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ORIGINAL PAPER

Application of gamma radiation and physicochemical treatment to improve the bioactive properties of chitosan extracted from shrimp shell

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Abstract. The aim of this study is to exploit a suitable chitosan extraction method from the chitin of indigenous shrimp shells by employing different physicochemical treatments and to improve different bioactive properties of this extracted chitosan (CS) by applying gamma radiation. Chitin was prepared from shrimp shell by pretreatment (deproteination, demineralization and oxidation). Chitosan was extracted from chitin by eight different methods varying different physicochemical parameters (reagent concentration, temperature and time) and assessed with respect to the degree of deacetylation, requirement of time and reagents. The method where chitin was repeatedly treated with 121°C for 30 min with 20 M NaOH, produced the highest degree of deacetylation (DD) value (92%) as measured by potentiometric titration, with the least consumption of time and chemicals, and thus, selected as the best suitable extraction method. For further quality improvement, chitosan with highest DD value was irradiated with different doses (i.e., 5, 10, 15, 20 and 50 kGy) of gamma radiation from cobalt-60 gamma irradiator. As the radiation dose was increased, the molecular weight of the wet irradiated chitosan, as measured by the viscosimetric method, decreased from 1.16×10^5 to 1.786×10^3, 1.518×10^3, 1.134×10^3, 1.046×10^3 and 8.23×10^2 dalton, respectively. The radiation treatment of chitosan samples increased the antimicrobial activity significantly in concentration dependent manner on both gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria, as determined by the well-diffusion method. Four to five percent wet chitosan treated with a radiation dose range of 5.0–10.0 kGy rendered the highest antimicrobial activity with least energy and time consumption. Solubility, water binding capacity (WBC) and fat binding capacity (FBC) also improved due to irradiation of chitosan.

Keywords: chitosan • gamma radiation • degree of deacetylation • antimicrobial activity

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Introduction

Chitin is the second most abundant natural biopolymer on earth after cellulose. It is ply-β-(1→4)--linked N-acetyl-D-glucosamine [1]. This is the major structural component of the exoskeleton of crustaceans, insects and the cell wall of certain fungi [1, 2]. Chitosan, a linear ply-β-(1→4)-linked--D-glucosamine, is a natural polysaccharide obtained from the deacetylation of chitin [3, 4]. Chitosan has two main advantages over chitin. Chitosan is readily dissolved in diluted acetic acid and possesses free amine groups ($-NH_2$) that are active site in many chemical reactions. Chitosan is recommended as suitable functional material for different uses because this polymer has excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties and so on [2]. These interesting properties make it an interesting polymer for several applications in agriculture, pharmaceutical, cosmetic, food, and textile industries, and in medicine as well [3].

Degree of deacetylation (DD) value of chitosan is very important because it affects many physicochemical properties of chitosan including solubility, chemical reactivity, and biodegradability, and hence determines its appropriate applications [5, 6]. No *et al*. [7] stated that DD value must be at least 85% in order to achieve the desired solubility. It also affects the biodegradability and immunological activities [8]. Poly-cationic properties of chitosan is also crucial in determining its bioactive properties. Especially for this unique nature, chitosan has been used for antifungal, antibacterial and antitumour activities [9].

Every year, approximately 100 billion tons of chitin is produced on the earth by crustaceans and other related organisms [10]. The chitin is currently sourced from crab and shrimp shells discarded as food industry wastes. The global annual estimate of shellfish processing discard is more than one million metric tons. Disposal of shell fish processing discards has thus been a challenge for most of the shellfish producing countries. Therefore, the production of value added products such as chitin, chitosan and their derivatives and application of products in different fields is of utmost interest [1].

Different methods have been used to produce chitosan from shell chitin. However, the variations in the method of preparation are likely to result in differences in the deacetylation degree, chain length or molecular weight and the conformational structure of chitosan and will thereby have an influence on the solubility, antimicrobial activity and other properties [4]. Therefore, different parameters for chitosan production need to be optimized carefully to obtain the chitosan with desired properties to fulfil the requirement with least expense.

