Antimicrobial Activity of Modified Shrimp Waste-derived Chitosan Films

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ABSTRACT

The addition of different antimicrobial agents to chitosan films has generally enhanced their antimicrobial activity and improved their physical and mechanical properties. The quality of the chitosan films was enhanced by incorporation of red ginger extract into the films. Ginger has the ability to inhibit the growth of pathogenic microorganisms such as bacteria, virus, protozoa and other parasitic organisms. In this work, antibacterial chitosan-starch-glycerol based films incorporated with ginger extract were prepared by thermal gelatinization method. The concentrations of ginger extract in the film forming solutions were varied using different volumes (0.0, 0.5, 1, 1.5 and 2.0 mL) of the extract. FTIR analysis was carried out in order to assess the functional group interactions between the matrix and the added agents. The antimicrobial activities of the modified chitosan films, carried out using Disc Diffusion method, showed that films incorporated with 2 mL of ginger extract had the largest zones of inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa* compared to the other films. Modified chitosan films without ginger extract showed minimal antimicrobial activities, while films with neither chitosan nor ginger extract showed no antimicrobial activities.

Keywords: Chitosan, edible film, ginger extract, antimicrobial

INTRODUCTION

Chitosan is a linear polysaccharide made up of (1-4)-linked 2-amino-2-deoxy-β-D-glucopyranose (Rinaudo, 2006). It consists of copolymers of glucosamine and N-acetyl glucosamine, and can be obtained by the partial deacetylation of chitin (Martino *et al.*, 2005; Aranaz *et al.*, 2009).

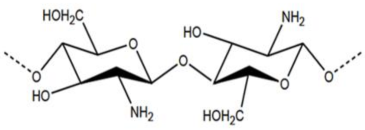

Figure 1. Chemical structure of chitosan

Chitin is vastly present in marine invertebrates, yeast, insect and fungi. It forms structural components of the cell walls of fungi, the exoskeletons of arthropods and insects. Percentage composition of chitin in shrimp, crab, crayfish and periwinkle sources in Nigeria as reported in the literature are; shrimp shells (8.15%), crab shells (7.80%), crayfish (2.88%), and periwinkle shells (0.44%) (Isa *et al.*, 2012), with shrimp having the highest yield of chitin. Chitosan is commonly obtained from chitin by deacetylation with solutions of sodium hydroxide or potassium hydroxide of 40 - 80% concentration at a temperature range (100-150^{o}C), and the time of treatment ranges from an hour to 24 hours. (Hossain and Iqbal, 2014; Abdou, 2008). In the quest to attain food security and sustainability, there is a growing interest in the development of natural biopolymers. Natural biopolymers as opposed to some synthetic materials used in food preservation are important due to their renewability, sustainability and biodegradability. The desirability to prolong the shelf life and enhance food quality as well as to minimise packaging waste has led to the exploration of new bio-based packaging materials, like edible and biodegradable films from renewable resources (Tharanathan and Kittur, 2003). Edible films, according to Mokrejs *et al.* (2009), are defined as a thin layer of material which can be consumed and provides a good barrier to moisture, oxygen and solute movement for the food. Edible films are non-toxic, non-polluting biodegradable natural biopolymers, such as polysaccharides (e.g. chitosan, cellulose, starch) and proteins (e.g. paraffin wax, beewax, candelilla wax) (Bergo *et al.*, 2010). The introduction of biocide into packaging material has been explored; the addition of other antimicrobial agents to chitosan films and coating has generally enhanced their antimicrobial activity and improved their physical and mechanical properties. Important factors to be considered in selection of natural antimicrobial agents are: the agents must satisfy consumer demands for healthy foods, chemical additives free, must have active effect against the targeted microorganism and limit the interactions between the film-forming biopolymer and the constituents of the food (Devlieghere *et al.*, 2004). About 400 different compounds are known to be present in ginger through chemical analysis. Ginger is reported to have the ability to inhibit the growth of pathogenic microorganisms such as bacteria, virus, protozoa and other parasitic organisms. Antibacterial analysis of root extracts of ginger found a positive activity against *Escherichia coli*,

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Staphylococcus aureus, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa* (Mahendran *et al.,* 2014). Therefore, addition of ginger extracts to the film-forming biopolymer was expected to enhance the antimicrobial activities of the chitosan-based films.

