A STUDY ON THE ANTIBACTERIAL PROPERTY OFACID-HYDROLYZED κ-CARRAGEENAN AGAINST *Bacillus subtilis* AND *Vibrio cholerae*

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ABSTRACT

Carrageenan oligosaccharides are produced from the hydrolysis of carrageenans from red seaweeds. It has been reported that there exists a correlation between hydrolysis time and oligosaccharide size. The antibacterial properties of acid-hydrolyzed kappa and carrageenan were pre-determined using the Kirby-Bauer method and turbidometric study. The best antibacterial agent was subjected to a 12-hr time-kill series. The 5% kappa carrageenan sample hydrolyzed for 30 minutes was added to cultures of *Bacillus subtilis* and *Vibrio cholerae*. Aliquots were taken from the cultures and plated during intervals over a 12-hr period. Populations of the bacterial cultures were estimated using the Miles and Misra technique. The results showed that the carrageenan oligosaccharides have antibacterial activity for both cultures. There was an initial decline in population from the period of second to sixth hour and logarithmic growth in population was observed from the seventh hour onwards. Statistical tests were used to correlate oligosaccharide size with antibacterial activity. The population of the treated *B. subtilis* culture was the same with the untreated culture at 12 hours. While the population of the treated *V. cholerae* culture was greater compared to the untreated culture at 12 hours. This study has concluded that only specific oligosaccharide sizes have optimal antibacterial activity against *B. subtilis* and *V. cholerae*.

INTRODUCTION

1.1 Background of the Study

The cholera epidemic caused by *Vibrio cholerae* O139 has been reported in 15 countries in South and Southeast Asia. The Center for Health Development of the Department of Health (DOH) in Region 1 had reported that there had been 103 cases of cholera contributing to 29 deaths from May to July 19, 2004.

Vibrio cholerae, a member of the family Vibrionaceae, is a facultative anaerobic, Gram-negative, non-spore forming curved rod-shaped bacteria. *V. cholerae,* a known marine and freshwater-borne human pathogen mutually associated with aquatic animals, is one of the most common organisms in surface waters of the world. *V. cholerae* is also a human pathogen causing non-invasive diarrhea affecting the small intestine through secretion of an enterotoxin. Transmission is by water or food. Its action on the mucosal epithelium is responsible for the characteristic diarrhea of the disease cholera and is one of the most rapidly fatal illnesses known (Todar, 2002).

Carrageenan oligosaccharides in a bacterial broth culture showed antibacterial activity against *Bacillus subtillis* (Tan and Marero, 2004). *Bacillus subtillis* is a Gram-positive, aerobic, rod-shaped spore-forming bacterium, and it often occurs as chain-like formations. Most bacilli are saprophytes. Each bacterium creates only one spore, which is resistant to heat, cold, radiation, dessication, and disinfection (Nature, 2003).

Carrageenan is defined as a group of cell wall galactan polysaccharides prepared by alkaline extraction from red seaweeds (*Rhodophyceae*) that have high sulfate contents (Canete and Montano, 2002). Carrageenan consists of alternating (1->3)-linked β-D-galactopyranose (Gal*p*) and (1->4)-linked α-D-galactopyranose. The commercially important k-carrageenan contains a 3,6-anhydro-α-D-galactopyranose (AnGal*p*) in place of α-D-galactopyranose, giving it gelling properties. The addition of a 2-*O*-sulfo group to the k-carrageenan sequence [->3)-β-D-

Gal*p*4S-(1->4)-α-D-AnGal*p*-(1->]$_n$ affords another commercially important carrageenan, i-carrageenan [->3)-β-D-Gal*p*4S-(1->4)-α-D-AnGal*p*2S-(1->]$_n$ (Yu *et al.*, 2002). Among their variety of applications, carrageenans may be used as a binder in cooked meats, to firm sausages and to make toothpaste, puddings, and other milk products (Chaplin, 2003). These carrageenans are commercially important because they are used as thickeners and gelling agents in food industry.

Carrageenan oligosaccharides are produced by the degradation of carrageenans using various methods such as hydrolysis using enzyme, organic acid or inorganic acid, and may be followed by physical processing, such as ultrasonicating, microwaving, and autoclaving (Cho *et al.*, 2002 and Yu *et al.*, 2002). Studies related to the antimicrobial properties of carrageenan are few; however there have been reports of its effectiveness against bacteria (Yu *et al.*, 2002). Carrageenan also has been reported to have medical properties such as: antigenic, stimulation of growth of connective tissue, antiviral agents against certain viruses, anticoagulant and antithrombic, and anti-ulcer (Solimabi and Das, 1980).

The use of carrageenan oligosaccharides is a novel approach. A synthesized oligosaccharide has been established as a potent antibiotic against Gram-positive bacteria (Jones *et al.*, 2001). This opens the door to the possibility of finding successful antibacterial agents in other oligosaccharides such as those of carrageenan (Tan and Marero, 2004).

1.2 Statement of the Problem

To determine the antibacterial properties of kappa carrageenan against *B. subtilis* and *V. cholerae*, it answered the following questions:

(1) Will the degradation time of acid-hydrolysis of the carrageenan oligosaccharides significantly affect its antibacterial property?

(2) Does the concentration of the acid-hydrolyzed carrageenan oligosaccharides significantly affect its antibacterial activity?

1.3 General Objectives

To determine if kappa carrageenan oligosaccharides possess antibacterial properties against *B. subtilis and V. cholerae* in a 12-hour time series study.

To determine the effect of the varying oligosaccharide sizes of the carrageenan influence the antibacterial property of Kappa carrageenan.

To determine the effect of varying concentrations of carrageenan oligosaccharide on its antibacterial property.

1.4 Significance of the Study

The emergence of cholera has been a significant public health problem around the world and battle to completely control this deadly disease continues (Soomro and Juneho, 2004). *V. cholerae* 0139 was first discovered as an epidemic organism which over the course of two years caused country-wide outbreaks in Asia, including the Philippines (Frischer, 1998). Thus, efforts to control and minimize the spread of *V. cholerae* 0139 must be continued and strengthened. Recent epidemics of cholera have emphasized the urgent need to find antibacterial agents that can inhibit the growth of *V. cholerae* and prevent the spread of these diseases.

The increasing incidence of Gram-negative pathogens resistant to multiple antibiotics intensifies the need for novel agents and therapeutic approaches for its treatment (Tan and Marero, 2004). Despite the progress in antibiotic chemotherapy, Gram-positive bacterial infections are continuously rising. This may be the result of the underestimation of its virulence compared to that of Gram-negative bacteria. Scientists in the 1970s and 1980s have been focusing their studies on the Gram-negative bacteria and consequently neglecting the evolution of resistant Gram-positive strains (Baquero, 1997). To be able to prevent the development of antibacterial resistance, novel antibacterial agents must be developed.

Our study supports previous claims of the antibacterial activity of carrageenan oligosaccharides. The identification of the antibacterial properties of the different types of carrageenan will provide the medical world different alternatives to use in the elimination of bacteria.

Since the Philippines is one of the major producers of carrageenan, manufacturing antibacterial agents from it would be more economical. And given the size of the Philippine carrageenan industry, the supply of this oligosaccharide is guaranteed unless abused.

1.5 Scope and Limitations

This experimental study focused on the antibacterial properties of carrageenan extracted from different species of red algae (Rhodophyceae). Based on the previous study conducted by Tan and Marero (2004), carrageenan oligosaccharides in a bacterial broth culture showed antibacterial activity against *B. subtillis*.

The antibacterial property of carrageenan oligosaccharides was only tested against *V. cholerae* and *B. subtillis*. The time series study underwent a 12-hour period.

Structural studies on carrageenan oligosaccharides that have direct effects on microbial physiology and growth are still unknown to the scientific community, though proposed mechanisms on microbial growth inhibition is discussed in passing.

The carrageenan powders used in the study were obtained from Sigma, Aldrich, Fluka, Sulpeco, and Riedel-de Haën Industries (Pasir Panjang, Singapore). The said company was responsible for the extraction and purification of κ-carrageenan from *Kappaphychus alvenzii*.

Crude extracts and hydrolyzed extracts were used in the experiment. Different concentrations of varying sizes of pure, concentrated hydrolyzed extracts of carrageenan oligosaccharides were prepared. Preliminary tests on the concentrations to be prepared for each oligosaccharide size were done through the disk-diffusion assay and the broth dilution test.

The experiment used triplicates for each bacterial strain.. The study was conducted for the period of October 2004 – March 2005, inclusive of the experimentation, interpretation of the results, and thesis paper writing.

The statistical test used in the experiment was the t test to compare the effectiveness of the antibacterial properties of the oligosaccharide samples on *V. cholerae* and *B. subtilis*.

REVIEW OF RELATED LITERATURE

2.1 Characterization of Carrageenans

Carrageenan is the generic name for a family of gel-forming, viscous polysaccharides that are obtained commercially by extraction from certain species of red seaweeds (Rhodophyceae). Carrageenans are composed of a linear galactose backbone with a varying degree of sulfation (from 15%-40%). Different carrageenan types differ in composition and conformation, resulting in a wide range of rheological and functional properties (De Velde and De Ruiter, 2004).

Carrageenan is a high molecular mass material with a high degree of polydispersity. The molecular mass distribution varies from sample to sample, depending upon the sample history, e.g., age of harvested seaweed, season of harvesting, way of extracting and duration of heat treatment. Commercial carrageenans have an average molecular mass ranging from 400-600 kDa with a minimum of 100 kDa. This minimum is set in response to reports of cecal and colonic ulceration induced by highly degraded carrageenan (De Velde and De Ruiter, 2004).

