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Research Paper

Preparation and evaluation of food grade preservatives from shells of locally available shellfishes

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Abstract. Chitosan, a derivative of chitin found in shells of shellfish was produced by the process of demineralization, deproteinization, decolorization and deacetylation of the dried and grinded shells of locally available shellfishes. The shellfish used include periwinkle, clam and whelk shells. The antimicrobial activities of the food grade preservatives from these shells were analyzed on common food borne pathogens. The tested food borne pathogens were *Staphylococcus aureus*, *Escherichia coli* and *Lactobacillus acidophilus* species. Chitosan concentration levels of 0.1, 0.5 and 1.0% were used on different colonies of the identified isolates. Results showed that zones of inhibition reduced with an increase in the level of chitosan concentration. Concentration level of 0.1% had more effect than the other concentrations, while 1.0% level of concentration had lesser effect and to some extent had no effect on *Escherichia coli* a gram negative *bacterium*. This study also showed that the chitosan produced from clam shell had more effect on these isolates, while chitosan from whelk had very low effect which may be due to differences in the composition of the shells, molecular weight and size. The study showed that preservatives from shells of locally available and affordable shellfish could replace the chemically synthesized preservative.

Keywords: Evaluation, chitosan, preservative, antimicrobial activity, shells, fish shell.

Introduction

A preservative is a substance or a chemical that is added to products such as food, beverages, pharmaceutical drugs, paints, biological samples, cosmetics, wood, and many other products to prevent decomposition by microbial growth or by undesirable chemical changes (Erich and Gert-Wolfhard, 2002). Food grade preservatives are additives that inhibit the growth of bacteria, yeasts, and molds in foods (Branen, 2002). They reduce the risk of food borne infections, decrease microbial spoilage, and preserve fresh attributes and nutritional quality.

The most commonly used antimicrobial preservative is lactic acid. Other common antimicrobial preservatives include sorbic acid, sodium sorbate and sorbates, nitrite, nitrate and lactic acid (Msagati, 2012; Dalton, 2002). Other preservatives include ethanol and natural preservatives like rosemary extract, salt, sugar, vinegar, alcohol and castor oil. Anti-oxidative preservatives slow the oxidation process that spoils most food, especially those with a high fat content. Some modern synthetic preservatives have become controversial because they have been shown to cause respiratory or other health problems (Pandey and Upadhyay, 2012).

Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of pollution in coastal areas. Chitin is widely distributed in marine invertebrates and usually isolated from the exoskeleton of crustaceans and more particularly from shrimps and crabs where α-chitin is produced (Minke and Blackwell, 1978). In terms of structure, chitin is associated with

proteins and therefore, high in protein content. Chitosan is a natural polysaccharide comprising copolymer of glucosamine and N-acetylglucosamine, and can be obtained by the deacetylation of chitin from crustacean shells such as crab, shrimp and crawfish, the second most abundant natural polymer after cellulose (No and Meyers, 1989). Chitosan is insoluble in water but soluble in organic acid solutions.

Water soluble chitosan can be produced in the form of oligosaccharide by enzymatic or chemical hydrolysis (Jeon *et al.*, 2000). Due to its biodegradability, biocompatibility and low toxicity, chitosan has received increased attention as one of the promising renewable polymeric materials for their various applications in the pharmaceutical, biomedical and in food industries for food formulations as binding, gelling, thickening and stabilizing agent (Knorr, 1984). The physicochemical characteristics of chitosan affect its functional properties, which differ with preparation methods.

Chitosan generally has a stronger antimicrobial activity against bacteria than fungi (Tsai *et al*., 2002). The antimicrobial effect of chitosan are reported to be dependent on its molecular weight (Uchida *et al*., 1989; Jeon *et al*., 2002), degree of deacetylation (Tsai *et al.*, 2002) and the type of bacterium (No *et al*., 2002).