In our study, firstly the pretreatments were applied involving deproteination with diluted alkali, demineralization with diluted acid and oxidation with hydrogen peroxide, for effective processing of chitin from shrimp shell. Then the resulting chitin was deacetylated with eight different physicochemical treatments varying the reagent concentration, treatment temperature, and time with a view to exploiting an economically feasible and time saving method for obtaining the chitosan with the desired quality, especially with a high degree of deacetylation. It has also been reported that the radiation processing of chitosan can enhance the antimicrobial activity [11]. Therefore, for further improvement of bioactive properties, especially the antimicrobial activity and solubility, chitosan was irradiated with different doses of gamma radiation at different conditions. Irradiated chitosan was assessed for its quality parameters, and thus, the most feasible and economical procedure of chitosan processing with irradiation was evaluated.

Materials and method

Shrimp shell collection and pretreatment

Shells were collected from the shrimp processing centre and transported to the laboratory. After washing, the shells were dried well and crushed into small pieces. The pretreatment of these processed shells was performed by deproteination with 4% NaOH, demineralization with 4% HCl and oxidation by 2% H_2O_2 (Sigma Aldrich).

Extraction of chitosan from shell chitin

After the pretreatment, chitin was treated with eight different alkalization methods varying temperature, time, reagent, and so on, to extract chitosan. Fifteen (15) g of chitin flakes were taken and moistened with 15 ml of distilled water and kept for 1 hour. Then the moistened chitin was treated by the following methods.

Method A. Chitin was heat treated at 121°C for 30 min by an autoclave (ALP Co. Japan, MC-40L) with 20 M NaOH and then washed and dried with a dryer at 50°C.

Method B. Chitin was heat treated at 121°C for 30 min with 20 M NaOH two times with an interval wash with distilled water. After the treatment, it was washed and dried with a dryer at 50°C.

Method C. Chitin was heat treated at 121°C for 30 min with 26 M NaOH and then washed and dried with a dryer at 50°C.

Method D. Chitin was heat treated at 121°C for 30 min with 26 M NaOH two times with an interval wash with distilled water. After the treatment, it was washed and dried with a dryer at 50°C.

Method E. Chitin was treated with 20 M NaOH at room temperature for 4 days in the presence of 0.2% H_2O_2 and then washed and dried with a dryer at 50°C.

Method F. Chitin was treated with 20 M NaOH at room temperature for 4 days followed by a wash. This product was again heat treated at 121°C for 30 min with 20 M NaOH in the presence of 0.2% H_2O_2 and then washed and dried with a dryer at 50°C.

Method G. Chitin was treated with 20 M NaOH at room temperature for 4 days two times in the presence of 0.2% H_2O_2 and then washed and dried with a dryer at 50°C.

Method H. Chitin was treated with 20 M NaOH at room temperature for 4 days two times in the presence of 0.2% H_2O_2. Then, it was washed and again heat treated at 121°C for 30 min with 20 M NaOH in the presence of 0.2% H_2O_2, and then washed again and dried with a dryer at 50°C.

Irradiation of chitosan

Chitosan samples of different concentration were irradiated with a series of radiation doses (5, 10, 15, 20, 30, 40 and 50 kGy) from a cobalt-60 gamma source (~65 kCi) of Gamma Source Division, Institute of Food and Radiation Biology. Ceric-cereus dosimetry was performed to measure the absorbed dose level.

Determination of degree of deacetylation

Potentiometric titration method was used for determining the degree and deacetylation as per

Tolaimate *et al*. [8] and Muzzarelli [12]. Chitosan solution (0.1%) was prepared by dissolving 0.05 g of chitosan in 50 ml of 0.1 M HCl solution. Titration of this chitosan solution was performed with 0.1 N NaOH with a pH meter, Jenway 3510 application. A curve with two inflexion points was obtained. The difference between the volumes of the two inflexion points corresponded to the acid consumption of the salification of the amine groups, which permitted the measurement of chitosan's deacetylation degree by the following calculation.

$$\text{Degree of deacetylation (DD value) \%} = 16.1 \times (V2 - V1) \times Mb/W$$

where, V1 – volume of NaOH at the first inflexion point; V2 – volume of NaOH at the second inflexion point; Mb – base molarity (molarity of NaOH); W – weight of chitosan.