Four ideal structures of the carrageenan exist: the kappa, iota, beta and lambda-carrageenan. Kappa carrageenan (κ-carrageenan) has a structure consisting of (1→3)-β-D-galactopyranose-4-sulfate-(1→4)-3,6-anhydro-D-galactopyranose-(1→3) molecules. Iota carrageenan (ι-carrageenan) has a (1→3)-β-D-galactopyranose-4-sulfate-(1→4)-3,6-anhydro-α-D-galactopyranose-2-sulfate-(1→3) molecular structure. Lambda carageenan (λ-carrageenan) has a molecular structure of (1→3)-β-D-galactopyranose-2-sulfate-(1→4)-α-D-galactopyranose-2,6-disulfate-(1→3) (Chaplin, 2003). Carrageenan oligosaccharides would vary in molecular weight and number but would contain these repeating units.

2.2 Antiviral Activities of Carrageenans

The heparin-like sulfated polysaccharides from red algae, or fractions thereof, have been found to be low-cost, broad-spectrum antiviral agents. Carrageenan, is co-internalized into infected cells with Herpes simplex virus (HSV), inhibiting viral activity. It interferes with fusion (synctium formation) between cells infected with the human immunodeficiency virus (HIV) and inhibits the specific retroviral enzyme reverse transcriptase (Neushul, 1995). Type of sugar chain, degree of sulfation and molecular weight are the main factors that influence the anti-HIV activity of carrageenan (Yamada *et al.*, 1997).

A microbicide called Carraguard is still undergoing clinical trials with promising results. It is a well-known topical vaginal gel with a strong potential against HIV. Because it forms a gel, which acts by coating the pathogen, it is not absorbed by the skin; therefore, there are no foreseen harmful side effects (Neushul, 1995 and Population Council, Inc., 2003). Carrageenan is available commercially and is inexpensive, safe, and water-soluble; these are the desirable characteristics for a vaginal formulation. Preliminary studies show that Carraguard is safe and non-irritating. Research on acceptability suggests that there would be a large demand of the product in both developing and developed countries (Population Council Inc., 2000)

2.3 Cancer Potency of Degraded Carrageenans

Researches have been focused on the safety of degraded carrageenan, also known as polygeenan (molecular weight > 40kDa), because reported harmful gastrointestinal effects in animal models. However, conflicting studies have not verified the validity of this claim (SFC, 2003). Polygeenan is formed by high-temperature heating and strong-acid hydrolysis of carrageenan. It may occur in the gastrointestinal tract with the aid of bacterial flora (Tobacman, 2001).

The harmful effects of carrageenan have been shown in numerous animal studies. Research has shown that carrageenan has been associated with the induction and promotion of

neoplasms and ulcerations. Polygeenan has been identified to cause these effects. The documented effects in rats include: intestinal lesions, neoplasia, promotion of neoplasia and ulceration (CEPA, 2001, Joint FAO/WHO, 2001 and Tobacman, 2001).

There is speculation that the digestion of carrageenan in food may result in the production of polygeenan due to the same physiological conditions inside the human digestive system. Scientists have proposed a mechanism for the activity of carrageenan. The ingested carrageenan can undergo hydrolysis in the stomach and is further broken down by intestinal bacteria. The intestinal epithelial cells take up the degraded carrageenan which could cause lysosomal disruption. The epithelial cells may subsequently undergo lysis, producing erosions. Polymorphonuclear cells and macrophages then infiltrate the site of intestinal inflammation. Lysosomal vacuolation occurs as well as lysosomal disruption with the release of intracellular enzymes from macrophage disruption (CEPA, 2001 and Joint FAO/WHIO, 2001).

2.4 Preparation and Antibacterial Activity of Carrageenan Oligosaccharides

The method of preparation of the carrageenan oligosaccharides contributes to the functionality of the oligosaccharides. The production and isolation of oligosaccharides is based on length and molecular weight. Due to the high cost of preparation of the algal oligosaccharides and unwanted side reactions Cho *et al.* (2002) have modified the process and are using organic acids, instead. This has resulted in a higher hydrolysis ration of carrageenan polysaccharide and higher yield of oligosaccharides. They also found out that increasing the reaction temperature through autoclaving improved the yield of desired oligosaccharides.

The oligosaccharides are generally prepared by degrading carrageenan with enzymes called carrageenases or highly concentrated inorganic acids. Many studies have shown that oligosaccharides obtained from the enzymatic cleavage of carrageenan have resulted in antimicrobial property. Carrageenan oligosaccharides obtained by using hydrolysis enzyme have important functional properties such as: anticoagulant, antibrowning, and antimicrobial

activities. Oligosaccharides produced using organic acid hydrolysis do not express antimicrobial properties (Joo, 2003) which is completely opposite with the studies of Cho *et al.* (2002). There was activity against *Escherichia coli* but only when the oligosaccharides were prepared by hydrolysis with organic acid at 120°C. They hypothesized that the radical scavenging ability of the algal oligosaccharides is the basis of their antimicrobial property.

2.5 Mechanisms of Other Antibacterial Oligosaccharides

A new oligosaccharide antibiotic is Evernimicin®, which was derived from everninomicin. Everninomicin is the name for a group of complex sugar-derived antibiotics, which are isolated from *Micromonospora carbonacea* (Champney and Tober, 2000). It has strong activity against aerobic Gram-positive bacteria, especially enterobacci, staphylococci, and streptococci (Marshall *et al.*, 1999 and Jones *et al.*, 1999). Due to its activity against a wide array of species, it may be used as a substitute for glycopeptides against Gram-positive bacteria (Jones *et al.*, 2001).

Champney and Tober (2000) found that translation and the formation of 50s subunit were inhibited in *S. aureus*. The inhibition of these vital processes is responsible for the detrimental effect on bacterial cells. It is the complete formation of the large ribosomal subunit that is prevented and thus inhibits both translation and assembly by the 50s particle (McNicholas *et al.*, 2000). Further biochemical analysis has determined that the 23s RNA is important for binding Evernimicin®. This would indicate that the nature of the oligosaccharide is not significant in the interference of the translation process, but rather the size of the sugar that produces the antimicrobial effect (Belova *et al.*, 2001).

An organic oligosaccharide with antimicrobial activity is chitosan. Chitosan is low acetyl-substituted form of chitin. It has exhibited activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Proteus vulgaris*. Although the exaxt mechanism for its antimicrobial property is still under research, researchers have proposed several hypotheses.

One is that the interaction between the positively-charged chitosan molecules and the negatively-charged microbial cell membrane leads to the leakage of proteinaceous and other intracellular components. Another is that chitosan is able to act as a chelating agent for trace metals which could then cause damage to the bacterial cell. They also have suggested that chitosan may be able to activate the defense process in the host tissue against the microorganisms. Some believe that chitosan is able to penetrate through the nuclear membrane and bind with tDNA and inhibit mRNA synthesis and subsequently protein synthesis causing cell damage or even cell death (Shahidi *et al.*, 1999).

2.6 Antibacterial Assays Used for Carrageenan Oligosaccharides

The antimicrobial activity of carrageenan oligosaccharides was assayed using the disk diffusion method. The activity was measured using the inhibition diameter. Among those tested were: *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Enterobacter aerogenes.* All carrageenan oligosaccharides had a strong antimicrobial activity against *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus,* and *Escherichia coli.* However, there was no antimicrobial activity against *Enterobacter aerogenes* (Cho *et al.*, 2002)

2.7 Characterization of *Vibrio cholerae*

The genus *Vibrio* consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum. Vibrios are capable of both respiratory and fermentative metabolism. They do not denitrify. Most species are oxidase-positive. In most ways vibrios are related to enteric bacteria, but they share some properties with pseudomonads a well. The Family Vibrionaceae is found in the "Facultatively Anaerobic Gram-negative Rods" in Bergey's Manual, on the level with the Family Enterobacteriaceae. Vibrios are distinguished from enterics by being oxidase-positive and motile by means of polar flagella. Of the vibrios that are clinically significant to humans, *Vibrio cholerae*, the agent of cholera, is the most important.

Most vibrios have relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. However, since vibrios are typically marine organisms, most species require 2-3% NaCl or a sea water base for optimal growth. In liquid media vibrios are motile by polar flagella that are enclosed in a sheath continuous with the outer membrane of the cell wall. On solid media they may synthesize numerous lateral flagella which are not sheathed.

V. cholerae is a pathogen of humans. It produces diarrhea and acute gastroenteritis *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin.

2.8 Cholera and Its Occurrence in the Philippines

Cholera, frequently called Asiatic cholera or epidemic cholera is a severe diarrheal disease caused by the bacterium *Vibrio cholerae*. Transmission to humans is by water or food. *V. cholerae* produces cholera toxin, the model for enterotoxins, whose action on the mucosal epithelium is responsible for the characteristic diarrhea of the disease cholera. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2-3 hours if no treatment is provided. More commonly, the disease progresses from the first liquid stool to shock in 4-12 hours, with death following in 18 hours to several days.

The clinical description of cholera begins with sudden onset of massive diarrhea. The patient may lose gallons of protein-free fluid and associated electrolytes, bicarbonates and ions within a day or two. This results from the activity of the cholera enterotoxin which activates the adenylate cyclase enzyme in the intestinal cells, converting them into pumps which extract water and electrolytes from blood and tissues and pump it into the lumen of the intestine. This loss of fluid leads to dehydration, anuria, acidosis and shock. The watery diarrhea is speckled with flakes of mucus and epithelial cells and contains enormous numbers of vibrios. The loss of

potassium ions may result in cardiac complications and circulatory failure. Untreated cholera frequently results in high (50-60%) mortality rates.

