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Comparison of disk diffusion, agar dilution, and broth microdilution for antimicrobial susceptibility testing of five chitosans

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COMPARISON OF DISK DIFFUSION, AGAR DILUTION, AND
BROTH MICRODILUTION FOR ANTIMICROBIAL
SUSCEPTIBILITY TESTING OF FIVE CHITOSANS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

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by

Lin Jiang

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Table of Contents

Acknowledgements.....	ii
List of Tables	v
List of Figures.....	vi
Abstract.....	vii
Chapter 1 - Introduction.....	1
Chapter 2 - Literature Review.....	4
2.1. Natural Antimicrobials.....	4
2.2. Chitin.....	4
2.3. Chitosan	7
2.4. Biological Properties of Chitosan	8
2.4.1. Biodegradability.....	8
2.4.2. Biocompatibility	10
2.4.3. Low Toxicity.....	10
2.5. Economic Aspects and Regulatory Status	10
2.5.1. Economic Aspects.....	10
2.5.2. Regulatory Status	11
2.6. Antimicrobial Activity of Chitosan	11
2.6.1. Overview.....	11
2.6.2. Factors Affecting the Antimicrobial Activity	13
2.6.2.1. Intrinsic Factors	13
2.6.2.2. Extrinsic Factors.....	14
2.6.3. Antimicrobial Mode of Action.....	15
2.7. Methods to Detect Antimicrobial Activity of Chitosan.....	16
2.7.1. Disk Diffusion.....	17
2.7.2. Agar Dilution	18
2.7.3. Broth Microdilution	18
2.7.4. Comparison of Disk Diffusion with Agar Dilution and Broth Microdilution	19
Chapter 3 - Materials and Methods.....	21
3.1. Bacteria Strains and Culture Conditions.....	21
3.2. Chitosan Preparation.....	21
3.3. Antimicrobial Susceptibility Testing	24
3.3.1. Disk Diffusion Assay.....	24
3.3.2. Agar Dilution Assay	25
3.3.3. Broth Microdilution Assay	26
3.4. Data Analysis	27
Chapter 4 - Results.....	28
4.1. Disk Diffusion.....	28

4.2. MICs Generated by Agar Dilution and Broth Microdilution.....	30
Chapter 5 - Discussion	42
Chapter 6 - Summary and Conclusion	48
References.....	50
Vita.....	58

List of Tables

Table 1. Representative Natural Antimicrobials and their Antimicrobial Activities.....	5
Table 2. Principal Sources of Chitin.....	6
Table 3. Representative Bacterial Strains from Different Species	22
Table 4. Five Chitosans with Different Mw and solubility	24
Table 5. Antimicrobial Activity of Chitosan Determined by Disk Diffusion.....	29
Table 6. MIC Range of Chitosan against 36 Bacterial Determined by Agar Dilution and Broth Microdilution	32
Table 7. <i>E. coli</i> MICs Determined by Broth Microdilution and Agar Dilution.....	33
Table 8. <i>Salmonella</i> MICs Determined by Broth Microdilution and Agar Dilution.....	34
Table 9. <i>Vibrio</i> MICs Determined by Broth Microdilution and Agar Dilution.....	35
Table 10. Gram-positive Bacteria MICs Determined by Broth Microdilution and Agar Dilution	36
Table 11. Comparison of Agar Dilution and Broth Microdilution MICs of Five Chitosans.....	40
Table 12. Comparison of Agar Dilution and Broth Microdilution MICs for Different Bacteria .	41

List of Figures

Figure 1. Structures of Chitin and Chitosan.....	8
Figure 2. Disk Diffusion Method Procedures	25
Figure 3. Agar Dilution Method Procedures.....	26
Figure 4. Broth Microdilution Method Procedures.....	27
Figure 5. Effect of Chitosan on the Growth of Different Bacteria by Disk Diffusion	30
Figure 6. MIC ₅₀ and MIC ₉₀ of Five Chitosan Determined by Broth Microdilution	37
Figure 7. MIC ₅₀ and MIC ₉₀ of Five chitosan Determined by Agar Dilution	38

Abstract

Chitosan is a polysaccharide biopolymer with excellent biocompatibility, biodegradability, and low toxicity, which allows for potential wide applications. Recently, antimicrobial activities of chitosan against foodborne pathogens have been studied; many used disk diffusion to determine the activity. However, this method is unable to obtain minimal inhibitory concentrations (MICs), i.e., not quantitative. The objective of this study was to compare disk diffusion with agar dilution and broth microdilution, two quantitative methods used routinely in clinical laboratories, to determine MICs of chitosan against foodborne pathogens. Five chitosan compounds with molecular weights ranging between 43 and 1,100 kDa were tested against 36 representative foodborne pathogens using the three methods. A water-soluble chitosan (43 kDa) was found to be the most effective one against *Escherichia coli* O157:H7 and *Salmonella enterica*, especially using the agar dilution method. The overall agreement of MICs (within 2-fold dilution) between agar dilution and broth microdilution was only 14.6% and MICs determined by broth microdilution were generally lower than those obtained by agar dilution. Among all strains tested, *Vibrio* spp. strains were most susceptible to chitosan whereas *Salmonella* serovars were least susceptible. The MIC values by either agar dilution or broth microdilution for *Vibrio* spp. were at least one dilution level lower than those for other bacteria. The effectiveness of chitosan against *Vibrio* spp. demonstrated in this study may prompt future applications of chitosan to control *Vibrio* spp. in foods, particularly raw oysters. The variability shown when different susceptibility testing methods were used suggests the need to apply multiple methods when conducting *in vitro* antimicrobial susceptibility testing of chitosans.

Chapter 1 - Introduction

Chitosan is a polysaccharide biopolymer derived primarily from chitin, which is widely present in the exoskeleton of crustaceans, such as crab, shrimp, and crawfish (Raafat *et al.*, 2008; Raafat & Sahl, 2009). Chitosan is commercially produced from the crustacean shell wastes through different degrees of deacetylation, which attribute to a variety of properties (Kumar, 2000). Due to its excellent biological characteristics, including biocompatibility, biodegradability, and low toxicity, over the past few years, chitosan has gained multiple applications ranging from pharmaceutical, cosmetic, medical, to food and agricultural field (No *et al.*, 2007; Raafat & Sahl, 2009).

The antimicrobial activities of chitosan have been demonstrated against foodborne Gram-positive and Gram-negative bacteria by many researchers (Tsai & Su, 1999; Jeon *et al.*, 2001; No *et al.*, 2002; Chhabra *et al.*, 2006; Ganan *et al.*, 2009). Because of the demonstrated broad spectrum of activities particularly the high killing rate toward bacteria, much more attention has been paid to the antimicrobial activity of chitosan in recent years (Kong *et al.*, 2010). There are numerous studies that have explored the antimicrobial activity of different chitosan compounds from various sources by employing diverse testing conditions. Therefore, discrepancies in the outcomes obtained in many instances were due to multiple intrinsic and extrinsic factors, such as molecular weight (Mw), the degree of deacetylation (DD), pH, test strains, and among others (Raafat & Sahl, 2009). On the other hand, the use of various testing methods may be yet another factor attributing to the discrepancies in the results of antimicrobial susceptibility testing of chitosan.

Currently, several methods have been applied to measure the *in vitro* susceptibility of bacteria to chitosan, such as agar dilution, broth microdilution, and disk diffusion, which are

standard methods recommended by the Clinical and Laboratories Standards Institute (CLSI) for measuring the *in vitro* susceptibility of bacteria to antimicrobial agents used in clinical settings (CLSI, 2009; CLSI, 2009). Among the three methods, disk diffusion seems to be the most popular method used to examine the antimicrobial activity of natural antimicrobials including chitosan. Although the method is relatively inexpensive and easy to perform, there are several disadvantages. Since disk diffusion measures the inhibition zone size which is then converted to categories of susceptible/intermediate/resistant based on CLSI recommendations (CLSI, 2009), this method is unable to obtain minimal inhibitory concentration (MIC) values (Dickert *et al.*, 1981). Secondly, different from antimicrobial agents used in clinical settings, there are currently no standard CLSI interpretive criteria of disk diffusion results to support natural antimicrobials susceptibility testing; thus, it is unable to explain the zone diameters generated by disk diffusion for natural antimicrobials. Besides, similar to other agar-based methods, disk diffusion is labor-intensive and time-consuming (Klancnik *et al.*, 2010).

In contrast, agar dilution and broth microdilution methods are able to overcome some of the limitations of the disk diffusion method. Not only are they more convenient for routine antimicrobial susceptibility testing of bacteria in clinical laboratories, they are capable of drawing quantitative conclusions by determining the MIC values for antimicrobials, as opposed to qualitative data generated by the disk diffusion method (Kim *et al.*, 2007). Considering the lack of standardized and reliable *in vitro* susceptibility testing methods for chitosan, direct comparison of the results obtained from numerous studies seems impossible. Currently, there is a scarcity of data on the comparative evaluation of these different susceptibility testing methods to determine the antimicrobial activity of chitosan in a single study. The study described in this thesis appears to be the first one where disk diffusion, agar dilution, and broth microdilution

were compared side by side regarding the determination of the antimicrobial activity of chitosan.

The aims of this study were 1) to determine the antimicrobial activity of five chitosan compounds against 36 representative foodborne Gram-positive and Gram-negative pathogens using disk diffusion, agar dilution, and broth microdilution; and 2) to comparatively evaluate the MIC values generated by agar dilution and broth microdilution.

Chapter 2 - Literature Review

2.1. Natural Antimicrobials

Traditional antimicrobials such as sorbate, benzoate, sulfite, etc. have been used as reliable preservatives to control microbial hazards in the food industry for decades (Raybaudi-Massilia *et al.*, 2009). However, these compounds do not satisfy the concept of “natural” or “healthy” foods that consumers are increasingly demanding. With consumers’ growing awareness and concerns regarding chemically synthesized preservatives (traditional antimicrobials), novel and safe natural antimicrobials targeting food pathogens with minimum adverse effects have attracted much more attention (Richard & Patel, 2005). On the other hand, natural antimicrobials can be used as a promising alternative of traditional antimicrobials for preserving foods such as in fresh-cut fruit and fruit juices (Raybaudi-Massilia *et al.*, 2009).

Natural antimicrobials are derived from many sources, ranging from animal (chitosan, lysozyme, and lactoperoxidase) to plant (essential oils, aldehydes, esters, herbs and spices) and to microbial origin (nisin) (Tiwari *et al.*, 2009). Table 1 summarizes representative natural antimicrobials from different origins and their antimicrobial activities.

Regarding the development of natural antimicrobials from the animal origin, many researchers focused on the potential use of chitosan in food preservation. Currently, chitosan has been approved as functional food in some Asian countries, such as Japan and Korea in the last decade (Aranaz *et al.*, 2009). However, it has not been officially proclaimed as GRAS (Generally Recognized As Safe) substances by the U.S. Food and Drug Administration (Raafat & Sahl, 2009).