The advantages of chitosan includes the characteristics such as biocompatibility, non-toxicity, low allergenicity and biodegradability allow it to be used in various applications (Kumar *et al*., 2004). Besides, chitosan is reported to have other biological properties, such as antimicrobial (Martins *et al*., 2014), and antioxidant (Ngo and Kim, 2014) activities. The degree of deacetylation, which is described by the molar fraction of deacetylated units or percentage of deacetylation, and the molecular weight of chitosan affect these properties (Aranaz *et al*., 2009).

Many foods available in the market contain different types of preservatives which are synthesized chemicals that can give rise to certain health problems such as Sulfites, nitrates, nitrites, benzoates and sorbates amongst others, are common preservatives used in various foods, and are well known to cause a variety of symptoms such as diarrhea, hypertension and abdominal pains. Nitrates and Nitrites additives have been used as curing agents in meat products, benzoates and sorbates as antimicrobial preservatives, with attendant problem.

Commercial chitosan is only manufactured from crustaceans such as crab, krill and crawfish primarily because a large amount of the crustacean exoskeleton are available as a by-product of food processing (Methacanon *et al*., 2003). Crustacean shells mainly consist of 30 to 40% protein, 30 to 50% calcium carbonate, and 20 to 30% chitin (Seung-wook, 2006). These proportions vary with species and with season. Thus, the method of chitin and chitosan preparation can vary with different sources.

The abundant shells of sea foods in the Niger Delta Area of Nigeria are ideally waste and at best used for road/building construction, but can be harnessed to better use thereby converting them to wealth of great economic value because of their availability. Therefore the objectives of the study are to produce food grade preservatives from locally available shellfish such as periwinkle, clam and whelk and to evaluate their effect on the growth of microorganisms.

MATERIALS AND METHODS

Sample collection

Shells of whelk, clams and periwinkle used for this work were obtained from Creek road market, Borikiri, Port Harcourt, Rivers State, Nigeria.

Reagent

All reagents used for this work were of analytical grade and were all obtained from the analytical laboratory, Department of Food Science and Technology, Rivers State University (RSU), Port Harcourt, Rivers State.

Preparation of samples

The raw materials were obtained in solid form from the different sources. All shells of the same size and species were grouped, thoroughly washed with clean water, sundried for 2 days and thereafter oven dried at 150°C to brittle them to fragments for a total time of 48 h. The shells were further size reduced, milled and stored at room temperature in airtight polyethylene bag for further analysis.

Preparation of chitin

The processes involved in the preparation of chitin to chitosan are majorly divided into deproteinization, demineralization, decolorization and deacetylation according to the method described by No *et al*. (2000). (Figure 1)

Deproteinization

Deproteinization (DP) for protein separation was done by using the following process. The milled shells were deproteinized by boiling in 3.5% aqueous sodium hydroxide for 3 h at 100°C with a solid to solvent ratio of 1:10 (w/v) with constant stirring, to extract the proteins present in the shells. This treatment was repeated twice to improve clarity of the solution. Absence of color