Treatment of cholera involves the rapid intravenous replacement of the lost fluid and ions. Following this replacement, administration of isotonic maintenance solution should continue until the diarrhea ceases. If glucose is added to the maintenance solution it may be administered orally, thereby eliminating the need for sterility and IV administration. By this simple treatment regimen, patients on the brink of death seem to be miraculously cured and the mortality rate of cholera can be reduced more than ten-fold. Most antibiotics and chemotherapeutic agents have no value in cholera therapy.

In 1961, the "El Tor" biotype of cholera vibrio emerged to produce a major epidemic in the Philippines and to initiate a seventh global pandemic. Recently, The Department of Health has declared an outbreak of acute gastroenteritis in six towns and a city in Pangasinan province. DOH records showed that 1,866 people have been stricken by acute gastroenteritis in 24 towns since the outbreak of the disease on May 31 in Malasiqui town. Seventeen deaths had been reported, 15 of them children. According to the 2001 DOH records, acute gastroenteritis and diarrheas are the top leading causes of morbidity in the Philippines; it is also the number five cause of infant deaths and number two cause of child mortality.

MATERIALS AND METHODS

3.1 Source of Bacterial Cultures

The microorganisms used in the experiment were *B. subtilis* , and *V. cholerae*. The *B. subtilis* culture was obtained from the National Science Research Institute – Department of Microbial Cultures of the University of the Philippines – Diliman. The serovar *V. cholerae* O139 culture was obtained from the Manila Health Department – Department of Enterics of the San Lazaro Hospital which was obtained from the recent July and August 2004 cholera outbreak in Metro Manila. The *B. subtilis* strains were maintained on nutrient agar and nutrient broth at 37°C and were periodically subcultured. The *Vibrio* strain was grown on marine broth and TCBS to simulate their natural growth which is on a marine environment and to provide the appropriate salt content.

3.2 Source of Carrageenan

The κ-carrageenan from *Kappaphychus alvarezii* was purchased from the Sigma, Aldrich, Fluka, Sulpeco, and Riedel-de Haën Industries (Pasir Panjang, Singapore).

3.3 Preparation of Carrageenan Oligosaccharides

Various concentrations of each carrageenan type (1%, 5% and 10% (w/v) in HCl), were prepared.

3.3.1 Mild-acid hydrolysis of the carrageenan

The procedure for mild-acid hydrolysis of the carrageenan was based on the published method of Yu *et al.* (2002).

The carrageenan (10mg/mL) was dissolved at 60°C in 0.1 M hydrochloric acid and was kept at varying periods namely; 0 minute, 10 minutes, 30 minutes and 60 minutes. The

degradation was terminated by neutralization with 0.1 M NaOH, then was filtered by Millipore membrane (GS, 0.45um). The filtered sample was then concentrated by rotary evaporation.

3.4 Preliminary Experimentation on Carrageenan Oligosaccharides

Preliminary studies on the different oligosaccharide sizes with varying concentrations and volumes determined the most efficient antibacterial agent to be used in the time series experiment.

3.4.1 Kirby-Bauer Method

The Kirby-Bauer technique is an agar diffusion test that provides useful semi-quantitative data on anti-microbic susceptibility.

Nutrient agar was prepared using deionized water. Autoclaved nutrient agar was poured on sterile Petri plates and was allowed to settle and solidify. A bacterial suspension was prepared by inoculating loopfuls of the stock culture of bacteria to a sterile test tube containing 5 mL of sterile nutrient broth. The test tube was placed on a vortex mixer for 3 minutes. The process was repeated until the suspension's turbidity was equal to a 1 MacFarland standard, which is approximately equivalent to 10^5 cfu/mL. Sterile cotton swabs were dipped on the bacterial suspension. The bacterial suspension was lawned on the agar in an evenly manner. 25 µL of each of the prepared carrageenan oligosaccharide were placed on three sterile paper discs and were positioned far from each other on the plate. The plates were allowed to dry for 30 minutes at room temperature and were incubated in an inverted position at room temperature for 24 hours. Zones of inhibitions were then observed and diameters were measured on each plate.

3.4.2 Turbidometric Method

Bacterial suspensions with turbidities equivalent to 1 MacFarland were prepared. Five hundred µL of the bacterial suspension were placed on microfuge tubes. Varying volumes (50 µL, 100 µL, 150 µL, 200 µL and 250 µL) of the previously prepared carrageenan

oligosaccharides were also placed on the microfuge tube. The microfuge tube was filled to 1 mL with nutrient broth for the *B. subtilis* tubes and with marine broth for the *V. cholerae tubes*. The tubes were placed on tube holders and were incubated at room temperature for 24 hours.

Two blanks for each concentration of each carrageenan oligosaccharide were also prepared to which the tubes would be compared. The first blank was composed of 500 µL of bacterial suspension and 500µL of nutrient broth for *B. subtilis* tubes and 500 µL of marine broth for *V. cholerae* tubes. The second blank was composed of 500 µL of the specific carrageenan oligosaccharide and 500 µL of nutrient broth for *B. subtilis* tubes and 500 µL of marine broth for *V. cholerae* tubes. The tubes were then compared to these blanks and were observed for its relative turbidity. All the samples were aseptically transferred to cuvettes and were subjected to a spectrophotometer for absorbance readings at 600 nm.

3.5 Preparation of Experimental Cultures

A loopful of the stock culture of bacteria was inoculated to a test tube containing 5-mL of sterile nutrient broth for 24 hours at 37°C. After which the full volume was aseptically transferred into a flask containing 50-mL sterile nutrient broth. The flask was placed on a rotary shaker for 2.5 hours followed by incubation at 37°C for 1.5 hours. When the flask became turbid, approximately equivalent to the count of 10^5 cfu/mL, the culture was used in the time-series experiment.

3.6 Preparation of Carrageenan Oligosaccharides and Inoculum Mixture

The concentration of each size of carrageenan oligosaccharide extract was varied; namely 1%v/v, 5%v/v, and 10% v/v of 4-hour broth culture. For the experiment, 0.25 mL of sterile carrageenan oligosaccharide extract for a particular size was aseptically added to 5 mL of the 4-hour sterile broth culture for the 5%w/v and same mathematical procedures shall be done for the rest of the concentrations.

3.7 Time Series Experiment for Determination of Antibacterial Activity

After the 4-hour initial incubation period, two 5-mL aliquots of the specimen was aseptically transferred into to sterile test tubes, one as a growth control and one for treatment. 0.25 mL of the carrageenan oligosaccharide extract was added to the "treatment" test tube. Both test tubes were incubated at 37°C, and the population count was determined at the following time intervals: 0 hour, 0.25 hour, 0.5 hour, 0.75 hour, 1 hour and every succeeding hour until 12 hours.

The population count was done using viable cell counts using the procedure by Miles and Misra (Brunel University, 1996). Ten-fold serial dilution was done to achieve a dilution series from 10^{-1} to 10^{-6} in eppendorf tubes using 0.85% NaCl solution as diluent. From each dilution 20(L was aseptically dispensed onto a section of a sterile nutrient agar plate. 6 divisions per plate corresponded to the dilutions 10^{-1} to 10^{-6}. The plates were allowed to dry for 30 minutes at room temperature and was incubated in an inverted position at room temperature for 24 hours.

The number of visible colonies was counted and the average per dilution of the two replicates was calculated. Microbial density was estimated using the following formula:

$$\textbf{Microbial Density (cfu/mL)} = \frac{\text{average no. of colonies}}{\text{volume plated in mL}} \times \text{dilution factor}$$

3.8 Statistical Tests

The standard t test with a 95% conficence interval was used to compare the effectiveness of the antibacterial properties of the different oligosaccharide samples on *V. cholerae* and *B. subtilis* only between 0hr and 24hrs incubation period. Effectivity of the different samples based on hydrolysis time, concentration and volume was also compared using the same t test.

RESULTS

The Kirby-Bauer test for the positive control, *Bacillus subtilis,* showed zones of inhibition in some plates, however there were no trends exhibited. Because the results were highly variable, *B. subtilis* was further subjected to a turbidometric test.

The preliminary results obtained for *Vibrio cholerae* were similar to that of *B. subtilis.* And because of this, *V. cholerae* was also subjected to a turbidometric test.

Based on the absorbance readings of *B. subtilis,* 50 µL – 100 µL of 1% kappa carrageenan, hydrolyzed for 10 minutes and 60 minutes showed immediate antibacterial activity (Figure 1). For 5% kappa carrageenan, all four samples showed similar results having immediate activity at 50 µL (Figure 2). 100 µL of 10% kappa carrageenan hydrolyzed for 0 minute, 10 miuntes and 30 minutes showed same antibacterial activity (Figure 3).

Based on the absorbance reading of *V. cholerae*, for 1% Kappa carrageenan, only the 30 minute hydrolyzed carrageenan showed immediate antibacterial activity at the low volume of 50 uL (Figure 10). For 5% Kappa carrageenan, all four samples showed similar results having immediate activity at 50 uL, however the 30 minute hydrolyzed sample exhibited the most activity (Figure 11). For 10% Kappa carrageenan, all four samples also showed similar results wherein immediate activity at 50 uL was observed, however the 30 minute hydrolyzed sample exhibited the most activity (Figure 12).

For *B. subtilis,* 1% kappa carrageenan hydrolyzed for 10 minutes and 30 minutes were better antibacterial agents compared to crude samples and 60-minute-hydrolyzed samples. 1% iota carrageenan hydrolyzed at 10 minutes was the best agent against *B. subtilis.*

For *V. cholerae,* 1% kappa carrageenan hydrolyzed for 30 minutes was the best antibacterial agent for this group. 5% kappa carrageenan hydrolyzed for 10 minutes was the best agent for all samples using *V. cholerae.* 10% kappa carrageenan samples hydrolyzed at 10 minutes were the best agent in this group.

