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EXTRACTION OF CHITIN/CHITOSAN FROM SHRIMP WASTES AND APPLICATION OF IRRADIATED CHITOSAN AS AN ANTI-BACTERIAL AGENT

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Abstract

Chitin, the second abundant naturally occurring polysaccharide, is found in the exoskeleton of insects and crustaceans (e.g. shrimps, lobsters, crabs, turtles etc.) and cell wall of fungi. The present investigation was carried out to extract chitin from shrimp wastes, convert chitin into chitosan, and evaluate the practicality of chitin-chitosan as an anti-microbial agent. Chitin was extracted from shrimp wastes. The conversion rate achieved was 28.26%. Both acetic acid and citric acid were found to be suitable for the solubility of chitosan, but acetic acid seemed to be a relatively better solvent for chitosan. Solubility was increased when chitosan was irradiated with 80 kGy gamma irradiation dose or more. Commercial chitosan and prepared chitosan were tested against *Staphylococcus aureus, Escherichia coli*, and *Bacillus* sp. isolated from spoiled mango and papaya. Experiments were conducted on radiation-induced enhancement of antimicrobial activity of chitosan. 0.01% chitosan (both prepared and commercial) was found to decrease the bacterial load of all the bacterial species and chitosan irradiated with 80 kGy or more had better antibacterial activity in this concentration, although this chitosan concentration was not effective enough to tota1 elimination of bacterial load. Chitosan concentration of 0.025% was found to be effective for total elimination of the bacteria.

Introduction

The yearly export of frozen shrimps and crabs/turtles in Bangladesh ranged between 15,000 and 26,000 metric tons for the last decade. About 50 - 60% body weight of shrimp head and shell are disposed as waste materials and 28% of these wastes materials are chitin. Chitin is composed with protein, minerals, fats etc. The structure of the copolymer chain of a chitin unit is β(1-4)-2-acetamide-2-deoxy-βD-glucan and a chitosan unit is β(1-4)-2-amino-2-deoxy-βD-glucan. Chitin-chitosan is unique owing to an additional nitrogen atom of acetamide and amine group at C-2 position. Basically, the nitrogen atom of chitin-chitosan acts as an electron donor and

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is presumably responsible for the selective chelation with metal ions.[1] The anhydroglucosamine chain makes chitin-chitosan behavior as a linear polyelectrolite at acidic pH. The free amino group in chitosan are considered to be much more effective for bonding metals ions than the acetyl group in chitin.[2] Due to the high density of amino group, chitosan can interact strongly with negatively charged substances, such as, proteins, dyes and polymers to give electric neutrality. Combining with the basic structure of polysaccharide, chitin-chitosan has two reactive sites, i.e., acetamide and amino group at C-2 position and primary alcohol at C-6 position. Both group are thus, important for physical and chemical modifications.

Application of gamma radiation on shrimp wastes reduces the treatment time that enhances the kinetics of chitin extraction and may also help to obtain chitosan with required molecular weight.[3] Irradiated chitosan increases the degree of deacetylation by 30% during conversion of chitin into chitosan.[4,5] Yoksan *et al.*[4] mentioned that molecular weight of gamma irradiated chitosan decrease up to 81.5%, which eventually enhances the reactivity of chitosan by 50 - 60% and degradability temperature of irradiated chitosan is much higher (309°C) than that of unirradiated chitosan. Gamma radiation (180 kGy) rarely affects the structure of chitosan but chitosan chain is formed by gamma radiation through decomposition of radicals to carbonyl compounds that play potential role for anti-microbial activity.[6]

The present investigation was carried out to extract chitin from shrimp wastes, standardize the method for extraction of chitin, convert chitin into chitosan, apply gamma radiation for the modification of chitin-chitosan to enhance its efficacy and evaluate the practicality of chitin-chitosan as an anti-bacterial agent.

Materials and Methods

Shrimp shells were collected from Bagerhat and washed thoroughly with distilled water. Shells were then dried at 70°C for 24 hrs. After drying, the shells were crushed into optimum particle size.

