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Chitosan-Containing Bread Made Using Marine Shellfishery Byproducts: Functional, Bioactive, and Quality Assessment of the End Product

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ABSTRACT: Chitosan is nature's second most abundant polymer after cellulose and forms the structural support in crustacean shell material and Basidomycete mushroom stalks. Chitosan is a known antimicrobial agent but, to date, was not examined as an antimicrobial agent in bread formulations for the prevention of mold or rope formation. The aim of this work was to investigate the effects of chitosan generated from prawn shell byproducts on the color, moisture, and texture and crumb formation of bread. A secondary aim of this work was to determine the antimicrobial effect of chitosan added to bread at a rate of 1% against the rope spoilage pathogen *Bacillus cereus* along with natural molds. The addition of chitosan to bread with a molecular mass of 124000 ± 10000 g/mol and 19% deacetylated was found to inhibit *B. cereus* growth and rope formation in bread when monitored over 3−5 days. Natural mold growth was also significantly delayed in bread made using chitosan substitution of flour at 1% compared to the control bread, where mold was observed growing on the bread surface after 72 h when bread was incubated at 30 °C.

KEYWORDS: *chitosan, bread, antimicrobial, crumb texture, hardness, loaf volume, Bacillus cereus*

INTRODUCTION

Sustainable food production and waste valorization are becoming important issues in the food industry, where high amounts of byproducts are generated. Expansion of the fishing industry worldwide has created a significant environmental and hygiene problem, and scientists have focused their efforts on byproduct utilization and total catch utilization.[1] In recent decades, functional foods have gained increased consumer interest, and the utilization of marine processing coproducts to generate bioactive ingredients for functional food use is both an economic and environmentally attractive option for marine processors. Processing of prawns for human consumption generates byproducts, and approximately 35% of the total mass of a prawn is discarded as waste.[2]

However, prawn exoskeleton contains between 20 and 50% chitin on a dry weight basis.[3] Chitin is the second most abundant biopolymer in nature and the most widespread amino-polysaccharide.[4,5] Chitin can be found in the cuticles of insects, the cell wall of fungi, yeasts, and green algae,[6] and the shell of crustaceans.[7] Chitin has an ordered, crystalline structure and has been found in three polymorphic forms, α-, β-, and γ-chitin.[8,9] At present, the majority of chitin and chitosan produced commercially is chemically extracted from crab, shrimp, and prawn exoskeleton waste.[10] α-Chitin, the polymorphic form found in prawn and crustacean shells with the exception of squid pen, is the most common form and is usually found when extreme hardness is required.[11,12] Polymorphic forms of chitin differ in the arrangement of the chains within the crystalline regions, in their degree of hydration, in the size of the unit cell, and in the number of chitin chains per unit cell.[11,13,14] In chitin, the degree of acetylation (DA) is typically 0.90, indicating the presence of some amino groups.[15] Chitin properties such as solubility, reactivity, biodegradability, and adsorption of different substances depend on the amount of protonated groups in the polymeric chain and, therefore, on the proportion of acetylated and nonacetylated glucosamine units.[16,17]

Chitosan is the N-deacetylated derivative of chitin, with a DA under 0.35.[15] In contrast to chitin, these free amino groups allow chitosan to dissolve in diluted aqueous acidic solvents due to the protonation of these groups.[14] Chitosan has been largely used in many areas such as biotechnology,[18] cosmetics and photography,[19] biomedical products,[20] water treatment,[21] the paper industry,[22] textile products,[23] and food processing.[14] Chitosan is biocompatible, biodegradable, and nontoxic, and it has been demonstrated to have fungistatic, spermicidal, antitumor, anticholesteremic, wound-healing, antimicrobial, antioxidative, and hemostatic properties.[14,24−26]

Foods ideally suited for use as bioactive delivery vehicles include those widely, easily, and regularly consumed. For example, bread was used previously for the delivery of omega-3 fatty acids.[27] Bread is also encouraged as part of a healthy diet and was the food vehicle of choice in this study. Indeed, chitosan breads have already been manufactured and assessed for antioxidative and anticholesterol effects.[26,28,29] Previously a bread formulation containing chitosan was evaluated in terms of its efficacy and safety in dyslipidemic type 2 diabetic subjects.[26] The results suggested that chitosan incorporated into bread formulations at 2% (w/w) improved the lipoprotein balance in consumers similar to typical biliary salt trappers. It also increased high-density lipoprotein (HDL-) and lowered low-density lipoprotein (LDL-) cholesterol without changing the triglyceride levels.[26]