2.2. Chitin

The name ‘chitin’ was first used by Bradconnot in 1811, which was derived from the

Table 1. Representative Natural Antimicrobials and their Antimicrobial Activities

Origin	Antimicrobials	Spectrum of activity	Application	Reference
Animal	Chitosan	Bacteria, yeast, and mold	Fruits, vegetables, meat, milk, and seafood	(No <i>et al.</i> , 2007)
	Lactoperoxidase	<i>S. Enteritidis</i> , <i>E. coli</i> O157:H7, <i>Shigella</i> spp.	Fruit and vegetable juices	(Touch <i>et al.</i> , 2004; Van Opstal <i>et al.</i> , 2006)
	Lysozyme	<i>L. monocytogenes</i> , <i>C. jejuni</i> , <i>B. cereus</i> and <i>S. Typhimurium</i>	Orange juice	(Liang <i>et al.</i> , 2002; Raybaudi-Massilia <i>et al.</i> , 2009)
Plant	Essential oils	<i>E. coli</i> O157:H7, <i>S. enterica</i>	Apple juice, Apple cider	(Friedman <i>et al.</i> , 2004; Liang <i>et al.</i> , 2006)
Microbial	Nisin	Only gram-positive bacteria, i. e. <i>L. monocytogenes</i> , <i>B. cereus</i> , <i>C.botulinum</i>	Fresh-cut water melon, orange juice	(Raybaudi-Massilia <i>et al.</i> , 2009)

Greek word ‘chiton’, meaning a coat of mail (Lower, 1984; Skaugrud & Sargent, 1990). Chitin, as the second most abundant natural polymers on Earth after cellulose (Shahidi *et al.*, 1999; Singla & Chawla, 2001; No *et al.*, 2007), distributes widely in nature and is mainly present as the structural component of crustacean shells (Raafat & Sahl, 2009). The principal sources of chitin are summarized in Table 2 (Felt *et al.*, 1998). Chitin is an insoluble linear mucopolysaccharide (Raafat & Sahl, 2009) (Fig. 1) composed of 2-acetamido-2-deoxy- β -D-glucose (N-acetylglucosamine) linked by a β (1 \rightarrow 4) bonds (Kumar, 2000). It can be regarded as cellulose with hydroxyl group at C-2 position replaced by acetamino group (Suzuki, 2000).

Table 2. Principal Sources of Chitin

Organism		Chitin content (%)
Crustacean	Crab ^a	72.1
	Shrimp ^a	69.1
	Lobster ^a	69.8
Insects	True fly ^a	54.8
	Sulphur butterfly ^a	64.0
Fungi	<i>Aspergillus niger</i> ^b	42.0
	<i>Mucor rouxii</i>	44.5

^aOrganic weight of cuticle ; ^bDry weight of the cell wall.

Chitin’s immunogenicity is exceptionally low, in spite of the presence of nitrogen. Chitin can be degraded by chitinase. Because of its high insolubility in ordinary solvents, chitin cannot

be isolated by the solvent extraction method. However, chitin is fairly stable in mild acidic and basic conditions, and thus may be obtained as the residue after decomposition of other components by acid and alkali (Kurita, 2006).

2.3. Chitosan

Chitosan is a biomaterial, primarily produced from the alkaline deacetylation (40-50% NaOH) of chitin. Since this N-deacetylation is almost never complete, chitosan is considered as a partially N-deacetylated derivative of chitin (Kumar, 2000). Advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan (Rabea *et al.*, 2003). Structurally, chitosan is a high-molecular-weight linear heteropolysaccharide composed of a β (1 \rightarrow 4) linked two monosaccharides, N-acetyl-D-glucosamine and D-glucosamine (Raafat & Sahl, 2009) (Fig. 1). Chitosan has three types of reactive functional groups, an amino group as well of both primary and secondary hydroxyl groups at the C-2, C-3, and C-6 positions, respectively (Furusaki *et al.*, 1996). The varied proportion of the two monosaccharides in chitosan contributes to different properties, such as degrees of deacetylation (DD; 75-95%), molecular weights (Mw; 50-2,000 kDa), viscosities, pKa values and so on (Singla & Chawla, 2001). However, these properties can greatly influence its physicochemical characteristics and directly affect its application (Raafat & Sahl, 2009). In contrast to chitin's insolubility, the presence of free amine groups along the chitosan chain allows it to dissolve in diluted acids such as acetic acid, lactic acid, and formic acid due to the protonation of these groups, rendering the corresponding chitosan salt soluble. Therefore, there are important experimental variables that should be taken into account when working with chitosan solutions such as the nature of the salt counter-ion, DD, Mw, pH, ionic strength, and the addition of non-aqueous solvents (Aranaz *et al.*, 2009).

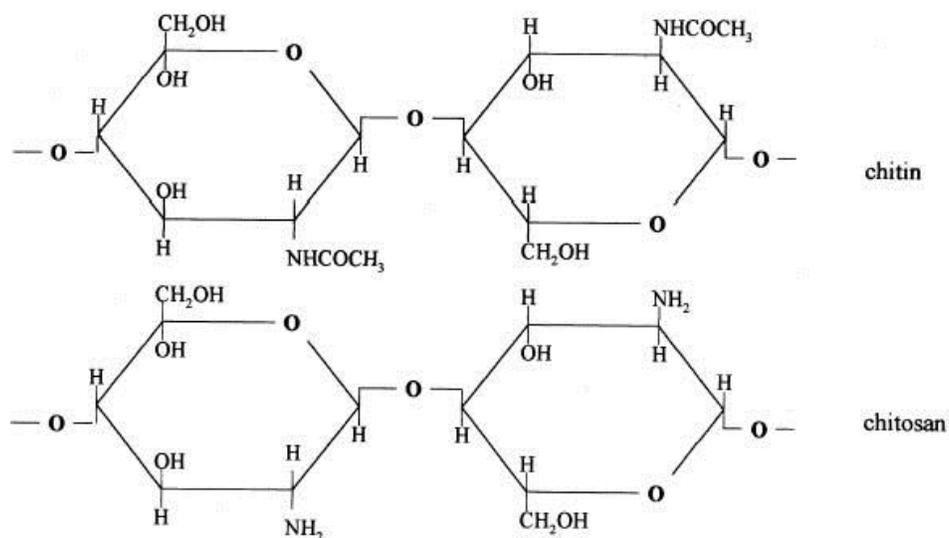


Figure 1. Structures of Chitin and Chitosan (Raafat & Sahl, 2009)

2.4. Biological Properties of Chitosan

Chitosan and its derivatives have attracted much commercial interest with regards to medical, pharmaceutical, and industrial applications due to the possession of several interesting properties: biodegradability, biocompatibility, and low toxicity (No *et al.*, 2007; Raafat & Sahl, 2009). Additionally, other properties such as analgesic, antitumor, hemostatic, hypocholesterolemic, antimicrobial, and antioxidant properties of chitosan have also been reported (Koide, 1998; Kumar, 2000; Kumar *et al.*, 2004).

2.4.1. Biodegradability

Traditionally, several methods can be used to produce chitosan oligomers, such as physical, chemical, and enzymatic methods. Physical methods including ozone treatment and ultraviolet radiation is preferred due to their accelerated degradation of chitosan (Yue *et al.*, 2009). Chitosan is absent from mammals but it can be degraded *in vitro* by several non-specific enzymes from a variety of sources, such as lysozymes, pepsin, papain, cellulase, pectinase,

proteases, and lipases (Pantaleone *et al.*, 1992; Darmadji & Izumimoto, 1994; Yalpani & Pantaleon, 1994; Kumar *et al.*, 2005). Moreover, it also can be catalyzed by chitosanases (chitosan *N*-acetyl-glucosamino-hydrolases). The biodegradation of chitosan leads to the release of non-toxic oligosaccharides of variable lengths which can be subsequently incorporated into glycosaminoglycans and glycoproteins, to metabolic pathways or be excreted (Pangburn *et al.*, 1982).

Chitosan degradation kinetics seems to be inversely related to the degree of crystallinity that is controlled mainly by the DD value. Moreover, the distribution of acetyl groups also affects biodegradability since the absence of acetyl groups or their homogeneous distribution (random rather than block) results in very low rates of enzymatic degradation (Aiba, 1992; Francis *et al.*, 2000).

Finally, several studies investigated the relationship between degradation and the DD value. It seems that degradation rate increases while the DD value was decreased. (Hirano *et al.*, 1989; Sashiwa *et al.*, 1991; Kurita *et al.*, 2000). For example, Kofuji *et al.* (2005) investigated the enzymatic behaviors of various chitosans by observing changes in the viscosity of chitosan solution in the presence of lysozyme, and found chitosan with a low DD tended to be degraded more rapidly. However, other authors reported that differences in degradation are due to variations in the distribution of acetamide groups in the chitosan molecule (Sashiwa *et al.*, 1991; Aiba, 1992; Shigemasa *et al.*, 1994). This occurs due to differences in deacetylation conditions, which influences viscosity of the chitosan solution by changing the inter- or intra-molecular repulsion forces (Sashiwa *et al.*, 1991). Therefore, the biodegradation rate of chitosan cannot be estimated from the DD value alone.

2.4.2. Biocompatibility

Another attractive biological property of chitosan is its biocompatibility. For example, the function of chitosan is not affected by the host and it does not elicit any undesirable local or systemic effects. Chitosan is well tolerated by live tissues, including the skin, ocular membranes, as well as the nasal epithelium (Shigemasa & Minami, 1995). However, this property also depends on the characteristics of the sample (natural source, method of preparation, Mw, and DD, etc.).

2.4.3. Low Toxicity

Low toxicity is another attractive feature of chitosan compared with other natural polysaccharides. Chitosan has an LD₅₀ of around 16 g/kg, very similar to that for salt and glucose by *in vivo* toxicity assays carried out on mice (Singla & Chawla, 2001). Nevertheless, people with shellfish allergy should be contraindicated. It is reported that toxicity of chitosan is dependent on the DD value. In a previous study, chitosans with DD values higher than 35% showed low toxicity, while a DD value under 35% caused dose-dependent toxicity (Aiba, 1992).

2.5. Economic Aspects and Regulatory Status

2.5.1. Economic Aspects

Louisiana boasts one of the most vibrant seafood industries in the nation. Annually, the economic benefits of commercial seafood in Louisiana amount to \$2.4 billion, contributing significantly to the state's economy (Louisiana Department of Wildlife and Fisheries, 2008). The newly released Louisiana Summary of Agriculture & Natural Resources reported that 107.5 million, 47.5 million, and 13.7 million pounds of shrimp, crabs, and oysters were landed in 2009, valued at \$115 million, \$33 million, and \$46.5 million, respectively (Louisiana State University Agricultural Center, 2011). The commercial production of chitin and chitosan is mostly obtained

from crustacean shells waste, such as shrimps, crabs, lobster, crawfish, etc. which is economically feasible and ecologically desirable because large amounts of shell wastes are available as a by-product of the seafood industry. Chitosan has been commercially produced in North America, India, Japan, Poland, Norway and Australia (Kumar, 2000; Raafat & Sahl, 2009).

2.5.2. Regulatory Status

Until now, chitosan has been approved as food additive or supplement in Japan, Korea, England, Italy, Portugal, and the United States (Novack et al., 2003; No et al., 2007). However, it has not been officially proclaimed as GRAS substances by the U.S. FDA (Raafat & Sahl, 2009).

