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# Evaluation of Antibacterial Property of Chitosan and Modified Chitosan from Cuttlefish (*Sepia spp.*) Bone Against *Vibrio* spp.

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# ABSTRACT

Cuttlefish bone is a common by-product that is considered waste in the environment without knowing the benefits it can offer. Cuttlefish bone is a good source of chitin by which chitosan is derived from. Chitosan is popularly known for its promising activity in inhibiting the growth of a wide range of microorganisms. This study further investigates the antibacterial activity of chitosan and modified chitosan, Chitosan-Glutaraldehyde Crosslink (CLCG), prepared from cuttlefish (Sepia spp.) bone. Test microorganisms were isolated from live fry and juvenile shrimp samples viz. Vibrio cholerae and V. parahaemolyticus or V. vulnificus. The structure of chitosan and modified chitosan were characterized through FT-IR spectroscopy. In this study, three different concentrations of filter paper discs of both chitosan and modified chitosan solutions (1.25mg ml<sup>-1</sup>, 3.75mg ml<sup>-1</sup> and 5mg ml<sup>-1</sup>, repectively) soaked at 90mins were used to determine its antibacterial property against the test organisms by disc diffusion method and the Minimum Inhibition Concentration (MIC) was then determined. Results from this study indicated that chitosan and modified chitosan solutions affect the inhibition zone at p<0.01 in V. cholerae and p<0.001 in V. parahaemolyticus or V. vulnificus. The antibacterial property of chitosan is said to be efficient at not greater than the concentration level of 3.75mg ml<sup>-1</sup> while in modified chitosan, the activity was found to be different with different bacterial strains. This indicates that chitosan and modified chitosan can be a possible antibacterial agent against the pathogenic human and animal bacteria.

Keywords: Cuttlefish bone, chitosan, modified chitosan, antibacterial property, Vibrio spp.

# **INTRODUCTION**

Chitosan is the deacetylated derivative of chitin, which is chemically defined as a copolymer of  $\dot{a}$ -(1,4) glucosamine ( $C_6H_{11}O_4N$ )n, having different number of N-acetyl groups<sup>1</sup>. It is a solid powder with white to light red color, insoluble in water but soluble in organic acids. Also, it is an abundant and renewable polysaccharide that is reported to exhibit a great variety of beneficial properties<sup>2</sup>. Chitosan can be used as a flocculant, as a processing aid and is being trialled for applications in fruit preservation, wound dressings, cosmetics, artificial organs and pharmaceuticals<sup>3</sup>.

Chitosan is usually prepared from chitin<sup>4</sup>. Chitin is present in a wide range of natural resources specifically in crustaceans, fungi, insects, annelids, molluscs, coelenterata etc. Apparently, chitosan is not only manufactured from crustaceans such as crab, krill and

crayfish, primarily because a large amount of the crustacean exoskeleton is available as a byproduct of food processing, but mainly also in squid and cuttlefishes. Though wide variety cephalopods are available, only limited studies have been carried out on these animals. Further the cuttlebone chitosan also reported antibacterial and antifungal activity against some of the human pathogenic microorganisms<sup>5</sup>.

*Vibrio* spp. are group of Gram- negative and rod-shaped bacteria that are natural inhabitants of freshwater, estuarine and marine environments. They are responsible for the majority of human diseases attributed to the natural microbiota of aquatic environments and seafood<sup>6</sup>. The most common pathogenic species are *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus*. *Vibrio also are important bacterial pathogens for animals reared in aquaculture. V. anguillarum, V. salmonicida*, and *V. vulnificus* are among the main bacterial pathogens of several fish species<sup>7</sup>, and *V. harveyi* is a major pathogen of shrimp, e.g., *Litopenaeusvannamei* and *Penaeus monodon*<sup>8</sup>. Mortality caused by *Vibrios* in reared fish and shellfish is very common during early larval stages and can occur suddenly, leading sometimes to death of the entire population<sup>9</sup>. Thus, the present study attempts to investigate the effectiveness of the antibacterial property of cuttlebone chitosan and its modified product to the growth of *Vibrio* species.

# **EXPERIMENTAL METHODS**

# Collection of cuttlebone

One kilogram of cuttlebone from different cuttlefish species was gathered from the Surigao City Public Market, selected seafood restaurants in Surigao City, Surigao del Norte, and from the cut lebone wastes of the selected houses in the Municipality of San Francisco (Anao-aon), Surigao del Norte. Gathering of cuttlebone was started from the month of May 2018 until December 2018. Cuttlebone was washed thoroughly then dried and pulverized before the conduct of experiment.

