Original Article

Extraction and Characterization of Chitin and Chitosan from *Tenebrio Molitor* Beetles and Investigation of its Antibacterial Effect Against *Pseudomonas aeruginosa*

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Abstract

Background: Chitin and chitosan are utilized in many industries such as pharmacy, biotechnology, and medicine. The mealworm beetle, *Tenebrio Molitor*, is simply breaded and does not require a vast production space.

Materials and Methods: In this study, we extracted chitin and chitosan using two different methods from *Tenebrio Molitor* adult beetles. Then we studied their physical and chemical properties along with their antibacterial effect.

Results: Using two new methods we extracted 13, 3%, and 17.7% chitin from the dry mealworm beetle which was higher than in previous studies. The chitosan yield of the extracted chitin was 78.26% and 76.43%, respectively. The observed FTIR peaks for chitin and chitosan in this study were in accordance with the characteristic peaks. The degree of acetylation of chitin was 95.09% and 92.55% and the degree of deacetylation was 75.84%, and 72.6% from the first and second methods, respectively. The extracted chitosan also showed an antibacterial effect against *Pseudomonas aeruginosa*.

Conclusions: Our study demonstrated that chitin and chitosan extracted from adult mealworm beetles could be considered as a replacement for commercial chitosan and needs further studies.

Keywords: Chitin, chitosan, *pseudomonas aeruginosa*

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Introduction

Chitin, a structural amino polysaccharide, exists abundantly as the primary nanostructured component of the skeletal structure of numerous unicellular and multicellular organisms.[1]

Chitosan, a derivative of chitin deacetylation, is the most significant chitin derivative. The presence of amine groups in chitosan, a derivative of chitin deacetylation, is a substantial advantage since it allows for a wide range of biological activities.[2]

Chitosan can be used in a variety of industries, including pharmacy, agriculture, biotechnology, and medicine. Due to its fascinating qualities such as biocompatibility, strong antibacterial activity wound healing properties, drug delivery, and each, the application of chitin and chitosan in medicine and pharmaceutical science has grown rapidly and is now of interest to many researchers around the globe.[3,4]

One of the issues that medical research is dealing with is wound healing. Chitin and chitosan have the potential to accelerate the wound healing process.[5] Chitosan is widely

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studied as a wound-healing agent because of its hemostatic impact in the early stages of wound formation, as well as its ability to prevent microbial growth maintenance of collagen deposition and fibroblast proliferation. Chitosan is mainly characterized by its degree of deacetylation, which affects various physical, chemical, and biological aspects such as solubility and hydrophilicity.[5,6]

To extract these chemicals, different sources including algae, bacteria, insects, fungus, yeasts, and other organisms have been investigated. Crustaceans' shells are currently the most common source of these minerals.[7]

Commercial chitosan is mostly obtained from a few species, notably crabs and shrimp, despite the fact that chitin is the second most prevalent natural polymer which is consistent in the exoskeleton of a wide range of living organisms. Due to its high cost and scarce resources, the usage of crab and shrimp shell waste has substantially reduced the amount of chitosan produced. Although chitin and chitosan are predicted to lead to the development of a new functional polymer, their use is limited and their potential has yet to be exploited. Chitin and its derivatives from insects have features that are similar to commercial chitin. It is also non-toxic and safe to use. Insects also provide a considerable resource for larger-scale chitin manufacturing due to their enormous numbers and ease of reproduction.[8,9]

Mealworms are simply breaded, do not require a vast production space, and have a high nutritional value. The mealworm beetle, *Tenebrio Molitor*, has previously been investigated as a source of chitin and chitosan production. The outcomes of the study were promising in terms of chitosan synthesis and antibacterial effects.[10] Although the yield of chitin in this study was lower than expected. In this study, we investigated alternative chitin production methods for *Tenebrio Molitor*, for the first time in Iran in order to find a superior substitute source of chitosan.

Materials and Methods

Sample collection

This study was approved by Police Headquarter with the ethical code (IR.BMSU.REC.1401.031). *Tenebrio Molitor* beetles were prepared from the breeding center and placed in the laboratory environment, without food for 72 hours to empty their intestinal contents.