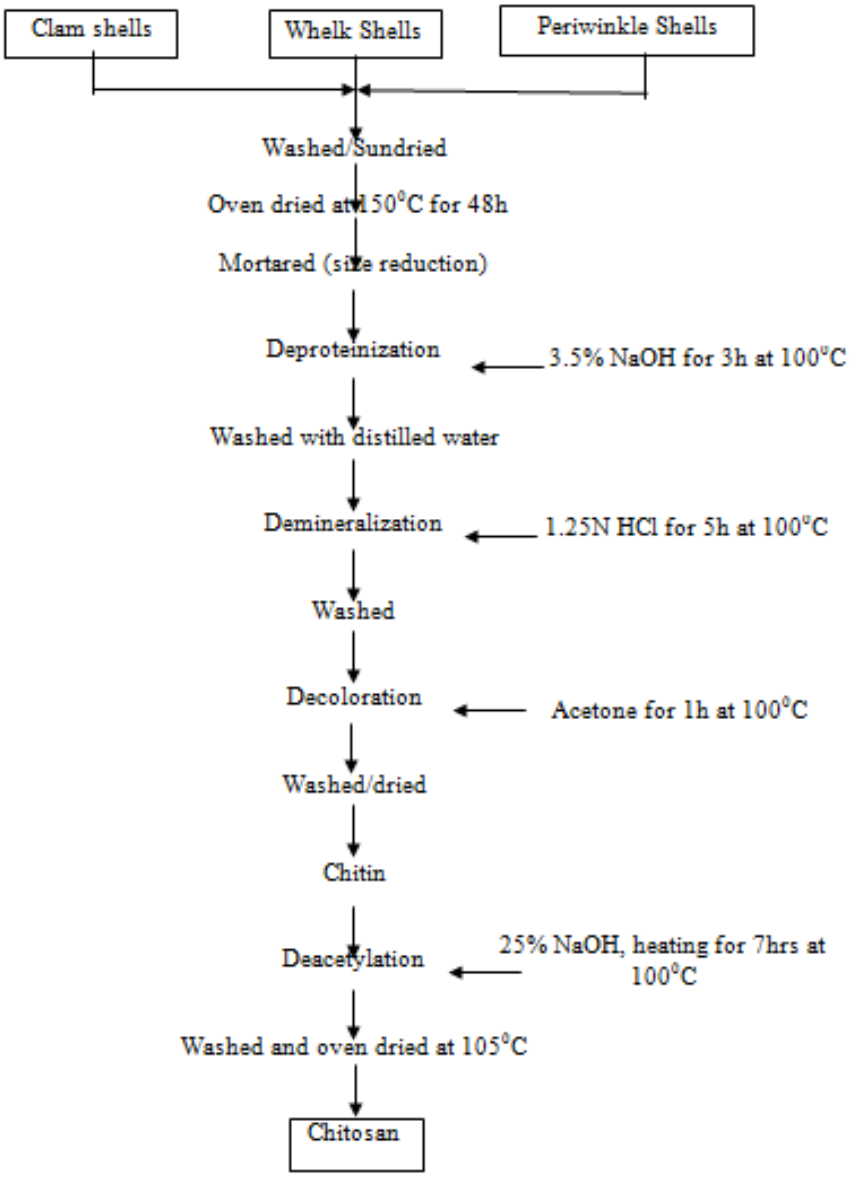

Figure 1. Flow chart for the production of chitosan (No *et al*., 2000).

indicated removal of protein at the end of the last treatment. After this, the alkaline was drained off from the shell and washed with distilled water up to neutrality and then dried at 90°C.

Demineralization (DM)

The deproteinzed samples were demineralized by using 1.25 N HCl and heating was done using water bath for 5hrs at higher temperature. This was to remove calcium carbonate. It was observed that the emission of CO_2 was an important indicator which was also dependent on the type of raw material used. The acid was drained off and washed thoroughly with distilled water.

Decolourization (DC)

Acid and alkali treatments alone produced a colored chitin product. Decolorization in high concentration of acetone and the chitosan was done followed by bleaching with 0.315 % (v/v) sodium hypochloride solution (containing 5.25% available chlorine) for 5 min with a solid to solvent ratio of 1:10 (w/v), based on dry shell. The decolorization process may be optional and used to bleach the chitin.

Deacetylation (DA)

Deacetylation is a process of removing acetyl groups from the chitin molecule and a process of conversion of

Table 1. Effect of chitosan from periwinkle on selected microorganisms.

Test organism	Zone of inhibition (cm)		
	0.10 (%)	0.50 (%)	1.00 (%)
L. acidophilus	2.5	2.0	1.0
E. coli	2.5	1.9	R
S. aureus	3.6	2.5	1.4

Key: L = Lactobacillus; E = Escherichia; S = Staphylococcus; R = Resistant.

Table 2. Effect of chitosan from clam on selected microorganisms.

Test organism	Zone of inhibition (cm)		
	0.10 (%)	0.50 (%)	1.00 (%)
L. acidophilus	2.4	2.3	R
E. coli	2.7	2.5	R
S. aureus	4.2	4.0	1.2

Key: L = Lactobacillus; E = Escherichia; S = Staphylococcus; R = Resistant.

chitin to chitosan. Deacetylation of chitin was done by treating with aqueous sodium hydroxide (1:1; w/w), 40 to 50% at 90 to 100°C for 7 h. After deacetylation the alkali was drained off and washed with tap water followed by distilled water. Finally, the chitosan was oven dried at 105°C for 1 h.

