Characterization of Chitosan Produced from Fermented Shrimp Shell Waste by Bacillus subtilis NA12 Using Gamma Radiation

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ABSTRACT

This study focused on characterization of chitosan obtained in a previous study from fermented shrimp shell waste (SSW) by Bacillus subtilis NA12. Extracted chitin was exposed to different gamma radiation doses (5-35 kGy). The molecular weight of the resultant chitosan decreased constantly with increasing radiation dose from 1.9 × 10^6 (g/mol) (non-irradiated) to 3.7 × 10^4 (g/mol) (at 35 kGy). The degree of deacetylation (DDA %) was determined by using potentiometric titration. The structural properties of chitin and chitosan were characterized by Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction analysis (XRD), and thermogravimetric analysis (TGA). The prepared chitosan has higher solubility and DDA % compared to the standard chitosan. FT-IR analysis clearly confirmed the successful extraction of pure chitin and chitosan. TGA showed that chitin exhibited a stable structure toward thermal decomposition than chitosan. XRD analysis revealed that extracted chitin was more crystalline than prepared chitosan. Chitosan with different molecular weights was evaluated as an antibacterial agent against representative pathogenic bacterial strains. Chitosan obtained at 35 kGy, with molecular weight 3.7 × 10^4 (g/mol) and DDA of 87.9 %, showed the highest antimicrobial activity against Escherichia coli, Klebsiella sp., Staphylococcus aureus and Pseudomonas aeruginosa. It revealed inhibition zone diameter of 5.4 ± 0.2, 5.4 ± 0.12, 3.5 ± 0.21 and 1.4 ± 0.06 cm, respectively.

Keywords: Bacillus subtilis / Shrimp Shell Waste / γ-Irradiation / Chitosan / Antibacterial Agent.

INTRODUCTION

Chitin is the most important natural polysaccharide after cellulose, it is found in exoskeleton of crustaceous shells as well as in cell walls of fungi. Ideally, chitin is a linear polysaccharide consisting of β-(1-4)-2-acetamido-2-deoxy-d-glucopyranose repeating units where the amine groups are entirely acetylated. The ideal structure of chitosan, the primary derivative of chitin, is comprised of linear β-(1-4)-2-amino-2-deoxy-d-glucopyranose repeating units where the N-acetylglucosamine residues in chitin macromolecular chain are fully deacetylated to become N-glucosamine residues. However, chitin is not widely used for industrial applications until now because it is insoluble in many solvents, relatively difficult to isolate from natural sources in pure form, and difficult to be reproduced under economic conditions. That is why it is also difficult to characterize this polysaccharide (1). Therefore, the chemical modifications of chitin were performed. The most common derivative is chitosan, derived by partial deacetylation of chitin (2).

After deacetylation of chitin, generally, the obtained chitosan has high molecular weight and viscosity, which limit its solubility. The use of chitosan in diverse areas is directly related to the polymer’s molecular weight and degree of deacetylation (3). Under these circumstances, reducing the molecular weight is very crucial for its use. According to the results reported by Wasikiewicz et al. (4), the most efficient way for the reduction of molecular weight of chitosan is gamma-irradiation. In a recent study, the degradation of chitosan by γ-irradiation to reduce its molecular weight was
investigated using different methods and under different conditions (5). Chitosan is biocompatible, biodegradable, nontoxic to human and environment, and shows antimicrobial in addition to antioxidant activities (6,7). These excellent physicochemical features have put chitosan in a unique position amongst biopolymers. It has found broader applications in a number of fields including pharmaceutics and medicine (8), food industry (9), textile (10) and water treatment (11).

Antimicrobial activity of chitosan and its derivatives against several bacterial species is considered as one of the most important properties linked directly to their possible biological applications. As a natural antimicrobial agent, the antibacterial and antifungal activities of chitosan have been widely reported (12,13). The antibacterial activity of these compounds is influenced by a number of factors such as the degree of polymerization (14,15), the level of deacetylation (16), the type of microorganism (17,18) and some other physicochemical properties.

