Bangladesh J Microbiol, Volume 37, Number 2, December 2020, pp 52-55

Original Article

Physicochemical Properties of Chitosan Extracted from *Pleurotus ostreatus* and Improvement of its Antibacterial Activity by Gamma Radiation

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This study was carried out to determine the physicochemical characteristics such as intrinsic viscosity (IV), molecular weight (MW), water binding capacity (WBC), and fat binding capacity (FBC) of chitosan extracted *Pleurotus ostreatus* fruit body. Antibacterial activity of the gamma irradiated chitosan was also determined. The intrinsic viscosity, MW, WBC and FBC of the produced chitosan were 769.69 ml/g, 1.8×10^5 Da, 408% and 234%, respectively. The fungal chitosan was subjected to different doses (*viz.*, 5, 10, 20, 30 and 40 kGy) of radiation from ^{60}Co gamma source to observe the effect of gamma radiation on its antibacterial activity. After irradiation, antibacterial activity was evaluated and compared with that of non-irradiated chitosan. Non-irradiated chitosan showed moderate antibacterial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 35150, and *Salmonella enteritidis* ATCC 13076 whereas chitosan samples treated with 5, 10 and 20 kGy gamma irradiation separately have shown enhanced antibacterial activity than that of non-irradiated chitosan against the above-mentioned bacteria. However, higher doses of gamma irradiation (30 kGy and 40 kGy) caused a gradual decline of the antibacterial activity.

Keywords: Chitosan, *Pleurotus ostreatus,* intrinsic viscosity, molecular weight, gamma radiation, antibacterial activity.

Introduction

Chitin is a structural component of the exoskeleton of crustaceans and insects, skeleton of sponges and inner skeleton of squid and cuttlefish, and cell wall of fungi. Chitosan is a deacetylated polymer (1-4) linked D-glucosamine and N-acetylglucosamine of chitin. Chitosan is a nontoxic, biodegradable polymer of high molecular weight (MW).

The MW of chitosan may vary depending on the sources and is generally greater than one million Daltons (Da). For example, commercially used chitosan have MW ranging from 1.0×10^5-1.2×10^6 Da[2]. The MW of chitosan can be determined by chromatography[3], light scattering[4] and viscometry[5]. among which, viscometry is the simplest and rapid method for the determination of MW of chitosan[5,6].

Different physicochemical factors (*viz.,* temperature, pH, concentration, molecular weight, ionic strength etc.) influence the viscosity of chitosan. The solubility, biodegradability, reactivity and adsorption of chitosan depend on the amount of protonated amino groups present in the polymeric chain. Chitosan is soluble in different acids such as acetic-, nitric-, hydrochloric-, perchloric- and phosphoric acid[7-12]. The biological activity of chitosan depends significantly on its MW and DA. MW and degree of acetylation (DA) may affect the antimicrobial activity of chitosan independently[13].

In previously published reports, it was shown that gamma irradiation; produced low MW chitosan with different surface charge and even different chemical structure which increases the water solubility of chitosan[14] and enhances its inhibitory effect against microorganisms[15].

Chitin and chitosan are used in biomedical, pharmaceutical, biotechnological and agriculture fields as bio-agents. Due to its unique polycationic nature, chitosan had been shown to possess antimicrobial activity against several microbes[16,17]. For antimicrobial property, chitosan may be used in feed and cosmetic products as alternative novel natural antimicrobial substance.

In Bangladesh, some studies were carried out on the production and characterization of chitosan from shrimp[18,19], but no study on production of chitosan from locally available fungi had been done so far in Bangladesh. Therefore, this study was performed to produce chitosan from a locally available fungus, *Pleurotus ostreatus* (oyster mushroom) belong to basidiomycete, and to determine its physicochemical characteristic and to evaluate the effect of radiation on its antibacterial activity. Besides, ionizing radiation was also applied to the fungal chitosan to improve its antibacterial activity.

Materials and Methods

Extraction of chitin and production of chitosan:

Oyster mushroom (*P. ostreatus*) was collected from Savar, Dhaka, Bangladesh and chitin was extracted from the fruiting bodies of

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this fungus. Chitosan was produced from the extracted chitin by alkalization method[20,21]. Fruit bodies of the fungus were dried in an oven set at 60°C for 2 days and the dried material was grinded to make powder. The powder was treated with 1N NaOH solution and kept at 100°C for 3h. The alkali insoluble materials (AIM) were collected by filtering the slurry. The AIM was dried in an oven at 40°C for 4 days. The dried AIM was then dissolved in 2% acetic acid and was kept in a water bath set at 100°C for 5h. The sample solution was centrifuged at 6,000 rpm for 10 minutes and the supernatant was decanted into a beaker. The solution was adjusted at pH 12.0 by 2N NaOH solution for precipitation. The precipitate was collected through centrifugation at 6000 rpm for 5 min. The precipitate was washed 4-5 times with distilled water by using the same centrifugation condition and dried in the oven at 45°C.