MATERIALS AND METHODS

Materials

Shrimp wastes used for production of chitosan were obtained from Oron, Akwa Ibom State of Nigeria. Chitosan with viscosity average molecular weight and degree of deacetylation (DDA) of 8,349.40 g/mol and 77.20% respectively was prepared from the shrimp wastes in our laboratory methods described by Abdou et al. (2008) and Hossain and Iqbal (2014), acetic acid (Sigma-Aldrich Chemical Company, 99.7%), glycerol (Sigma-Aldrich Chemical Company, 99.5%), nutrient agar (Sigma-Aldrich Chemical Company), and starch (locally sourced) were used in this work. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus cereus* used for antimicrobial studies were obtained from Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

Extraction of ginger

Fresh ginger rhizomes were thoroughly washed with distilled water to remove contaminants. The non-edible parts were scraped free from the edible parts. Exactly 200 g of the sample were chopped into small pieces and ground to a smaller size with the aid of an electric blender. The ginger extract was obtained by hydrodistillation for 4 hours using a Clevenger apparatus.

Film preparation

Starch (4 g), glycerol (2 mL 90% v/v), and ginger extract (0.5 mL, 1 mL, 1.5 mL, 2 mL) were blended at different combinations as shown in Table 1. Each combination was dissolved in 1% chitosan solution of 1% acetic acid to obtain 100 mL of film forming solution (FFS). FFS was thoroughly mixed with the aid of magnetic stirrer with the stirring speed of 12000 rpm for 30 minutes. The FFS was then heated on a hot plate with a continuous stirring at the temperature of 95^oC until the starch gelatinized and continued for ten more minutes. Some portions of gelatinized FFS were poured into plastic Petri dishes of diameter 8.5 cm and oven dried at 30^oC for 24 hours according to Sanyang *et al.* (2015) with modifications.

Fourier Transform Infrared spectroscopy (FTIR)

FTIR was performed in order to evaluate the functional groups interaction, compatibility and uniformity of the films. The spectra of the films were carried out using FTIR spectroscopy in the range of 4000 – 650 cm^{-1} using Agilent FTIR spectrometer (CARRY 630, Agilent Technology, USA).

Antimicrobial Assay

The Disc Diffusion assay was used to evaluate the antimicrobial activity of the films. The films produced with and without (control) ginger extract and chitosan were aseptically cut into 12 mm discs and placed on plates containing nutrient-agar, which had been previously spread with 0.1 mL of inoculums, each containing 10^7 CFU mL^{-1} of bacterial cultures. The plates were incubated at 37^oC for 24 h. The diameter of the growth inhibition zones around the discs was measured using a millimetre rule.

Statistical analysis

Films Fb1-Fb5 and Fbc were tested with the following microorganisms: *Staphylococcus aureus* (SA), *Pseudomonas aeruginosa* (PA), *Salmonella typhimurium* (ST), *Bacillus cereus* (BC). The tests were carried out in triplicate for each formulation and the results were presented in mm as mean ± SD. The zones of inhibition means were analysed by analysis of variance (one-way ANOVA), Levene's Test and post hoc multiple comparison tests (Tukey's test), statistical significance was identified at 95% confidence level ($P < 0.05$) using SPSS V20 software.