In a previous work (7); a high potent proteolytic local isolate was identified as Bacillus subtilis NA12 by using 16S rRNA. Chitin was extracted from shrimp shell waste (SSW) by the fermentation activity of B. subtilis NA12. The extracted chitin was subjected to different doses of γ-radiation (5-35 kGy) in a 60Co gamma source at radiation dose rate of 3.35 kGy/h. Then, chitin was converted to chitosan by deacetylation according to the method described by Kurita (19). In the present study, the resultant chitin and chitosan were characterized by using FT-IR, TGA, and XRD. The degree of deacetylation (DDA %) and the average molecular weight of chitosan were determined. Moreover, the antimicrobial activity of the produced chitosan (with different molecular weight and degree of deacetylation) was investigated against various pathogenic bacterial isolates.

MATERIALS AND METHODS

Microorganisms

Bacillus subtilis strain previously isolated from soil was used in fermentation of shrimp shell waste. The bacterial strain was maintained on nutrient agar slants at 4 °C and subcultured at monthly intervals.

Shrimp Shells Waste

Shrimp shells waste (SSW) was purchased from Al-Obour fish market, Cairo, Egypt. The shells were kept frozen during transportation and preservation till processing in different experiments. The shells were firstly soaked in a 1% (v/v) chlorine solution for 30 min, to remove any impurities, then washed with tap water several times to get rid of the residual chlorine. The washed shells were then dried at 50 °C overnight in a drying oven and preserved at 4 °C in tightly closed plastic bags until being used.

Extraction and Gamma Irradiation of Chitin

At the end of fermentation period, the fermented SSW was collected and filtered out using a cheese cloth. The remaining residue (crude chitin) was washed with distilled water several times, dried in an oven at 80 °C for 3 h, and stored for further studies. Later, the extracted chitin was divided into 4 parts and treated with H2O2 (10 %). The four parts were exposed to different doses of γ-radiation (5, 15, 25, and 35 kGy) as mentioned in previous work (7), irradiation was carried out in a 60Co gamma source at NCRRT; the radiation dose rate at the time of experiment was 3.35 kGy/h.

Converting Chitin to Chitosan by Deacetylation

The conversion process was carried out by steeping the different chitin samples for one day in a strong sodium hydroxide (NaOH) solution (40 %, w/v) at room temperature, then refluxing in the same alkali solution at 135 ± 2 °C. The refluxing time was 9, 6, 4, 3, and 2 h for non-irradiated chitin, irradiated chitin at 5, 15, 25, and 35 kGy, respectively. The samples were then washed with distilled water until neutral. Finally, they were washed with hot ethanol and boiled in acetone to remove any impurities. The purified chitosan was then dried at room temperature and weighed (19).
Determination of the Degree of Deacetylation

Potentiometric titration method according to Hua-Cai and Deng-Ke (20) and Gupta and Jabrail (21) was used to determine the degree of acetylation of chitosan obtained from extracted chitin and irradiated at different levels of γ-radiations (5, 15, 25, and 35 kGy) (7). Each chitosan sample, 0.1 g (dry weight) was dissolved in 20 ml of 0.1 M standard hydrochloric acid (HCl) solution and adjusted to pH < 2, which was considered as the starting point. Then, titrated against 0.05 M standard NaOH solution; NaOH was added stepwise and the pH values of the solution were recorded. The degree of deacetylation (DDA %) was calculated using the following equation:

\[ DDA(\%) = \left(\frac{203}{Q_1 + 42} Q_2\right) \times 100 \]  

(1)

Where, \( Q = N \times \Delta V/m \), \( \Delta V \) is the volume difference (in liter) of the NaOH solution between the two inflection points, \( N \) is the normality of the standard NaOH solution (0.05 mol/L), and \( m \) is the dry weight of chitosan (g/L), the values of 203 and 42 are the respective molecular weight of \( N \)-acetyl glucosamine (chitin skeleton unit) and one acetyl group, respectively.