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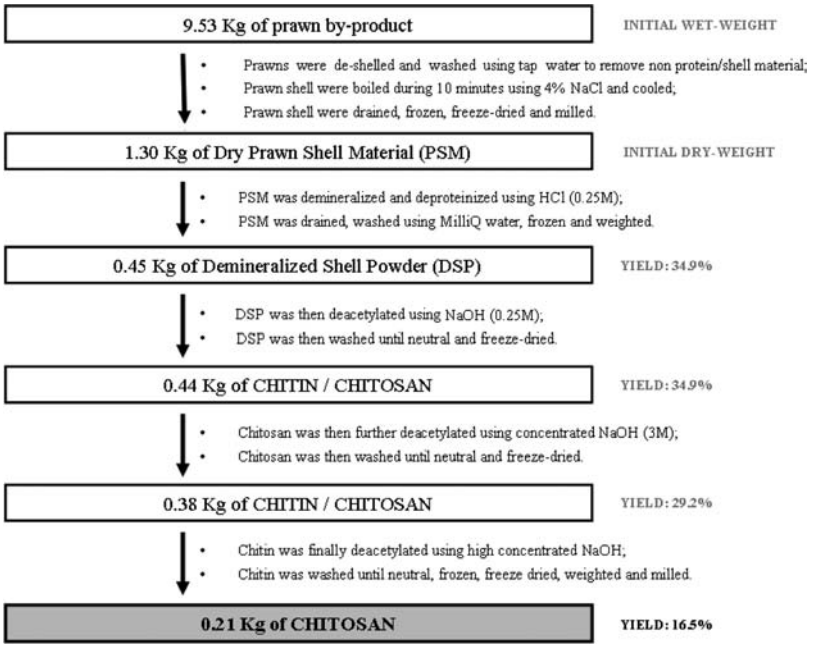

Figure 1. Chitosan generation from prawn byproduct material.

Loss of bread quality is generally associated with microbial spoilage, staling, and moisture loss. The most common sources of microbial spoilage of bread are mold and bacterial growth.[29] Consumer shopping patterns have changed during the past decades such that the daily trip to the bakery is not the norm and as such, consumers expect bread products to remain spoilage free for significant periods of time.[30] Bread is a highly perishable product, and to achieve an acceptable shelf life, it is necessary to introduce additives. The bread industry has been working for the past 30 years to reduce the number of additives and so-called synthetic preservatives in bread, in a genuine effort to make it as natural and fresh as possible.[31]

Rope spoilage is one of the most common bacterial decompositions of bread. Spoilage organisms include different *Bacillus* species, which are Gram-positive, spore-forming bacteria widely distributed in nature and whose heat-resistant spores can survive pasteurization and baking processes.[32] Ropiness is caused mainly by *Bacillus subtilis* but also by other *Bacillus* species such as *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus cereus*.[33] *Bacillus* contamination in the bakery industry was previously reported to be due to contamination of raw materials and bakery equipment.[34] Rope occurs particularly when warm (25−30 °C) and humid environmental conditions allow germination of *Bacillus* spores.[34] This may result in economic losses as well as foodborne illnesses.[35−37]

The objective of this work was to produce chitosan-containing bread, using chitosan generated from prawn (*Nephrops norvegicus*) shell byproducts, and to study the influence of its inclusion on bread quality. Chitosan bread was made with the potential to extend the shelf life of bread without adversely affecting the taste and structure of bread. The effects of chitosan inclusion in bread on parameters including color, volume, texture, moisture, crumb grain structure, and shelf life were studied over a 5 day period. Furthermore, the antimicrobial properties of bread containing 1% chitosan were studied for mold prevention and against *B. cereus*.

MATERIALS AND METHODS

Materials. Prawn material (approximately 10 kg) was kindly supplied by Clanawley Kilmore International (CKI). The chemical extraction of chitin is based on a series of demineralization and deproteinization steps using acid and base. Chitin was deacetylated into chitosan using concentrated alkali sodium hydroxide.[14,19] In this study, HCl, NaOH, and all other chemicals used were supplied by Sigma (Aldrich, Dublin, Ireland).