2.6. Antimicrobial Activity of Chitosan

2.6.1. Overview

With the consumers' increasing demand for more natural and safer food without chemical preservatives, the applications of novel natural antimicrobials has attracted much more attention in recent years. In that regards, much attention has been focused on the safety and efficiency of chitosans from animal origin as the natural antimicrobials. The antimicrobial activity of chitosan has been observed in a wide variety of microorganisms, including bacteria, yeast, and fungi (Rabea *et al.*, 2003). Moreover, chitosan has several advantages over other types of natural antimicrobials, such as higher antimicrobial activity, broader spectrum of activity, higher killing rate and lower toxicity toward mammalian cells (Rabea *et al.*, 2003). For example, one study suggested that chitosan could be used as an antimicrobial preservative in emulsion formulations for mucosal as well as for parenteral applications (Jumaa *et al.*, 2002). Similarly, another study proposed that chitosan be used as an adjunct in the potentiation of antimicrobial effect of benzonates and others (Sagoo *et al.*, 2002). In addition, chitosan was found capable of

potentiating the antimicrobial activity of a number of preservatives, such as phenethyl alcohol, benzoic acid, and phenylmercuric acetate against numerous bacteria strains (Raafat & Sahl, 2009).

The reported minimum inhibitory concentrations (MICs) of chitosan vary widely with the bacteria, ranging from 0.005% to 1.5% (w/v) (Shahidi *et al.*, 1999; Jeon & Kim, 2000; No *et al.*, 2002). Chitosan has been shown to inhibit both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Vibrio* spp., and *Salmonella* Typhimurium. Wang *et al.* (1992) reported that a much higher concentration of chitosan (1-1.5%) is required for complete inhibition of *S. aureus* after two days of incubation. Another study found that chitosan concentrations (>0.005%) were sufficient to complete inactivation of *S. aureus* (Shahidi *et al.*, 1999). Simpson *et al.* (1997) found that 0.02% of chitosan was required to inhibit *B. cereus* growth, while it also reported that this bacteria can be inactivated by chitosan in another study (Shahidi *et al.*, 1999). Numerous studies have shown the effect of chitosan on *E. coli* inhibition. Darmadji and Izumimoto *et al.* (1994) reported that chitosan with concentration of 0.1% was required to inhibit *E. coli* growth in meat preservation. But another showed that lower concentration (0.0075%) of chitosan was enough to inhibit the *E. coli* growth (Simpson *et al.*, 1997). Moreover, concentrations of 0.5 or 1% of chitosan was capable of completely inactivating the *E. coli* growth at pH 5.5 (Wang, 1992). Besides, No *et al.* (2002) found that *V. parahaemolyticus* growth was effectively inhibited by 1-3 log cycles at a 0.1% concentration of chitosan with molecular weight of 470 kDa while *L. monocytogenes* was completely inhibited at 0.1% concentration by chitosan of Mw=746 kDa.

2.6.2. Factors Affecting the Antimicrobial Activity

There are numerous studies exploring the antimicrobial activity of different chitosans from various sources using diverse testing conditions. Discrepancies in the results obtained in those studies were observed. No *et al.* (2002) reported that chitosan (0.1%) was more effective in inhibiting Gram-positive than Gram-negative bacteria. In another study, chitosan had stronger antimicrobial activity against *S. aureus* than *S. enterica* and *V. vulnificus*, suggesting that chitosan is more effective at inhibiting Gram-positive than Gram-negative bacteria (Chhabra *et al.*, 2006). In direct contrast, Helander *et al.* (2001) demonstrated that chitosan presented a higher antimicrobial activity against Gram-negative than Gram-positive bacteria.

On the one hand, chitosan's *in vitro* antimicrobial activity is dependent upon various intrinsic and extrinsic factors, such as Mw, DD, viscosity, solvent, pH, test strains, temperature, and metal ions etc. (Raafat & Sahl, 2009). On the other hand, methodologies applied in varied studies will be another factor contributing to different results of antimicrobial activity of chitosan.

2.6.2.1. Intrinsic Factors

Although many studies have explored the intrinsic and extrinsic factors affecting the antimicrobial activity of chitosan, it is still difficult to pinpoint the influence of Mw or the DD value on the antimicrobial activity of chitosan. For example, it was reported that chitosan possessed a higher antimicrobial activities with decreasing Mw for gram-negative bacteria, not for gram-positive bacteria (No *et al.*, 2002). In the same study, it found that the minimal inhibitory concentrations (MICs) of chitosans ranged from 0.05% to above 0.1% for different bacteria tested and Mw of chitosan used. It was suggested that chitosan with Mw of 470 kDa was more effective for inhibiting the growth of Gram-negative bacteria, whereas that with 1,106 kDa

was less effective. For Gram-positive bacteria, chitosan with Mw of 470 kDa was less effective. Another study (Jeon *et al.*, 2001) demonstrated that low Mw chitosans (5-10 kDa) showed the strongest antimicrobial activity against pathogenic bacteria. Zheng *et al.* (2003) reported that among 5 chitosans with Mw less than 300 kDa, the antimicrobial activity against *S. aureus* was strengthened as the Mw increased, while the effect on *E. coli* was weakened which was in agreement with Chen *et al.* (1998). 2006 study that the antimicrobial activity of low Mw chitosan is higher than the high Mw samples against *E. coli*. Another study (Shin *et al.*, 1997) showed that chitosan with Mw of 40 kDa could inhibit the growth of 90% of *S. aureus* and *E. coli* at a concentration of 0.5% and chitosan with Mw of 180 kDa could almost completely inhibit the growth of *S. aureus* and *E. coli* at a concentration of 0.05%. It has been suggested by Jeon *et al.* (2001) that an Mw of more than 10 kDa is required for proper inhibition of microorganisms by chitosans. It was also reported that *Campylobacter* spp. were the most sensitive microorganisms to chitosan, and the MIC of chitosan for *Campylobacter* ranged from 0.005 to 0.05% (Raybaudi-Massilia *et al.*, 2009). Moreover, according to studies by Shigemasa *et al.* (1995) and Liu *et al.* (2001), chitosans with a high DD were more effective than those with a low DD value in inhibiting the growth of microorganisms, which probably was due to the higher percentage of protonated amine groups.

2.6.2.2. Extrinsic Factors

The antimicrobial activity of chitosan is inversely influenced by pH, i.e., stronger antimicrobial activity was observed at lower pH. Based on the studies of Liu *et al.* (2006), No *et al.* (2002), and Rabea *et al.* (2003), chitosan showed its antimicrobial activity only in an acidic medium, which was caused by the poor solubility of chitosan at pH above 6.5. The reason that requiring a pH at least 6.5 for chitosan to maintain its antimicrobial activity may be due to the

presence of predominantly positive-uncharged amino groups as well as poor solubility of chitosan (Papineau *et al.*, 1991; Sudharshan *et al.*, 1992).

The alternation of ionic strength in a medium may affect the antimicrobial activity of chitosan (Raafat & Sahl, 2009). However, results varied from different studies. For example, one study reported that the presence of divalent cations reduces the antimicrobial activity of shrimp chitosan against *E. coli* (Tsai & Su, 1999), likely because the increase of metal ions, especially divalent ions, could decrease the chelating capacity of chitosan (Kong *et al.*, 2010). In contrast, Chung *et al.* (2003) suggested that the higher ionic strength could enhance the solubility of chitosan and therefore increase its antimicrobial activity. It is probably due to existing cations in medium may interact with the negative-charged components mainly on the cell wall of bacteria besides polycationic chitosan, consequently weakening the antimicrobial activity.

2.6.3. Antimicrobial Mode of Action

The exact mechanism of the antimicrobial action of chitosan remains to be elucidated, but several factors influence the antimicrobial activities of chitosan. The mode of antimicrobial action of chitosan is discussed below.

The polycationic nature of chitosan ($pK_a = 6.3$) is prerequisite for antimicrobial activity. As pH is below the pK_a of chitosan, electrostatic interaction between the polycationic structure (NH_3^+ groups of glucosamine) and the predominantly anionic components of the microorganisms' surface (such as Gram-negative lipopolysaccharides and cell surface proteins) plays a very important role in the antimicrobial activity of chitosan. Eventually, the interaction between the positively charged NH_3^+ groups and the negatively charged microbial cell surface contribute to the leakage of protein and other intracellular components of the microbial cells, ultimately resulting in the impairment of vital bacteria activities (Muzzarelli *et al.*, 1990;

Helander *et al.*, 2001; Je & Kim, 2006; Raafat *et al.*, 2008; Kong *et al.*, 2010). The number of amino groups linking to C-2 on chitosan backbones is important in electrostatic interaction, which indicate that large amount of amino groups are capable of enhancing the antimicrobial activity. Therefore, chitosan with higher DD shows a stronger inhibitory effect than that of a lower DD chitosan (Kong *et al.*, 2010).

The different MWs of chitosan and its physical states render distinctive modes of antimicrobial action. LMw water-soluble chitosan was able to penetrate cell wall of bacteria and then interact with DNA and inhibit synthesis of mRNA and DNA transcription (Sudharshan *et al.*, 1992). For HMw water-soluble chitosan and solid chitosan could only interact with cell surface without penetrating into the cell wall and lead to altering cell permeability or form an impermeable layer around the cell, thus blocking the transport of essential solutes into the cell (Kong *et al.*, 2010).

2.7. Methods to Detect Antimicrobial Activity of Chitosan

The antimicrobial activities of chitosan may be determined using three main methods, agar dilution, broth microdilution, and disk diffusion which are standard methods recommended by Clinical and Laboratories Standards Institute (CLSI) for measuring *in vitro* susceptibility of bacteria to antimicrobial agents used in clinical settings (CLSI, 2009; CLSI, 2009). Since these methods apply different principles, the results obtained may differ. Besides methods, antimicrobial susceptibility testing results can also be affected by many other factors, such as the microorganisms tested and the degree of solubility of each test-compound (Valgas *et al.*, 2007). Among the three methods, disk diffusion has been the most popular one used to examine the antimicrobial activity of natural antimicrobials including chitosan (Kim & Kim, 2007; Mayachiew *et al.*, 2010). Below is an overview of the methods.

2.7.1. Disk Diffusion

The disk diffusion method allows for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner. In this method, the bacterial inoculum is adjusted to certain concentration, inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate with a sterile cotton-tipped swab to form an even lawn. The paper disks (6 mm in diameter; BD Diagnostic Systems) impregnated with diluted antibiotic solution was placed on the surface of each MHA plate using a sterile pair of forceps. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler or caliper. Based on the diameter of the inhibition zone and the CLSI interpretative criteria, the results are then assigned to three categories, susceptible, intermediate, or resistant. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial. The major disadvantages of this method are unable to generate the MIC value (i.e., not quantitative) and difficult to examine the susceptibility of fastidious and slow-growing bacteria (Wilkins & Thiel, 1973; Dickert *et al.*, 1981). Moreover, different from antimicrobial agents used in clinical settings, there are currently no standard CLSI interpretive criteria of disk diffusion results to support natural antimicrobials susceptibility testing; thus, it is unable to explain the zone diameter generated by disk diffusion for natural antimicrobials. Besides, similar with other agar-based methods, disk diffusion is labor-intensive and time-consuming (Klancnik *et al.*, 2010). In many previous studies, disk diffusion was used to determine the antimicrobial activities of chitosan (Kulkarni *et al.*, 2005; Pranoto *et al.*, 2005; Coma *et al.*, 2006; Kim & Kim, 2007; Cao *et al.*, 2009; Mayachiew *et al.*, 2010); however, chitosan was reported to be effective against bacteria, yeast, and fungi without mentioning MIC values. Also, it has been reported (Klancnik *et al.*, 2010) that this method is not always reliable for determining the antimicrobial activity of

natural antimicrobials, i.e., plant extract, because the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium. Compounds with less polarity diffused slower than more polar ones (Moreno *et al.*, 2006). Due to these concerns, disk diffusion may not be a suitable one to determine the antimicrobial activity of natural compounds.