Both bacterial species demonstrated reduction in population count in the presence of carrageenan oligosaccharides. After an initial increase in population (Figures 7, 8, 9), the viable cell counts of the control of *B.subtilis* leveled at 10^9 cfu/mL for all replicates (Figures 7, 8, 9). During the first hour, the graphs of *B. subtilis* showed decreasing populations (Figures 7, 8, 9). The populations reached around 5.0 x 10^8 cfu/mL on the first hour for all replicates. It dropped up to 2.5 x 10^8 cfu/mL, the lowest population count, on the ninth hour for all replicates. On the tenth hour, population count was re-established at 10^9 cfu/mL and the population increased from the tenth hour onwards.

Viable cell counts of the control of *V. cholerae* leveled at 10^7 cfu/mL for all replicates (Figures 16, 17, 18). Gradual decease in population was observed until the first hour after the bacterial suspension was exposed to carrageenan oligosaccharides (Figures 16, 17, 18). Populations dropped lowest on the sixth hour with a population count of 3-4 x 10^6 cfu/mL. After the sixth hour, populations started to increase again until it reached a count of 10^7 cfu/mL on the ninth hour for the first replicate, eleventh hour for the second replicate and tenth hour for the third replicate. Thereafter, populations increased greatly.

All sizes of κ carrageenan oligosaccharides except for those rendered by the 1%, 50 mL and 10%, 250mL preparations possess effective antibacterial properties against *B. subtilis*. κ carrageenan oligosaccharides produced by the preparation of 50-200mL of 1% κ carrageenan, 50 mL of 5% κ carrageenan and 100-150 mL of 10% kappa carrageenan are ineffective antibacterial agents against *V. cholerae*.

Populations of the treatment set-up are significantly different from the control set-up except at 0h,11h and 12h for *B. subtilis* (Table 19) and at 0h and 12 h for *V. cholerae* (Table 20).

A significant decrease in *B. subtilis* population was observed from 0h to 0.75h (Table 21); followed by an abrupt slight increase in population from 0.75h to 1h; then a significant decrease again in its population from1h to 3h ; again, a significant slight increase from 3h to 4h.

A major significant decrease in population is observed from the 4th to 7th hour. From the 7th hour onwards, logarithmic increase in population is observed again.

Vibrio cholerae observes a significant slump in population from 0h to 2h (Table 22). A relatively constant population is observed from the 2nd hour until the 6th hour. Substantial logarithmic growth in population is observed from the 6th hour onwards.

DISCUSSION

The variable results obtained from the Kirby-Bauer method of both the *B. subtilis* and *V. cholerae* may be attributed to the volume of the carregeenan introduced which may not have been enough to cause significant antibacterial activity; thus the need for the turbidimetric tests in which varied volumes of carrageenan, ranging from 50 uL to 250 uL, were used. However, the results obtained from the turbidimetric tests may not be totally accurate because the carrageenan samples have variable colors that may interfere and affect the absorbance readings. Therefore our criteria for choosing the specific carrageenan sample were based on both the turbidimetric test and the qualitative test. Based on the aforementioned criteria we were able to single out the 5% Kappa carrageenan hydrolyzed for 10 minutes.

Having subcultured both bacterial cultures 24 hours prior to its use in the time series study, the initial population may be attributed to the ending logarithmic phase of the bacteria. The stationary phase began approximately around the first hour. The treatment populations exhibited a decreasing pattern from 0 minutes to the first hour for *B. subtilis* and from 0 minutes to the third hour for *V. cholerae*. The decrease in the population is evident between the first and second hour of exposure to the carrageenan. It may be inferred that the abrupt decrease in population was caused by the presence of the carrageenan oligosaccharides. However, the population decline was not consistent and prolonged exposure caused the bacterial population to increase.

The exact mechanism of action of the carrageenan oligosaccharides has not been studied. We propose that it may be through the imbalance produced by the presence of an increased amount of carbohydrates in the broth. This may disrupt the osmoregulation of the bacteria and cause leakage of water and solutes, thus disrupting normal physiological functions. Cho *et al.* (2002) proposed that the activity was due to the radical scavenging ability brought by the sulfate groups. It is also be taken into consideration that the amount of sulfate groups was positively correlated with anti-HIV activities (Yamada *et al.*) The suggested mode of action of Shahidi *et al.* (1999) was through penetration of the nuclear membrane, binding with DNA,

inhibiting mRNA synthesis, and subsequently protein synthesis causing damage, or even death, to the cell.

The proposed mechanism by which the carrageenan oligosaccharides act as an antibacterial agent is through the sulfate groups present in its molecular structure. The carrageenan oligosacchrides surround the bacteria and encysts the entire bacterial cell making it immobile and thereby unable to proceed with its normal physiological activities. It is through the sulfate groups that the carrageenan oligosaccharides are broken according to the length which is enough to encyst the bacterial cell. The bacterial cell's immobility will weaken the cell and eventually lead to cell death. (Dr. Coke Montaño, personal communication, 2005)

Monitoring and adapting to changes in environmental conditions are critical processes that determine the survival of microorganisms and their successful long-term competition for a given habitat. All microorganisms must cope with changes in water availability in their milieu since the concentration of solutes within the cell is higher than that in the environment. Changes in the external osmolarity trigger rapid water fluxes along the osmotic gradient and thus cause either swelling and eventually rupture of the cell in hypotonic environments or plasmolysis and dehydration of the cytoplasm under hypertonic conditions (Bremer 1998). This change in external osmolarity is brought about by the neutralization process of the hydrolysis of carrageenan brought about by the production of NaCl ions. Plasmolysis may contribute to the low account of bacterial population. Choline, the precursor of an osmoprotectant; glycine betaine is scarcely present in carrageenan (http://www.pharmcast.com, 1999). With glycine betaine's presence in the growth medium, *B. subtilis* could perform osmoregulation via the ABC transporters of the cell. However, the very same compounds that protect *B. subtilis* from the detrimental effects of high osmolarity become a threat to its survival when the cells are exposed to hypoosmotic conditions since this will trigger a rapid influx of water into the cell. The cell must therefore rid itself rapidly of ions and organic osmolytes to curb the increase in turgor. *B. subtilis* accomplishes this via mechanosensitive channels; MscL and MscS channels; exhibiting

different levels of conductances. *B. subtilis* mutants lacking the MscL and MscS proteins can not survive severe osmotic down-shocks. The *B. subtilis* used in the experiment may have these proteins for there is a logarithmic growth on the seventh hour after osmoregulation of NaCl ions.

B. subtilis cells that were exposed to sulfide stress brought about by carrageenan induces the stringent response. (Leichert et.al 2002). Proteomic and the transcriptomic patterns showed a massive downregulation of a large number of vegetative genes, which in turn could be a cause of the decrease in bacterial population. Sulfide stress also induces oxidative stress genes which downregulates oxygen-reactive species causing strong cytotoxic effects by modifying major cellular components resulting in functional inactivation and eventually death.

Since, carrageenan has the precursor component of glycine betaine, which prevents improper osmoregulation and maybe the strain of *B. subtili* used in the experiment has genes that is antagonistic to sulfide stress and oxidative stress; these two factors may be accountable for the regrowth of the remaining viable *B. subtilis* after the fluctuation in the population.

Vibrio cholerae can shift to a "rugose" phenotype, thereby producing copious exopolysaccharide (EPS), which promotes its environmental survival and persistence (Ali, A. A. *et al.*, 2002). It is through this adaptation technique that they are able to survive energy- and nutrient-deprived conditions. The exopolysaccharide material serve a variety of purposes including structural role, benefiting the bacterium by enabling attachment to surfaces, improving nutrient acquisition, or providing protection from environmental stresses and host defenses (Wai, S. N., *et al.*, 1998). By having exopolysaccharide materials, the rugose strains acquired resistance to osmotic and oxidative stress (Wai, S. N., *et al.*, 1998).

From the study conducted by Tan and Marero (2004), the proposed mechanism by which the carrageenan oligosaccharides have affected the *B. subtilis* was through the imbalance produced by the presence of an increased amount of carbohydrates. The imbalance caused by the increase in the amount of carbohydrate may disrupt the osmoregulation of the

bacteria (Tan and Marero, 2004). It is possible that the *V. cholerae* may have been affected by the carrageenan oligosaccharides in the initial period because of the carbohydrate imbalance. But having been exposed for a prolonged period, they may have been able to adapt to their environment by producing exopolysaccharide materials.

CONCLUSION

Based on the results of the experiment, all of the hydrolyzed carrageenan samples exhibited antibacterial activity on *B. subtilis and V. cholerae*. This was demonstrated in the difference in the population densities of the control and treated bacterial culture. This was also exhibited in the absorbance readings of the samples. Bactericidal activity was achieved 30 minutes to 6 hours upon application of the oligosaccharide, though it doesn't show trends. From the seventh hour onwards, bacteria become acclimatized with the presence of the carrageenan oligosaccharide and regains logarithmic growth phase. The 5% kappa carrageenan sample hydrolyzed for 10 minutes exhibited the best antibacterial activity for both *B. subtilis* and *V. cholerae*.

RECOMMENDATIONS

If we were given enough time and resources, we would have included the comparison of the antibacterial properties of all four types of carrageenan (Kappa, Iota, Lambda, and Beta) in our study. We would have also included techniques (such as PAGE analysis) that may qualitatively and quantitatively assess the exact measurements of each oligosaccharide.

Studies on the effect of the type of hydrolysis (acid hydrolysis or enzyme hydrolysis) employed to obtain carrageenan oligosaccharides to its antibacterial activity. Other bacteria may be used to further determine the extent of their antibacterial activity. Further studies may be done to determine whether these are bacteriostatic or bactericidal. More extensive studies may be done to determine the exact mechanism by which carrageenan oligosaccharides work. Studies involving the effect of sulfation to the antibacterial properties of carrageenan oligosaccharides may also be performed.