A**ntibacterial activities**

Three different microorganisms (bacteria) were tested for the antibacterial activity with the produced chitosan (preservative) using the spot on – lawn method. Three different concentrations (0.1, 0.5 and 1.0%) of the chitosan (pH 5.5) were prepared in 1% v/v acetic acid. Blank sample (without chitosan) was also prepared for comparison. The chitosan solutions were filtered with Whatman filter paper to remove impurities. The bacteria culture were grown in an incubator for 16 h at 37°C. After incubation 0.1 ml of the bacteria culture were inoculated onto the agar overlay plates and the plates were incubated at 37°C for 24 h. After incubation, the plates were examined for zone of inhibition and zones greater than 2mm were measured.

RESULTS AND DISCUSSION

Antimicrobial activity of chitosan

A total of three bacteria were tested for the antimicrobial activity of the zone of inhibition treated with chitosan produced from three different shells (periwinkle, clam and whelk) at three concentrations, 0.1, 0.5 and 1%, respectively. Organisms used were *L. acidophilus*, *E. coli* and *S. aureus*.

Table 1 shows the effect of chitosan produced from periwinkle shell on selected microorganism. The zone of inhibition for the three organisms ranged from 2.5 to 3.6 cm with *L. acidophilus* and *E. coli* having least and S. aureus having the highest zone of inhibition at 0.1% concentration. At 0.5% concentration of chitosan, the zone of inhibition ranged from 1.9 to 2.5 cm with *E. coli* having the least and S. aureus having the highest, while at 1% concentration of chitosan, the zone of inhibition ranged from 1.0 to 1.4 cm with *L. acidophilus* as least and S. aureus as the highest with *E. coli* being resistant. Results has shown that the chitosan from periwinkle has more effect on a Gram positive bacteria (S. aureus) than a Gram negative bacteria (*E. coli*) since the least zones of inhibition were on *E. coli* at the three concentration level. This result disagrees with the result reported by Chung *et al*. (2004) and Chen *et al*. (2002) who stated that chitosan or its derivatives have been proven more effective for Gram-negative bacteria than Gram-positive bacteria.

The size of the zone of inhibition is usually related to the level of antimicrobial activity present in the sample (Figure 2). A larger zone of inhibition indicates that the antimicrobial is more potent. According to Jeon et al. (2001) and Ueno *et al*. (1997), chitosan possesses antimicrobial activity against a number of Gram-negative and Gram-positive bacteria. The effect of chitosan from periwinkle at different concentration level (0.1, 0.5 and 1.0%) showed that zone of inhibition reduced with increase in concentration with S. aureus as most potent. At concentration of 0.1%, the antimicrobial activity of chitosan from periwinkle showed a zone of inhibition ranging from 2.5 to 3.6 cm. *L. acidophilus* and *E. coli* had the zones inhibition of 2.5 cm while S. aureus had the zone in inhibition of 3.6 cm. The former mentioned organisms are less sensitive to the chitosan from periwinkle than the latter meaning that S. aureus was inhibited more at the three different levels of concentration.

Table 2 shows the effect of chitosan produced from clam shell on selected microorganism (*L. acidophilus*, *E. coli* and *S. aureus*). The zone of inhibition at 0.1 and 0.5% concentrations, ranged from 2.4 to 4.2 cm and from 2.3 to 4.0 cm respectively, with *L. acidophilus* having the least zone of inhibition and S, aureus having the highest zone of inhibition in both cases (Figure 3). At 1% level of concentration of chitosan results showed that *L.*