2.7.2. Agar Dilution

Agar dilution is a quantitative susceptibility testing method because MIC values can be obtained using the method. In this method, two-fold serial dilutions of an antibiotic made in Mueller-Hinton agar (MHA) medium and then bacterial suspensions were inoculated on the MHA using a Cathra replicator with 1 mm pins, as recommended by the CLSI (CLSI, 2009). It has been studied extensively as a recommended reference method for the bacteria growing aerobically (CLSI, 2009). The advantages of agar dilution include the ability to simultaneously test the susceptibility of a number of bacteria in one plate and the ability to test susceptibility of fastidious organisms since the agar with supplements is able to adequately support the bacteria growth. Moreover, as mentioned above, the test results yield MIC values for testing bacteria. However, agar dilution is not commonly used in most microbiology laboratories due to the time-consuming and labor-intensive.

2.7.3. Broth Microdilution

Broth microdilution is another quantitative reference method routinely used in clinical laboratories. In this method, susceptibility panel in 96-well microtiter plates were containing various concentration of antimicrobial agents. Then, standardized numbers of bacteria was inoculated into the wells of 96-well microtiter and incubate overnight at 35°C. The MIC value was observed as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation. It is a widely utilized method, allowing for the simultaneous

testing of multiple antimicrobials with ease particularly when commercially prepared microtiter trays are used. Compared with agar-based method, broth microdilution can decrease much labor and time. However, limitations of the method primarily are associated with the lack of or poor growth of many anaerobic microorganisms. Testing some fastidious anaerobes gives inconsistent and unreliable results because of poor growth of strains due to excessive exposure to oxygen during the set-up procedure (CLSI, 2009).

2.7.4. Comparison of Disk Diffusion with Agar Dilution and Broth Microdilution

Compared with disk diffusion, agar dilution and broth microdilution are found to overcome some limitations of the disk diffusion method, primarily that capability to draw quantitative conclusion by determining the MIC value for antimicrobials (Kim *et al.*, 2007). As such, both agar dilution and broth microdilution are conveniently used for routine antimicrobial susceptibility testing in clinical laboratory.

In studies examining antimicrobial susceptibility of natural antimicrobials including chitosans, disk diffusion seems to be the most popular method used (Kulkarni *et al.*, 2005; Pranoto *et al.*, 2005; Coma *et al.*, 2006; Kim & Kim, 2007; Cao *et al.*, 2009; Mayachiew *et al.*, 2010). For example, Kim *et al.* (2007) evaluated the inhibitory effect of chitosan against *E. coli* and *S. Typhimurium* with disk diffusion method, which suggested that chitosan as a natural bioactive was able to use as a natural antimicrobial for improvement of food safety. Another study adopted the disk diffusion method to determine the antimicrobial activity of chitosan in oyster preservation and the data showed that the chitosan treatment could extend the shelf-life of oysters from 8-9 days to 14-15 days (Cao *et al.*, 2009). Moreover, Mayachiew *et al.* (2010) studied the antimicrobial activity of chitosan film enriched with the galangal extract by disk diffusion and broth macrodilution methods. However, agar dilution and broth microdilution were

also used to examine the antimicrobial activity of chitosan, especially for the MIC determination. In a recent study (Limam *et al.*, 2011), the antimicrobial susceptibility testing of chitosan against *E. coli*, *P. aeruginosa*, *S. aureus* was carried out by broth microdilution method (MIC, 0.156 to 5mg/ml), except *P. aeruginos* which was the most resistant bacteria tested. Furthermore, by dilution method used in the study (No *et al.*, 2002) to determine the antimicrobial activity of chitosan and oligomers, MIC of chitosans ranged from 0.05% ->0.1% varied from the bacteria and Mws of chitosan.

However, considering the lack of standardized and reliable *in vitro* susceptibility testing methods for chitosan, direct comparison of the results obtained from numerous studies seems impossible. Currently, there is a scarcity of data on the comparative evaluation of these different susceptibility testing methods to determine the antimicrobial activity of chitosan in a single study. Additionally, previous studies determining antimicrobial activity of chitosan used only small numbers of bacterial strains in each genus/species.

The aims of this study were 1) to determine the antimicrobial activity of five chitosan compounds (three acid-soluble chitosans with Mws of 1100, 444, and 223 kDa and two water-soluble chitosans with Mws of 67 and 43 kDa) against 36 representative foodborne Gram-positive and Gram-negative pathogens using agar dilution, broth microdilution, and disk diffusion; and 2) to comparatively evaluate the MIC values generated by agar dilution and broth microdilution.

Chapter 3 - Materials and Methods

3.1. Bacteria Strains and Culture Conditions

The thirty-six bacterial strains (Table 3) used in this study were selected from our strain collection, which included 31 Gram-negative bacteria (*Acinetobacter calcoacticus*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Vibrio cholera*, *Vibrio fluvialis*, *Vibrio harveyi*, *Vibrio mimicus*, *Vibrio parahaemolyticus*, and *V. vulnificus*) and 5 Gram-positive bacteria (*Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococci faecalis*). All *Vibrio* strains were grown on tryptic soy agar (TSA; BD Diagnostic Systems, Sparks, MD) supplemented with 2% NaCl, while the rest strains were grown on TSA. Cultures were incubated at 35°C for 24 h.

3.2. Chitosan Preparation

Five chitosan compounds (Mw = 1,100, 444, 223, 67, 43 kDa; designated 1-5; Table 4) prepared from crab shell waste were purchased from Kitto Life (1-3; Seoul, Republic of Korea), and Keumho Chemical (4 and 5; Seoul, Republic of Korea). The first three compounds (1-3) were acid-soluble while the last two (4 and 5) were water-soluble. The compounds were placed in separate vials and dried in an oven under 60°C before the experiment.

Chitosans were dissolved in lactic acid (1%, v/v) or water to obtain a stock solution (10%, w/v). The pH values for all chitosan solutions were adjusted to 5.9 (No *et al.*, 2002) with 1 N HCl (Sigma-Aldrich, St. Louis, MO) and 1 N NaOH. The stock solutions were filter sterilized through a 0.2 µm filter (BD Diagnostic Systems), kept in the refrigerator, and diluted in water and acid before use.

Table 3. Representative Bacterial Strains from Different Species

Strain group	Strain ID and serotype	Source and reference
Gram-negative		
<i>Acintobacter calcoacticus</i>	ATCC 19606	Unknown
<i>Citrobacter freundii</i>	ATCC 8090	Unknown
<i>Enterobacter aerogenes</i>	ATCC 13048	Sputum, South Carolina Dept. of Health and Environmental Control
<i>Escherichia coli</i>	P132	Unknown
<i>Escherichia coli</i>	K-12	Unknown
<i>Escherichia coli</i>	HB101	Unknown
<i>Escherichia coli</i>	B6914; O157:H7	Unknown
<i>Escherichia coli</i>	933; O157:H7	Unknown
<i>Escherichia coli</i>	EC06; O157:H7	Unknown
<i>Escherichia coli</i>	EC13; O157:H7	Human
<i>Escherichia coli</i>	UMD 66; O157:H7	Unknown
<i>Escherichia coli</i>	ATCC 25922	Clinical isolate
<i>Escherichia coli</i>	ATCC 35218	Canine, Tennessee
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Blood culture
<i>Salmonella enterica</i>	H9812; Braenderup	Unknown
<i>Salmonella enterica</i>	LT2; Typhimurium	Unknown
<i>Salmonella enterica</i>	UMD373; Typhimurium	Unknown
<i>Salmonella enterica</i>	50; Enteritidis	Chicken isolate, our lab

(Table 3 continued)

<i>Salmonella enterica</i>	119; Kentucky	Chicken isolate, our lab
<i>Vibrio cholera</i>	ATCC 14035	Unknown
<i>Vibrio fluvialis</i>	ATCC 33809	Unknown
<i>Vibrio harveyi</i>	BB120	Unknown
<i>Vibrio harveyi</i>	BB152	Unknown
<i>Vibrio mimicus</i>	ATCC 33653	Ear, 35-year-old female, North Carolina
<i>Vibrio parahaemolyticus</i>	ATCC 33847	Gastroenteritis, Maryland
<i>Vibrio parahaemolyticus</i>	ATCC 49529	Feces, Berkeley, CA
<i>Vibrio parahaemolyticus</i>	NY 477; O4:K8	Oyster, New York
<i>Vibrio vulnificus</i>	ATCC 27562	Blood, Florida
<i>Vibrio vulnificus</i>	ATCC 33815	Ulcer of cornea
<i>Vibrio vulnificus</i>	WR1	Sea water, Washington
<i>Vibrio vulnificus</i>	225	Our oyster isolate, Louisiana
Gram-positive		
<i>Enterococcus faecalis</i>	ATCC 29212	Urine
<i>Enterococcus faecalis</i>	ATCC 19433	Unknown
<i>Listeria monocytogenes</i>	ATCC 19112; 2	Spinal fluid of man, Scotland
<i>Listeria monocytogenes</i>	ATCC 13932; 4b	Spinal fluid, Germany
<i>Staphylococcus aureus</i>	ATCC 29213	Wound

Table 4. Five Chitosans with Different Mw and solubility

ID	Mw (kDa)	Solution	Stock conc.
1	1,100	Lactic acid	10%
2	444	Lactic acid	10%
3	223	Lactic acid	10%
4	67	Water	10%
5	43	Water	10%

3.3. Antimicrobial Susceptibility Testing

For antimicrobial susceptibility methods described below, the bacterial suspensions were prepared by suspending 3-5 well-isolated colonies from appropriate agar plates into 3 ml cation-adjusted Mueller Hinton broth (CAMHB; BD Diagnostic Systems, adjusted to pH 5.9) (No *et al.*, 2002) and the turbidity was adjusted equivalent to a 0.5 McFarland standard. For disk diffusion and broth microdilution, 100 μ l of the 0.5 McFarland suspension was further diluted into 10 ml of CAMHB, which was used as the final inoculum. For agar dilution, no further dilution was applied. Based on the preliminary test data (not shown), concentrations of chitosan ranged from 0.03-1% for agar dilution. For broth microdilution, concentrations of three acid-soluble chitosans were 0.0015-0.4%, while the two water-soluble chitosans were 0.003~1.6% (no. 4, Mw of 67 kDa) and 0.003-0.8% (no. 5, Mw of 43 kDa).

3.3.1. Disk Diffusion Assay

For the disk diffusion method (Figure 2), the bacterial suspension prepared above was inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate (pH 5.9) with a sterile cotton-tipped swab to form an even lawn. Eight sterile paper disks (6 mm in diameter; BD

Diagnostic Systems) impregnated with 20 µl diluted chitosan solution (0.4 and 0.8% for no.1-3, 3.2% for no. 4 and 1.6% for no.5 chitosan, respectively) were placed on the surface of each MHA plate using a sterile pair of forceps. The plates were incubated aerobically at 37°C for 24 h. The diameter of inhibition zone was measured after 24 h incubation using a ruler or caliper.