DATA REPRESENTATION

TABLES

Table 1. Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 1% κ- Carrageenan Incubated at 37°C for 24 Hours

	Volume of κ- Carrageenan				
Hydrolysis Time of κ - Carrageenan	50 µL	100 µL	150 µL	200 µL	250 µL
0 minute	0.149	0.150	0.153	0.154	0.157
10 minutes	0.148	0.118	0.122	0.126	0.174
30 minutes	0.146	0.162	0.164	0.141	0.167
60 minutes	0.106	0.131	0.135	0.150	0.183

Table 2. Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 5% κ- Carrageenan Incubated at 37°C for 24 Hours

	Volume of κ- Carrageenan				
Hydrolysis Time of κ - Carrageenan	50 µL	100 µL	150 µL	200 µL	250 µL
0 minute	0.142	0.179	0.241	0.309	0.365
10 minutes	0.141	0.175	0.234	0.296	0.356
30 minutes	0.129	0.172	0.231	0.293	0.355
60 minutes	0.139	0.176	0.236	0.297	0.358

Table 3. Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 10% κ- Carrageenan Incubated at 37°C for 24 Hours

	Volume of κ- Carrageenan				
Hydrolysis Time of κ - Carrageenan	50 µL	100 µL	150 µL	200 µL	250 µL
0 minute	0.204	0.233	0.247	0.280	0.296
10 minutes	0.193	0.223	0.244	0.223	0.291
30 minutes	0.197	0.220	0.241	0.321	0.290
60 minutes	0.189	0.224	0.246	0.329	0.292

Table 4. t values of Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 1% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

Absorbance Values of Treatment after 24h Incubation	0.149	0.15	0.153	0.154	0.157
	0.148	0.118	0.122	0.126	0.174
	0.146	0.162	0.164	0.141	0.167
	0.106	0.131	0.135	0.15	0.183
Absorbance Values of Treatment at 0h	0.15	0.153	0.154	0.157	0.158
	0.158	0.124	0.13	0.134	0.184
	0.15	0.167	0.168	0.148	0.175
	0.111	0.134	0.139	0.156	0.188
SD	0.0037	0.0015	0.0029	0.0022	0.0039
t	2.673	5.667	2.959	5.555	3.065

Table 5. t values of Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 5% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

Absorbance Values of Treatment after 24h Incubation	0.142	0.179	0.241	0.309	0.365
	0.141	0.175	0.234	0.296	0.356
	0.129	0.172	0.231	0.293	0.355
	0.139	0.176	0.236	0.297	0.358
Absorbance Values of Treatment at 0h	0.145	0.181	0.244	0.311	0.367
	0.15	0.185	0.242	0.302	0.361
	0.134	0.18	0.236	0.3	0.36
	0.145	0.182	0.238	0.299	0.36
SD	0.0025	0.0034	0.0026	0.0026	0.0017
t	4.6	3.806	3.402	3.232	4.041

Table 6. t values of Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 10% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

Absorbance Values of Treatment after 24h Incubation	0.204	0.233	0.247	0.28	0.296
	0.193	0.223	0.244	0.223	0.291
	0.197	0.22	0.241	0.321	0.29
	0.189	0.224	0.246	0.329	0.292
Absorbance Values of Treatment at 0h	0.206	0.235	0.25	0.283	0.297
	0.203	0.233	0.252	0.23	0.299
	0.204	0.225	0.247	0.326	0.297
	0.195	0.228	0.25	0.334	0.295
SD	0.0033	0.0031	0.0022	0.0016	0.0033
t	3.783	3.085	4.735	3.124	2.875

Table 7. Viable Cell Counts for the First Replicate of *Bacillus subtilis* Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours

Time (hr)	Negative Control (cfu/mL)	Negative Control Log Population	Treatment with Carrageenan Oligosaccharides (cfu/mL)	Treatment with Carrageenan Oligosaccharides Log Population
0	2.51×10^9	9.3997	2.50×10^9	9.3979
0.25	2.80×10^9	9.4472	1.59×10^9	9.2014
0.50	2.71×10^9	9.4330	1.36×10^9	9.1335
0.75	2.45×10^9	9.3892	4.00×10^8	8.6021
1	2.54×10^9	9.4048	6.50×10^8	8.8129
2	2.16×10^9	9.3344	5.00×10^8	8.6990
3	2.50×10^9	9.3979	3.50×10^8	8.5440
4	2.35×10^9	9.3711	5.50×10^8	8.7404
5	2.14×10^9	9.3304	5.00×10^8	8.6990
6	2.09×10^9	9.3201	4.00×10^8	8.6021
7	2.13×10^9	9.3284	1.70×10^8	8.2304
8	1.76×10^9	9.2455	2.50×10^8	8.3979
9	2.22×10^9	9.3463	2.50×10^8	8.3979
10	1.85×10^9	9.2672	1.20×10^9	9.0414
11	1.74×10^9	9.2405	1.10×10^9	9.0792
12	1.36×10^9	9.1335	1.30×10^9	9.0969

Table 8. Viable Cell Counts for the Second Replicate of *Bacillus subtilis* Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours

Time (hr)	Negative Control (cfu/mL)	Negative Control Log Population	Treatment with Carrageenan Oligosaccharides (cfu/mL)	Treatment with Carrageenan Oligosaccharides Log Population
0	2.51×10^9	9.3997	3.50×10^9	9.5441
0.25	2.80×10^9	9.4472	1.36×10^9	9.1335
0.50	2.71×10^9	9.4330	1.50×10^8	8.1761
0.75	2.45×10^9	9.3892	4.00×10^8	8.6021
1	2.54×10^9	9.4048	6.50×10^8	8.8129
2	2.16×10^9	9.3344	5.00×10^8	8.6990
3	2.50×10^9	9.3979	3.50×10^8	8.5441
4	2.35×10^9	9.3711	5.00×10^8	8.6990
5	2.14×10^9	9.3304	4.50×10^8	8.6532
6	2.09×10^9	9.3201	4.00×10^8	8.6021
7	2.13×10^9	9.3284	1.70×10^8	8.2304
8	1.76×10^9	9.2455	2.50×10^8	8.3979
9	2.22×10^9	9.3463	2.50×10^8	8.3979
10	1.85×10^9	9.2672	1.20×10^9	9.0414
11	1.74×10^9	9.2405	1.10×10^9	9.0792
12	1.36×10^9	9.1335	1.40×10^9	9.1461

Table 9. Viable Cell Counts for the Third Replicate of *Bacillus subtilis* Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours

Time (hr)	Negative Control (cfu/mL)	Negative Control Log Population	Treatment with Carrageenan Oligosaccharides (cfu/mL)	Treatment with Carrageenan Oligosaccharides Log Population
0	2.51×10^9	9.3997	3.50×10^9	9.5441
0.25	2.80×10^9	9.4472	1.14×10^9	9.0569
0.50	2.71×10^9	9.4330	1.50×10^8	8.1761
0.75	2.45×10^9	9.3892	3.50×10^8	8.5441
1	2.54×10^9	9.4048	6.00×10^8	8.7782
2	2.16×10^9	9.3344	5.00×10^8	8.6990
3	2.50×10^9	9.3979	3.50×10^8	8.5441
4	2.35×10^9	9.3711	5.00×10^8	8.6990
5	2.14×10^9	9.3304	4.50×10^8	8.6532
6	2.09×10^9	9.3201	3.50×10^8	8.5441
7	2.13×10^9	9.3284	1.65×10^8	8.2175
8	1.76×10^9	9.2455	2.00×10^8	8.3010
9	2.22×10^9	9.3463	2.50×10^8	8.3979
10	1.85×10^9	9.2672	1.15×10^9	9.0414
11	1.74×10^9	9.2405	1.10×10^9	9.0607
12	1.36×10^9	9.1335	1.40×10^9	9.1461

Table 10. Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 1% κ- Carrageenan Incubated at 37°C for 24 Hours

	Volume of κ- Carrageenan				
Hydrolysis Time of κ - Carrageenan	50 µL	100 µL	150 µL	200 µL	250 µL
0 minute	0.202	0.207	0.201	0.220	0.235
10 minutes	0.199	0.204	0.213	0.219	0.218
30 minutes	0.162	0.139	0.137	0.138	0.153
60 minutes	0.195	0.198	0.121	0.118	0.109

Table 11. Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 5% κ- Carrageenan Incubated at 37°C for 24 Hours

	Volume of κ- Carrageenan				
Hydrolysis Time of κ - Carrageenan	50 µL	100 µL	150 µL	200 µL	250 µL
0 minute	0.215	0.212	0.210	0.209	0.207
10 minutes	0.191	0.189	0.185	0.181	0.182
30 minutes	0.187	0.195	0.231	0.243	0.256
60 minutes	0.205	0.225	0.254	0.273	0.283

Table 12. Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 10% κ- Carrageenan Incubated at 37°C for 24 Hours

	Volume of κ- Carrageenan				
Hydrolysis Time of κ - Carrageenan	50 µL	100 µL	150 µL	200 µL	250 µL
0 minute	0.232	0.221	0.222	0.232	0.229
10 minutes	0.207	0.195	0.205	0.211	0.213
30 minutes	0.196	0.193	0.204	0.204	0.201
60 minutes	0.222	0.278	0.302	0.344	0.316

Table 13. t values of Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 1% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