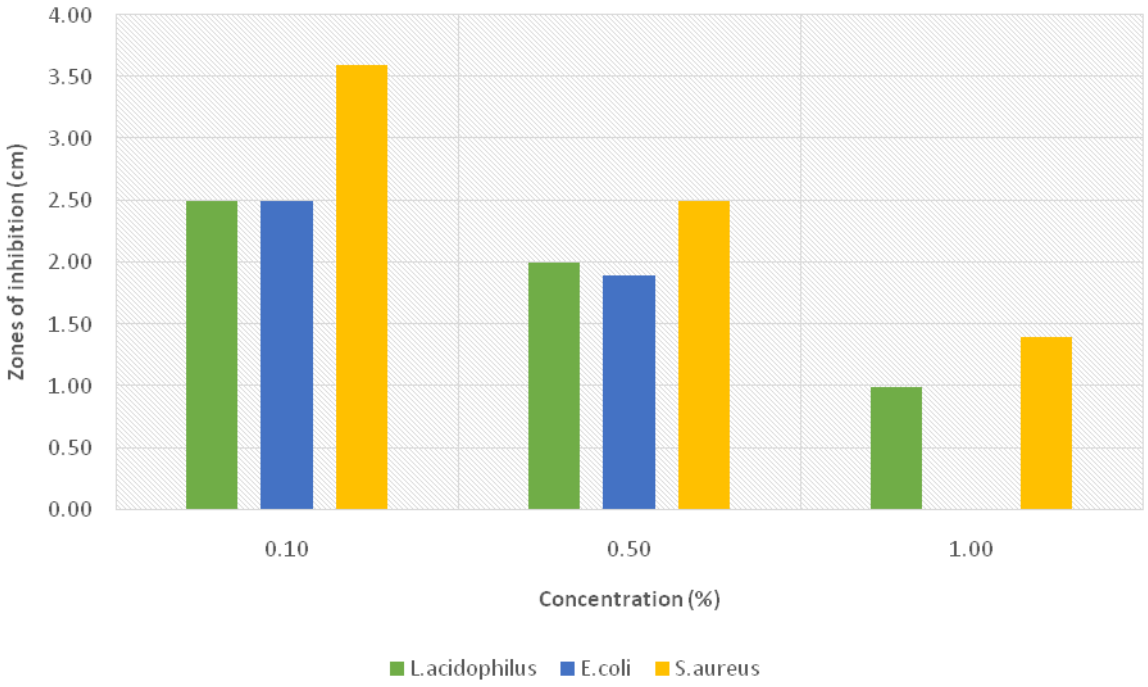

Figure 2. Effect of chitosan from periwinkle shell on three (3) selected microorganism.