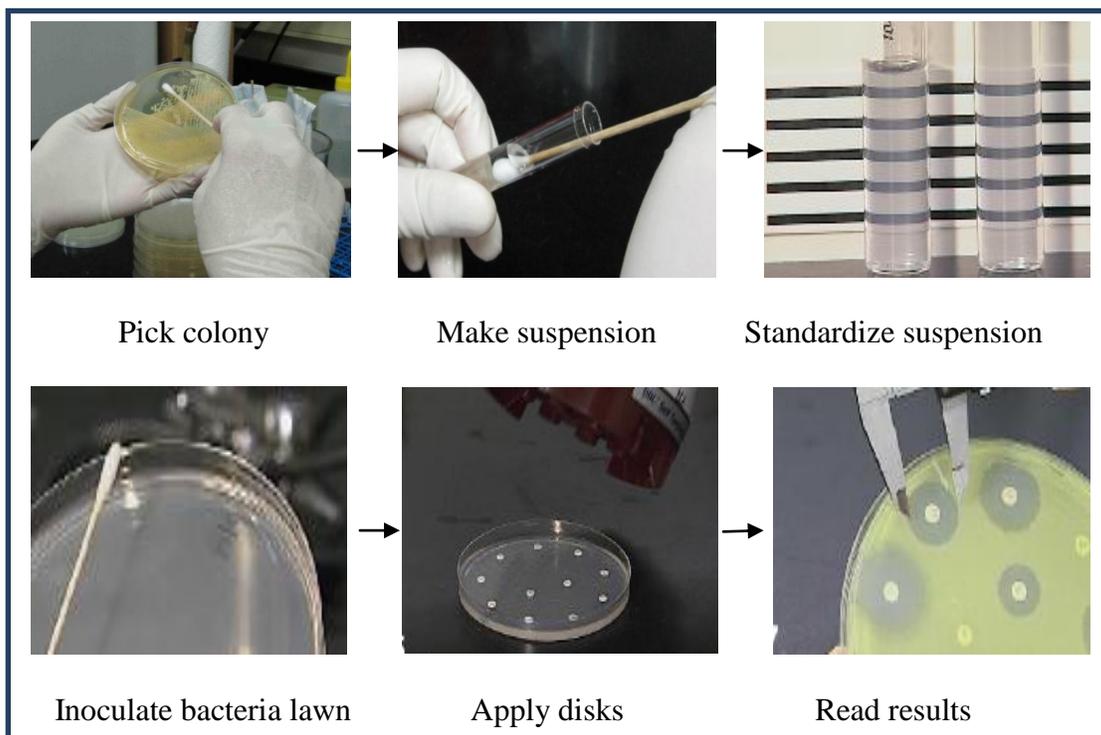


Figure 2. Disk Diffusion Method Procedures

3.3.2. Agar Dilution Assay

For agar dilution (Figure 3), two-fold serial dilutions of chitosan were made in molten tempered (45°C) Mueller-Hinton agar (MHA) medium (pH 5.9) to obtain the desired final concentrations of 1%, 0.5%, 0.25%, 0.125%, 0.06%, and 0.03% by mixing the agar and chitosan solutions thoroughly. Bacterial suspensions were inoculated on the MHA plates using a Cathra replicator (Oxoid, Lenexa, KS) with 1 mm pins, as recommended by the CLSI (CLSI, 2009). The plates were incubated aerobically at 37°C for 24 h. MICs of chitosans were recorded as the lowest concentration of chitosan that completely inhibit bacterial growth.

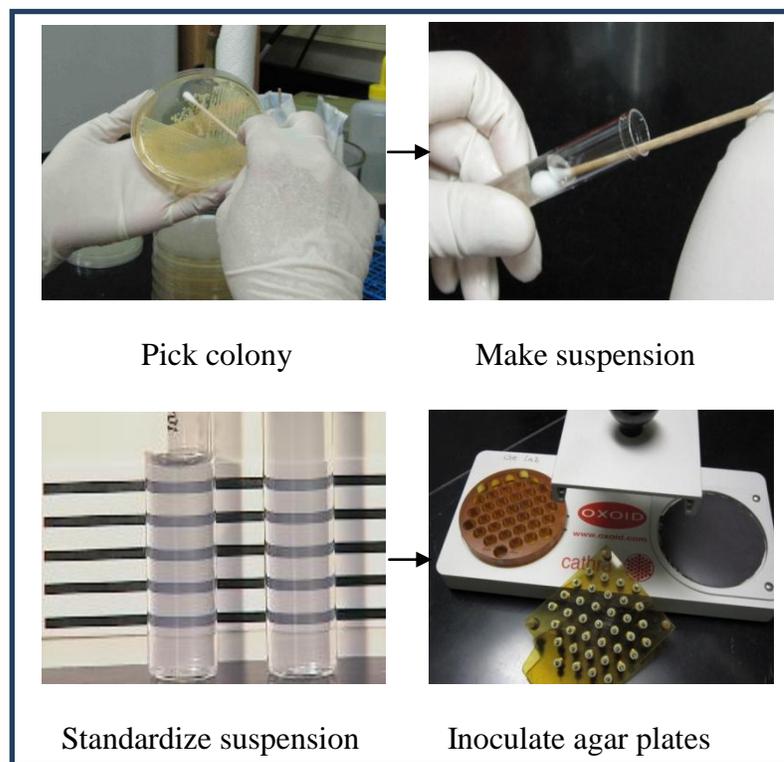


Figure 3. Agar Dilution Method Procedures

3.3.3. Broth Microdilution Assay

For broth microdilution (Figure 4), susceptibility panel in 96-well microtiter plates (Fisher Scientific, Illinois, IL) were prepared by dispensing 100 μ l of chitosan solutions with the highest concentrations into the first column wells and 50 μ l of CAMHB (pH 5.9) into the rest wells by an 8-channel pipette. Then, the two-fold serial dilutions of chitosan solutions were made by drawing up 50 μ l of chitosan solution in the first column wells into the second column and then move on to the next column to achieve the final concentrations. Aliquots (50 μ l) of each bacterial suspension were inoculated into wells of the microtiter plates to obtain a final volume of 100 μ l in each well of the plate. The last two wells were positive and negative controls, respectively. The positive control was inoculated with bacterial suspension only, while the negative well was left blank without inoculation. The 96-microwell plates were sealed using a perforated plate seal (TREK Diagnostic Systems Inc., Cleveland, OH) and incubated at 37°C for

24 h. The MICs of chitosans were recorded as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation for 24 h.

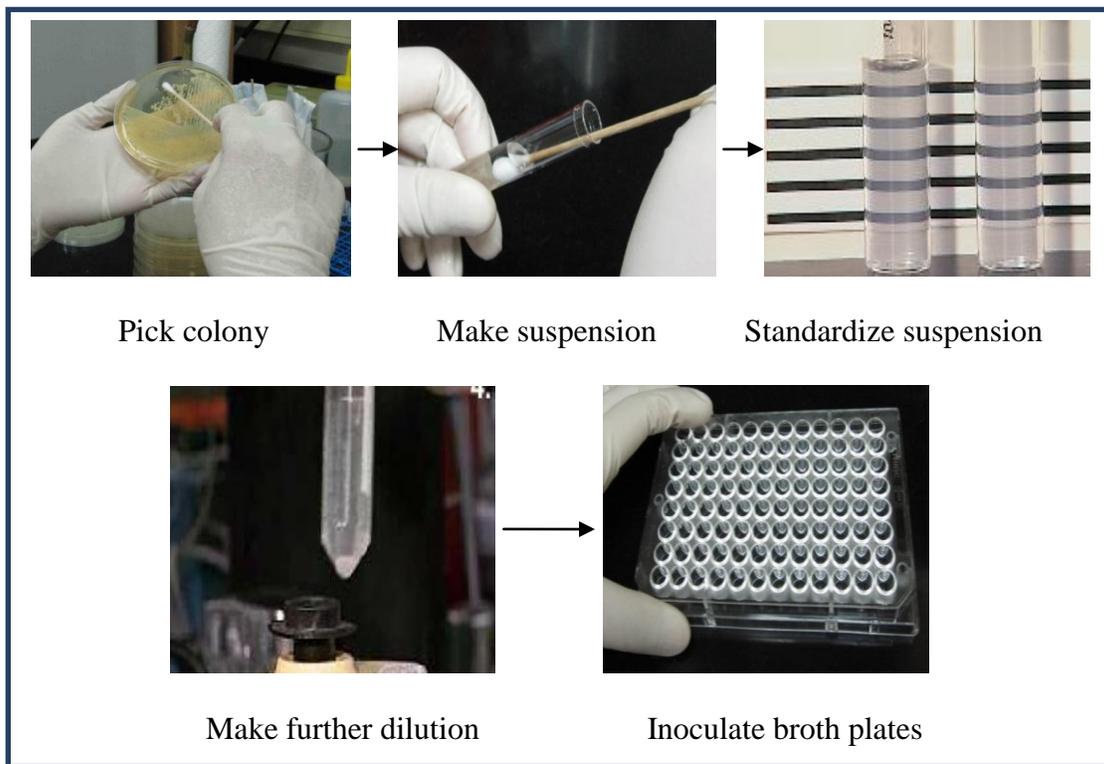


Figure 4. Broth Microdilution Method Procedures

3.4. Data Analysis

All experiments were conducted in triplicate, and mean values and standard deviations of the diameter of inhibition zone in disk diffusion assay were calculated from the experimental data obtained. Mean significance of inhibition zone diameter for different bacterial groups was analyzed using analysis of variance (ANOVA; SAS for Windows, version 9; SAS Institute Inc., Cary, NC). Differences between the mean values were considered significant when $P < 0.05$. MIC agreement between agar dilution and broth microdilution was defined as the same MIC $\pm 2 \log_2$ dilution. Off-scale MIC results obtained from both methods were not included in the agreement calculation.

Chapter 4 - Results

4.1. Disk Diffusion

Antimicrobial activity of chitosans was evaluated based on the diameters of clear inhibition zone surrounding the paper disks. If there is no inhibition zone, it is assumed that there is no antimicrobial activity. Fig. 5 shows representative disk diffusion plates with different bacteria after 24 h incubation. The diameter of inhibition zone of *Vibrio parahaemolyticus* is larger than that of *Escherichia coli*, indicating *V. parahaemolyticus* is more susceptible to chitosan solution than *E. coli*. Table 5 showed the antimicrobial activity of five chitosans with different Mw against *E. coli* strains, *Salmonella enterica* serovars, *Vibrio* spp. and other Gram-negative and Gram-positive bacterial strains by disk diffusion. With regards to diameters of the inhibition zones, chitosans 1-5 all demonstrated effective inhibition on the growth of these bacteria. Among 36 bacteria, *Vibrio* spp. strains were significantly more susceptible while *Salmonella enterica* serovars were more resistant ($P < 0.05$). The average size of inhibition zones varied from 7.12 to 10.68 mm against *E. coli*, 9.39 to 11.44 mm against *Vibrio* spp., and 9.08 to 11.45 mm against Gram-positive bacteria. However, chitosans 1-4 showed weak to no inhibition effect on the growth of *Salmonella enterica* serovars, because small or no inhibition zone was observed. In contrast, chitosan 5 (Mw = 43 kDa) showed a higher antimicrobial activity ($P < 0.05$) at concentration of 1.6 % had clear inhibition zones. The inhibition zone diameter was not significant different ($P > 0.05$) for *Vibrio* spp. among five chitosans.