Wheat flour (Shackleton's Millers, Co. Meath, Ireland), salt, emulsified bread fat (Irish Bakels Ltd., Dublin, Ireland), and dried yeast (Doves Farm, UK) were required for the breadmaking process.

Chitosan Generation. Initially, 9.53 kg of prawn (*N. norvegicus*) byproduct consisting of shell and protein material was collected and frozen in a Styrofoam box. This byproduct was kept frozen and stored in the dark during transportation. The prawn shell material was thawed and separated into heads and claws. Chitosan was generated following the procedure described in Figure 1. Prawn shell material was heated in boiling sodium chloride (4% NaCl) for 10 min and cooled in tap water to remove prawn protein material. Visible meat was also removed from the shell material by hand, and clean shells were washed extensively in tap water and freeze-dried. Clean shells were weighed, milled, and sieved, and 1.30 kg of material labeled as prawn shell material (PSM) was obtained. PSM was demineralized and deproteinized using a BioFlo 110 Modular Bioreactor (New Brunswick Scientific). HCl (0.25 M) was added to the PSM in a 1:40 w/v ratio. The temperature of the reaction was maintained at 40.0 °C for 6 h. PSM was drained, washed until pH 7.0 (neutral) using Milli-Q water, frozen, freeze-dried, and weighed. A yield of 0.45 kg was obtained, and this material was referred to as demineralized shell powder (DSP). DSP was deacetylated and further deproteinized using 0.25 N NaOH at a shell to solvent ratio of 1:40 w/v. The reactor conditions were kept constant at 40 °C for 4 h. A yield of 0.44 kg of chitin was obtained. Chitosan was prepared by hydrolysis of the acetamide groups of chitin, using a severe alkaline treatment.[14] In this work, chitin was further deacetylated using 3.0 M NaOH at a chitin to solvent ratio of 1:40 w/v. The reaction was maintained at 70.0 °C for 6 h. Chitin was then washed until neutral, frozen, freeze-dried, and weighed. A yield of 0.38 kg chitosan was obtained. A final deacetylation step was carried out by subjecting chitosan to treatment with 45% NaOH at 100 °C for 6 h. The final product, chitosan, was washed until neutral using Milli-Q water, frozen, freeze-dried, weighed, and milled. A final yield of 0.22 kg of chitosan was obtained. Chitosan

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was milled and sieved, and chitin/chitosan particles >500 μm in size were discarded.

Chitosan Assessment. Molecular weight (MW) analysis and sedimentation studies were performed on generated prawn chitosan samples. The sedimentation and molecular weight data for the generated chitosan were analyzed with SEDFIT-MSTAR, a modern version of the MSTAR program.[38] The degree of acetylation was measured by nuclear magnetic resonance (NMR).

Breadmaking. Bread loaves were produced following a straight dough baking procedure. The doughs were prepared for mixing according to the formulations listed in Table 1.

Table 1. Composition of Bread Made Using 1% Chitosan

ingredient	white bread (g)	chitosan bread (g)
flour	180.0	178.2
chitosan	0.0	1.8
salt	1.8	1.8
fat	1.8	1.8
yeast	2.7	2.7
improver	0.9	0.9
water	111.4	111.4

The amount of water added and the optimal mixing time were calculated using a Chopin MixOlab. After mixing, the dough was divided into 290 g pieces and placed in a proofer (Koma SDCC-1P/W, Koma Koeltechnische Industrie B.V., The Netherlands) at 35.0 °C and 80% relative humidity for 15 min. The bread pieces were then molded by hand, placed in tins (15 × 10 × 07 cm), and proofed for a further 45 min. The loaves were baked at 220.0 °C for 25 min. Four control and four chitosan loaves were produced per batch. The loaves were allowed to cool for 2 h, placed into plastic bags, and stored at room temperature. The loaves were tested at days 1 and 5 postbaking.