Table 1: Compositions of chitosan/ginger extract/starch/glycerol blends for antimicrobial analysis

Formulation	Chitosan (%)	Ginger Extract (mL)	Starch (%)	Glycerol (mL)
Fb1	1	2	4	2
Fb2	1	1.5	4	2
Fb3	1	1	4	2
Fb4	1	0.5	4	2
Fb5	1	0	4	2
Fbc	0	0	4	2

Fb1	**Film formulation (1)**	**Fb4**	**Film formulation (4)**
Fb2	**Film formulation (2)**	**Fb5**	**Film formulation (5)**
Fb3	**Film formulation (3)**	**Fbc**	**Film formulation (control)**

RESULTS

FTIR Spectra

To investigate the interactions of the constituents of the blends, FTIR spectroscopy was used. In Figure 2, "A" represents the film composed of chitosan, starch, glycerol and ginger extract, and "B" is the spectrum of film comprising of all the constituents except ginger extract, while C shows the spectrum of the film without chitosan and ginger extract.

FTIR spectra of the films

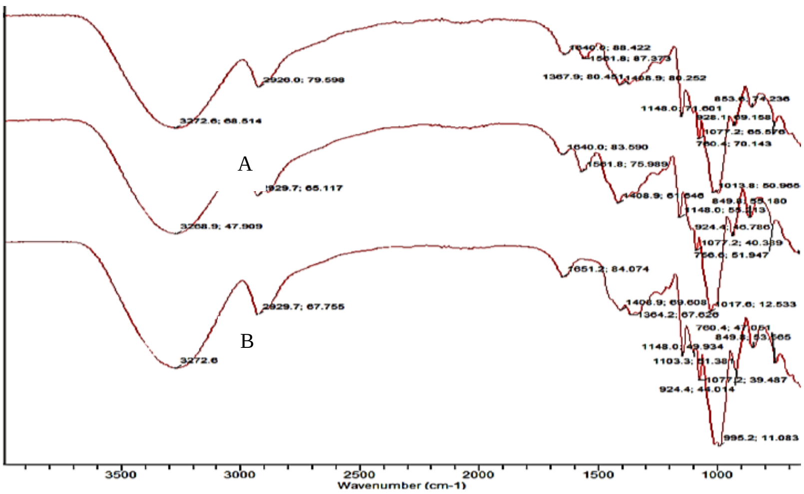

Figure 2. FTIR spectra of chitosan/starch/ginger extract/glycerol blend films

Antimicrobial Activity

Table 2 shows the result of antimicrobial activities of chitosan films (Fb1-Fb5, and Fbc) and ciprofloxacin with their zones of inhibition as mean ± SD in mm. The films had no effect on *Bacillus cereus* and *Salmonella typhimurium,* and Fbc which served as a negative control had no effect on all the tested microorganisms (*Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus and Salmonella typhimurium*) (Plate 1) and Ciprofloxacin which served as a positive control showed activity in all the tested microorganisms (Plate 2). The antimicrobial activity of the chitosan blend incorporated with ginger extract is presented in the Table 2. In the disc diffusion assay, all chitosan film blends incorporated with ginger showed antimicrobial activities and chitosan film without ginger extract showed minimal antimicrobial activity, while starch-only films which served as a negative control showed no antimicrobial activities In order to determine the potential antimicrobial effects of chitosan film blends, ciprofloxacin was used as a positive control for comparison

Table 2: Measure of the zones of inhibition of tested microorganisms

Film	Zone of inhibition (mm)			
	Staphylococcus aureus	**Pseudomonas aeruginosa**	**B. cereus**	**S. typhimurium**
F_{b1}	**19.333 ± 1.155**	**16.333 ± 1.528**	-	-
F_{b2}	**14.667 ± 1.155**	**14.667 ± 1.155**	-	-
F_{b3}	**13.000 ± 1.000**	**13.333 ± 0.577**	-	-
F_{b4}	**11.333 ± 0.577**	**12.333 ± 0.577**	-	-
F_{b5}	**13.667 ± 0.577**	**12.333 ± 0.577**	-	-
F_{bc}	-	-	-	-
Ciprofloxacin	**23**	**23**	**22**	**19**