Determination of the average molecular weight of chitosan

Chitosan samples were dissolved in a solvent mixture consisting of acetic acid (0.3 M) and sodium acetate (0.2 M). The viscosity of the samples was measured in Ubbelhode capillary viscometer. First, the capillary tube was filled with the pure solvent to a given level and the drain time (\( t_0 \)) for this solvent was measured. Then, the capillary was filled with the same solvent containing five different concentrations of chitosan polymer (0.02-0.1 g/ml), then, the drain time (\( t \)) was measured; the capillary was filled with a sample that passed through the capillary twice, before the running time was measured again. Each sample was measured three times (3) and the relative viscosity \( \eta_{rel} \) of the polymer in (ml/g) was calculated from the following equation:

\[ \eta_{rel} = \frac{t}{t_0} \]  

(2)

The inherent viscosity \( \eta_{inh} \) of the polymer in (ml/g) was calculated from the following equation:

\[ \eta_{inh} = \ln \eta_{rel}/c \]  

(3)

Where, \( c \) is the concentration of the prepared polymer solution in (g/ml).

Also, the specific viscosity \( \eta_{sp} \) of the polymer was calculated from the following equation:

\[ \eta_{sp} = \eta_{rel} - 1 \]  

(4)

And the reduced viscosity \( \eta_{red} \) of the polymer in (ml/g) was calculated from the following equation:

\[ \eta_{red} = \frac{\eta_{sp}}{c} \]  

(5)

A graph of \( \eta_{inh} \) and \( \eta_{red} \) on the y-axis in relation to the solution's concentration (C) on the x-axis was plotted. The intrinsic viscosity of solution \( \eta_i \) in (ml/g) was determined by extrapolation of the two straight lines obtained by linear regression to zero concentration (C equal 0) as presented by Al-Sarra et al. (22). The average molecular weight of chitosan (M) was determined by using Mark-Houwink-Sakurada equation reported by Roberts and Domszy (23) that relates the intrinsic viscosity to the polymer's molecular weight as presented in the following equation:

\[ \eta_i = kM^\alpha \]  

(6)

Where, \( M \) is the average molecular weight, \( k \) and \( \alpha \) are constants that depend on the solvent-polymer system [\( k = 0.076 \) (ml/g) and \( \alpha = 0.76 \)] (24).
Fourier Transform Infrared (FT-IR) Spectroscopy

Chitin and chitosan samples were prepared in the forms of potassium bromide (KBr) disks according to Khan et al. (25). Briefly, the disk was made from 40 mg of chitin or chitosan powder and 120 mg of KBr. The spectra of chitin and chitosan samples were obtained by using FT-IR spectrophotometer (JASCO FT-IR-6300, Japan) in the range of 4000-400 cm⁻¹.

X-Ray Diffraction Analysis (XRD)

The XRD patterns of the prepared chitin and chitosan were measured using Shimadzu XRD 6000 diffractometer with Cu target. The XRD runs were carried out over the 2θ ranging from 10° to 80° at a scan speed of 8°/min.

Thermal Gravimetical Analysis (TGA)

The thermal stability and weight loss of the prepared chitin and chitosan, as a function of temperature from ambient to 600 °C at a heating rate of 10 °C/min, were determined by using Shimadzu TGA system (TGA-50) under a nitrogen atmosphere.

Antibacterial Activity of Chitosan

Different chitosan samples, obtained from irradiated chitin at 5, 15, 25, and 35 kGy, with different molecular weights and degree of deacetylation (DDA%), were tested for the antibacterial activity. The tested bacterial pathogens were Escherichia coli, Pseudomonas aeruginosa, Klebsiella sp. (Gram-negative) and Staphylococcus aureus (Gram-positive). The antibacterial activity assay was performed according to the method described by Berghe and Vlietinck (26).