Absorbance Values of Treatment after 24h Incubation	0.202	0.207	0.201	0.22	0.235
	0.199	0.204	0.213	0.219	0.218
	0.162	0.139	0.137	0.138	0.153
	0.195	0.198	0.121	0.118	0.109
Absorbance Values of Treatment at 0h	0.202	0.21	0.203	0.223	0.236
	0.207	0.211	0.221	0.224	0.224
	0.166	0.146	0.143	0.146	0.159
	0.2	0.202	0.123	0.224	0.113
SD	0.0033	0.0021	0.0030	0.0504	0.0024
t	2.573	5.093	3	1.211	3.598

Table 14. t values of Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 5% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

Absorbance Values of Treatment after 24h Incubation	0.215	0.212	0.21	0.209	0.207
	0.191	0.189	0.185	0.181	0.182
	0.187	0.195	0.231	0.243	0.256
	0.205	0.225	0.254	0.273	0.283
Absorbance Values of Treatment at 0h	0.216	0.215	0.213	0.211	0.209
	0.199	0.196	0.19	0.191	0.191
	0.194	0.201	0 238	0.251	0.263
	0.207	0.227	0.26	0.278	0.286
SD	0.0035	0.0024	0.0017	0.0035	0.0033
t	2.563	3.781	6.148	3.51	3.178

Table 15. t values of Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 10% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

Absorbance Values of Treatment after 24h Incubation	0.232	0.221	0.222	0.232	0.229
	0.207	0.195	0.205	0.211	0.213
	0.196	0.193	0.204	0.204	0.201
	0.222	0.278	0.302	0.344	0.316
Absorbance Values of Treatment at 0h	0.232	0.221	0.222	0.233	0.232
	0.212	0.202	0.214	0.22	0.221
	0.202	0.203	0.209	0.212	0.205
	0.227	0.28	0.307	0.349	0.318
SD	0.0027	0.0046	0.0037	0.0036	0.0026
t	2.954	2.077	2.578	3.12	3.232

Table 16. Viable Cell Counts for the First Replicate of *Vibrio cholerae* Plated on Marine Agar Plates Incubated at 37°C for 24 Hours

Time (hr)	Negative Control (cfu/mL)	Negative Control Log Population	Treatment with Carrageenan Oligosaccharides (cfu/mL)	Treatment with Carrageenan Oligosaccharides Log Population
0	5.66×10^7	7.7528	5.91×10^7	7.7716
0.25	5.97×10^7	7.7760	4.50×10^7	7.6532
0.50	2.96×10^7	7.4713	1.05×10^7	7.0212
0.75	3.51×10^7	7.5453	3.00×10^7	7.4771
1	4.02×10^7	7.6042	1.10×10^7	7.0414
2	2.57×10^7	7.4099	4.00×10^6	6.6021
3	3.12×10^7	7.4942	5.00×10^6	6.6990
4	2.48×10^7	7.3945	5.50×10^6	6.7401
5	2.33×10^7	7.3674	5.00×10^6	6.6990
6	2.45×10^7	7.3892	4.00×10^6	6.6021
7	2.43×10^7	7.3856	7.00×10^6	6.8451
8	1.79×10^7	7.2529	9.00×10^6	6.9542
9	1.36×10^7	7.1335	1.10×10^7	7.0414
10	1.67×10^7	7.2227	1.20×10^7	7.0792
11	2.30×10^7	7.3617	1.65×10^7	7.2175
12	2.44×10^7	7.3874	9.50×10^7	7.9777

Table 17. Viable Cell Counts for the Second Replicate of *Vibrio cholerae* Plated on Marine Agar Plates Incubated at 37°C for 24 Hours

Time (hr)	Negative Control (cfu/mL)	Negative Control Log Population	Treatment with Carrageenan Oligosaccharides (cfu/mL)	Treatment with Carrageenan Oligosaccharides Log Population
0	5.66×10^7	7.7528	3.41×10^7	7.5327
0.25	5.97×10^7	7.7760	2.50×10^7	7.3979
0.50	2.96×10^7	7.4713	9.00×10^6	6.9542
0.75	3.51×10^7	7.5453	2.00×10^7	7.3010
1	4.02×10^7	7.6042	9.00×10^6	6.9542
2	2.57×10^7	7.4099	2.50×10^6	6.3979
3	3.12×10^7	7.4942	2.50×10^6	6.3979
4	2.48×10^7	7.3945	4.50×10^6	6.6532
5	2.33×10^7	7.3674	5.00×10^6	6.6990
6	2.45×10^7	7.3892	3.50×10^6	6.5441
7	2.43×10^7	7.3856	6.00×10^6	6.7782
8	1.79×10^7	7.2529	8.00×10^6	6.9031
9	1.36×10^7	7.1335	8.50×10^6	6.9294
10	1.67×10^7	7.2227	9.00×10^6	6.9542
11	2.30×10^7	7.3617	1.45×10^7	7.1614
12	2.44×10^7	7.3874	7.00×10^7	7.8451

Table 18. Viable Cell Counts for the Third Replicate of *Vibrio cholerae* Plated on Marine Agar Plates Incubated at 37°C for 24 Hours

Time (hr)	Negative Control (cfu/mL)	Negative Control Log Population	Treatment with Carrageenan Oligosaccharides (cfu/mL)	Treatment with Carrageenan Oligosaccharides Log Population
0	5.66×10^7	7.7528	4.55×10^7	7.6580
0.25	5.97×10^7	7.7760	1.50×10^7	7.1761
0.50	2.96×10^7	7.4713	1.15×10^7	7.0607
0.75	3.51×10^7	7.5453	2.50×10^7	7.3979
1	4.02×10^7	7.6042	9.50×10^6	6.9777
2	2.57×10^7	7.4099	3.00×10^6	6.4771
3	3.12×10^7	7.4942	3.50×10^6	6.5441
4	2.48×10^7	7.3945	4.50×10^6	6.6532
5	2.33×10^7	7.3674	5.00×10^6	6.6990
6	2.45×10^7	7.3892	4.00×10^6	6.6021
7	2.43×10^7	7.3856	7.00×10^6	6.8451
8	1.79×10^7	7.2529	8.00×10^6	6.9031
9	1.36×10^7	7.1335	9.50×10^6	6.9777
10	1.67×10^7	7.2227	1.05×10^7	7.0212
11	2.30×10^7	7.3617	1.55×10^7	7.1903
12	2.44×10^7	7.3874	8.00×10^7	7.9031

Table 19. t values of 0h to 12h of *Bacillus subtilis* with κ Carrageenan Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours (t > 2.920)

0 hour			
Control Population	Treatment Population	SD	t value
2.51E+09	2.50E+09	5.77E+08	-1.97
2.51E+09	3.50E+09		
2.51E+09	3.50E+09		

0.25 hour			
Control Population	Treatment Population	SD	t value
2.80E+09	1.59E+09	2.25E+08	11.059
2.80E+09	1.36E+09		
2.80E+09	1.14E+09		

0.5 hour			
Control Population	Treatment Population	SD	t value
2.71E+09	1.36E+09	6.99E+08	5.3471
2.71E+09	1.50E+08		
2.71E+09	1.50E+08		

0.75 hour			
Control Population	Treatment Population	SD	t value
2.45E+09	4.00E+08	2.89E+07	124
2.45E+09	4.00E+08		
2.45E+09	3.50E+08		

1 hour			
Control Population	Treatment Population	SD	t value
2.54E+09	6.50E+08	2.89E+07	114.4
2.54E+09	6.50E+08		
2.54E+09	6.00E+08		

2 hour			
Control Population	Treatment Population	SD	t value
2.16E+09	5.00E+08	0.00E+00	Undefined
2.16E+09	5.00E+08		
2.16E+09	5.00E+08		

3 hour			
Control Population	Treatment Population	SD	t value
2.50E+09	3.50E+08	0.00E+00	Undefined
2.50E+09	3.50E+08		
2.50E+09	3.50E+08		

4 hour			
Control Population	Treatment Population	SD	t value
2.35E+09	5.50E+08	2.89E+07	110
2.35E+09	5.00E+08		
2.35E+09	5.00E+08		

5 hour			
Control Population	Treatment Population	SD	t value
2.14E+09	5.00E+08	2.89E+07	100.4
2.14E+09	4.50E+08		
2.14E+09	4.50E+08		

6 hour			
Control Population	Treatment Population	SD	t value
2.09E+09	4.00E+08	2.89E+07	102.4
2.09E+09	4.00E+08		
2.09E+09	3.50E+08		

7 hour			
Control Population	Treatment Population	SD	t value
2.13E+09	1.70E+08	5.77E+08	5.885
2.13E+09	1.70E+08		
2.13E+09	1.65E+08		

8 hour			
Control Population	Treatment Population	SD	t value
1.76E+09	2.50E+08	2.89E+07	91.6
1.76E+09	2.50E+08		
1.76E+09	2.00E+08		

9 hour			
Control Population	Treatment Population	SD	t value
2.22E+09	2.50E+08	0.00E+00	Undefined
2.22E+09	2.50E+08		
2.22E+09	2.50E+08		

10 hour			
Control Population	Treatment Population	SD	t value
1.85E+09	1.20E+09	2.89E+07	40
1.85E+09	1.20E+09		
1.85E+09	1.15E+09		

11 hour			
Control Population	Treatment Population	SD	t value
1.74E+09	1.10E+09	0.00E+00	undefined
1.74E+09	1.10E+09		
1.74E+09	1.10E+09		

12 hour			
Control Population	Treatment Population	SD	t value
1.36E+09	1.30E+09	5.74E+07	-0.200
1.36E+09	1.40E+09		
1.36E+09	1.40E+09		

Table 20. t values of 0h to 12h of *Vibrio cholerae* with κ Carrageenan Plated on Marine Agar Plates Incubated at 37°C for 24 Hours (t > 2.920)

0 hour			
Control Population	Treatment Population	SD	t value
5.66E+07	5.91E+07	1.25E+07	1.435
5.66E+07	3.41E+07		
5.66E+07	4.55E+07		