# Isolation of Vibrio spp.

*Vibrio* spp. was isolated from the live fry and juvenile shrimp samples caught from the selected pond of Abuyog, Leyte. Bacterial isolation process was conducted at the Microbiology Laboratory of the Visayas State University-Tolosa, Tanghas, Tolosa, Leyte. The process for bacterial isolation from fry and juvenile samples was carried out using the method of Peeler and Maturin, 1992 (Fig. 1 and 2). *Vibrio* spp. were identified according to the appearance and characteristics of their colony according to Frankelstein and Mukerjee (1963).



#### Figure 2. Bacterial isolation process for

#### Extraction and Preparation of Chitosan and Modified Chitosan

Chitin was extracted from the pulverized cuttlebone by demineralization (using 5% Acetic acid for 24hrs) and deproteinization (using 4% NaOH boiling for 2-3hrs at 100°C) and converted into chitosan through deacetylation process using 50% NaOH and heating at 100°C for 2hrs<sup>10,11,12</sup>. Modified chitosan which was the Cross-Linking of Chitosan and Glutaraldehyde (CLCG) was synthesized using the method of Li et. al with modification. It was done by dissolving 10.0g of chitosan into 150.0 ml of 5.0% acetic acid and then adding 150.0ml of 25% glutaraldehyde into chitosan solution to form a water gel after 24hrs of stirring at room temperature by using a magnetic stirrer. After stirring, the noncross-linked glutaraldehyde was removed by washing the cross-linked complex more than eight times with double distilled water. The obtained CLCG undergone air and oven drying at 60°C and was stored at room temperature. The percentage yield of CLCG also was calculated using the same mentioned formula. Each concentration of chitosan and CLCG powder (1.5mg, 3.75mg, and 5mg) was dissolved in every 1 milliliter of 5% CH<sub>3</sub>COOH. Three solutions of chitosan were designated as Treatment 1, 2, and 3, respectively. While the three CLCG solutions were labelled as Treatment 4, Treatment 5, and Treatment 6, respectively. The production flow scheme of cuttlebone chitosan and modified chitosan and its application is shown in Figure 3.



Figure 3. Cuttlebone chitosan and modified chitosan production flow scheme (Takiguchi, 1991a, b; Xing *et al.*, 2005; Li *et. al.*,2013)

# Fourier Transform Infrared (FT-IR) Spectral Analysis

The chitosan and its modified product from Cuttlebone of Sepia spp. was sent to the Department of Pure and Applied Chemistry at the Visayas State University, VisCa, Baybay City, Leyte for FT-IR spectral analysis.

#### Determination of Antibacterial Activity by Disc Diffusion Method

Bacterial isolates (*Vibrio* spp.) were tested for antimicrobial sensitivity using the disc diffusion method<sup>13</sup>. Peptone water with 3% NaCl was prepared and sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes. Bacterial strain was inoculated in the sterilized peptone water with 3% NaCl and incubated at 37°C for 24h. Mueller Hinton Agar (MHA, Scharlau) with 3% NaCl was prepared, sterilized in an autoclave at 15lbs pressure for 15 minutes. It was poured into sterile petridishes and was allowed to solidify. Sterile filter papers having diameter of approximately 6mm was prepared and was soaked in the different level of chitosan and modified chitosan solution (1.5, 3.75, and 5mg ml<sup>-1</sup>), tetracycline solution (1mg ml<sup>-1</sup>) being the standard or positive control (Treatment 7), and 5% acetic acid solution (negative control) served as Treatment 8, with soaking time of 90 minutes. Around 24 hour old culture from the peptone water were spread as thick straight lines across the surface of pre-set & dried plates of MHA using sterile cotton swabs, which were then left to dry for 10 minutes before placing the antimicrobial sensitivity discs (filter paper). Plates were incubated for 24hrs at 37°C in an inverted position. The experiment was carried out in triplicates and the zone of inhibition was recorded or measured using a ruler.

#### Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of chitosan and its modified form (CLCG) were determined by broth dilution method<sup>14</sup>. In this method, a stock solution of  $100\mu g m l^{-1}$  (400µg) was prepared. This was serially diluted to obtain various ranges of concentrations between  $25\mu g m l^{-1}$  and  $200\mu g m l^{-1}$ . To 2ml of each of the dilutions of different concentrations was transferred into sterile test tube containing 2ml of nutrient broth. The 0.2ml of test organism was introduced into the test tubes. A test tube containing only broth and test organism was used as the positive control while the tube with nutrient broth alone was the negative control. All the test tubes and control was incubated at  $37^{\circ}$ C for 18h. The tubes were studied for the visible signs of growth or turbidity after the period of incubation. The lowest concentration of chitosan and modified chitosan that inhibit the growth of bacteria will be considered as the minimum inhibitory concentration.

#### Statistical Analysis

Data on the inhibitory effects of chitosan and CLCG was analyzed by one-way analysis of variance (ANOVA) using SPSS-20 version software followed by Duncan's Multiple Range Test (DMRT) for values at p<0.01.