RESULTS AND DISCUSSION

Physical Properties of the Prepared Chitosan

Determination of DDA (%) of Chitosan

Typical pH-potentiometric titration curves are shown in Fig. 1. Precipitation occurred at around 70% degree of titration in the solutions, where in this range, the response of the glass electrode became sluggish. However, after the second equivalence point, a reasonably fast electrode response was achieved again indicating that proton-exchange processes within chitosan were over in this pH-range. The procedure resulted in a titration curve with two inflection points: the first corresponded to excess HCl, while the second referred to the protonated chitosan. The difference between the two inflection points yields the number of moles of H⁺ required for the protonation of the free (deacetylated) amino groups and corresponds to the amount of D-glucosamine (D-GlcN) in the titrant solution (27). Assuming that the rest of the sample is N-acetyl-D-glucosamine (NAc-D-Glc), the DDA% values were recorded in Table 1. It was noted that chitosan sample obtained from the irradiated sample at 35 kGy have a high degree of deacetylation and this can be interpreted as a result of radiation-induced controlled decrease in molecular weight which is mainly due to scission of the glycosidic bond by radiation (28). Thus, the chain length of the polymer was reduced rendering it more flexible. Flexible chains of polymer facilitate mobility and removal of acetyl groups from the polymer leading to increasing the number of NH₂ groups, thus DDA % increased.

FT-IR Spectroscopy of Chitin and Chitosan

The FT-IR spectra of the extracted chitin in comparison with a standard chitin sample are presented in (Fig. 2). The bands observed at 3271 and 2894 cm⁻¹ correspond to the vibrational stretching of the hydroxyl groups and –CH– stretch chitin, respectively. Another absorption band
appeared at 1656 and 1420 cm\(^{-1}\) corresponding to the (amide I) stretching of C=O bonds of the acetamide groups and symmetric deformation of CH\(_3\). The band at 1316 cm\(^{-1}\) due to the formation of CO–NH group, while the at 1560 cm\(^{-1}\) corresponds to N–H deformation of the amino group (amine II) \(^{(29)}\). The peaks observed at 1070 and 1029 cm\(^{-1}\) were the secondary hydroxyl group (characteristic peak of –CH–OH in cyclic alcohol, C–O stretch) and the primary hydroxyl group (characteristic peak of –CH\(_2\)–OH in primary alcohol, C–O stretch), respectively. The absorption band at 1153 cm\(^{-1}\) corresponds to the asymmetric stretching of the C–O–C bridge. In a similar study, it was observed that chitin prepared from the irradiated sample has broader hydroxyl band at 3200-3500 cm\(^{-1}\) due to glycosidic bonds (C\(_1\)-O-C\(_4\) group) that decreased with increasing radiation doses leading to hydroxyl group formation \(^{(30)}\).

![Fig. (1): Potentiometric titration curves of chitosan obtained from chitin irradiated at 5 kGy (○), 15 kGy (△), 25 kGy (□), and 35 kGy (◇)](image)

**Table (1):** DDA values of chitosan prepared from both non-irradiated and irradiated chitin determined by the potentiometric method

<table>
<thead>
<tr>
<th>Sample</th>
<th>DDA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan standard</td>
<td>65.7</td>
</tr>
<tr>
<td>Chitosan from non-irradiated chitin</td>
<td>80.4</td>
</tr>
<tr>
<td>Chitosan from chitin irradiated at 5 kGy</td>
<td>84.4</td>
</tr>
<tr>
<td>Chitosan from chitin irradiated at 15 kGy</td>
<td>85.9</td>
</tr>
<tr>
<td>Chitosan from chitin irradiated at 25 kGy</td>
<td>87.4</td>
</tr>
<tr>
<td>Chitosan from chitin irradiated at 35 kGy</td>
<td>87.9</td>
</tr>
</tbody>
</table>

The FT-IR spectra of chitosan prepared from non-irradiated as well as irradiated chitin at 35 kGy compared to standard chitosan are illustrated in Fig. 3. The appearance of a strong and broad band is attributed to the axial stretching of O–H and N–H bond observed between 3500 and 3100 cm\(^{-1}\), centered at 3400 cm\(^{-1}\), the band at 1590 cm\(^{-1}\) has a larger intensity than at 1655 cm\(^{-1}\), which suggests effective deacetylation. When chitin deacetylation occurs, the band observed at 1655 cm\(^{-1}\) decreases, while a growth at 1590 cm\(^{-1}\) occurs, indicating the prevalence of NH\(_2\) groups \(^{(31)}\). When the same spectrum is observed in which the band from 1500 to 1700 cm\(^{-1}\) is stressed, this indicates that there was an intensification of the peak at 1590 cm\(^{-1}\) and a decrease at 1655 cm\(^{-1}\), which suggests the occurrence of deacetylation. It was also noted that the intensity of the peak at 1590 cm\(^{-1}\)
increased by increasing irradiation dose due to increasing the degree of deacetylation \(^{(32)}\). Pires et al. \(^{(33)}\) found that the samples of chitin/chitosan infrared absorption spectra of characteristic bands, such as C-H stretchings, located around 2920 and 2850 cm\(^{-1}\), for O–H and N–H stretchings, which are overlapped and centered around 3400 cm\(^{-1}\), the band at 1320 cm\(^{-1}\) is due to aliphatic CH bending vibrations. The chitin presence is associated with acetamide group, whose bands are present at 1655 and 1380 cm\(^{-1}\), attributed to C=O and C–H deformation bands, respectively. Bands between 1250 and 800 cm\(^{-1}\) are related to the pyranosidic ring, reflecting C–O–C and \(\beta\)-glycosidic linkages and also C–O bond from primary and secondary alcohols. Thus, the main dissimilarity among those spectra concerns the relative transmittance of acetamide groups.