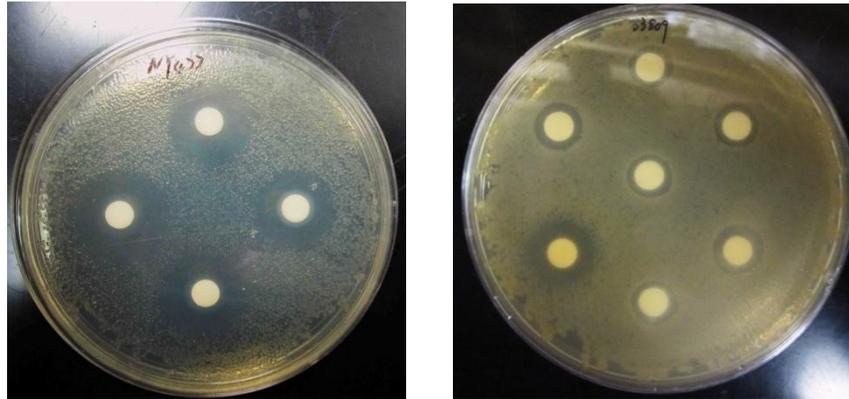
For the first three (1-3) chitosans, inhibition zone sizes increased at a higher chitosan concentration (0.8%) for most of the bacteria tested ($P < 0.05$), which indicated chitosan was more effective at higher concentration.

Table 5. Antimicrobial Activity of Chitosan Determined by Disk Diffusion

Chitosan ID	Concentration (%)	Inhibition Zone Diameter (mm)				
		<i>E. coli</i> (n=10)	<i>Salmonella enterica</i> (n=5)	<i>Vibrio</i> spp. (n=12)	G ⁺ (n=5)	Any bacteria (n=36)
1	0.4	7.46 ± 1.43 ^a	6.15 ± 0.34 ^a	10.73 ± 2.41 ^a	9.08 ± 0.56 ^a	8.74 ± 2.43 ^a
	0.8	8.51 ± 1.06 ^b	7.35 ± 1.90 ^a	9.39 ± 0.98 ^b	9.09 ± 0.69 ^a	8.75 ± 1.30 ^b
2	0.4	7.44 ± 1.69 ^a	N	10.71 ± 1.98 ^a	9.77 ± 0.68 ^a	8.87 ± 1.41 ^a
	0.8	8.88 ± 1.15 ^b	N	9.74 ± 0.71 ^a	10.17 ± 0.52 ^a	8.89 ± 1.54 ^b
3	0.4	7.12 ± 1.66 ^a	N	11.05 ± 3.06 ^a	9.30 ± 1.21 ^a	8.86 ± 2.90 ^a
	0.8	6.89 ± 1.44 ^a	6.12 ± 0.27 ^a	11.44 ± 2.77 ^a	9.86 ± 0.74 ^a	8.83 ± 2.95 ^b
4	3.2	10.26 ± 1.63 ^{ab}	N	11.34 ± 1.09 ^a	11.45 ± 0.41 ^b	10.14 ± 2.65 ^b
5	1.6	10.68 ± 0.34 ^c	9.82 ± 1.86 ^b	11.09 ± 0.59 ^a	10.81 ± 0.64 ^a	10.72 ± 0.76 ^b
Combined (1-5)		8.33 ± 2.09 ^b	6.79 ± 1.59 ^a	10.69 ± 1.98 ^a	9.94 ± 1.03 ^a	9.19 ± 2.19 ^b

Means in same column with different superscript letters are significantly different (p<0.05)

N means no inhibition zone were observed



(a) *Vibrio parahaemolyticus*

(b) *Escherichia coli*

Figure 5. Effect of Chitosan on the Growth of Different Bacteria by Disk Diffusion

4.2. MICs Generated by Agar Dilution and Broth Microdilution

Table 6 shows the MIC ranges of five chitosans with different molecular weights generated by agar dilution and broth microdilution for different bacteria groups. MIC generated by broth microdilution ranged from 0.0015% to 1.6%, all within the ranges for the five chitosans tested. While the agar dilution MICs for chitosans 1-4 ranged from 0.03% to above 1% except for those of chitosan 5 (Mw = 43 kDa), which were within the test range of 0.03-1%.

In general, MICs generated by broth microdilution were almost always one to several times lower than those obtained by agar dilution, dependent upon the bacterial strains tested and Mws of chitosan used. Table 7 shows the *E. coli* MICs determined by broth microdilution and agar dilution. MICs of broth microdilution ranged from 0.003-1.6% for five chitosans while the agar dilution MICs ranged from 0.06-0.25% for the two water-soluble chitosan 4-5 (Mw = 67 and 43kDa). However, the MICs generated by agar dilution for three acid-soluble chitosan were out of the test range (>1%), especially for five *E. coli* O157:H7 strains. In contrast, all of *E. coli* O157:H7 strains were effectively inhibited by chitosan no.5 (Mw = 43kDa) in agar dilution with

concentration of 0.125-0.25% and four of five *E. coli* O157:H7 strains were inhibited by chitosan no.4 (Mw = 67), which indicated that no.5 and no.4 chitosans were more effective against the *E. coli* O157:H7 growth.

A similar observation (Table 8) was made for *Salmonella* tested by agar dilution, because all of the MICs were beyond the highest concentration (1%) for four of five chitosan, except for chitosan no.5 (Mw = 43kDa). It showed a higher antimicrobial activity in inhibiting the growth of *Salmonella* species. However, in broth microdilution, only chitosan no.2 (Mw = 444kDa) showed no antimicrobial activity against this bacteria within the test range, while other four chitosans no. 1, 3, 4, 5 were all able to inhibit the bacteria growth with concentration of 0.4%, 0.1%, 1.6% and 0.2%, respectively.

For *Vibrio* spp., Table 9 shows the MIC value obtained from broth microdilution and agar dilution. The MIC generated by broth microdilution ranging from 0.003-0.025%, which is about 10 times lower than those of agar dilution (0.03-0.125%). Besides, from Table 6, it seems that the *Vibrio* MICs obtained by both agar dilution and broth microdilution were lower than those of other Gram-negative bacteria, which demonstrated that *Vibrio* spp. were more susceptible, while *Salmonella* spp. and *E. coli* O157:H7 proved to be more resistant to chitosan.

With Gram-positive bacteria, MICs were all within test range either by agar dilution or broth microdilution from table 10. And the assay showed that in broth microdilution chitosan exhibited stronger antimicrobial activity against gram-positive bacteria than most of gram-negative bacteria, including *E. coli*, *Salomonella*, *A. calcoacticus*, *C. freundii* and *E. aerogenes*. But it showed a similar MIC range (0.003-0.025%) with *Vibrio* species.

Table 6. MIC Range of Chitosan against 36 Bacterial Determined by Agar Dilution and Broth Microdilution

Bacteria	MIC range (%)									
	1		2		3		4		5	
	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
<i>Vibrio</i>	0.003-0.006	0.03-1	0.003-0.006	0.03-0.5	0.003-0.0125	0.03-0.5	0.003-0.0125	0.06-0.125	0.003-0.025	0.06-0.125
<i>E. coli</i>	0.003-0.4	0.5->1	0.003->0.4	0.5->1	0.003->0.4	0.5->1	0.006-1.6	0.06->1	0.0015-0.4	0.06-0.5
<i>Salmonella</i>	0.4-0.4	>1	>0.4	>1	0.4-0.4	>1	1.6-1.6	>1	0.2-0.4	0.25-0.5
G+	0.003-0.006	0.03-1	0.003-0.0125	0.03-1	0.003-0.025	0.125-1	0.0125-0.025	0.06-0.25	0.0125-0.2	0.03-0.5

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa

Table 7. *E. coli* MICs Determined by Broth Microdilution and Agar Dilution

Strain ID and serotype	MIC (%)									
	1		2		3		4		5	
	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
P132	0.006	0.5	0.003	0.5	0.006	0.5	0.006	0.06	0.003	0.06
K-12	0.006	0.5	0.003	0.5	0.006	0.5	0.025	0.125	0.003	0.06
HB101	0.006	0.5	0.006	0.5	0.006	0.5	0.025	0.125	0.003	0.06
ATCC 25922	0.1	>1	0.1	>1	0.1	>1	0.1	0.125	0.0125	0.06
ATCC 35218	0.1	1	0.1	>1	0.4	1	0.2	0.125	0.025	0.125
B6914; O157:H7	0.1	>1	0.1	>1	0.4	>1	1.6	0.125	0.0125	0.125
933; O157:H7	0.1	>1	0.1	>1	0.4	>1	1.6	0.125	0.025	0.125
EC06; O157:H7	0.1	>1	0.1	>1	0.4	>1	1.6	0.125	0.006	0.125
EC13; O157:H7	0.1	>1	0.1	>1	0.4	>1	1.6	0.125	0.025	0.125
UMD 66; O157:H7	0.4	>1	>0.4	>1	0.4	>1	1.6	>1	0.2	0.25

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa

Table 8. *Salmonella* MICs Determined by Broth Microdilution and Agar Dilution

Strain ID and serotype	MIC (%)									
	1		2		3		4		5	
	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
H9812; Braenderup	0.4	>1	>0.4	>1	0.1	>1	0.8	>1	0.2	0.25
LT2; Typhimurium	0.4	>1	>0.4	>1	0.1	>1	1.6	>1	0.2	0.25
UMD373; Typhimurium	0.4	>1	>0.4	>1	0.1	>1	1.6	>1	0.2	0.25
50; Enteritidis	0.4	>1	>0.4	>1	0.1	>1	1.6	>1	0.2	0.25
119; Kentucky	0.4	>1	>0.4	>1	0.1	>1	1.6	>1	0.2	0.25

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa

Table 9. *Vibrio* MICs Determined by Broth Microdilution and Agar Dilution

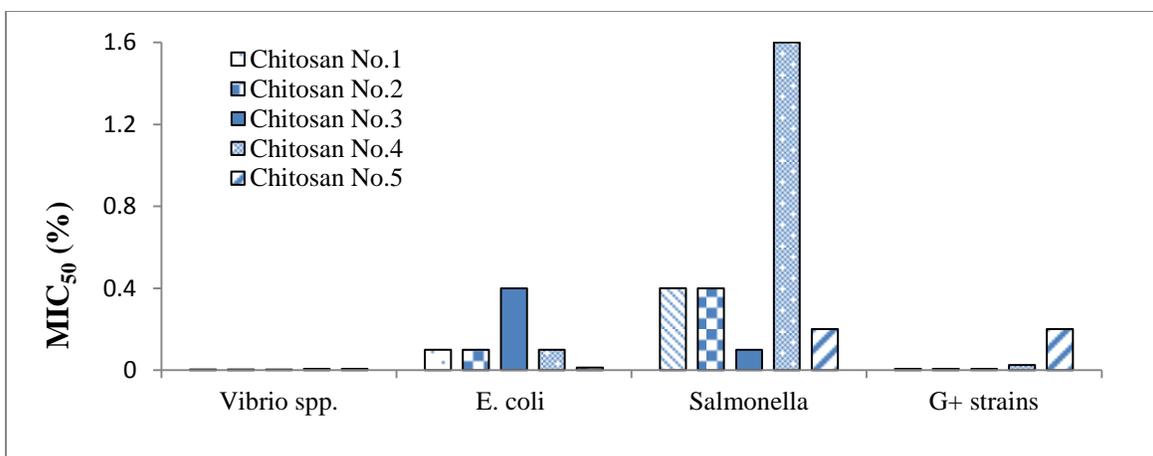
Bacteria	Strain ID and Serotype	MIC (%)									
		1		2		3		4		5	
		Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
<i>V. cholera</i>	ATCC 14035	0.003	0.03	0.003	0.03	0.006	0.03	0.0125	0.125	0.025	0.125
<i>V. fluvialis</i>	ATCC 33809	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.06	0.003	0.06
<i>V. harveyi</i>	BB120	0.006	0.06	0.006	0.06	0.006	0.5	0.0125	0.125	0.003	0.06
<i>V. harveyi</i>	BB152	0.006	0.06	0.003	0.03	0.006	0.5	0.0125	0.125	0.003	0.06
<i>V. mimicus</i>	ATCC 33653	0.003	0.03	0.003	0.03	0.025	0.03	0.0125	0.125	0.025	0.125
<i>V. parahaemolyticus</i>	ATCC 33847	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.125	0.006	0.06
<i>V. parahaemolyticus</i>	ATCC 49529	0.003	0.03	0.003	0.03	0.006	0.03	0.006	0.125	0.003	0.06
<i>V. parahaemolyticus</i>	NY 477; O4:K8	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.125	0.003	0.06
<i>V. vulnificus</i>	ATCC 27562	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.125	0.006	0.06
<i>V. vulnificus</i>	ATCC 33815	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.06	0.003	0.06
<i>V. vulnificus</i>	WR1	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.125	0.006	0.06
<i>V. vulnificus</i>	225	0.003	0.03	0.003	0.03	0.003	0.03	0.006	0.125	0.006	0.125