Preparation of chitin from shrimp shells : To extract chitin from shrimp shells, the following three basic steps[3] were followed : (i) Demineralization of the crushed shells in 2N HCl for 24 hrs at room temperature, (ii) removal of fats from the wastes by adding 1 N NaOH (solid: liquid = 1 : 20) keeping at room temperature for 24 hrs, and (iii) deproteination was carried out by heating the treated shells at 110°C for 8 hrs keeping in 1N NaOH. The contents were washed thoroughly with distilled water in every step. A second treatment cycle was then carried out by repeating the three basic steps to remove the remaining impurities.

Conversion of chitin into chitosan : Prepared chitin was taken in a volumetric flask. 50% NaOH was then added and heated at 103°C for 3 hours (S : L = 1 : 20). Chitin was deacetylated and washed thoroughly with distilled water. Boiling with double distilled water for 3 hours to remove the impurities. Prepared chitosan was then dried at 105°C for 24 hours. A second lot of chitosan was prepared from commercial chitin (Sigma) using the same method mentioned above. This second lot will be described as commercial chitosan throughout this paper.

Chitosan was irradiated at 60, 70, 80, 90, 100 and 120 kGy at room temperature using a ^{60}CO gamma source. Acetic acid and citric acid and their different concentrative were used for determining the better solubility of irradiated and unirradiated chitosan. Microbiological analyses were carried out according to the Manual for the Identification of Medical Bacteria.(7)

Determination of anti-bacterial activity of chitosan : Bacterial species were isolated from mango and papaya and identified according to the Manual for the Identification of Medical Bacteria.(7) The isolates were used for determining the antibacterial activity throughout the study. Different percentage of chitosan such as 0.5, 1.5, 3.0 and 5.0 of non-irradiated and irradiated (0, 60, 70, 90 and 120 kGy) chitosan (both prepared and commercial) were prepared with 2% acetic acid and taken in 20 ml test tubes and autoc1aved. After autoc1aving 0.5 ml of different percentages of chitosan were spread over the surface of nutrient agar plates. In control plates only 0.5 ml of 2% acetic acid was spread. Then 0.1 ml bacterial suspension (turbidity adjusted to McFarland nephelometer standard No. 0.5) of selected bacterial species were spread over the plates gently. The plates were then incubated at 37°C for 24 hour. After incubation the presence of bacterial number were counted.

To determine the zone of inhibition produced by chitosan, a sterile cotton swab was socked with the suspension of above mentioned isolated bacteria and spread over the nutrient agar plates. Then 10 µl chitosan solution of different concentration were added on blank disks placed on nutrient agar plates. Diameters of zone of inhibitions were estimated after 24 h incubation at 37°C.

Results and Discussion

Thirteen gm chitin were extracted after two cycle treatment from 46 gm dried and crushed shrimp shells (conversion rate = 28.26%). This conversion rate was similar as those reported by Ramnani *et al.*(3) Extracted and commercial chitin was subjected for deacetylation to convert chitin into chitosan. Since the solubility of chitosan is the crucial property to act as an anti-microbial agent, so both prepared and commercial chitosan i.e., which was prepared from commercial chitin, were

tested for solubility in acetic acid and citric acid. Although both the acids were found to be suitable for the solubility of chitosan, 2% acetic acid was shown to be relatively better solvent for chitosan (Table 1). Along with the changes of the organic acids, different concentrations (0.5, 1.5, 3.0, 5.0 %) of prepared and commercial chitosans (non-irradiated and irradiated) were also tested to find out the maximum amount of chitosan readily soluble in 2% acetic acid. All of the tested concentrations of chitosan was found to be readily soluble in 2% acetic acid. Again, though both the prepared and commercial chitosan were shown to have almost similar solubility, chitosan irradiated with 80 kGy or more gamma radiation was found to be relatively better soluble (Table 2).

Table 1. Solubility test of chitosan (0.5%) in organic acid.