![Fig. (2): FT-IR spectrum of extracted chitin in comparison with a standard chitin sample](image)

![Fig. (3): FT-IR spectra of standard chitosan (a), chitosan obtained from non-irradiated chitin (b), and chitosan from irradiated chitin at 35 kGy (c)](image)

**TGA of obtained chitin and chitosan**

TGA analysis was carried out to study the thermal stability of the extracted chitin and chitosan. Fig. (4a) clearly shows the TGA curves of the extracted chitin in comparison with a standard chitin sample. Both curves show that weight loss takes place in two stages. The first stage starts around 90 °C and the second stage starts around 280 °C. The first stage is assigned to the loss of water, because
polysaccharides usually have a strong affinity for water, and therefore may be easily hydrated. The second one corresponds to the thermal decomposition of the main chain of chitin, vaporization and elimination of a volatile product (34). The decomposition temperature of chitin standard was higher than that of the prepared chitin samples and this could be attributed to different extraction sources, as chitin standard was extracted from crayfish while in this work chitin was extracted from shrimp shells. Fig. (4b) shows the TGA curves of chitosan prepared from irradiated chitin at 35 kGy and standard chitosan, the curves show that weight loss occurs in two stages. The first stage starts around 110 °C and the second stage starts around 251 °C. The first stage is assigned water loss as polysaccharides are characterized by having a strong affinity for water and therefore may be easily hydrated. The second one corresponds to the thermal decomposition of the main chitosan chain, vaporization and elimination of volatile products (34). Comparing Fig. (4a) and (4b) it is evident that the decomposition temperature of chitin is higher than that of the corresponding chitosan. Such result indicates that chitin exists as a stable structure toward thermal decomposition than chitosan (35). On the other hand, there is no difference between the thermal stability for fermented chitin and chitosan prepared from non-irradiated and irradiated chitin.

![Graph showing TGA trace of the extracted chitin (…) in comparison with a standard chitin (---) (a), and chitosan prepared from chitin irradiated at 35 kGy in comparison with a standard chitosan (b).](image_url)

**X-Ray Diffraction of Obtained Chitin and Chitosan**

X-ray diffraction pattern (XRD) was analyzed to study the crystallinity of chitin that was extracted from fermented SSW. The represented data illustrated in Fig. (5) show strong reflections at \(2\theta = 9.6^\circ\) and \(19.2^\circ\). The band at \(9.6^\circ\) is due to the incorporation of bound water molecules into the crystal lattice. The intensity of the peak at \(2\theta = 9.6^\circ\) increases with increasing radiation dose due to hydroxyl group formation (36). Also, the crystallinity of the obtained chitosan was confirmed by XRD analysis. Fig. 6 represents the XRD patterns of obtained chitosan in comparison with standard chitosan. It is observed that the sharpness of the bands is higher in the chitin samples than in their chitosan analogue with a slight decrease in the crystallinity percentage of chitin after deacetylation reaction. Peaks corresponding to the angle \(2\theta = 20.1^\circ\) in XRD of chitosan were less resolved and shifted to higher \(2\theta\) (37). Also, it could be observed that chitosan prepared from non-irradiated chitin exhibited higher crystallinity than that prepared from irradiated one. The above-mentioned data collected from FT-IR, TGA and XRD analyses clearly confirm the successful extraction of pure chitin and the corresponding chitosan.
Determination of Average Molecular Weight (MW) for Chitosan