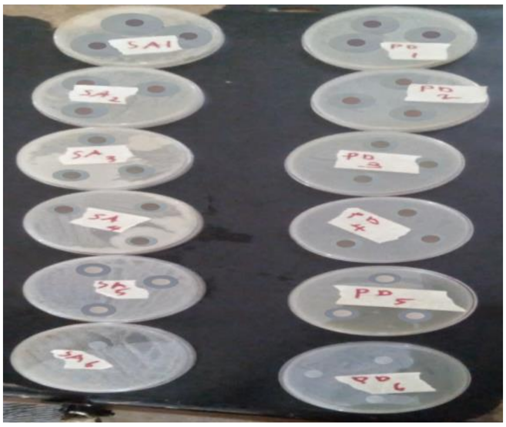

Plate 1: Petri dish for antimicrobial test of the Films against Staphylococcus aureus, Pseudomonas aeruginosa

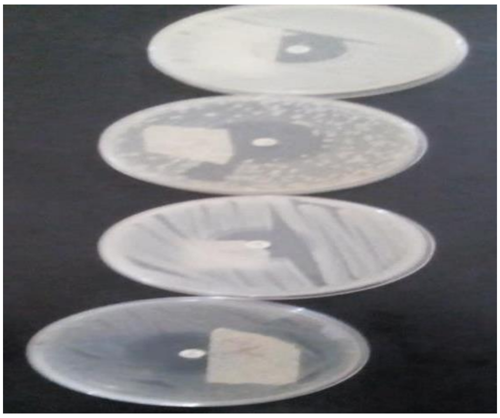

Plate 2: Petri dish for the antimicrobial test of Ciprofloxacin against Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus cereus.

One-way analysis of variance was conducted at 95% Confidence Level (CL) to evaluate the assumption that there was no significant difference in the efficacy of the chitosan blend formulations against targeted microorganism (SA and PA). 'Factors' were the different films (Fa1 –Fa5), while 'variables' were zones of inhibitions, included two groups: SA (Mean = 14.400, Std. Deviation = 2.898) and PA (Mean = 13.800, Std. Deviation = 1.781).

Firstly, The ANOVA was significant, $F(2, 10) = 32.423$, $p = 0.000$ at 95% CL. Thus, there was significant difference in the efficacy of the chitosan blend films against targeted microorganism 'SA'.

Secondly, The ANOVA was significant, $F(2, 10) = 9.393$, $p = 0.002$ at 95% CL. Thus, there is significant difference in the efficacy of the chitosan blend films against targeted microorganism 'PA'.

The assumption of homogeneity of variances was tested for SA and found to be tenable using Levene's Test, $F(2, 10) = 1.053$, $p = 0.428$ at 95% CL. The assumption of homogeneity of variances was also tested for PA and found to be tenable using Levene's Test, $F(2, 10) = 2.000$, $p = 0.171$ at 95% CL. (Table 3)

Table 3: Test of homogeneity of variances between SA and PA

	Levene Statistic	df1	df2	Sig.
SA	**1.053**	**4**	**10**	**0.428**
PA	**2.000**	**4**	**10**	**0.171**

Post hoc comparisons to evaluate pairwise differences among group means were conducted with the use of Tukey HSD test since equal variances were tenable as proved from Levene's Test. For SA, the tests revealed that there was significant difference between Fb1 and the rest of the Factors in the group (Fb2, Fb3, Fb4 and Fb5), significant difference between Fb2 and 'Fb1 and Fb4', significant difference between Fb3 and Fb5 against Fb1 and significant difference between Fb4 and 'Fb1 and Fb2' (Table 4). For PA, the tests revealed that there was significant difference between Fb1 and the rest of the Factors in the group (Fb2, Fb3, Fb4 and Fb5), no significant difference between Fb2 with the rest of the factors in the group (Fb1, Fb3, Fb4 and Fb5) and significant difference between Fb3, Fb4 and Fb5 against Fb1 (Table 4).