Chitosan	Control (Distilled water)	Organic acid	Concentration of organic acid (%)			
			0.5	1.0	2.0	4.0
Prepared	Insoluble	Acetic acid	+	++	+++	+++
		Citric acid	+	+	++	+++
Commercial	Insoluble	Acetic acid	+	++	+++	+++
		Citric acid	+	+	++	+++

$^{+}$ Slightly soluble, $^{++}$ relatively more soluble, $^{+++}$ better soluble.

Table 2. Solubility of irradiated and non-irradiarted chitosan (prepared and commercial).

Radiation dose (kGy)	Type of chitosan	Concentration of organic acid (%)			
		0.5	1.0	2.0	4.0
0	Prepared	++	++	++	++
	Commercial	++	++	++	++
60	Prepared	++	++	++	++
	Commercial	++	++	++	++
70	Prepared	++	++	++	++
	Commercial	++	++	++	++
80	Prepared	+++	+++	+++	+++
	Commercial	+++	+++	+++	+++
90	Prepared	+++	+++	+++	+++
	Commercial	+++	+++	+++	+++

$^{++}$Relatively more soluble, $^{+++}$ better soluble.

Major bacteria isolated from spoiled mango and papaya was *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sp., *Bacillus* sp., *Escherichia coli* and *Aeromonas* sp. Among these bacterial species, *Staphylococcus aureus* (Gram positive

cocci), *Escherichia coli* (Gram negative rod) and *Bacillus* sp. (Gram positive rod) were selected for testing the anti-bacterial activity of prepared and commercial chitosan.

Tables 3, 4 and 5 suggest that, one log of the bacterial load of *Staphylococcus aureus*, *E. coli* and *Bacillus* sp respectively was decreased by 2% acetic acid than the respective positive control (106 to 107 cfu/ml). 0.01% chitosan (both prepared and commercial) was found to decrease the bacterial load of all the mentioned bacterial species and chitosan irradiated with 80 kGy or more had better antibacterial activity in this concentration, although this chitosan concentration was not effective enough to total elimination of bacterial load. Commercial chitosan showed better result in all cases.

Table 3. Anti-bacterial (*Staphylococcus aureus*) activity of irradiated and non-irradiated chitosan (prepared and commercial).

Radiation doses (kGy)	Type of chitosan	Control (cfu/ml)		Chitosan concentration in 2.0% acetic acid (cfu/ml)				
		+ve	Acetic acid	0.01%	0.025%	0.050%	0.075%	0.1%
0	P	1.4×10^5	2.3×10^4	2.5×10^2	0	0	0	0
	C	1.4×10^5	2.3×10^4	2.1×10^2	0	0	0	0
60	P	1.4×10^5	2.3×10^4	6.2×10^1	0	0	0	0
	C	1.4×10^5	2.3×10^4	5.5×10^1	0	0	0	0
70	P	1.4×10^5	2.3×10^4	5.8×10^1	0	0	0	0
	C	1.4×10^5	2.3×10^4	5.2×10^1	0	0	0	0
80	P	1.4×10^5	2.3×10^4	5.4×10^1	0	0	0	0
	C	1.4×10^5	2.3×10^4	4.8×10^1	0	0	0	0
90	P	1.4×10^5	2.3×10^4	5.2×10^1	0	0	0	0
	C	1.4×10^5	2.3×10^4	4.8×10^1	0	0	0	0
120	P	1.4×10^5	2.3×10^4	5.2×10^1	0	0	0	0
	C	1.4×10^5	2.3×10^4	4.7×10^1	0	0	0	0

P = Prepared. C = Commercia1.

Chitosan concentration of 0.025% was found to be effective for total elimination of the bacteria. The results agreed with the findings of Chowdhury.[8] Finally the anti-bacterial activity of different concentration of chitosan dissolved in 2% acetic acid was measured by estimating the zone of inhibition they produced (Table 6). Only isolated *E. coli* and *S. aureus* were used for this investigation. Acetic acid itself showed to have anti-bacterial activity because of its low pH value. However, all the tested concentration of prepared chitosan produced zone of inhibition greater than those produced by acetic acid. The result indicates the presence of anti-bacterial activity of prepared chitosan. Between the tested organisms *S. aureus* was found to be more sensitive than *E. coli.*

Table 4. Anti-bacterial (*E. coli*) activity of irradiated and non-irradiated chitosan (prepared and commercial).