0.25 hour			
Control Population	Treatment Population	SD	t value
5.97E+07	4.50E+07	1.53E+07	3.557
5.97E+07	2.50E+07		
5.97E+07	1.50E+07		

0.5 hour			
Control Population	Treatment Population	SD	t value
2.96E+07	1.05E+07	1.26E+06	26.52
2.96E+07	9.00E+06		
2.96E+07	1.15E+07		

0.75 hour			
Control Population	Treatment Population	SD	t value
3.51E+07	3.00E+07	5.00E+06	3.499
3.51E+07	2.00E+07		
3.51E+07	2.50E+07		

1 hour			
Control Population	Treatment Population	SD	t value
4.02E+07	1.10E+07	1.04E+06	50.533
4.02E+07	9.00E+06		
4.02E+07	9.50E+06		

2 hour			
Control Population	Treatment Population	SD	t value
2.57E+07	4.00E+06	7.64E+05	51.101
2.57E+07	2.50E+06		
2.57E+07	3.00E+06		

3 hour			
Control Population	Treatment Population	SD	t value
3.12E+07	5.00E+06	1.26E+06	37.899
3.12E+07	2.50E+06		
3.12E+07	3.50E+06		

4 hour			
Control Population	Treatment Population	SD	t value
2.48E+07	5.50E+06	5.74E+05	59.9
2.48E+07	4.50E+06		
2.48E+07	4.50E+06		

5 hour			
Control Population	Treatment Population	SD	t value
2.33E+07	5.00E+06	0.00E+00	undefined
2.33E+07	5.00E+06		
2.33E+07	5.00E+06		

6 hour			
Control Population	Treatment Population	SD	t value
2.45E+07	4.00E+06	2.89E+05	124
2.45E+07	3.50E+06		
2.45E+07	4.00E+06		

7 hour			
Control Population	Treatment Population	SD	t value
2.43E+07	7.00E+06	5.77E+05	52.9
2.43E+07	6.00E+06		
2.43E+07	7.00E+06		

8 hour			
Control Population	Treatment Population	SD	t value
1.79E+07	9.00E+06	5.74E+05	28.7
1.79E+07	8.00E+06		
1.79E+07	8.00E+06		

9 hour			
Control Population	Treatment Population	SD	t value
1.36E+07	1.10E+07	1.26E+06	5.414
1.36E+07	8.50E+06		
1.36E+07	9.50E+06		

10 hour			
Control Population	Treatment Population	SD	t value
1.67E+07	1.20E+07	1.50E+06	7.159
1.67E+07	9.00E+06		
1.67E+07	1.05E+07		

11hour			
Control Population	Treatment Population	SD	t value
2.30E+07	1.65E+07	1.00E+06	12.99
2.30E+07	1.45E+07		
2.30E+07	1.55E+07		

12 hour			
Control Population	Treatment Population	SD	t value
2.44E+07	9.50E+07	1.26E+07	-7.883
2.44E+07	7.00E+07		
2.44E+07	8.00E+07		

Table 21. t values of Time Frames from 0h to 12h of *Bacillus subtilis* with κ Carrageenan Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours (t > 2.920)

0-0.25 hour			
0h Population	0.25h Population	SD	t value
2.50E+09	1.59E+09	7.81E+08	3.99
3.50E+09	1.36E+09		
3.50E+09	1.14E+09		

0.25-0.5 hour			
0.25h Population	0.5h Population	SD	t value
1.59E+09	1.36E+09	5.14E+08	2.73
1.36E+09	1.50E+08		
1.14E+09	1.50E+08		

0.5-0.75 hour			
0.5h Population	0.75 Population	SD	t value
1.36E+09	4.00E+08	6.85E+08	0.43
1.50E+08	4.00E+08		
1.50E+08	3.50E+08		

0.75-1 hour			
0.75 Population	1h Population	SD	t value
4.00E+08	6.50E+08	0.00E+00	undefined
4.00E+08	6.50E+08		
3.50E+08	6.00E+08		

1-2 hour			
1h Population	2h Population	SD	t value
6.50E+08	5.00E+08	2.89E+07	8.00
6.50E+08	5.00E+08		
6.00E+08	5.00E+08		

2-3 hour			
2h Population	3h Population	SD	t value
5.00E+08	3.50E+08	0.00E+00	undefined
5.00E+08	3.50E+08		
5.00E+08	3.50E+08		

3-4 hour			
3h Population	4h Population	SD	t value
3.50E+08	5.50E+08	2.89E+07	-10.00
3.50E+08	5.00E+08		
3.50E+08	5.00E+08		

4-5 hour			
4h Population	5h Population	SD	t value
5.50E+08	5.00E+08	0.00E+00	undefined
5.00E+08	4.50E+08		
5.00E+08	4.50E+08		

5-6 hour			
5h Population	6h Population	SD	t value
5.00E+08	4.00E+08	2.89E+07	5.00
4.50E+08	4.00E+08		
4.50E+08	3.50E+08		

6-7 hour			
6h Population	7h Population	SD	t value
4.00E+08	1.70E+08	2.60E+07	14.33
4.00E+08	1.70E+08		
3.50E+08	1.65E+08		

7-8 hour			
7h Population	8h Population	SD	t value
1.70E+08	2.50E+08	2.60E+07	-4.33
1.70E+08	2.50E+08		
1.65E+08	2.00E+08		

8-9 hour			
8h Population	9h Population	SD	t value
2.50E+08	2.50E+08	2.89E+07	-1.00
2.50E+08	2.50E+08		
2.00E+08	2.50E+08		

9-10 hour			
9h Population	10h Population	SD	t value
2.50E+08	1.20E+09	-2.89E+07	-56.00
2.50E+08	1.20E+09		
2.50E+08	1.15E+09		

10-11 hour			
10h Population	11h Population	SD	t value
1.20E+09	1.10E+09	2.89E+07	5.00
1.20E+09	1.10E+09		
1.15E+09	1.10E+09		

11-12 hour			
11h Population	12h Population	SD	t value
1.10E+09	1.30E+09	5.00E+05	-17.32
1.10E+09	1.40E+09		
1.10E+09	1.40E+09		

Table 22. t values of Time Frames from 0h to 12h of *Vibrio cholerae* with κ Carrageenan Plated on Marine Agar Plates Incubated at 37°C for 24 Hours (t > 2.920)

0-0.25 hour			
0h Population	0.25h Population	SD	t value
5.91E+07	4.50E+07	1.12E+07	2.77
3.41E+07	2.50E+07		
4.55E+07	1.50E+07		

0.25-0.5 hour			

0.25h Population	0.5h Population	SD	t value
4.50E+07	1.05E+07	1.56E+07	1.99
2.50E+07	9.00E+06		
1.50E+07	1.15E+07		

0.5-0.75 hour			
0.5h Population	0.75h Population	SD	t value
1.05E+07	3.00E+07	4.37E+06	-5.815
9.00E+06	2.00E+07		
1.15E+07	2.50E+07		

0.75-1 hour			
0.75h Population	1h Population	SD	t value
3.00E+07	1.10E+07	4.01E+06	6.55
2.00E+07	9.00E+06		
2.50E+07	9.50E+06		

1-2 hour			
1h Population	2h Population	SD	t value
1.10E+07	4.00E+06	2.89E+05	40
9.00E+06	2.50E+06		
9.50E+06	3.00E+06		

2-3 hour			
2h Population	3h Population	SD	t value
4.00E+06	5.00E+06	5.00E+05	-1.732
2.50E+06	2.50E+06		
3.00E+06	3.50E+06		

3-4 hour			
3h Population	4h Population	SD	t value
5.00E+06	5.50E+06	7.37E+05	-2.646
2.50E+06	4.50E+06		
3.50E+06	4.50E+06		

4-5 hour			
4h Population	5h Population	SD	t value
5.50E+06	5.00E+06	5.77E+05	-0.5
4.50E+06	5.00E+06		
4.50E+06	5.00E+06		

5-6 hour			

5h Population	6h Population	SD	t value
5.00E+06	4.00E+06	2.89E+05	7
5.00E+06	3.50E+06		
5.00E+06	4.00E+06		

6-7 hour			
6h Population	7h Population	SD	t value
4.00E+06	7.00E+06	2.89E+05	-17
3.50E+06	6.00E+06		
4.00E+06	7.00E+06		

7-8 hour			
7h Population	8h Population	SD	t value
7.00E+06	9.00E+06	5.74E+05	-5
6.00E+06	8.00E+06		
7.00E+06	8.00E+06		

8-9 hour			
8h Population	9h Population	SD	t value
9.00E+06	1.10E+07	7.64E+05	-3.024
8.00E+06	8.50E+06		
8.00E+06	9.50E+06		

9-10 hour			
9h Population	10h Population	SD	t value
1.10E+07	1.20E+07	2.89E+05	-5
8.50E+06	9.00E+06		
9.50E+06	1.05E+07		

10-11 hour			
10h Population	11h Population	SD	t value
1.20E+07	1.65E+07	5.00E+05	-17.32
9.00E+06	1.45E+07		
1.05E+07	1.55E+07		

11-12 hour			
11h Population	12h Population	SD	t value
1.65E+07	9.50E+07	1.16E+07	-9.88
1.45E+07	7.00E+07		
1.55E+07	8.00E+07		

FIGURES

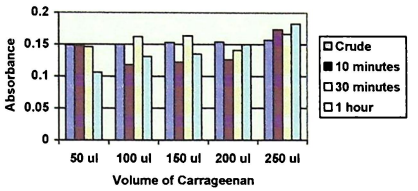

Figure 1. Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 1% κ- Carrageenan Incubated at 37°C for 24 Hours