#### **RESULTS AND DISCUSSION**

The percentage yield of chitin and chitosan in this study which is 38.32% and 12.14%, respectively, is relatively smaller compared to the extracted chitosan from the study of Barwin Vino *et*, *al.* (1954) having 21% and 49.71% for chitin and chitosan, respectively. The reduction can be possible due to the depolymerisation of chitosan polymer, loss of mass or weight of the sample from excessive removal of acetyl groups from the polymer during deacetylation and loss of chitin and chitosan particles during washing (decantation). The modified chitosan has 36.9% yield obtained from 10g of chitosan.





Figure 5. Bacterial colonies isolated from shrimp fry and juvenile samples. Large yellow colony represents growth of *V. cholerae* (a) and small green colonies represent *V. parahaemolyticus* or *V. vulnificus*(b).

and Modified Chitosan

V. V. c Find FTTatR spectra of chitosan and modified chitosan are depicted in Figure 8a and b, respecti (a) n modified chitosan (CL(GG), the peaks found at 1033.16 cm-1 corresponds to CN stretcning, a NH bending was observed at 1555.33 cm-1, an OH stretching was also observed at 3262.05 cm-1, and the peaks found at 2931.8-2868 cm-1 corresponds to CH3 symmetric stretch. However, peaks that can correspond to the functional groups in the structure of chitosan were not present. This may be due to the high susceptibility of chitosan to environmental factors and processing conditions, which includes heating or freezing that could impose stress on its structure and cause polymer degradation (Szymańska and Winnicka, 2012). Moreover, this result can be attributed to the purification level and degree of deacetylation (DD) of chitosan. According to the study of Szymańska and Winnicka preparation involves basic purification (2012). although chitosan methods like demineralization and deproteinization, chitosan material may contain some impurities, such as ash, heavy metals, or proteins. Also, there is a possibility that the degree of deacetylation of chitosan is relatively low which leads to the degradation and absence of peaks. Same results were obtained in the study of Kurita et, al. (2002), wherein polymer with low DD was found to have a fast rate of degradation.



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Figure 6. FT-IR Spectrum of chitosan (a) and modified chitosan (b) from cuttlefish (Sepia spp.) bone.

#### Antibacterial Property of Chitosanand Modified Chitosan

Antibacterial property of chitosan and modified chitosan (CLCG) were tested against the isolated microorganisms from shrimp fry and hepatopancreas of juvenile shrimp samples displaying the colonies of Vibrio cholerae and V. parahaemolyticus or V. vulnificus. The results shown in Figure 5 indicate that Treatment 2 with 3.75mg ml<sup>-1</sup> of chitosan had the highest mean zone of inhibition in millimeter for both V. cholerae and V. parahaemolyticus or V. vulnificus having mean average of 17.33mm and 16.67mm. While, as shown in Figure 6, Treatment 2 with 3.75mg ml<sup>-1</sup> of modified chitosan had the highest zone of inhibition with a mean average of 15.67 for V. cholerae and Treatment 1 with 1.25mg ml<sup>-1</sup> of modified chitosan got the highest zone of inhibition having mean average of 17.33mm for V. parahaemolyticus or V. vulnificus. This study showed that chitosan and modified chitosan have a promising effect in inhibiting the growth of the isolated Vibrio spp. Similar results were obtained by Li et, al. (2013) indicated that, modified chitosan markedly inhibited the growth of antibiotic-resistant Burkholderiacepacia complex (Bcc) regardless of bacterial species and incubation time while bacterial growth was unaffected by solid chitosan. Also, the study of Jeon et, al. (2001) suggests that the activity was found to be different with different bacterial strains. However, the result of this study was inconsistent from the results obtained by Shanmugamet, al. 2015 which state that, the antimicrobial activity of cuttlebone chitosan against pathogenic strains was found concentration dependent. While in this study, higher zone of inhibition lays on the second concentration level of chitosan which is 3.75mg  $ml^{-1}$  than in the 5mg  $ml^{-1}$  for both V. cholerae and V. parahaemolyticus or V. vulnificus. This means that, 3.75mg ml<sup>-1</sup> of chitosan could be the highest possible concentration that is efficient in inhibiting the growth of the isolated Vibrio spp. and the activity will be lessen in the higher concentrations.

The zone of inhibition recorded from chitosan and modified chitosan was much higher compared to the negative control (Treatment 8) with 5% CH<sub>3</sub>COOH with mean average ranging from 7.67mm to 8mm in both Figure 5 and Figure 6. However, the zone of inhibition was smaller compared to the positive control which is the tetracycline (Treatment 7) with mean average ranges from 20.67mm to 21.33mm. This result was almost similar to the work of Shanmugam et. al. (2015), by which the control treatment which is also acetic acid solution shows almost no effect to the growth of some pathogenic bacteria and tetracycline showed the maximum zone of inhibition of 25mm.