In order to evaluate the molecular weight of chitosan, viscometry is commonly selected as it is one of the simplest and most rapid methods for determining molecular weights (24). The viscometric constant $a$ and $K$ in the Mark-Houwink-Sakurada equation were determined in 0.3 M acetic acid and 0.2 M sodium acetate. The data presented in Table 2, show the change in the molecular weights of different chitosan samples obtained from non-irradiated chitin and irradiated chitin at 5, 15, 25, and 35 kGy. It could be stated that the molecular weight of chitosan decreases constantly with increasing the radiation dose from $1.9 \times 10^6$ to $3.7 \times 10^4$ g/mol. This could be mainly due to chain scission at $\beta$-1,4-glycosidic bonds by radiation. The prepared chitosan dissolved completely in acetic acid to a clear solution, this implies that gamma radiation induces chain degradation (30).

![Fig. (5): X-ray diffraction patterns of standard chitin (a), non-irradiated extracted chitin (b), extracted chitin exposed to 15 kGy (c), and extracted chitin exposed to 35 kGy (d)](image)

![Fig. (6): X-ray diffraction patterns of standard chitosan (a), chitosan obtained from non-irradiated chitin (b), chitosan obtained from chitin irradiated at 15 kGy (c), and chitosan obtained from chitin irradiated at 35 kGy (d)](image)
Table (2): Average molecular weight (MW) of obtained chitosan

<table>
<thead>
<tr>
<th>Sample</th>
<th>MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard chitosan</td>
<td>1.3 × 10⁶</td>
</tr>
<tr>
<td>Chitosan from non-irradiated chitin</td>
<td>1.9 × 10⁶</td>
</tr>
<tr>
<td>Chitosan from irradiated chitin at 5 kGy</td>
<td>4.8 × 10⁵</td>
</tr>
<tr>
<td>Chitosan from irradiated chitin at 15 kGy</td>
<td>11.2 × 10⁴</td>
</tr>
<tr>
<td>Chitosan from irradiated chitin at 25 kGy</td>
<td>6.4 × 10⁴</td>
</tr>
<tr>
<td>Chitosan from irradiated chitin at 35 kGy</td>
<td>3.7 × 10⁴</td>
</tr>
</tbody>
</table>

Antimicrobial Activity of Obtained Chitosan

The antimicrobial activity of chitin, chitosan and their derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi, has received considerable attention in recent years\(^{38}\). Several mechanisms were proposed for the antimicrobial activity of chitosan: (1) polycationic structure of chitosan\(^{39}\), (2) chitosan can form a polymer membrane on the surface of the cell preventing nutrients from entering the cell\(^{40}\), (3) chitosan of low molecular weight (LMW) enters the cell, binding to DNA and inhibiting RNA and protein synthesis\(^{41}\), (4) since chitosan could adsorb the electronegative substance in the cell and flocculate them, it disturbs the physiological activities of the microorganism leading to cell death\(^{42}\). Chitosan owns a broad spectrum of antimicrobial activity, but exhibits inhibitory efficiency against different fungi, Gram-positive and Gram-negative bacteria. Data in Table (3) show the antibacterial activity of different chitosan samples (CS) obtained from irradiated chitin at 5, 15, 25, and 35 kGy with different MW [4.8 × 10⁵ - 3.7 × 10⁴ (g/mol)] and different DDA values (84.4-87.9%). The results were statistically analyzed by ANOVA (Duncan's test), it could be noticed that the antibacterial activity was strengthened as the MW and DDA of chitosan decreased. CS4 (chitosan obtained at 35 kGy with MW 3.7 × 10⁴ (g/mol) and DDA 87.9 %) revealed the highest antimicrobial activity against E. coli (5.4 ± 0.2 cm), Klebsiella sp. (5.4 ± 0.12 cm), S. aureus (3.5 ± 0.21 cm) and P. aeruginosa (1.4 ± 0.06 cm).