Determination of molecular weight

For this study, the viscometer method [13] was used for the determination of the viscosity average molecular weight of chitosan. In this method, different concentration of chitosan solutions were prepared using the solvent, 0.3 M acetic acid/0.2 M sodium acetate. The flow time of solvent ($t_{solvent}$) and sample solution (t_{sample}) were measured at 25 ± 0.1°C. From these flow times, relative viscosity (η_{rel}), specific viscosity (η_{spe}) and reduced viscosity (η_{red}) were calculated. A graph was plotted using the different concentrations of chitosan and corresponding reduced viscosity at X and Y co-ordinates, respectively. An extrapolation plot of reduced viscosity against chitosan concentration was made to find out the intrinsic viscosity [η], which is equal to the Y-intercept. Then, the viscosity average molecular weight was calculated using the classical Mark-Houwink equation:

$$[\eta] = KMv^{\alpha}$$

here, [η] – intrinsic viscosity, defined as reduced viscosity extrapolated to chitosan concentration of zero; Mv – molecular weight; K and α – constant (K = 0.078 and α = 0.76).

Determination of antimicrobial activity

Agar well diffusion, a widely used method was applied to evaluate the antimicrobial activity of chitosan. In this method, the Mueller Hinton (Difco, USA) agar plate surface was inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then a hole of diameter 6.8 mm was punched aseptically with a sterile cork borer. Different concentrations of chitosan solutions (1.5, 1.0 and 0.5%) were prepared with 1% acetic acid. A volume of 100 µl of chitosan was introduced into the well. Then, the agar plates were incubated at 37°C suitable conditions for 24 hours. The antimicrobial agent diffused in the agar medium and inhibited the growth of the microbial strain forming a clear holo zone. The diameter of the holo zone was measured in mm as the zone of inhibition.

Measurement of water binding capacity (WBC) and fat binding capacity (FBC)

WBC of chitosan was determined by a modified method of Knorr [14]. A centrifuge tube containing 0.5 g of chitosan sample was weighed (A) and 10 ml of distilled water was added and mixed with a vortex (Heidolph, Reax 2000) for 1 min. Then the centrifuge tube was kept at ambient temperatures for 30 min with intermittent shaking for 5 s every 10 min. The tube was then centrifuged for 25 min at 3000 rpm. After the supernatant was decanted, the tube was weighed (B) again and WBC was calculated as follows.

$$\text{WBC [\%]} = \frac{\text{water bound [g]},(B - A)}{\text{initial sample weight [g]}} \times 100$$

FBC% of chitosan was determined by the similar method of WBC except one where oil (soybean) was used in lieu of water.

$$\text{FBC [\%]} = \frac{\text{fat bound [g]},(B - A)}{\text{initial sample weight [g]}} \times 100$$

Result and discussion

Chitin processing from shrimp shell and parameter optimization to exploit robust and suitable method for extraction of chitosan from shell chitin

Chitin from shrimp shell was processed by the conventional method employing the three steps deproteinization, demineralization and oxidation, and 31.4% chitin was obtained from the crude shrimp shell. To explore a suitable method for obtaining high quality chitosan, deacetylation of chitin was performed by alkali treatment applying eight different procedures (designated as A, B, C, D, E, F, G and H), which differed with respect to the treatment time, temperature, reagent concentration and so on, as mentioned in the method section. The conversion rate of chitosan from chitin were 85.53, 76.26, 79.13, 78.53, 85.73, 81.53, 80.66 and 77.46%, respectively. Degree of deacetylation (DD value) % of extracted chitosan was assessed as its major quality parameter. And the method B, where chitin was repeatedly treated with 121°C for 30 min with 20 M NaOH, produced the highest degree of deacetylation value (92.0 ± 0.34%) with the least consumption of time (~4 h) and chemicals (Fig. 1), and thus, selected as the most suitable extraction method. It was noted that the conventional method produced chitosan with DD value 70–75%.