The moisture content of beetles

After washing three times with distilled water, the beetles were dried in an oven at 60°C for 1 week. The weight of the beetles was measured before and after drying.[11] The body moisture of the beetles was calculated based on the following formula:

Percentage of moisture content = 100% (wet weight-dry weight)/(wet weight)

Extraction of chitin

The dried beetles were powdered and passed through a 250 m sieve before being stored at 4°C until the experiment. In this study, two different methods were employed for chitin extraction. In the first method, 10 grams of dry powder was demineralized in 100 ml of 2 M hydrochloric acid for 2 hours at 65 to 75°C for chitin extraction. After that, the solution was filtered using filter paper before being washed with deionized water. Then, for 16 hours, the filtrates were deposited in 50 ml of 2 M NaOH at 80 to 90°C and then filtered again and washed with deionized water to eliminate any protein residues. To decolorize the filtered materials, they were placed in a solution comprising chloroform, methanol, and water (ratios 1, 2, and 4, respectively) for 1 hour. After decolorization, the mixture was filtered and rinsed with distilled water for the last time. The extracted chitin was then placed in an oven at 60°C.[12]

In the second method, 10 grams of sample was refluxed at 100°C for 10 minutes in a 100 mL sodium hypochlorite (NaClO) solution (3%, v: v) after rinsing with distilled water this step was repeated. The samples were then refluxed for 15 minutes in 50 mL of 1 M HCl at 75°C to demineralize. For the deproteinization step, the samples were refluxed in 50 mL of 1 M NaOH solution at 100°C for 20 minutes. Finally, after washing, the extracts were dried at 60°C.[13]

The following formula was used to determine the dry weight chitin content of beetles:

The chitin yield = 100× (produced chitin weight)/(initial weight)

Chitosan extraction process

One gram of each of the extracted chitin from adult beetles was converted to chitosan by deacetylation. The deacetylation process was performed by treating the sample with 50% NaOH (weight/volume 1:20) at 100°C for 3 hours. The samples were then rinsed using deionized water to reach pH 7. The chitosan samples obtained were then dried at 40°C for 24 hours.

The following formula was used to determine the dry weight chitosan content of chitin:

The chitosan yield = 100 × (produced chitosan weight)/(initial chitin weight)

Fat and water binding capacity

A tube was weighed and then 0. 5 gm. chitosan was added. 10 ml water was added to the tube and vortexed for 1 min then every ten minutes this step was repeated for 5 seconds. After 30 minutes the tube was centrifuged at 3000 rpm for 25 minutes. To determine fat binding capacity the last step was then repeated by substitution of soybean oil with water. After the supernatant was poured out, the tube was weighed again.[14] Finally, the water and fat binding capacity was calculated from the following equations:

Fat or water binding capacity: 100 × (water or fat bound)/(Initial sample weight)

Fourier-transform infrared spectroscopy

Using a Thermo Nicolet FTIR Spectrometer, extracted chitin and chitosan samples from *Tenebrio Molitor* beetles were

examined at 4000 to 400/cm. The following formula was used to compute the degree of acetylation (DA) of chitin and the degree of deacetylation (DD) of chitosan[15]:

DA (%) = (A1655/A3450) × 100

DD (%) = 100 -[(A1658/A3450) × 115]

Solubility

In an incubator shaker set to 240 rpm at 25°C, 0.1 g chitosan was dissolved in 10 ml of 1% acetic acid for 30 minutes in a weighed tube. The mixture was heated for 10 minutes in a boiling water bath, cooled, and then centrifuged for 10 minutes at 5000 rpm. The filtrate was rinsed with distilled water and dried at 60°C after the supernatant was removed.[16] The following equation was used to estimate the solubility:

Solubility = 100 × (Initial sample weight – Final sample weight)/(Initial sample weight – tube weight)

Antimicrobial activity

The 4%, and 8% solution of chitosan in 1% acetic acid was prepared, sterilized (by autoclaving for 20 minutes at 121°C) and then placed on 6 mm sterile filter paper discs. Anti-*pseudomonas aeruginosa* activity was determined using disc diffusion. A 0.5 McFarland bacterial suspensions (approximately 10^8 CFU/ml) were prepared freshly in Muller Hilton Agar broth and added to the petri dish. The 4 and 8% chitosan solution (20 µL) was placed on a petri dish and incubated at 37°C for 24 h. The antibiotics Ampicillin (1 mg/ml) was used as positive controls and PBS was used as a negative control. The results were also compared with 4 and 8% commercial chitosan (75-85% deacetylation, Sigma Aldrich). Experiments were run in duplicate. The antimicrobial activities were assessed as the diameter of growth inhibition.[10,11,17]

Results

Moisture content

After washing and drying the beetles were powdered for chitin extraction. The moisture content of the beetles was obtained by reducing the body weight of beetles before and after drying them in the oven. The results showed that there was 21.1% ± 0.7 moisture in the beetles' bodies.

Chitin content

For chitin extraction, two methods were used and demineralization and deproteinization of samples were done with HCl and NaOH. The results showed that the chitin extracted with the first method (12) yield was 17.7% while the second method (13) yielded chitin of 13.3%.