Color Measurement. Color recordings were taken on bread loaves using a Hunter Lab colorimeter (Hunter Lab, Ultrascan XE, Reston, VA, USA). Crust and crumb CIE values were recorded: L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness) (Table 2). The UltraScan XE was standardized using black and white

Table 2. CIE Color Values of Control Crust and Crumb and Chitosan (1%)-Containing Bread Crust and Crumb

	L^*	a^*	b^*
Color Recordings, Day 1			
control (crust)	54.24 ± 0.42	12.49 ± 0.08	20.01 ± 0.35
control (crumb)	80.52 ± 0.21	0.49 ± 0.08	16.29 ± 0.13
chitosan (crust)	54.18 ± 0.46	12.36 ± 0.11	20.12 ± 0.37
chitosan (crumb)	79.32 ± 0.20	0.53 ± 0.02	16.61 ± 0.07
Color Recordings, Day 5			
control (crust)	52.06 ± 0.34	11.75 ± 0.13	17.54 ± 0.36
control (crumb)	80.52 ± 0.36	0.49 ± 0.03	16.29 ± 0.13
chitosan (crust)	52.00 ± 0.36	10.58 ± 0.10	16.95 ± 0.35
chitosan (crumb)	79.32 ± 0.37	0.53 ± 0.02	16.61 ± 0.07

tiles. Illumination (D65, 10°) with an 8° viewing angle and a 10 mm port size was used. D65 was the chosen illuminant as it approximates to daylight. All samples were analyzed in triplicate on days 1 and 5.

Volume Measurement. The volume of the bread loaves was calculated using a volume measurer (BVM-L370, TexVol Instruments, Vike, Sweden). The results are the average of three samples and were measured at days 1 and 5 postbaking.

C-Cell. For sample preparation, the loaf was sliced by hand to 10 mm thickness. Image analysis of bread slices was conducted by C-Cell (Calibre Control International Ltd., UK). The instrument was connected to a PC running C-Cell software version 2.0. The samples were measured at day 1 postbaking.

Texture Analysis. Texture profile analysis of bread slices was measured using a Texture Analyzer (TA-XT2i; Stable Microsystems, Surrey, UK), equipped with a P/20P 20 mm Perspex cylinder probe. The test was performed at days 1 and 5 postbaking .

Moisture Measurement. Bread crumb moisture was calculated at 1 and 5 days after baking with a two-step oven method, AACC air-oven method 44-15 (1983). This method determines moisture content as loss in weight of a sample when heated under specified conditions. The moisture oven (Brabender Corp., Duisburg, Germany) was used to heat 10 g of milled crumb, cut from a central plug from the center slices, at 130.0 °C for 1 h.

Antimicrobial Analysis of Chitosan Bread. The antimicrobial effect of chitosan addition to bread was studied by assessing the variation in natural mold growth and intentional rope development on both the control and chitosan-containing bread. *B. cereus* strain NCTC 7464 was inoculated into 25 mL of Nutrient Broth (NB) (Oxoid Ltd., UK) and incubated at 37 °C for 24 h. The overnight culture was serially diluted in NB to give the required numbers of ca. 10^6 cfu mL^{-1} in the solution used for inoculation of the bread. Cell numbers were confirmed by plating. Three slices (10 mm thickness) of the control and chitosan-containing bread were cut into 6 cm diameter circles and placed into sterile Petri dishes. For both breads, one slice was inoculated with a 1 mL aliquot of the diluted *B. cereus* solution (A), whereas two slices were set up as controls and inoculated with either 1 mL of sterile nutrient broth (B) or left uninoculated (C). All Petri dishes were introduced in plastic bags and incubated at 30 °C for 72 h. Visible mold growth and rope formation were graded as either none (−), questionable (±), slight rope (+), moderate rope (++), or significant rope (+++) .

Statistical Analysis. All tests were replicated three times, and mean values and standard deviations were calculated. The experimental data were subjected to an analysis of variance using a Statistical Analysis System (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Chitosan Characterization. Sedimentation velocity analysis of generated chitosan samples gave sedimentation coefficient (S) and concentration dependence of sedimentation coefficient (k_S) values in the ranges expected for chitosan. Sedimentation velocities were measured at several concentrations and extrapolated back to zero concentration, obtaining $S^o_{20,W}$ = (1.8 + 0.1) S and k_S = (100 + 50) mL/g (Figure 2). The sedimentation equilibrium data were generated using the new software SEDFIT-MSTAR, a modern version of the MSTAR program described by Colfen and Harding.[38] Chitosan