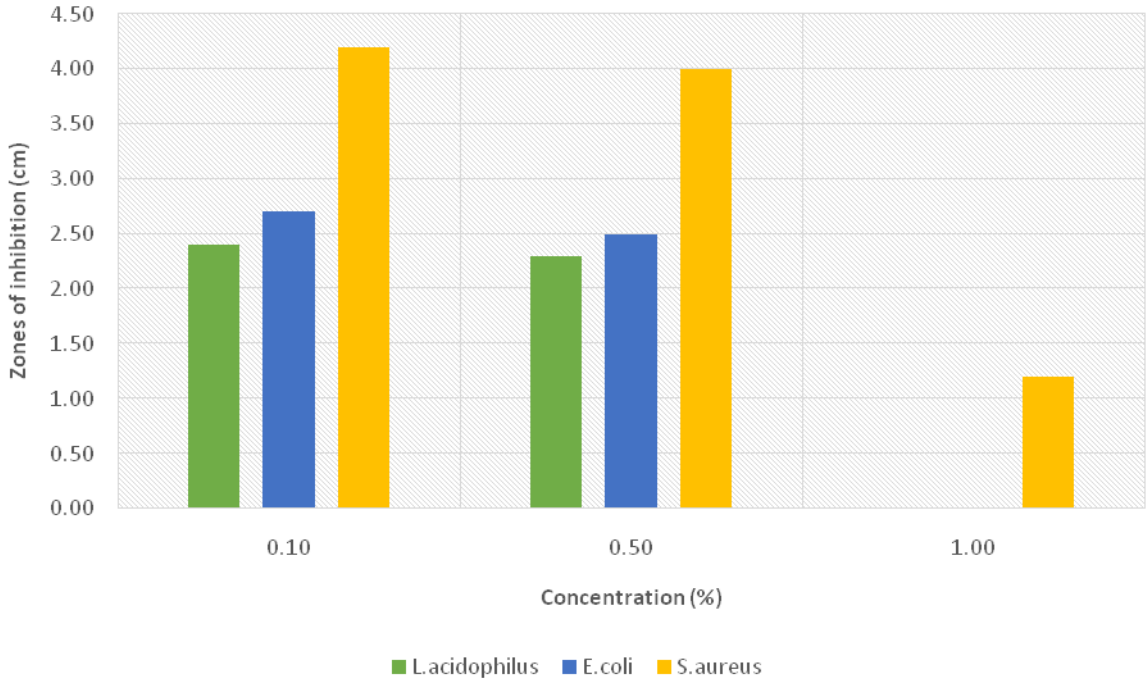

Figure 3. Effect of chitosan from clam shell on three (3) selected microorganism.

acidophilus and *E. coli* were resistant (there were no zones of inhibition), while S. aureus had a zone inhibition of 1.2 cm. Zone of inhibition decreased with an increase in the concentration of chitosan. This is in agreement with the findings of Rejane *et al*. (2015) who evaluated the antimicrobial activity of chitosan and its quaternized derivative on *E. coli* and *S. aureus* growth. *E.coli* has more zone inhibition than *L. acidophilus* at two different

Table 3. Effect of chitosan from whelk on selected microorganisms.

Test organism	Zone of inhibition (cm)		
	0.10 (%)	0.50 (%)	1.00(%)
L. acidophlus	2.9	2.5	1.1
E. coli	2.5	3.0	1.0
S. aureus	3.5	2.5	2.0

Key: L = Lactobacillus; E = Escherichia; S = Staphylococcus; R = Resistant.

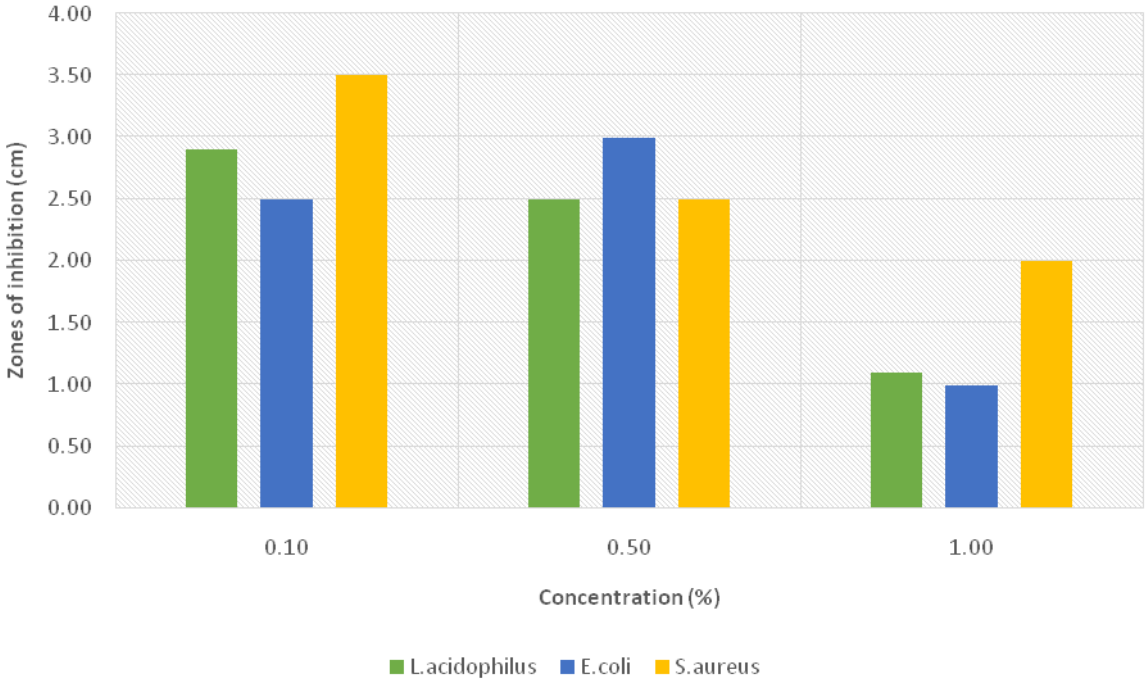

Figure 4. Effect of chitosan from whelk shell on three (3) selected microorganism.