Radiation (kGy)	Type of chitosan	Control (cfu/ml)		Chitosan concentration in 2.0% acetic acid (cfu/ml)				
		+ve	Acetic acid	0.01%	0.025%	0.050%	0.075%	0.1%
0	P	1.2×10^4	1.6×10^3	1.4×10^2	0	0	0	0
	C	1.2×10^4	1.6×10^3	1.1×10^2	0	0	0	0
60	P	1.2×10^4	1.6×10^3	4.8×10^1	0	0	0	0
	C	1.2×10^4	1.6×10^3	4.2×10^1	0	0	0	0
70	P	1.2×10^4	1.6×10^3	4.5×10^1	0	0	0	0
	C	1.2×10^4	1.6×10^3	3.8×10^1	0	0	0	0
80	P	1.2×10^4	1.6×10^3	4.6×10^1	0	0	0	0
	C	1.2×10^4	1.6×10^3	4.1×10^1	0	0	0	0
90	P	1.2×10^4	1.6×10^3	4.4×10^1	0	0	0	0
	C	1.2×10^4	1.6×10^3	4.2×10^1	0	0	0	0
120	P	1.2×10^4	1.6×10^3	4.1×10^1	0	0	0	0
	C	1.2×10^4	1.6×10^3	4.0×10^1	0	0	0	0

P = Prepared. C = Commercial.

Table 5. Anti-bacterial (*Bacillus* sp.) activity of irradiated and non-irradiated chitosan (prepared and commercial).

Radiation (kGy)	Type of chitosan	Control (cfu/ml)		Chitosan concentration in 2.0% acetic acid (cfu/ml)				
		+ve	Acetic acid	0.01%	0.025%	0.050%	0.075%	0.1%
0	P	2.5×10^5	1.8×10^4	1.8×10^2	0	0	0	0
	C	2.5×10^5	1.8×10^4	1.5×10^2	0	0	0	0
60	P	2.5×10^5	1.8×10^4	6.3×10^1	0	0	0	0
	C	2.5×10^5	1.8×10^4	6.1×10^1	0	0	0	0
70	P	2.5×10^5	1.8×10^4	5.8×10^1	0	0	0	0
	C	2.5×10^5	1.8×10^4	5.1×10^1	0	0	0	0
80	P	2.5×10^5	1.8×10^4	5.6×10^1	0	0	0	0
	C	2.5×10^5	1.8×10^4	4.8×10^1	0	0	0	0
90	P	2.5×10^5	1.8×10^4	4.7×10^1	0	0	0	0
	C	2.5×10^5	1.8×10^4	4.3×10^1	0	0	0	0
120	P	2.5×10^5	1.8×10^4	4.6×10^1	0	0	0	0
	C	2.5×10^5	1.8×10^4	4.3×10^1	0	0	0	0

P = Prepared. C = Commercial.

Table 6. Anti-bacterial properties of chitosan dissolved in 2% acetic acid against *E. coli*

and *S. aureus*.

% of chitosan (w/w)	Zone of inhibition (mm)	
	E. coli	*S. aureus*
Control (2% acetic acid)	11	16
0.05	12	17
0.15	12	18
0.25	13	18
0.35	15	18
0.45	15	18
0.60	15	19

Reviewing the above findings, it can be concluded that prepared irradiated chitosan can be used as an anti-bacterial agent. As it was found to readily soluble in acetic acid in a low concentration, the chitosan solution can be thought to use as a preservative in food items. But before doing that its anti-fungal activity should be measured first which would be our next target. There is a great possibility that, being a natural polymer chitosan will make its own place as a preservative in food industry by replacing chemical preservatives.

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