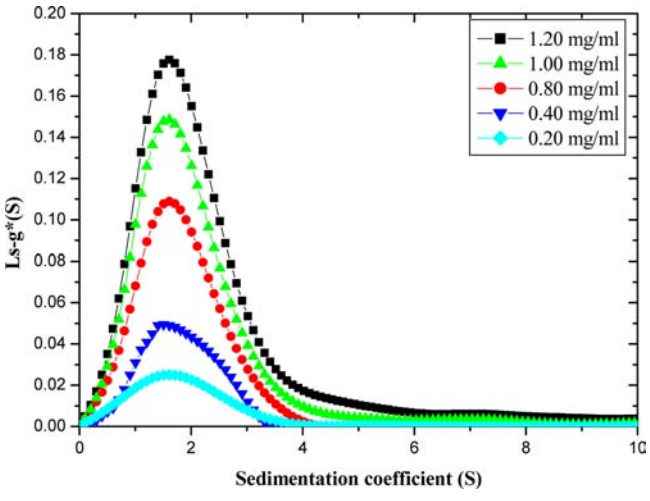

Figure 2. Sedimentation coefficient values for chitosan generated from prawn shell material.

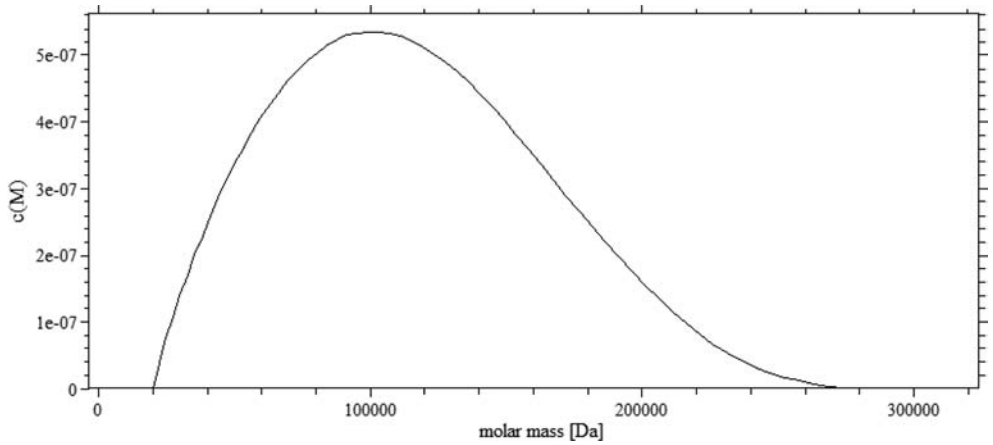

Figure 3. Molar mass weight distribution found for chitosan generated from Irish prawn shell materials.

was analyzed using a rotor speed of 40000 rpm and a temperature of 20.0 °C in an acetate buffer at pH 4.3. The partial specific volume was 0.57 mL/g. The sedimentation equilibrium results were obtained using a rotor speed of 10000 rpm, at 20.0 °C, and in an acetate buffer at pH 4.3. The partial specific volume was 0.57 mL/g, and a loading concentration of 0.5 mg/mL was used. The results showed an average molecular mass of 124000 ± 10000 g/mol. The generated chitosan was reasonably unimodal, as shown in Figure 3. The DA of chitosan was 19% and was calculated using NMR.

Bread Properties. In addition to its nutritional and health beneficial properties, chitosan was reported previously to have antifungal and antimicrobial effects and was demonstrated to alter yeast activity previously.[39,40] MixOlab results showed a significant difference in dough development time and stability following the inclusion of 2% chitosan. A slight variation in the water absorption percentage was observed in the rheological tests compared to the 100% flour formulation. Following a number of preliminary baking trials, the standard breadmaking procedure was not significantly modified, apart from increased intermediate and final proofing times. The result was a symmetrical loaf, well proportioned, with a well-rounded top free from streaks and an even, rich brown crust color (CIE color values shown in Table 2). Preliminary baking tests also showed how inclusions of chitosan at concentrations >1% significantly negated the quality of the bread, particularly in terms of loaf volume and crumb texture, even when optimum water absorption and dough development time were applied.

The addition of chitosan produced a decrease in loaf specific volume and slice area compared to the control ($p < 0.05$), as shown in Table 3. Indeed, a decrease in specific volume in bread loaves was observed in previous studies as a result of the addition of other fibrous ingredients.[13,32] Chitosan was also found to alter yeast activity previously,[26] affecting the specific volume and slice area even in low concentrations. This is why a number of preliminary proofing and baking trials were carried out prior to the present trial.