Effect of gamma radiation on the molecular weight of extracted chitosan

Chitosan of best quality was irradiated at different doses, both in dry and wet conditions, with a view

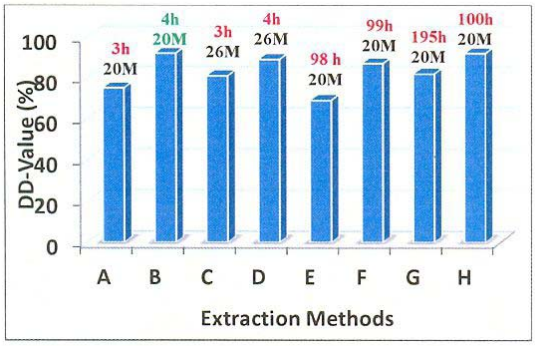

Fig. 1. Degree of deacetylation (DD value) % obtained by eight different extraction methods.

to improving different quality parameters including antimicrobial activity, solubility with decreased molecular weight. Molecular weight of non-irradiated chitosan was 1.16×10^5 dalton. The highest and lowest MW of chitosan were 8.75×10^4 and 3.1×10^4 dalton when irradiated in dry condition at doses of 5 and 50 kGy, respectively (Fig. 2A) where as the highest and lowest MW of chitosan irradiated in wet condition were 1.79×10^3 and 0.98×10^3 dalton, respectively at the same doses by viscosity average molecular weight (Fig. 2B).

Similar type effect of gamma radiation on molecular weight of chitosan was found by Gryczka *et al.* [15] where gel permeable chromatography (GPC) method was applied for determining molecular weight. Detection of double peaks in bimodal distribution curve of GPC of non-irradiated chitosan depicted that some degradation already occurred during chitosan processing (deacetylation) [15].

Gryczka *et al.* also investigated the mechanism of radiolytic degradation of dry chitosan in a wide dose range by electron paramagnetic resonance (EPR) spectroscopy coupled with FTIR and GPC analysis. Deconvolution of the EPR spectra suggested the production of some intermediate radicals during irradiation which helped reveal the mechanism of radiolysis assessed with respect to free radicals. Nitroxyl radicals were identified with EPR spectra while sample was irradiated in the presence of oxygen which provided the evidence of the involvement of amino group in radiolysis mechanism [15]. Our results clearly showed that radiation induced degradation rate of chitosan in dry condition is much lower than that in wet condition. In wet condition, gamma radiation also causes radiolysis of water molecule producing a pool of H_2O_2, highly reactive hydroxyl radicals and other related products [16]. We suggested that products form radiolysis of water further augment the scission of β-(1→4)-glycosidic linkage of the chitosan molecule along with other reactive nitrogenous intermediate radicals leading to higher rate of radiolysis of chitosan in wet condition.

Radiation increased the antimicrobial activity of chitosan gradually in dry condition

Radiation treatment improved the antimicrobial activity of chitosan significantly in dry condition as

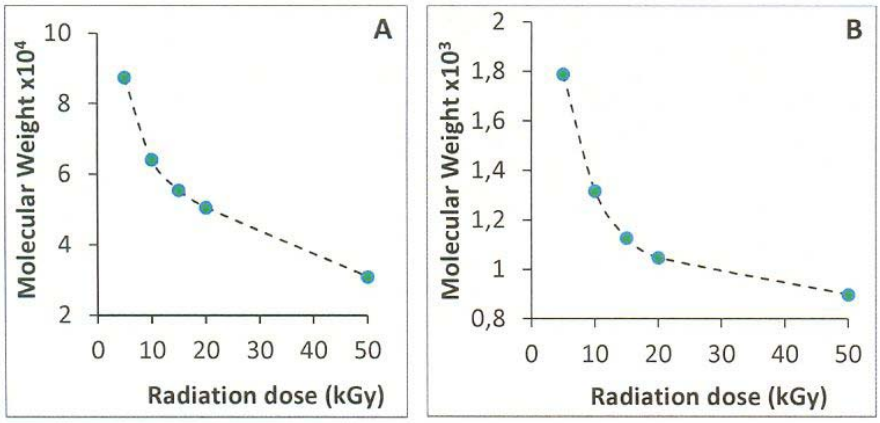

Fig. 2. Effect of gamma radiation on the molecular weight (MW) of chitosan irradiated in (A) dry and (B) wet condition.