Table 4: Multiple comparisons
Tukey Honest Significant Difference

Film	Zone of inhibition (mm)	
	Staphylococcus aureus	**Pseudomonas aeruginosa**
F_{b1}	**19.333 ± (1.155)**^{a}	**16.333 ± (1.528)**^{a}
F_{b2}	**14.667 ± (1.155)**^{b,c}	**14.667 ± (1.155)**
F_{b3}	**13.000 ± (1.000)**^{b}	**13.333 ± (0.577)**^{b}
F_{b4}	**11.333 ± (0.577)**^{b,d}	**12.333 ± (0.577)**^{b}
F_{b5}	**13.667 ± (0.577)**^{b}	**12.333 ± (0.577)**^{b}

***Values in parenthesis are standard deviation, n = 3. Means with the different letter in the same column indicate significant differences** ($p < 0.05$).

DISCUSSION

Pure chitosan has four main distinctive bands. Firstly, broad band ranging from around 3500–3100 cm^{-1} which is attributed to N–H and OH–O stretching vibration. The intermolecular hydrogen bonding of chitosan molecules also to a certain degree plays a role in the absorption at this band (De Vasconcelos *et al*., 2006). Secondly, band located at 2877.5 cm^{-1} is attributed to CH stretching (Zivanovic et al., 2007). Thirdly, the band at 1654.9 cm^{-1} is assigned to amide-I band (Wan *et al.*, 2006). Lastly, the bond around 1580.4 cm^{-1} is the amide-NH_2 band (Duan *et al.,* 2004). N-H and OH….O stretching vibrations, and intermolecular hydrogen bonding of chitosan molecules were detected as broad bands around 3500-3100 cm^{-1} (De Vasconcelos *et al.,* 2006; Li, 2008). Band between 2929.7 – 2922.2 cm^{-1} was from CH stretch; the bands at 1632.6 cm^{-1} and 1640 cm^{-1} were assigned to amide I (Wan *et al.,* 2003). Band at 1408.9 is associated with CH_2 bending and CH_3 deformation, band at 1543.1 cm^{-1} is assigned to amide II which is noticeably absent in the spectra "C", due to the absence of chitosan in the films. The observable changes in the spectral peaks wavenumbers can be attributed to the interaction taking place in a definite system (Yin *et al.,* 1999). The shifts from lower to higher wavenumber and vice versa, indicates that interactions have taken place. In antimicrobial assay using film disc method, the size, shape, polarity of the diffusing molecule, and the chemical structure of the film play a crucial role (Cagri *et al.,* 2001). Chitosan films were found to only inhibit the organisms that were in direct contact with the active sites (Coma *et al*., 2002; Li, 2008). One of the limitations of the detection of antimicrobial activity of chitosan films is the hydrophilic nature of chitosan and chitosan-starch-glycerol blend which could result in the swelling and bending of the film, thus preventing the film to stay in full contact with the inoculated agar (Zivanovic *et al*., 2005; Rhim *et al*., 2006;). Overall, Fb1 film (containing 2 mL of ginger extract) was the most effective, others also showed antimicrobial activities but there were no much significant difference with the rest of the members of the group. Fb5, which lacked ginger extract also showed antimicrobial activity as the result of the present of chitosan which was greater or equal to Fb4. The use of crude ginger extract (which contains limited amount of active antimicrobial agents) as against using refined ginger extract enriched with its essential oil, in addition to limited contact areas between the films and the inoculated agar may contribute to the minimal antimicrobial activity of some of the tested films.

CONCLUSION

Edible films comprising of chitosan, starch, glycerol and ginger extract mixed at varying proportions were prepared and analysed. FTIR was used to determine the functional group interactions between the matrix and the added agents, and the antimicrobial activities of the films were also determined. Production and characterization of chitosan edible films to test the physical and antimicrobial properties of the films were carried out. Chitosan films incorporated with 2 mL of ginger extract were found to inhibit the activities of *Staphylococcus aureus* and *Pseudomonas aeruginosa* more compared to other films. Chitosan films without ginger extract were also found contain some antimicrobial activity.

REFERENCES

Abdou, E.S., Nagy, K.S.A., Elsabee, M.Z. (2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresources Technology*, 99, 1359-1367.

Aranaz, I., Mengíbar, M., Harris, R., Paños, I., Miralles, B., Acosta, N., ... & Heras, Á. (2009). Functional characterization of chitin and chitosan. *Current chemical biology*, *3*(2), 203-230.