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa

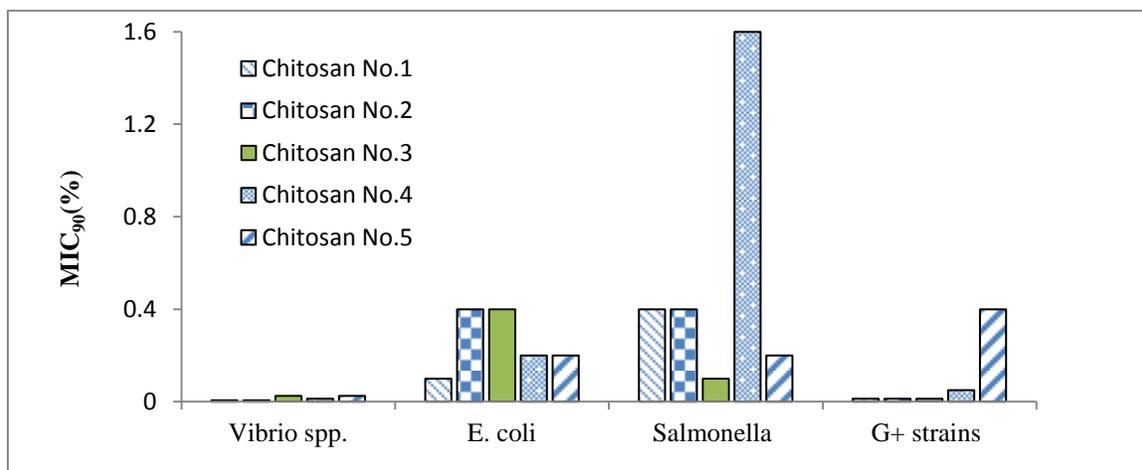
Table 10. Gram-positive Bacteria MICs Determined by Broth Microdilution and Agar Dilution

Strain	MIC (%)									
	1		2		3		4		5	
	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
<i>E. faecalis</i>	0.006	1	0.003	1	0.006	1	0.025	0.25	0.2	0.25
<i>L. monocytogenes</i>	0.006	0.125	0.003	0.03	0.006	0.125	0.025	0.06	0.2	0.06
<i>L. monocytogenes</i>	0.006	0.125	0.003	0.06	0.006	0.125	0.025	0.06	0.2	0.06
<i>S. aureus</i>	0.003	1	0.003	1	0.006	1	0.006	0.125	0.025	0.125
<i>S. faecalis</i>	0.003	1	0.003	1	0.006	1	0.006	0.125	0.025	0.25

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa



(a) MIC₅₀ (MIC causing inhibition of 50% of bacterial)

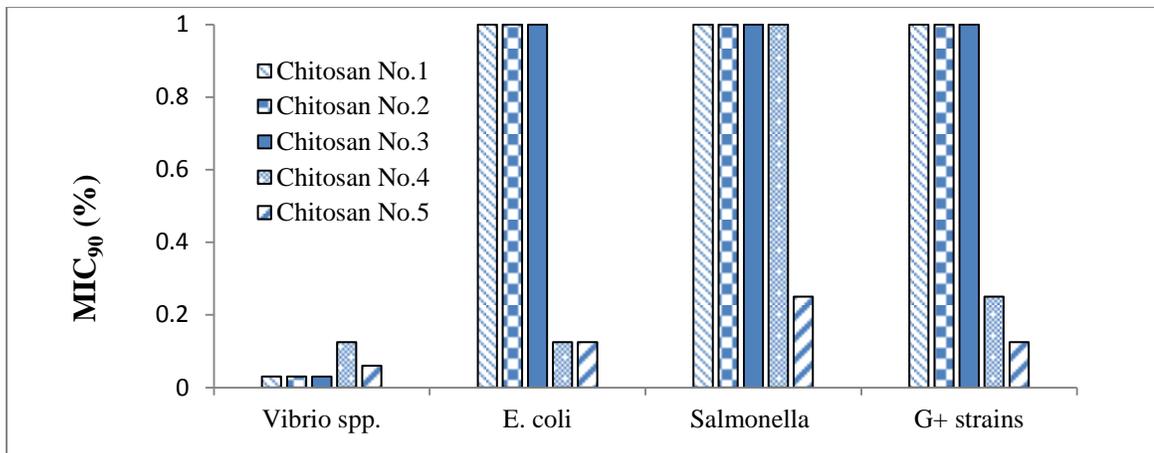


(b) MIC₉₀ (MIC causing inhibition of 90% of bacterial)

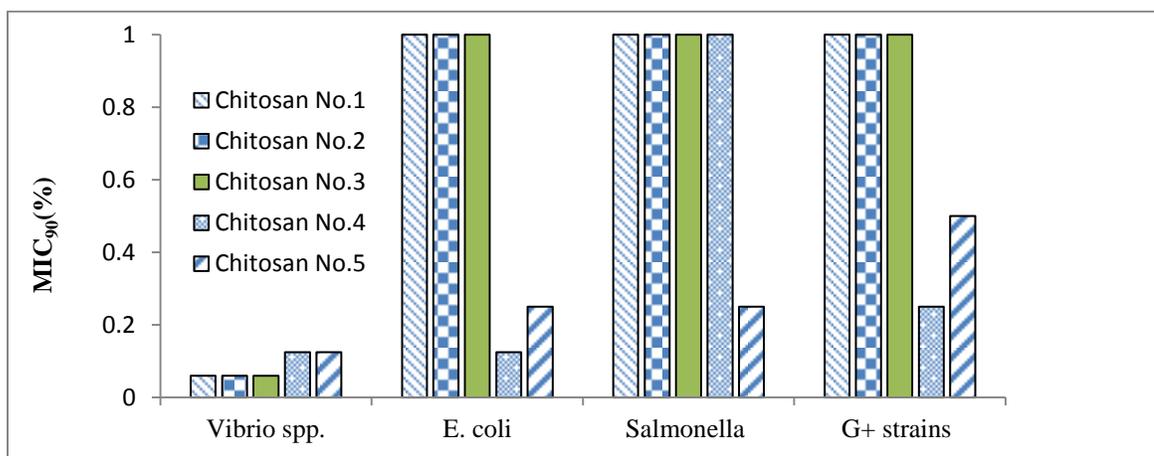
Figure 6. MIC₅₀ and MIC₉₀ of Five Chitosan Determined by Broth Microdilution

Figure 6 shows the MIC₅₀ and MIC₉₀ values of five chitosans against different bacterial groups by broth microdilution method. From (a), chitosan markedly inhibited the growth of most bacteria tested within the concentration of 0.4% using broth microdilution, except for the case of *Salmonella enterica* (1.6%) with chitosan no. 4. The inhibitory effects were varying according to the type of bacteria. MIC₅₀ values of *Vibrio* spp. were between 0.003%-0.006%, regardless of chitosan used. For *E. coli* spp., all the chitosans showed inhibitory effect on their growth at concentration of 0.1%, except chitosan no.3 and no. 5 with concentration of 0.4% and 0.0125%,

respectively. And MIC₅₀ of *Salmonella enterica* were higher than other gram-negative bacteria at concentration of 0.4-1.6%, except for chitosan no. 3 (0.1%). With gram-positive bacteria, four of five chitosan showed higher antimicrobial activity than most of gram-negative bacteria, including *E. coli* and *Salmonella enterica*, but with similar MIC₅₀ values as *Vibrio*. A similar observation was found for MIC₉₀ values of five chitosans for different bacteria.



(a) MIC₅₀ (MIC causing inhibition of 50% of bacterial)



(b) MIC₉₀ (MIC causing inhibition of 90% of bacterial)

Figure 7. MIC₅₀ and MIC₉₀ of Five chitosan Determined by Agar Dilution

Figure 7 shows the MIC₅₀ and MIC₉₀ values of five chitosans against different bacterial groups by agar dilution method. MIC₉₀ values for chitosan no.1-3 were obvious higher than those

obtained from broth microdilution method. *Vibrio* spp. was still the bacteria with lowest MIC₅₀ value for five chitosans from (a). But for other bacteria, MIC₅₀ values obtained by this method were higher than those of broth microdilution, especially for *E. coli* and *Salmonella enterica* (some of MIC were >1%). But from this result, chitosan no.5 (Mw= 43 kDa) showed a higher antimicrobial activity on any bacteria with a lower MIC₉₀ value compared with other four chitsoans, MIC₉₀ values (b) also showed the same trend with MIC₅₀ of five chitosans against different bacteria.

Table 11 summarizes the agreement of MICs of five chitosans for the thirty-six bacteria between agar dilution and broth microdilution. The overall agreement of MICs ($\pm 2 \log_2$ dilution) between these two methods when testing the five chitosans was 14.6%, which indicated the two methods had very poor agreement with each other. The level of agreement between the two methods ranged from 32.3% with chitosan 4 (Mw = 67 kDa) to 0% with chitosan 1 and 2 (Mw = 1100 and 444 kDa). Chitosan 4 (Mw = 67 kDa) MIC between the two methods was 32.3%, followed by chitosan 5 (Mw = 43 kDa) (27.8%), chitosan 3 (Mw = 223 kDa) (13.0%), chitosan 1 and 2 (Mw = 1100 and 444 kDa) (0%). From table 12, the agreement of the agar dilution and broth microdilution method was higher (60%) than 50% only for G⁺ bacteria with chitosan no.5, while others MIC were all with very low agreement by the two methods for five chitosans. Therefore, based on the agreement data, it suggested that agar dilution and broth microdiution methods had very poor agreement with each other on MIC determination of chitosan.