Chitosan content

Following the chitin extraction, chitosan was produced with obtained chitin from each method. The deacetylation was carried out with the treatment of samples with NaOH. The chitosan yield of the extracted chitin from the first method was 78.26% while the second chitin yielded 76.43% chitosan.

Fat and water binding capacity

The capability of water to attach to hydrophilic substances is known as its water-binding capacity. The amount of oil absorbed per unit weight is measured by fat binding capacity. Two distinct isolated chitosan had water and fat binding capacities of 632–609% and 419–386%, respectively.

FTIR, degree of acetylation, and deacetylation

Three significant amide bands at 1648, 1621, and 1558 cm^{-1} were found in the FTIR of adult *Tenebrio Molitor* chitin using the first method, which is an indicator of the C = O secondary amide stretch (Amide I), C = O secondary amide stretch (Amide I), and N-H bend and C-N stretch (Amide II), respectively. The C = O secondary amide stretch (Amide I) and N-H bend were measured at 1650 cm^{-1}, C = O secondary amide stretch (Amide I) and N-H bend at 1620 cm^{-1}, and C = O secondary amide stretch (Amide I) and C-N stretch (Amide II) at 1559 cm^{-1} for the chitin isolated using the second approach. These peaks are indicative of chitin and indicate that the structure was in the alpha form.

The DA of chitin from *Tenebrio Molitor* beetles obtained using the first approach (95.09%) was found to be greater than that of chitin obtained using the second method (92.55%). Mineral residues may be found in chitin with a DA value greater than 100% and protein residues may be present with a value less than 100% [Figure 1].

Figure 2 shows the FT-IR spectrums of chitosan extracted from *Tenebrio Molitor* beetles using two different chitin extraction techniques. The amide I (C = O-NHR) and amine group (-NH2) bands, which are indicative of chitosan derived from *Tenebrio Molitor* beetles, were 1652 cm^{-1} and 1588 cm^{-1} in the FT-IR spectrums, respectively. The peaks for the (C = O- NHR) and amine group (-NH2) bands in the second chitosan were 1650 and 1591 cm^{-1}, respectively. Furthermore, the strong FT-IR absorption band of chitosan derived from two different extracted chitin of *Tenebrio Molitor* beetles in 3455 and 3418 cm^{-1}, OH and amine N-H symmetrical stretching vibrations. FTIR was used to determine the degree of acetylation of chitosan produced from adult *Tenebrio Molitor* beetles. The chitosan formed from the obtained chitin from the first approach had a degree of deacetylation (DD) of 75.84%, whereas the chitosan from the second method had a DD of 72.6%.

Solubility

Chitosan's solubility is mostly determined by its biological species, degree of deacetylation, and Mv. In this study, the solubility of chitosan made from two distinct chitin sources was 95.3 ± 0.4% and 92.3 ± 0.2%, respectively.

Antimicrobial activity

In this study, the 8% chitosan obtained from *Tenebrio Molitor* beetles showed an inhibitory zone of 4 mm against *pseudomonas aeruginosa*. The antimicrobial activity of *Tenebrio Molitor* beetles chitosan was lower compared to ampicillin (10 mm) but higher than the 8% commercial