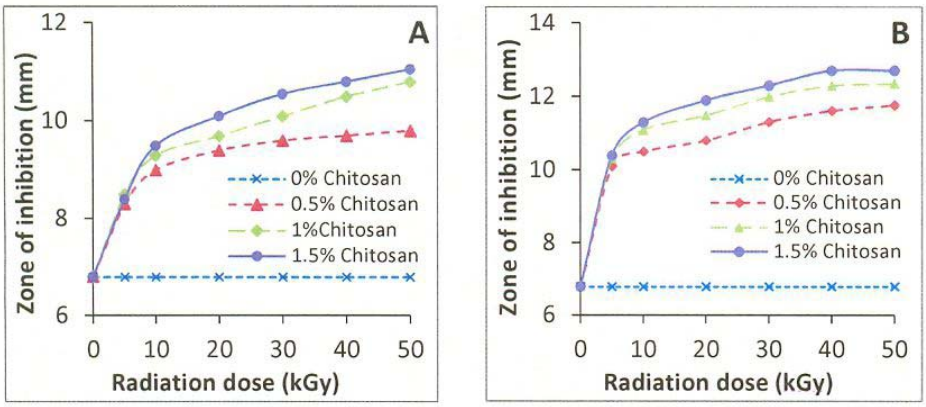

Fig. 3. Antimicrobial activity of irradiated dry chitosan on (A) *Escherichia coli* and (B) *Staphylococcus aureus*.

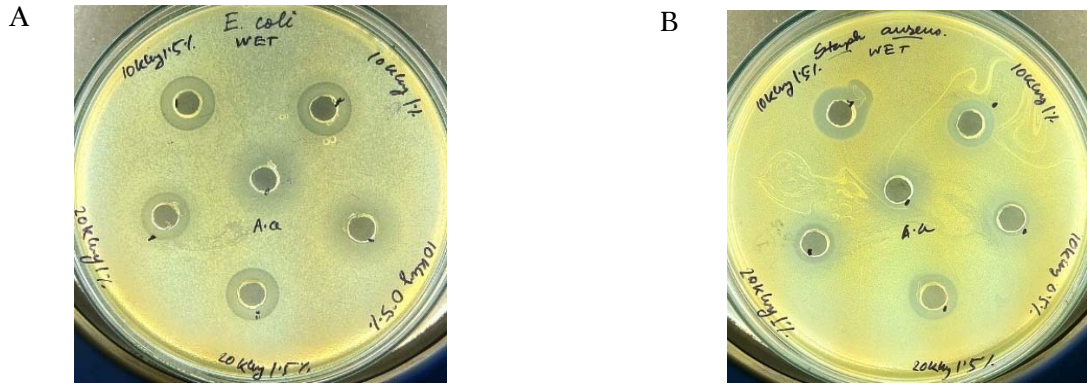

Fig. 4. Well diffusion method for antimicrobial activity of wet chitosan irradiated with 10 and 20 kGy dose at 0.5, 1.0 and 1.5% concentration on (A) *Escherichia coli* and (B) *Staphylococcus aureus*.

measured by the well diffusion method. When dry chitosan was irradiated, its antimicrobial activity on both gram-negative (*E. coli*) and gram-positive bacteria (*S. aureus*) gradually increased, as the dose increased up to the last dose applied (Fig. 3). To obtain the maximum antimicrobial activity in dry chitosan, application of a higher radiation dose is required, which leads to the involvement of a time-consuming process with high energy consumption.