Determination of intrinsic viscosity and molecular weight (MW) of chitosan:

The molecular weight of chitosan was determined by Ostwald viscometer[6]. In this method, the viscosity of a liquid is measured by comparing the viscosity of an unknown liquid (sample solution) with the viscosity of a known solvent (0.3 M acetic acid + 0.2 M sodium acetate). The viscosity of the liquid was measured by comparing the flow times of two liquids ($t_{solvent}$= flow time of solvent and t_{sample} = flow time of sample) of equal volume using the same viscometer. On the basis of these flow times, average runtime, specific viscosity ($\bullet_{spe}$) and reduced viscosity ($\bullet_{red}$) were calculated. A graph was prepared using concentration and reduced viscosity of chitosan at X and Y co-ordinates, respectively. Then an extrapolation plot of reduced viscosity against chitosan concentration was made using trend line to find out the intrinsic viscosity [•], which is equal to Y-intercept. The intrinsic viscosity of the sample was calculated using the following formula:

Average runtime = (Runtime A + Runtime B + Runtime C)/3

Specific viscosity ($\bullet_{spe}$) = (Sample runtime- Solvent runtime)/ Solvent runtime

Reduced viscosity ($\bullet_{red}$) = Specific viscosity/ Sample concentration

Intrinsic viscosity = Y-intercept of the plot

The molecular weight was calculated by Mark-Houwink equation[22].

$[\bullet] = KM^{\pm}$ where, {[•] = Intrinsic viscosity, M= Molecular weight, K and ± = Constant (K= 0.078 and ±= 0.76)}

Determination of water binding capacity (WBC):

WBC of chitosan was measured using the method described by Knorr[23]. WBC was determined by weighing a tube containing 0.5 g of chitosan. Ten milliliter water was added to it and mixed by a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with intermittent shaking for 5 sec after every 10 min and finally centrifuged at 3000 rpm for 25 min. Then the supernatant was decanted, the tube was weighed again. Bound water (g) was determined by subtracting the weight of centrifuge tube containing only chitosan from the weight of the tube containing water plus chitosan. WBC was calculated as follows:

WBC (%) = [Water bound (g) / Initial sample weight (g)] × 100

Determination of fat binding capacity (FBC): FBC of chitosan was measured using the method described by Knorr[23]. For the determination of FBC, same protocol described in WBC was followed and soybean oil was used instead of water. FBC was calculated as follows:

FBC (%) = [Fat bound (g)/ Initial sample weight (g)] × 100

Irradiation of chitosan with gamma ray:

Chitosan solution was irradiated with a series of radiation doses (*i.e.*, 5, 10, 20, 30 and 40 kGy) at room temperature using a ^{60}Co Gamma source (Gamma beam, 650, AECL, Canada), situated at Atomic Energy Research Establishment (AERE), Savar, Dhaka, Bangladesh. Ceric-cereus dosimetry was performed to measure the absorbed dose level.

Determination of antibacterial activity of chitosan:

Agar well diffusion method was used for determination of the antimicrobial activity of chitosan[24,25]. To determine the antibacterial activity of chitosan, 0.5% chitosan solution was prepared in 1% acetic acid solution. The antibacterial activity of irradiated and non-irradiated chitosan samples were tested against *Bacillus subtilis* ATCC 6633*, Staphylococcus aureus* ATCC 6538, *E. coli* ATCC 35150, and *Salmonella enteritidis* ATCC 13076. The test organisms were separately cultured in nutrient broth at 37°C for 6 to 8 hours. A sterile cotton swab was dipped in the test tube containing the bacterial culture and then spread uniformly onto previously prepared Muller Hilton Agar plates. Wells of uniform diameter (7.0 mm) were bored in the MHA medium. Aliquots (50µl) of the gamma irradiated chitosan solution; non-irradiated chitosan solution and 1% acetic acid (control) were dispensed in separate wells of the inoculated MHA plates. The plates were then incubated at 37°C for 20 hours. After incubation, the diameters of the zone of inhibition (in mm) were measured.

Results and Discussion

In this study, the physicochemical characteristics and antibacterial activity of a fungal chitosan were determined. To the best of our knowledge, this is the first report on the antibacterial activity evaluation of chitosan produced from a fungus available in Bangladesh.