Table 11. Comparison of Agar Dilution and Broth Microdilution MICs of Five Chitosans

Chitosan ID	No. of strains	MIC comparison, agar dilution against broth microdilution (log2 scale)										% Agreement ^a
		-3~-2	-2~-1	same	1-2	2-3	3-4	4-5	5-6	6-7	>7	
1	22						13	1		2	6	0
2	21						14	1		1	5	0
3	23				2	7	1	3	4	3	3	13.0
4	31			9	1	2	10	8	1			32.3
5	36	1		8	2	4	6	7	7	1		27.8

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa

Table 12. Comparison of Agar Dilution and Broth Microdilution MICs for Different Bacteria

Bacteria	Agreement (%) by 5 chitosans				
	1	2	3	4	5
<i>Vibrio</i> (n=12)	0	0	8.33	0	0
<i>E. coli</i> (n=9)	0	0	11.1	33.3	11.1
G ⁺ (n=5)	0	0	0	40	60
All (n=30)	0	0	6.7	20	20

1-5 means chitosan no.1 to no.5, with Mws of 1100, 444, 223, 67, and 43 kDa

Chapter 5 - Discussion

There are numerous studies that have explored the antimicrobial activity of different chitosan compounds from various sources by employing diverse testing conditions. However, the discrepancies in the results obtained in many instances were observed, partially because chitosan's *in vitro* antimicrobial activity is relied on various intrinsic and extrinsic factors, such as molecular weight (Mw), degree of deacetylation (DD), pH, and test strains (Raafat & Sahl, 2009). Many researches are mainly focused on exploring the intrinsic and extrinsic factors effect on the antimicrobial activity of chitosan. For example, No et al. (2002) revealed that chitosan showed stronger inhibition effects for Gram-positive bacteria than Gram-negative bacteria and chitosan showed a higher antimicrobial activity than chitosan oligomers. Another study reported that the antimicrobial activity of chitosan with Mw of less than 300 kDa was strengthened as the Mw increased, while the effect on *E. coli* strains was weakened (Zheng & Zhu, 2003).

On the other hand, the different methodologies applied in the *in vitro* susceptibility testing are also contributing to these discrepancies. Disk diffusion, agar dilution and broth microdilution methods currently are all available to examine the antimicrobial activity of chitosan in different studies (Liu *et al.*, 2004; Kim & Kim, 2007; Raafat *et al.*, 2008). However, the lack of standardized and reliable *in vitro* susceptibility method makes direct comparison of the results obtained among the numerous studies impossible. There is few data on comparison and evaluation the different methods to determine the antimicrobial activity of chitosan in one study. Therefore, this study appears to be the first study where disk diffusion, agar dilution and broth microdilution

were used to determine the antimicrobial activity of chitosan against total 36 gram-positive and gram-negative bacteria, then comparing the MICs obtained by agar dilution and broth microdilution and also evaluating the results generated by the three methods in order to obtain more reliable results and suggest a proper and uniform method of testing chitosan's antimicrobial activity.

The antimicrobial activity of chitosan tested in this study differed with the molecular weight of chitosan and type of bacteria species. According to disk diffusion data, a higher inhibition activity was observed for chitosan 5 (Mw = 43 kDa) against *Salmonella enterica* bacteria. Similarly, chitosan 5 (Mw = 43 kDa) was the most effective in inhibiting the growth of *Salmonella* strains (0.25%), while other chitosan were all beyond the test range (>1%) based on the MIC value from agar dilution. Differences in molecular weight of chitosan could be the reason resulting in these variations in antimicrobial activity of chitosan and its derivatives. Some study reported that the antimicrobial activity of chitosan with low Mw is higher than the high Mw chitosan against *E. coil* (Liu *et al.*, 2006), while the antimicrobial activity was strengthened with lower Mw chitosan found in present study. Another study demonstrated that low molecular weight chitosan (5-10 KDa) showed the highest antimicrobial activity against pathogenic bacteria (Jeon *et al.*, 2001). That's probably because small molecule with lower Mw is easier to penetrate the cell membrane of bacteria than large Mw of chitosan, which contribute to the leakage of protein and other intracellular components of the microbial cells, ultimately resulting in the impairment of vital bacteria activities. In our study, the antimicrobial activity of chitosan varied from their Mws and the bacteria

tested, except for chitosan with Mw of 43 kDa showed relatively higher antimicrobial activity against most of gram-negative bacteria, such as *E. coli* O157:H7 and *Salmonella*.

In present study, the inhibition effects differed with regard to the molecular weight of chitosan and the type of bacterial by using three methods. MICs of *Salmonella* were mostly out of test range in agar dilution method and there is no clear inhibition zone in disk diffusion for this bacterial proved that *Salmonella* were more resistant compared with other bacterial species. In previous study revealed that chitosan of Mw = 1106 and 224 kDa showed weak or no inhibition effect on *Salmonella* at 0.1% concentration (No *et al.*, 2002), which has the same trend with our current study, where suggested that four of five chitosans, including chitsoan (Mw =1100 and 223 kDa), processed very weak and even no antimicrobial activity against *Salmonella*.

Numerous studies have shown the effect of chitosan on *E. coli* inhibition. Our study found that chitosan with Mw of 1100 and 444 kDa was able to inhibit the growth of *E. coli* strains at 0.1% concentration which is in accordance with findings of Darmadji and Izumimoto on the effect of chitosan in meat preservation (Darmadji & Izumimoto, 1994). But another showed that lower concentration (0.0075%) of chitosan was enough to inhibit the *E. coli* growth (Simpson *et al.*, 1997). Moreover, Wang found that concentrations of 0.5 or 1% of chitosan was capable to complete inactivate the *E. coli* growth at pH 5.5 (Wang, 1992).

Vibrio spp. was demonstrated to be most susceptible bacteria among the diversity of microorganisms based on its lower MIC values examined by both agar dilution and broth microdilution, while clear inhibition zone were also observed in disk diffusion. Since *V. parahaemolytics* and *V. vulnificus* are the major factors to cause infection of

consumption of raw or undercooked seafood, including oysters, mussels, etc. and lead to clinical manifestations ranging from mild diarrhea to death (Vior, 2003; Butt *et al.*, 2004). Based on our study, chitosan possessed a high antimicrobial activity against *V. parahaemolyticus* and *V. vulnificus* even at low concentrations depending on its molecular weights, which can be applied to inactivate *V. parahaemolyticus* and *V. vulnificus* in live oyster maintaining its sensory quality and also increase the oyster safety and shelf life of shucked raw oysters. In addition, according to the MIC values in the current study, five chitosans showed a higher or similar antimicrobial activity against gram-negative bacteria, including *E. coli*, *Salmonella*, *A. calcoacticus*, *C. freundii*, *E. aerogenes* and *Vibrio*. Similarly, No *et al.* (2002) found that chitosan generally showed stronger antimicrobial activity with gram-positive bacteria than gram-negative bacteria.

Disk diffusion method allows to simultaneously testing a large number of antimicrobials in a relatively easy and inexpensive manner. However, the results of disk diffusion are considered as qualitative because it can only reveal the susceptibility of antimicrobials against the bacteria tested, which described as susceptible, intermediate, and resistant correlated with diameter of inhibition zone. The major disadvantage of this method is unable to generate the MIC value and difficult to examine the susceptibility of fastidious and slow-growing bacteria (Wilkins & Thiel, 1973). Besides, similar with other agar-based methods, it is labor-intensive and time-consuming (Klancnik *et al.*, 2010). In many studies disk diffusion was used to determine the antimicrobial activities of chitosan (Kulkarni *et al.*, 2005; Pranoto *et al.*, 2005; Coma *et al.*, 2006; Kim & Kim, 2007; Cao *et al.*, 2009; Mayachiew *et al.*, 2010), however, they can only demonstrate that chitosan was effective against bacteria, yeast, and fungi. Similar in the present study, the inhibition

activity of chitosan was demonstrated by observing the clear inhibition zone on the plate and there is no way to further examine the MIC value of chitosans. Moreover, this method is not always reliable for determining the antimicrobial activity of chitosan, because the incompletely diffusion of chitosan solution on the culture medium.

Agar dilution is a quantitative susceptibility testing method using two fold dilutions of an antibiotic. The advantages of agar dilution include the ability to simultaneously test the susceptibility of a number of bacteria in one plate and the ability to test susceptibility testing for fastidious organisms since the agar is able to adequately support the bacteria growth. Moreover, the result of the test yields an exact MIC for testing bacteria. However, agar dilution cost intensively labor and time due to the preparation of agar plate mixed with chitosan solution.

Broth microdilution is another quantitative reference method routinely used in clinical laboratories. The advantages of the method include considerable savings in media usage, requirement of a small quantity of sample, and test the susceptibility of multiple antimicrobials at the same time. Moreover, it decreased the intensive labor and time cost compared with agar-based method.

Compared with disk diffusion method, agar dilution and broth microdilution are quantitative methods, which is able to determine the MIC value. However, the MICs value of five chitosan in present study varied from methods adopted, where broth microdilution MICs were generally lower than those from agar dilution regardless of bacteria species tested. In one previous study comparing the agar dilution and broth microdilution to examine the antimicrobial activity of natural antimicrobials, the broth microdilution tended to give lower MIC readings than agar dilution for gram-negative

bacteria (Klancnik *et al.*, 2010). However, the antibiotic susceptibility testing by comparing agar dilution and broth microdilution in many studies was demonstrated a good correlation between the two method (Luber *et al.*, 2003; Amsler *et al.*, 2010). One of the reasons why there is poor agreement relationship between agar dilution and broth microdilution on examining the MICs of chitosan is probably that the chitosan solutions probably had a closer contact with bacteria growing in the well of 96-well microplate in broth microdilution method; therefore, chitosan could inhibit the bacteria growth completely and effectively. In contrast, in agar dilution method, the bacteria were inoculated with a replicator with 1 mm pins, which may not have a completely contact with chitosan mixing in the agar. That will contribute to the higher MIC value.

As several methods for determining the antimicrobial activity of chitosan is available but there is no standardized and validated procedure of the methods for it as antibiotics used in clinical laboratories, since it is difficult to compare the results from numerous studies in different laboratories. Based on the present study, broth microdilution method can be recommended as a fast screening method for MIC determination. However, there is few study are presently on the evaluation of the methodologies used for measurement the antimicrobial activity of chitosan, indicating that continuation of our study is needed for future work.

Chapter 6 - Summary and Conclusion

Five chitosan compounds with molecular weights ranging between 43 and 1,100 kDa were tested against 36 representative foodborne pathogens using the three methods. A water-soluble chitosan (43 kDa) was found to be the most effective against *E. coli* O157:H7 and *Salmonella enterica*.

Compared with disk diffusion, agar dilution and broth microdilution were more appropriate for quantitatively determining the antimicrobial activity of chitosan, which were able to determine the MIC values. However, the MIC values of five chitosans varied between the two methods, chitosan compounds, as well as the bacterial strains tested. The data demonstrated that broth microdilution MICs were generally lower than those from agar dilution regardless of bacterial species tested. The overall agreement of MICs (± 2 log₂ dilution) between agar dilution and broth microdilution was only 14.6% when testing the five chitosans, suggesting poor agreement, which were differed with methodologies that applied in the *in vitro* susceptibility testing, bacteria species, and molecular weights of chitosan. But broth microdilution is more economical of time and resources and is well suitable for screening many combinations of bacteria and chitosans.

Besides, the results showed that MIC₅₀ and MIC₉₀ values were at least one dilution (2-fold) lower using the broth microdilution for *Vibrio* spp. compared with other bacteria, suggesting *Vibrio* strains were more susceptible. Therefore, the application of chitosan in inhibiting this bacterial genus in a food system is needed in future work. In contrast, *Salmonella* species were proved to be more resistant compared with other bacteria examined by the three methods.

In conclusion, this study appears to be the first one where disk diffusion, agar dilution and broth microdilution were used side by side to determine the antimicrobial activity of chitosan. The variability shown when different susceptibility testing methods were used suggests the need to apply multiple methods when conducting *in vitro* antimicrobial susceptibility testing of chitosans. Since the antimicrobial activity results from numerous studies need to be comparable, our study suggested that broth microdilution would be a suitable and fast screening method for MIC determination compared to other methods.

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