Gellan Gum display at what carbohydrate concentrations gelation occurs in an eppendorf tube when left at 4°C for 15 minutes. Gel firmness was rated as 1-no gel, 2-viscous, 3-semi solid, 4-solid. No rigid gel formation occurred at carbohydrate concentrations less than 0.50% (w/v). This data was used to develop a standard gelation procedure used with mill isolate carbohydrates.

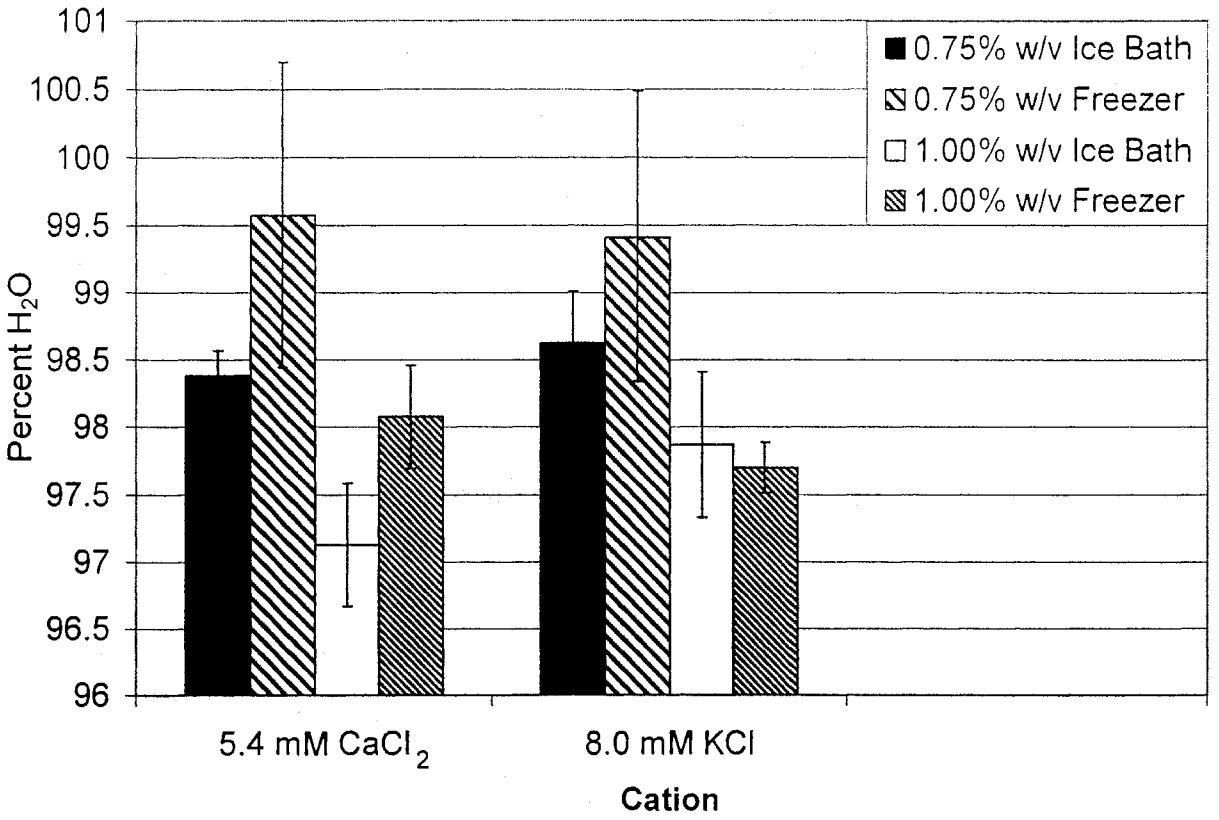

Figure 4.2: Further experiments conducted using Kelcogel® Gellan Gum along with cations $CaCl_2$ and KCl concluded that 1.00% (w/v) carbohydrate gels contained less water and thus formed a more rigid gel. This data was also used to develop a standard gelation procedure used with mill isolate carbohydrates.

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Chapter 5: Summary and Recommendations

Bacteria were isolated from pulp and paper mill biofilms and screened to find isolates capable of producing carbohydrate materials potentially useful for encapsulation. Diversity of bacteria indicated that amounts of bacteria found in these biofilms may be influenced by the seasonal factors. A total of 55 isolates were preliminarily screened based on quantity and flocculation ability of the carbohydrate that they produced because it has been reported that there is a correlation between the amount of bacterial carbohydrate and the ability for this carbohydrate to form aggregates in solution (Tenny, 1973). Flocculation ability of two known bacterial polysaccharides, Xanthan and Gellan gum, was also tested and compared to the carbohydrates produced by the 55 isolates. Xanthan and Gellan had high flocculation abilities at low carbohydrate concentrations. A range of different carbohydrate quantities and flocculation abilities were observed for the 55 isolates. Our study suggests that the average flocculation ability of the 55 isolates did not correlate with the carbohydrate quantity produced by these 55 isolates.