Figure 7. Mean zone of inhibition of the different concentration levels of chitosan, positive control and negative control against *V. cholerae* and *V.parahaemolyticus* or *V. vulnificus* 



# *Effects of Chitosan and Modified Chitosan against V. cholerae and V. parahaemolyticus or V. vulnificus*

In this study, the chitosan and modified chitosan had shown to have and antibacterial properties against both in V. cholerae and V. parahaemolyticus or V. vulnificus at 1% significance. As shown in Table 1a and b, Treatment 8 is significantly different to Treatment 1 to Treatment 7. This means that negative control which is the 5% CH3COOH has lower antibacterial activity compared to those of different concentration levels of chitosan and modified chitosan. However, Treatment 1 is not significantly different to Treatment 2, 3, 5 and 6; Treatment 2, 3, 4, 5, and 6; and Treatment 2, 4 and 7 (1mg/ml tetracycline) were also not significantly different from each other. These only proved that they have the same effect in inhibiting the growth of V. cholerae and V. parahaemolyticus or V. vulnificus as what the antibiotic, tetracycline, can do.

Table 1. Summary of Analysis of Variance (ANOVA) for the zone of inhibition for V.	•
choleraeand V. parahaemolyticusor V. vulnificus	

1	J J	
	V. cholerae	V. parahaemolyticus or V.
		vulnificus
Between Groups	$0.002^{**}$	$0.000^{***}$
Within Groups		

Legend: ns=not significant \*\*=p<0.01 \*\*\*=p<0.001

Treatment	Mean zone of inhibition (mm)	Treatment	Mean zone of inhibition (mm)
1	15.0000 <sup>b</sup>	1	12.3333 <sup>b</sup>
2	17.3333 <sup>bcd</sup>	2	16.6667 <sup>bcd</sup>
3	15.3333 <sup>bc</sup>	3	14.3333 <sup>bc</sup>
4	14.3333 <sup>cd</sup>	4	17.3333 <sup>cd</sup>
5	15.6667 <sup>bc</sup>	5	14.6667 <sup>bc</sup>
6	15.6667 <sup>bc</sup>	6	15.6667 <sup>bc</sup>
7	21.3333 <sup>d</sup>	7	20.6667 <sup>d</sup>
8	8.0000 <sup>a</sup>	8	7.6667 <sup>a</sup>
	(a)	(	b)

Table 2. Antibacterial activity of chitosan and modified chitosan against V. cholerae (a) and V. *parahaemolyticus* or V. *vulnificus(b)*.

# Minimum Inhibitory Concentration (MIC)

Estimation of MIC is defined as the lowest concentration of an antimicrobial that will exhibit the visible growth of a microorganism after overnight incubation. It is extensively used in the comparative testing of new drugs. In clinical laboratories, it is used to establish the susceptibility of organisms that is required for clinical management<sup>20</sup>. In this study, the MIC values for chitosan and modified chitosan from *V. cholera* and *V. parahaemolyticus* or *V. vulnificus* were reported as 100 $\mu$ g ml<sup>-1</sup> and 200 $\mu$ g ml<sup>-1</sup> and 200 $\mu$ g ml<sup>-1</sup> and 200 $\mu$ g ml<sup>-1</sup>, respectively.

Table 3. Minimum Inhibition Concentration of Chitosan and Modified chitosan ( $\mu$ g/ml) on isolated *Vibrio* spp.

Vibrio spp.	Chitosan (µg/ml)			Modified chitosan (µg/ml)				
	200	100	50	25	200	100	50	25
V. cholerae	-	-	+	+	-	+	+	+

<i>V</i> .								
parahaemolyticusor	-	+	+	+	-	+	+	+
V. vulnificus								
$\mathbf{T} = 1 + 1 + 1$	1 • 1 ( . )							

Legend: clear (-) and turbid (+)

# CONCLUSION

The results from the present study have shown the antibacterial property of chitosan and modified chitosan against the isolated pathogenic bacteria. The study proved that the discs soaked in chitosan and modified chitosan solutions affect the inhibition zone at p<0.01 for *V. cholerae* and p<0.001 in *V. parahaemolyticus* or *V. vulnificus*. In this study, the antibacterial property of chitosan is said to be efficient at not greater than concentration level of 3.75mg ml<sup>-1</sup>. While in modified chitosan, the activity was found to be different with different bacterial strains. Thus, the present study carries out the greater opportunities of utilizing the internal bone of the cuttlefish that is thrown and consider as waste at home, restaurants and seafood processing industries are excellent and promising source of antibacterial.

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