Table 3. Specific Volume and Slice Area at Day 1 Postbaking of Chitosan (1%)-Containing and Control Breads

	batch 1	batch 2	batch 3
	Specific Volume (mL/g)		
control	3.90 ± 0.05	4.03 ± 0.01	3.97 ± 0.02
chitosan	3.71 ± 0.02	3.60 ± 0.04	3.45 ± 0.03
	Slice Area (mm^2)		
control	7393.5 ± 47.0	7249.0 ± 49.1	7568.0 ± 175.1
chitosan	7218.0 ± 62.5	6740.5 ± 45.8	6799.8 ± 52.2

Color is one of the most important quality parameters in bread products, as it has a striking effect on the perception of the product by consumers and influences them on other factors such as aroma or flavor. Previous studies have indicated that the addition of chitosan to bread formulations resulted in a darker color and that this may be due to increased intensity of Maillard reactions.[28] However, in this work, this was not the case as indicated in Table 2. The chitosan bread made using 1% chitosan was found to have a crust color showing no significant variations compared to the control. A positive correlation was found between crust concavity and crumb L^* value (0.87, $p < 0.05$), as a greater degree of concavity results in a more compact crumb. A more compact crumb alters the light reflection, varying the perception of the color. A smaller area of cells and cell diameter may also alter color perception.

Chitosan was found to affect the redistribution and state of water in bread and to facilitate the dehydration of starch and gluten, and moisture migration from crumb to crust in higher concentrations.[28,40] According to the results, substituting 1% of flour for chitosan produced no significant variation in moisture content between the chitosan-containing bread and the control. Moisture loss was observed between days 1 and 5, as expected, due to bread staling (Table 4).

Table 4. Total Moisture Content at Days 1 and 5 Postbaking in Control and Chitosan (1%)-Containing Bread

	moisture, day 1 (%)	moisture, day 5 (%)
control bread	44.26 ± 0.47	41.68 ± 0.40
chitosan bread	43.95 ± 0.16	41.10 ± 0.71

C-Cell results showed significant differences between chitosan-containing bread and the control for some parameters (Figure 4). The addition of chitosan to the bread formulation decreased the number of holes and the total area of holes in bread slices, as shown in Table 5 ($p < 0.05$). The cell diameter and the total area of cells also decreased (Table 6), generally showing a more even crumb grain throughout the loaf ($p < 0.05$).

Chitosan-containing bread and the control had similar texture profiles. There is an indirect correlation between the specific volume and the slice area and crumb hardness (−0.90,

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Figure 4. C-CELL image of white bread (A) and 1% chitosan-containing bread (B).

Table 5. Number of Holes and Total Area of Holes in Control and Chitosan (1%)-Containing Bread

	no. of holes	total area of holes (%)
control	0.69 ± 0.22	1.25 ± 0.43
chitosan	0.51 ± 0.15	0.83 ± 0.27

Table 6. Cell Diameter and Total Area of Cells of Control and Chitosan (1%)-Containing Bread

	area of cells (%)	cell diameter (mm)
control	54.34 ± 0.20	2.89 ± 0.07
chitosan	52.35 ± 0.14	2.57 ± 0.04

$p < 0.05$; −0.83, $p < 0.05$), as a lower volume has a more compact crumb texture. Even though the specific volume was lower, no significant difference in hardness was observed between the chitosan-containing bread and the control ($p < 0.05$) as shown in Table 7. In addition, a decrease in springiness

Table 7. Hardness, Springiness, and Resilience at Days 1 and 5 Postbaking of Chitosan (1%)-Containing Bread and Control Bread

	hardness (g)	springiness	resilience
		Day 1	
control	242.0 ± 8.7	0.937 ± 0.004	0.372 ± 0.010
chitosan	293.7 ± 13.5	0.926 ± 0.005	0.347 ± 0.003
		Day 5	
control	463.2 ± 15.8	0.912 ± 0.006	0.232 ± 0.008
chitosan	524.0 ± 37.3	0.898 ± 0.005	0.231 ± 0.014

at day 5 was observed, due to chitosan addition ($p < 0.05$). As expected, a change in hardness, springiness, and resilience was observed between days 1 and 5 in the control bread and in the chitosan-containing bread due to bread staling ($p < 0.05$). Texture data are shown in Table 7.

These results showed that the inclusion of chitosan (1%) in bread formulations produced a slight decrease in loaf specific volume but has no significant effect on the overall quality or acceptance of bread.