Table (3): Inhibition zone diameters of different chitosan samples, obtained from irradiated chitin at 5, 15, 25 and 35 kGy with different degree of deacetylation (DDA), against some pathogenic bacterial strains

<table>
<thead>
<tr>
<th>Chitosan sample (CS)</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>Klebsiella sp.</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS1 (5 kGy, DDA 84.4%)</td>
<td>1.1 ± 0.1²</td>
<td>0.03 ± 0.06⁹</td>
<td>1.07 ± 0.12⁴</td>
<td>0.83 ± 0.15⁴</td>
</tr>
<tr>
<td>CS2 (15 kGy, DDA 85.9%)</td>
<td>2.0 ± 0.15¹</td>
<td>0.77 ± 0.12¹¹</td>
<td>2.5 ± 0.1³</td>
<td>1.7 ± 0.1¹</td>
</tr>
<tr>
<td>CS3 (25 kGy, DDA 87.4%)</td>
<td>4.1 ± 0.1³</td>
<td>1.07 ± 0.06⁶</td>
<td>3.67 ± 0.15⁸</td>
<td>2.63 ± 0.15⁸</td>
</tr>
<tr>
<td>CS4 (35 kGy, DDA 87.9%)</td>
<td>5.4 ± 0.2²</td>
<td>1.4 ± 0.06²</td>
<td>5.4 ± 0.12²</td>
<td>3.5 ± 0.21²</td>
</tr>
</tbody>
</table>

Values which are the means of three determinations in a vertical column followed by different superscripted letters are significantly different at p≤ 0.05 by Duncan's test. The values are the means of three replicates ± standard deviation.

Devlieghere et al.\(^{43}\) explained that the antibacterial activity of chitosan increases in the order of chitin deacetylation degree (DDA). This inhibition is more important against Gram-negative than Gram-positive bacteria tested. Gram-negative bacteria, with lipopolysaccharide at the outer surface providing negative charges, seemed to be very sensitive to chitosan while the sensitivity of Gram-positive bacteria that may possess variable amounts of negatively charged teichoic acids at their outer surface varied greatly. However, No et al.\(^{44}\) showed stronger bactericidal effects of chitosan toward Gram-positive than Gram-negative bacteria.
Benhabiles et al. (45) studied the antimicrobial activity of chito-oligosaccharide (COS), chitosan and chitin (0.1 %, w/v) against four Gram-positive (S. aureus ATCC 25923, S. aureus ATCC 43300, B. subtilis and B. cereus) and seven Gram-negative bacteria (E. coli ATCC 25922, P. aeruginosa ATCC 27853, Salmonella typhimurium, Vibrio cholerae, Shigella dysenteriae, Prevotella melaninogenica and Bacteroides fragilis) with an emphasis on the effects of biopolymer MW and DDA. They found that chitin exhibited a bacteriostatic effect on the Gram-negative bacteria namely, E. coli ATCC 25922, V. cholerae, S. disenteriae and B. fragilis. While chitosan exhibited a bacteriostatic effect on all bacteria strains tested, except S. typhimurium. The antibacterial activities of chitosan had exceeded the action of chitin, in particular, against Gram-negative bacterial strains because chitosan possesses a number of polycationic amines which can interact with the negatively charged residues of carbohydrates, lipids and proteins located on the cell surface of bacteria, which subsequently inhibit the growth of bacteria. In addition, if the molecular weight of chitosan is low, its polymer chains have greater flexibility to bind more than one cell. Thus, bridges between bacterial cells and polymer chains of chitosan are quickly formed so that the bacteria are immediately inactivated. The chito-oligosaccharide exhibited a bactericidal effect on all bacteria strains tested.

CONCLUSION

Low molecular weight chitosan with a high degree of deacetylation was produced from chitin which was previously extracted from shrimp shell waste by fermentation using locally isolated Bacillus subtilis NA12 strain. Results of characterization of the chitin and chitosan collected from FT-IR, TGA and XRD clearly confirmed the successful extraction of pure chitin and chitosan. The results of XRD analysis revealed that chitin is more crystalline than chitosan. The chitosan prepared at 35 kGy showed the highest antibacterial activity against different bacterial pathogens selected for this study.

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