Level of concentration 0.1 and 0.5%, respectively. This result is in agreement with the statement made by Chen *et al*. (2002).

Table 3 shows the effect of chitosan produced from whelk shell on selected microorganism (*L. acidophilus*, *E. coli* and *S. aureus*). There were zones of inhibition across all tested microorganisms at the different level of concentrations. At 0.1% concentration the zones of inhibition ranged from 2.5 to 3.5 cm with S aureus having the highest zone of inhibition while *E. coli* having the least zone of inhibition. *L. acidophilus* had 2.9 cm zone inhibition. The zone of inhibition for the three organisms ranged from 2.5 to 3.0 cm with *L. acidophilus* and *S. aureus* having least zone of inhibition and *E. coli* having the highest zone of inhibition at 0.5% concentration. At 1.00% concentration, the zones of inhibition ranged from 1.0 to 2.0 cm with *E. coli* having the least zone inhibition and S. aureus having the highest zone inhibition. *L. acidophilus* had a zone inhibition of 1.10 cm.

The results of the effect of chitosan from whelk on the selected microorganisms show that at 0.1 and 1.0% *E. coli* was more resistive to the effect of the chitosan when compared to zone of inhibition of *L. acidophilus* and *S. aureus* at the same concentrations but was more susceptible at 0.5% concentration with a zone inhibition of 3.0 cm. At this concentration level the result is in agree with the reports of Chen *et al*. (2002), Chung *et al*. (2004) and Seung-wook (2006) who stated chitosan has more effect on Gram negative bacteria than Gram positive bacteria. The results as shown in Table 3 and Figure 4 reported that the least concentration level being 0.1% had more effect on the selected isolate but mostly on S aureus. This trend seen in this study disagrees with the trend reported by Seung-wook (2006), who reported that the higher the concentration the more zones inhibited.

Antibacterial activity of chitosan is influenced by its molecular weight, degree of deacetylation, concentration in solution, and pH of the medium (Lim and Hudson, 2003). No *et al*. (2003) reported that elimination of the deproteinization stage in chitosan production yields a chitosan with low degree of deacetylation, but higher molecular weight and viscosity than those of deproteinized

deproteinized chitosan. To support the finding therefore, the present study produced chitosan that involved the process of deproteinization, this means that the resultant chitosan is a product with a high degree of deacetylation and lower molecular weight and viscosity. The results in the present study showed minimum inhibitory concentration (MIC) at 1.00% level of concentration for all the organisms under review. This could have been due to the species of the selected shellfish, their molecular weight and the effect of the reagents used for chitosan extraction. In all the results S. aureus was greatly affected by the chitosan up to a zone inhibition of 4.2 cm.

CONCLUSION

The quality and properties of the various chitosan varied due to the differences in the shells used for preparation. The success of production of chitosan can be attributed to the high temperature treatment of the shells before deproteinization process, which is in agreement with earlier reports. The present study showed the possibility of producing good quality and acceptable chitosan from locally available shells in Rivers State, South-South, Nigeria. The study showed that shells of periwinkle, clam and whelk which constitute waste and pollution could be an economic source of chitin which could further be treated chemically to obtain chitosan with high antimicrobial activities. The chitosan produced in this study has shown good minimum inhibitory concentration (MIC) effects at 0.1, 0.5 and 1% levels respectively, with chitosan from clam shell having the highest effect on the selected isolates and on Gram positive bacteria at low concentrations. Therefore, the use of chisotan against selected pathogens can be an additional breakthrough in the future as extracts from these shells could be a natural source that can be freely used in the food industry.

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