Bergo, P., Sobral, P. J. A., & Prison, J. M. (2010). Effect of glycerol on physical properties of cassava starch films. *Journal of Food Processing and Preservation*, *34*(s2), 401-410.

Cagri, A., Ustunol, Z., & Ryser, E. T. (2001). Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing p-aminobenzoic or sorbic acids. *Journal of Food Science*, *66*(6), 865-870.

Coma V, Gros MA, Garreau S, Copinet A, Salin F, Deschamps A. (2002). Edible antimicrobial films based on chitosan matrix. J Food Sci. 67(3): 1162-1169.

De Vasconcelos, C., Bezerril, P., Dos Santos, D., Dantas, T., Pereira, M., Fonseca, J., (2006). Effect of molecular weight and ionic strength on the formation of polyelectrolyte complexes based on poly (methacrylic acid) and chitosan. Biomacromolecules 7 (4), 1245–1252.

Devlieghere, F., Vermeiren, L., & Debevere, J. (2004). New preservation technologies: Possibilities and limitations. International Dairy Journal, 14, 273–285.

Duan, B., Dong, C., Yuan, X., Yao, K. (2004). Electrospinning of chitosan solutions in acetic acid with poly (ethylene oxide). Journal of Biomaterials Science, Polymer Edition 15 (6), 797–811.

Hossain, M. S., & Iqbal, A. (2014). Production and characterization of chitosan from shrimp waste. *Journal of Bangladesh Agriculture Univ*ersity, *12*(1), 153-160.

Li, J. (2008). Characterization and Performance Improvement of Chitosan Films as Affected by Preparation Method, Synthetic Polymers, and Blend Ratios. PhD dissertation, University of Tennessee.

Mahendran S., Keeran N. *et al*. (2014). Comparative evaluation of antimicrobial properties of red and white ginger. Asian Journal of Pharmaceutical and Clinical.

Martino, A.D., Sittinger, M. and Risbud, M.V. (2005) 'Chitosan: A versatile biopolymer for orthopaedic tissue engineering', *Biomaterials*, 5983-5990.

Mokrejs, P., Langmaier, F., Mladek, M., Janacova, D., Kolomaznik, K. and Vasek, V. 2009. Extraction of collagen and gelatine from meat industry by-products for food and non food uses. Waste Management and Research. 27(1): 31–37.

Rhim JW, Hong SI, Park HW, Ng PKW. (2006). Preparation and characterization of chitosanbased nanocomposite films with antimicrobial properties. *Journal of Agricultural and Food Chemistry*, 54: 5814- 5822.

Rinaudo, M. (2006). Chitin and chitosan: properties and applications. *Progress in polymer science*, *31*(7), 603-632.

Sanyang, M.L.; Sapuan, S.M.; Jawaid, M.; Ishak, M.R.; Sahari, J. (2015) Effect of plasticizer type and concentration on physical properties of sugar palm starch (Arenga pinnata) films. Ind. CropsProd., under review.

Tharanathan, R.N. and Kittur, F.S. (2003). Chitin - The undisputed biomolecule of great potential. *Critical Reviews in Food Science and Nutrition*, vol. 43, no. 1, p. 61-87.

Wan, Y., Creber, K.A.M., Pepply, B., Bui, V.T., (2003). Ionic conductivity of chitosan membrane. Polymer 44, 1057–1085.

Wan, Y., Wu, H., Yu, A., Wen, D., (2006). Biodegradable polylactide/chitosan blend membranes. Biomacromolecules 7 (4), 1362–1372.

Yin, Y.J., Yao, K.D., Cheng, G.X., Ma, J.B., 1999. Properties of polyelectrolyte complex films of chitosan and gelatin. Polymer international 48 (6), 429–432.

Zivanovic, S., Li, J., Davidson, P.M., Kit, K., 2007. Physical, mechanical, and antibacterial properties of chitosan/PEO blend films. Biomacromolecules 8 (5), 1505–1510.