Radiation attributed to the antimicrobial activity of chitosan in wet condition critically

Radiation treatment significantly improved the antimicrobial activity of chitosan in wet conditions. When chitosan was irradiated at 0.5% concentration with different doses, the antimicrobial activity increased initially at 5 kGy, but later decreased rapidly to almost nil as the dose was increased to or above 20 kGy (Figs. 4 and 5).

When chitosan was irradiated at 1.0% concentration with different doses, after increasing antimicrobial activity at 5 and 10 kGy, it started losing its potency slowly as the dose increased gradually and finally become 0 at 40–50 kGy. Interestingly, when the sample was irradiated at 1.5% concentration, the antimicrobial activity on gram-negative bacteria substantially increased at 5–10 kGy and sustained up to the highest dose (50 kGy) applied. On the other hand, potency seemed to decrease against gram-positive bacteria (Fig. 5).

When chitosan was irradiated at 4% concentration, antimicrobial activity increased significantly at the dose of 5 and 10 kGy and the activity persisted up to 40 kGy with slow increment, which was still active on both gram-positive and gram-negative bacteria (Fig. 6).

When chitosan was irradiated at 10% concentration, antimicrobial activity increased steeply at 10 kGy. At the later doses, the activity gradually increased but very slowly up to the last dose applied, that is, 50 kGy. But the level of antimicrobial activity at this dose was still less than that of irradiated at 4% concentration (Fig. 7). Therefore, if the chitosan is irradiated at the high concentration (>4%), a higher radiation dose is required to obtain the maximum antimicrobial potency, which ultimately requires more energy and time leading to a non-economic process.

Irradiation improved water and fat binding capacity (WBC and FBC) of chitosan

Gamma radiation also influenced both water and fat binding capacity (WBC and FBC) of chitosan. WBC of non-irradiated chitosan was 568% and it was increased gradually up to 752% as the radiation dose increased to 50 kGy (Fig. 8). FBC of non-irradiated chitosan was 554%. It was also increased gradually up to 704% as the dose increased up to 50 kGy. The solubility of chitosan also increased as the dose increased gradually over the applied range.

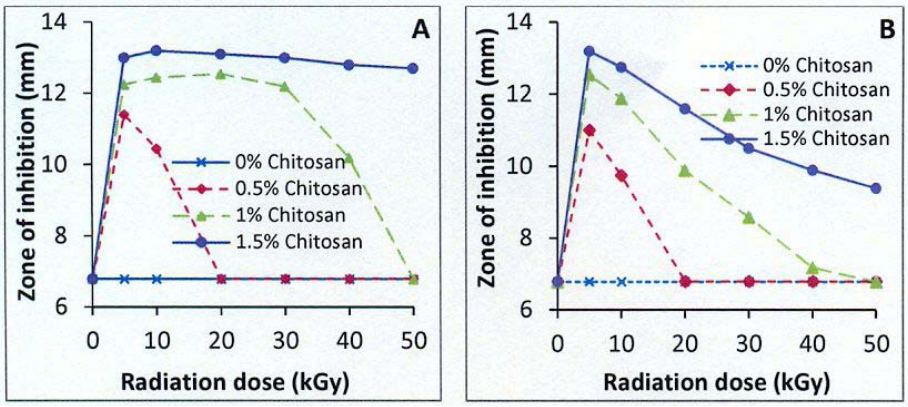

Fig. 5. Antimicrobial effect of chitosan concentration (0.5, 1.0 and 1.5%) maintained during irradiation of chitosan on (A) *E. coli* and (B) *S. aureus*.