Antimicrobial Properties. Fungal spoilage is the main cause of substantial economic losses in packaged bakery products and can also be regarded as a source of pathogens that may result in public health problems.[41] The efficiency of chitosan for use as an antimicrobial agent was confirmed previously. Low molecular weight chitosan was shown to exhibit high antimicrobial activity against yeasts and bacteria after a short exposure time.[42] In this study, a decrease in yeast activity was observed when 1% of flour was substituted with chitosan, and complete inhibition of yeast activity was observed when chitosan was substituted for flour in the bread formulation at concentrations >4%.

When directly inoculated with *B. cereus*, chitosan bread strongly inhibited the growth of *B. cereus* and subsequent rope formation in bread over a 3 day period (Figure 5). Control

Figure 5. Inhibitory effects of chitosan bread against *B. cereus* and rope formation when directly inoculated in control and chitosan-containing bread (A).

bread samples developed a typical, high-fruity odor after only 24 h of incubation. In chitosan bread spiked with *B. cereus*, the development of ropiness and a fruity odor was delayed. The difference in growth, under the same conditions, was easily observed after 24 h of incubation of samples at 30 °C. When chitosan bread was inoculated with a sterile nutrient broth (Figure 6) or was left uninoculated, a delay in the development of natural mold growth was observed compared to the control bread (Table 8). The substitution of flour with chitosan (1%)

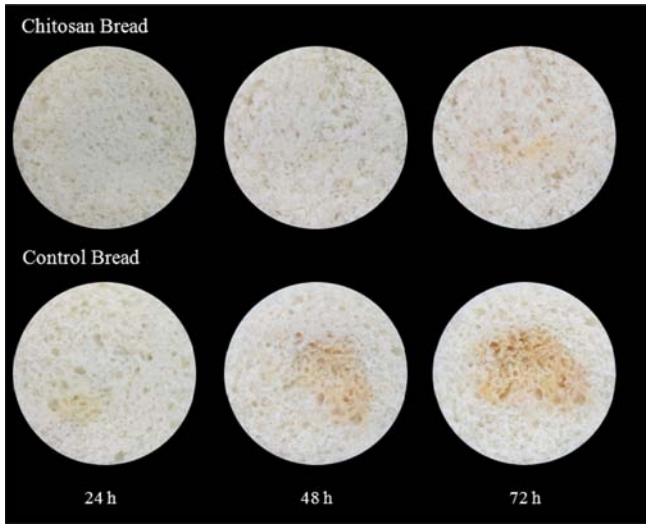

Figure 6. Inhibitory effects of chitosan bread against bacteria and mold in bread when inoculated with 1 mL of NB (B).

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Table 8. Bread Spoilage of Control and Chitosan (1%)-Containing Bread Slices Following Incubation at 30 °C for 96 h

loaf	slice	24 h	48 h	72 h	96 h
		Inoculation A			
control bread	1	±	++	+++	+++
	2	±	+	++	+++
	3	−	++	++	+++
chitosan bread	1	−	±	+	+++
	2	−	−	+	+
	3	−	±	+	++
		Inoculation B			
control bread	1	±	±	+	+
	2	−	±	++	++
	3	−	±	++	++
chitosan bread	1	−	−	±	+
	2	−	−	±	+
	3	−	±	+	++
		Inoculation C			
control bread	1	−	−	−	±
	2	−	±	±	±
	3	−	±	±	+
chitosan bread	1	−	−	±	+
	2	−	−	−	−
	3	−	−	−	±

in bread formulations produced a slight decrease in loaf specific volume, as a result of a decrease in yeast activity, but had no significant effect on the overall quality or acceptance of bread. White bread and chitosan containing-bread had comparable profiles.

During wheat cultivation, *Bacillus* strains attach to the ears of wheat and pass into flour during the milling process.[43] *B. cereus* was reported to be a causative agent of food poisoning.[36,37] In this work, chitosan was found to inhibit *B. cereus* growth and rope formation in bread when monitored over a 5 day period. Natural mold growth was also significantly inhibited in bread made using chitosan substitution of flour at 1% compared to the control bread, where higher mold was observed growing on the bread surface (Figure 6). In summary, chitosan extracted from prawn shell byproducts showed potential for use as an antimicrobial bread ingredient that could be incorporated into bread at low percent volumes to prevent rope formation.

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Notes

The authors declare no competing financial interest.

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