The MW of the produced chitosan was determined by viscosity-average molecular weight determination process[6]. In this process, reduced viscosity was determined by using Ostwald viscometer and then intrinsic viscosity was determined from the graph (Fig.1).

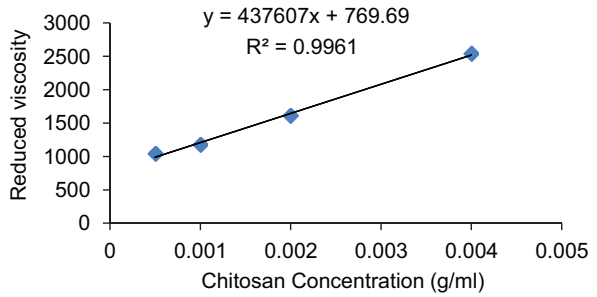

Figure 1. *Determination of intrinsic viscosity of chitosan*

In this case, Y-intercept of the graph suggested that the intrinsic viscosity of the fungal chitosan was 769.69 ml/g. The MW was calculated by Mark-Houwink equation using this intrinsic viscosity as described previously by Wang *et al*.[22]. The MW of the chitosan from *P. ostreatus* was found 1.8×10^5 Da. This finding was correlated with the finding of Pochanavanich and Suntornsuk (2002) [26].

From the Table 1, it was observed that the water binding capacity of the extracted chitosan ranged from 380% to 430% (Average 408±25.80). This finding did not correlate the findings of Cho, *et al*. (1998); they reported 458% to 805% water binding capacity of chitosan extracted from shrimp and crab shell[27] .

From the Table 1, it was apparent that the fat binding capacity of the extracted chitosan ranged from 196% to 262% (Average 234±33.85%). This finding did not correlate the findings of No *et al*. (2000); they reported 314% to 535% fat binding capacity of chitosan[28].

In a previous study it was found that chitosan has antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*[22] and gamma-irradiated shrimp chitosan showed elevated antibacterial activity[19]. Therefore, the fungal chitosan extracted from *Pleurotus ostreatus* was irradiated with different doses of gamma radiation to observe the effect of gamma radiation on its antibacterial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *E. coli* ATCC 35150, and *Salmonella enteritidis* ATCC 13076.

From the Table 2, it was found that non-irradiated chitosan sample showed moderate antimicrobial activity where the zones of inhibition were 11.3, 12.0, 8.9 and 11.0 mm, against *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis,* respectively, Irradiated chitosan sample showed significant increase in activity with maximum activity 14.3, 16.0, 12.1 and 14.1 mm, against *Bacillus subtilis* and *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidi*, respectively at 20 kGy radiation applied. However, antibacterial activity of chitosan samples was decreasing gradually after 20 kGy radiation applied.

Results from this study corroborate with the previous study where antibacterial activity of chitosan extracted from shrimp shells was increased as the irradiation dose increased up to a certain level[29]. A limited dose level decreased the molecular weight of chitosan in such a way that might have caused the increased membrane permeability of bacterial cells and thus inhibit their growth[13,16]. Therefore, dose optimization for achieving maximum antimicrobial activity is crucial and our study suggested that 20 kGy is the optimum irradiation dose for obtaining maximum antimicrobial activity of this fungal chitosan against different Gram positive and Gram negative bacteria including food borne pathogens.

Table 1. *Water binding (WB) and fat binding (FB) capacities of chitosan*

Expt. No.	Initial weight (g)	*Water binding (WB) capacity of chitosan*			Fat binding (FB) capacity of chitosan		
		Weight of water bound	WBC (%)	Ave. WBC (%)	Weight of fat bound	WBC (%)	Ave. FBC (%)
1	0.5	2.08	416		1.48	196	
2	0.5	2.40	380	408±25.80	1.71	242	234±33.85
3	0.5	2.65	430		1.81	262	

Table 2. *Antibacterial activity of gamma-irradiated chitosan against four common bacteria.*

Test organisms	Zone of inhibition (mm) Doses of gamma-radiation (kGy)					
	0	5	10	20	30	40
Bacillus cereus ATCC 6633	11.3	12.8	13.5	14.3	12.3	11.8
Staphylococcus aureus ATCC 6538	12.0	13.1	13.7	16.0	13.3	12.9
Escherichia coli ATCC 35150	8.9	10.3	10.4	12.1	11.3	10.3
Salmonella enteritidis ATCC 13076	11.0	11.9	12.9	14.1	12.8	12.0

Acknowledgements

The authors would like to express heartfelt thanks to the personnel of Gamma Source Division of Atomic Energy Research Establishment (AERE), Savar, Dhaka for technical help.

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