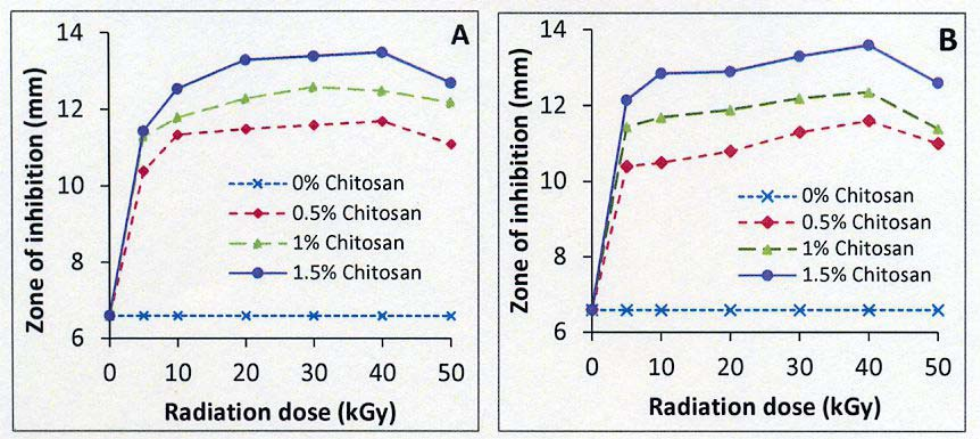

Fig. 6. Antimicrobial activity of chitosan irradiated at 4.0% concentration on (A) *E. coli* and (B) *S. aureus*.

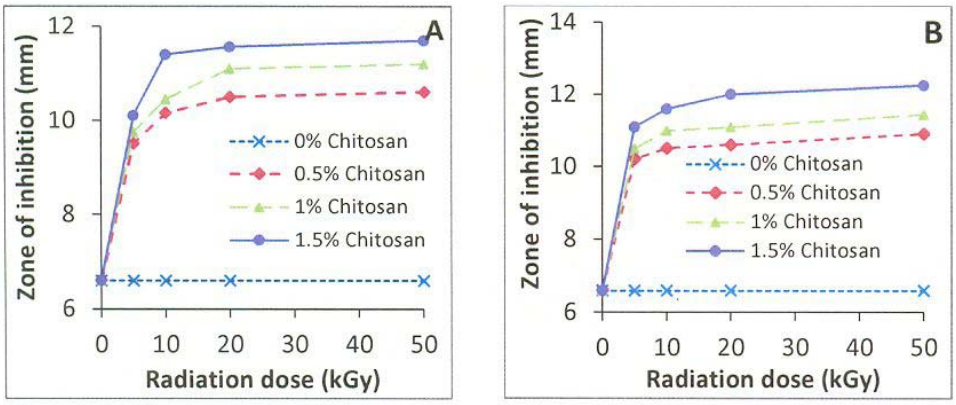

Fig. 7. Antimicrobial activity of chitosan irradiated at 10.0% concentration on (A) *E. coli* and (B) *S. aureus*.

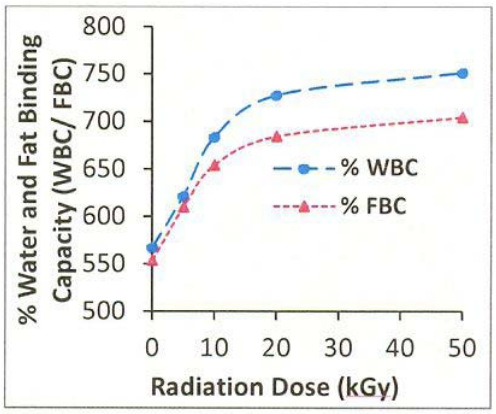

Fig. 8. Effect of gamma radiation on water binding capacity (WBC) and fat binding capacity (FBC) of irradiated chitosan.

Conclusion

Repeated alkali treatment (20 M NaOH) at 121°C for 30 min with an intermittent washing was found to be the best production method of chitosan from shrimp chitin. Molecular weight of chitosan decreased as the radiation dose increased gradually. Gamma radiation, depending on the radiation dose and chitosan concentration, critically influences the antimicrobial activity of chitosan on organism type. It is more feasible and economic to irradiate the product at 5–10 kGy at a concentration of 4–5% of chitosan for substantial improvement of its antimicrobial potency, as this product is active against both gram-positive and gram-negative bacteria with minimum energy and time consumption. WBC, FBC and solubility of chitosan can also be increased by applying gamma radiation, which are also important properties of chitosan for different uses.

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