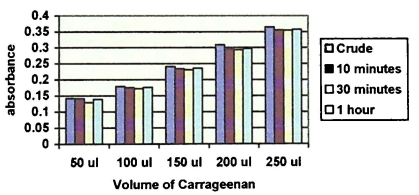

Figure 2. Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 5% κ- Carrageenan Incubated at 37°C for 24 Hours

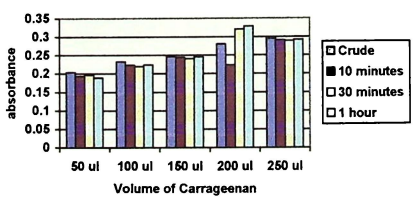

Figure 3. Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 10% κ- Carrageenan Incubated at 37°C for 24 Hours

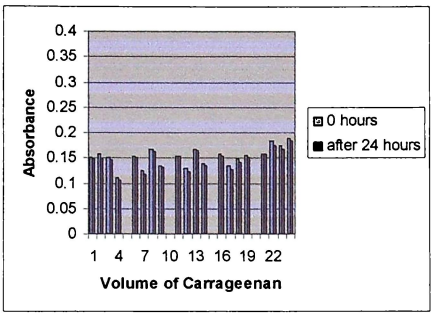

Table 4. t values of Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 1% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

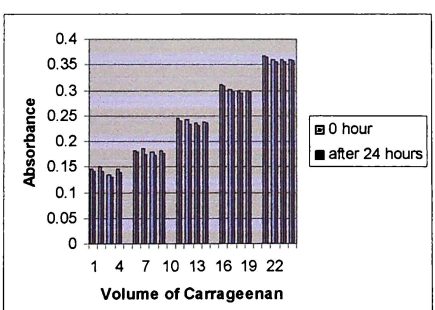

Table 5. t values of Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 5% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

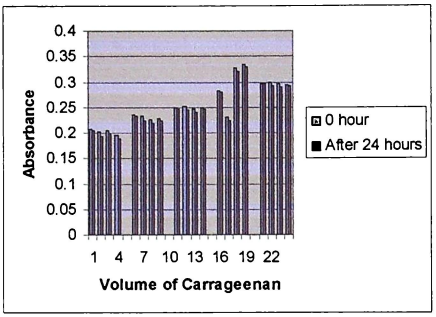

Table 6. t values of Absorbance Readings of *Bacillus subtilis* Cultured in Nutrient Broth with 10% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

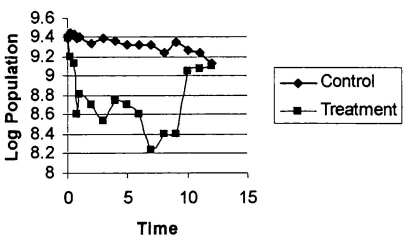

Figure 7. Comparison of Viable Cell Counts for the First Replicate of *Bacillus subtilis* Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours

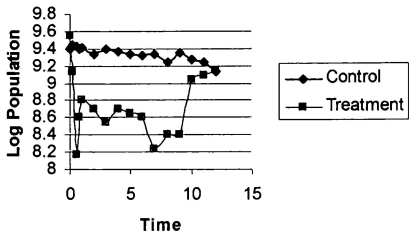

Figure 8. Comparison of Viable Cell Counts for the Second Replicate of *Bacillus subtilis* Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hours

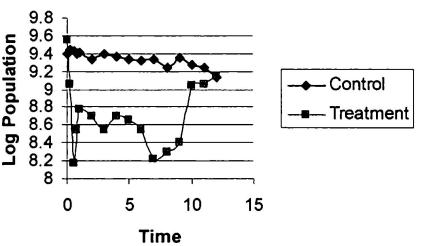

Figure 9. Comparison of Viable Cell Counts for the Third Replicate of *Bacillus subtilis* Plated on Nutrient Agar Plates Incubated at 37°C for 24 Hou

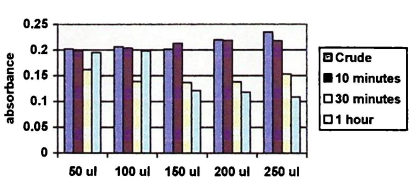

Figure 10 . Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 1% κ- Carrageenan Incubated at 37°C for 24 Hours

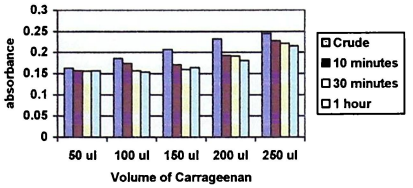

Figure 11. Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 5% κ- Carrageenan Incubated at 37°C for 24 Hours

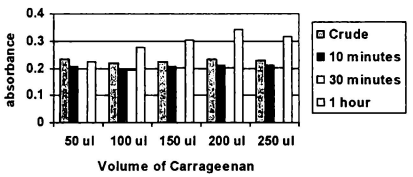

Figure 12. Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 10% κ- Carrageenan Incubated at 37°C for 24 Hours

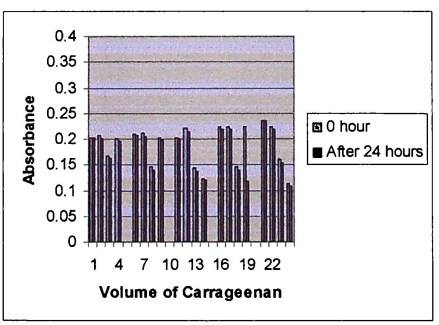

Table 13. t values of Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 1% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

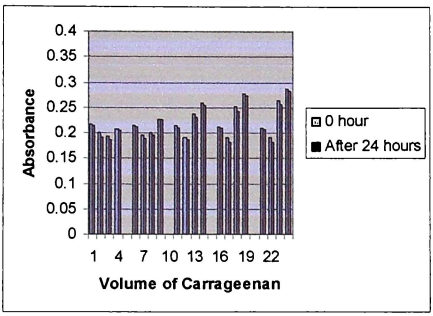

Table 14. t values of Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 5% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

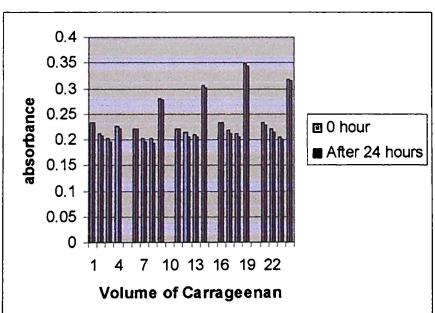

Table 15. t values of Absorbance Readings of *Vibrio cholerae* Cultured in Marine Broth with 10% κ Carrageenan Incubated at 37°C for 24 Hours (t > 2.920)

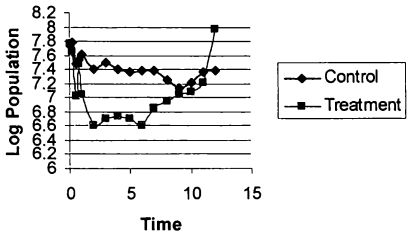

Figure 16. Comparison of Viable Cell Counts for the First Replicate of *Vibrio cholerae* Plated on Marine Agar Plates Incubated at 37°C for 24 Hours

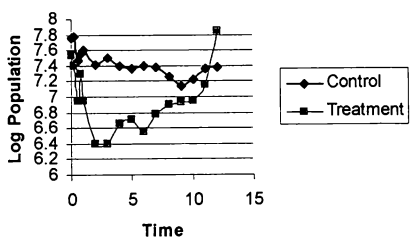

Figure 17. Comparison of Viable Cell Counts for the Second Replicate of *Vibrio cholerae* Plated on Marine Agar Plates Incubated at 37°C for 24 Hours

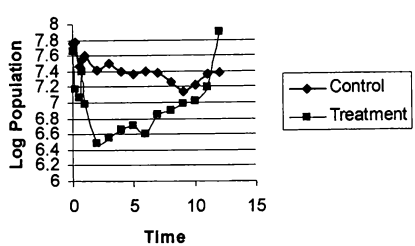

Figure 18. Comparison of Viable Cell Counts for the Third Replicate of *Vibrio cholerae* Plated on Marine Agar Plates Incubated at 37°C for 24 Hours

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6.3 Personal Interview

Montano, Coke. Personal Interview. 22 November 2004

Appendix

Source of Carrageenan
Powdered carrageenan obtained from Sigma, aldrich, Fluka.

Source of Bacterial Cultures
B. Subtilis- NSRI
V. cholerae- Quezon City Health Department

Preparation of Carrageenan Oligosaccharides
1%, 5%, 10% concentrations were prepared based on the published methods of Yu *et. al.*2002

Preliminary Experimentation on Carrageenan Oligossacharides
The concentrations and volumes were varied and used to determine the most efficient antibacterial agent to be used in the time series study.

Preparation of Experimental Cultures
1 loopful of bacterial culture + 5mL nutrient broth incubated at 37°C for 24 h then full volume + 50mL nutrient broth subjected to rotary shaker and incubated at 37°C for 24 h when equivalent to count of 10^5 cfu/mL: the culture was used in the time-series study

Statistical Test
t test was used to compare the effectiveness of the antibacterial properties of the different oligosaccharide samples on *V. cholerae* and *B. subtilis*.

Time Series Study for Determination of Antibacterial Activity
After 4 hr incubation, 0.25 mL carrageenan oligosaccharide extract+ treatment, control & treatment incubated at 37°C. Population count determined at following time intervals: 0, 0.25, 0.50, 0.75, 1 hr & every succeeding hr until 12 hrs using Miles and Misra

Preparation of Carrageenan Oligosacharides and Inoculum Mixture
0.25 mL carrageenan oligosaccharide extract+ 5 mL 4-hr broth culture 5%w/v.