There were however some isolates that acted as outliers and they were isolates 1 and 34 which were included in the selection for further study. A total of four isolates were chosen for investigation of their carbohydrate gelation. Isolate 1 was selected because it had high carbohydrate production and high flocculation ability, isolates 2 and 8 were selected because they represented the average of the 55 isolates with respect to carbohydrate production and flocculation ability, and isolate 34 was selected because it

produced very large amounts of carbohydrate in a short period of time, but had a low flocculation ability.

Isolates 1, 2, 8, and 34 were identified using 16s rDNA sequencing along with biochemical tests. Isolate 1 was identified as a *Flavobacteria* spp. (98% identification), isolate 2 as a *Pseudomonas* spp. (99% identification), and isolate 34 as a *Sphingomonas* spp. (99% identification). Unfortunately, isolate 8 could not be conclusively identified. Using 16s rDNA, isolate 8 was identified as Bacterium N25 (99% identification) or *Mangroveibacter plantisponsor* (99% identification). Since biochemical information is lacking for these species, the molecular identification could not be confirmed with biochemical tests.

Several batches of carbohydrate were harvested for each of the four isolates for gelation experiments. The isolates had their inocula (OD_{600}) adjusted to 0.06 in PBS before carbohydrate was batched because this value that was expected to correspond to approximately 10^7 CFU/mL. Adjusting initial inocula to $OD_{600} = 0.06$ did not appear to result in 10^7 CFU/mL for each of our isolates. Isolate 1 and 34 had lower CFU/mL inocula than expected, while isolates 2 and 8 had expected inoculum concentration of 10^7 CFU/mL. Isolates 1, 2, and 8 all showed very little between batch variance of carbohydrate production. Interestingly however, the amount of carbohydrate produced by isolate 34 varied considerably between batches. For all isolates, it appeared that a relationship existed between inoculum concentration and carbohydrate production. As inoculum concentration decreased, carbohydrate concentration increased, thus variance of isolate 34 inocula may be the reason for the variance in its carbohydrate production. Once enough carbohydrate was harvested, gelation experiments were performed.

A range of gelation experiments were conducted using carbohydrates harvested from isolates 1 and 34. Xanthan and Gellan gum were included as controls and used to compare to our mill isolate carbohydrates. Gelation experiments were conducted only with isolate 1 and 34 carbohydrate because these particular isolates showed thickening properties at room temperature when creating stock solutions of carbohydrate. Isolate 1 carbohydrate produced a semi-solid gel at carbohydrate concentrations of 2.0 and 1.0% w/v when 0.25 M $FeCl_3$ was used as a cation source, while 5.4 mM $CaCl_2$ appeared to inhibit gel activity. When isolate 1 carbohydrate was mixed with non-gelling concentrations of the known gelling agents Xanthan (0.025% w/v) or Gellan (0.05% w/v) semi-solid gels were produced at isolate 1 carbohydrate concentrations of 2.0 and 1.0% w/v. When a combination of all three carbohydrates along with the addition of 5.4 mM $CaCl_2$ plus 0.25 M $FeCl_3$ was tested, semi-solid gels were produced at even lower isolate 1 carbohydrate concentrations (0.5% w/v), suggesting that isolate 1 carbohydrate may be useful in enhancing non-gelling concentrations of other carbohydrates.

Isolate 34 carbohydrate was then exposed to the same gelling conditions as isolate 1. No gelling activity was recorded for isolate 34 carbohydrate at 0.5-2.0% w/v even when $CaCl_2$, and $FeCl_3$ were present. There was an increase in gelling activity when non-gelling concentrations of Xanthan, Gellan, and a mixture of the two were added to our isolate 34 carbohydrate (2.0, 1.0, and 0.5% w/v) and semi-solid gels were produced. Again this may suggest that isolate 34 carbohydrate may amplify gelling activity of other non-gelling carbohydrates.

This study successfully isolated and identified two bacteria from pulp and paper mill biofilms which may be potential sources of novel encapsulation materials. Both

isolates produce carbohydrates that may be useful for encapsulation purposes, although further investigation is needed. First and foremost, optimization of growth conditions and optimal carbohydrate production should be examined for isolate 1 and isolate 34 in order to produce maximum amounts of carbohydrate. Carbohydrate structure should also be examined using analytical methods such as gas chromatography/mass spectrometry as the structures of the carbohydrates produced by the two isolates are unknown. Finally, additional encapsulation experiments should be conducted to investigate the usefulness of these carbohydrates.