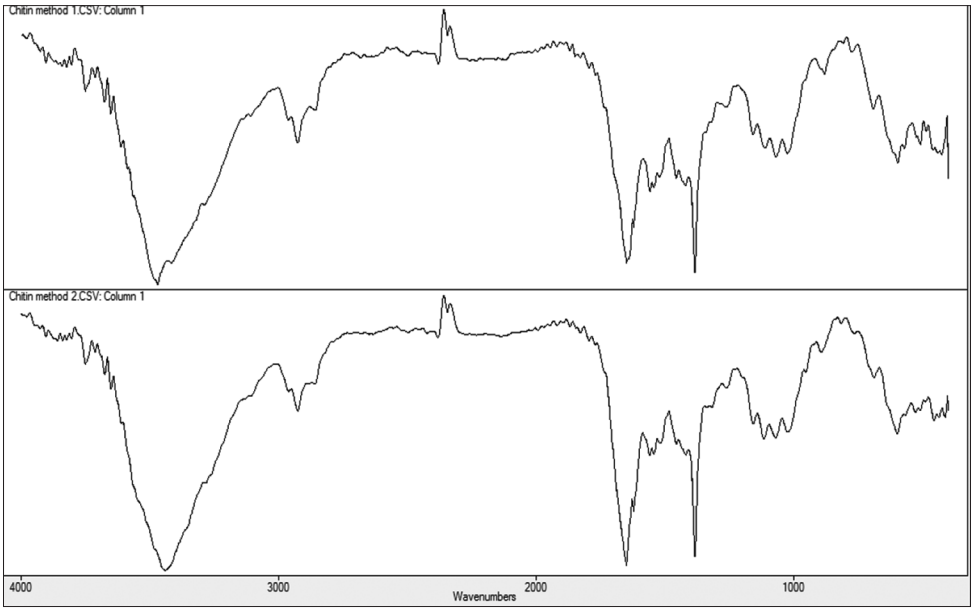

Figure 1: FTIR spectrums of chitin

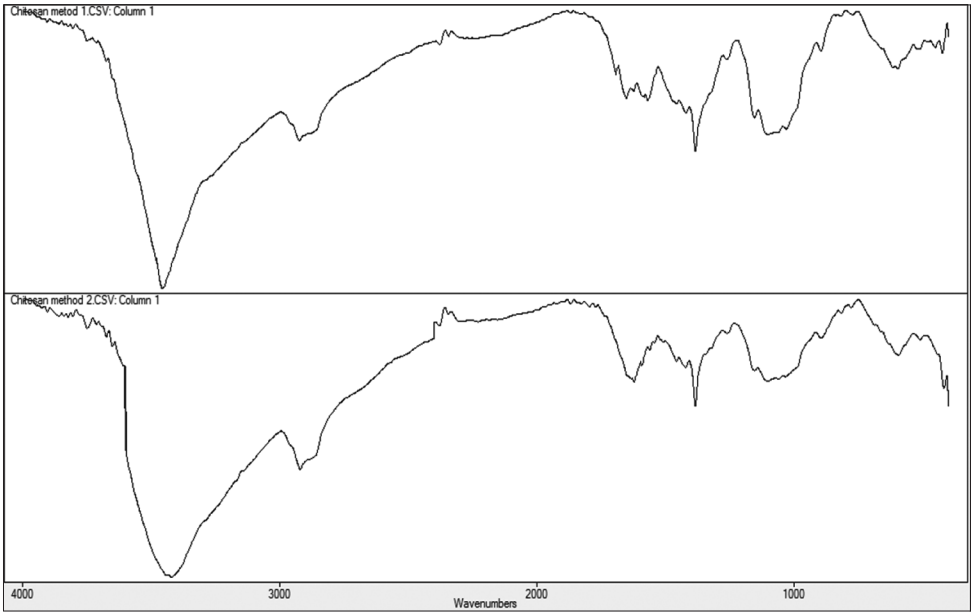

Figure 2: The FTIR spectrums of chitosan

chitosan (2 mm). The acetic acid and 4% chitosans showed no antimicrobial activity.

Discussion

In this study after drying the beetles, the moister content of their body was calculated. Our results demonstrated that only 21.1% of their body consisted of water which was lower than the blue crab shell and shells of Litopenaeus vannamei and Portunus pelagicus which ranged from 37 to 50% which indicate a higher dry weight and therefore chitin yield.[18,19]

After that chitin was extracted, and our results demonstrated a chitin yield of 17.7% from *Tenebrio Molitor* beetles using a method suggested by Kaya *et al*.[12] in 2014 which was performed on Colorado potato beetle which was similar to our samples. The chitin yield in the kaya study was 20% which was similar to our results. The chitin yield in the second method proposed by Kaya *et al*.[13] 2015 was 13.3% which was in the same line as the extracted chitin from crab, crayfish, and shrimp shells from the kaya study. In a previous study by Shin CS *et al.,*[10] the chitin extracted from *Tenebrio Molitor* beetles was 8.4% which was lower than our study. However, the chitosan content of chitin was 78.33% which was similar to our study.

The chitin yield from other insects, varies for instance seven Orthoptera contained between 5.3 and 8.9%,[17] Bombyx mori and Holotrichia parallela contained 15%,[20,21] bat guano

contained 28%,[22] Hydrophilus piceus contained 19–20%,[23] and cicada had 36.6%.[24] Crustacean shells contain 15% to 20% of chitin which is similar to the amount of chitin extracted from the *Tenebrio Molitor* beetle in our study.[25]

In the next step, chitosan was extracted by deacetylation of chitin. The chitosan extracted from chitin using the first method yielded 78.26% while the second method yielded 76.43% chitosan. These results demonstrate high chitosan extracted compared to many other species such as Sepia prashadi, Sepiella inermis, Sepioteuthis lessoniana, Doriprismatica sibogae of cephalopod mollusks, and bat guano (15, 18.75, 57.14, 78.57, and 22%, respectively)[22] and as the same pattern as other studies such as crabs, lobsters of Crustacea, L. lessoniana, and Lozotaeniodes formosana (74.6, 74.3, 77.57, and 77.21%, respectively).[26]

The degree of acetylation of the extracted chitins was calculated by their FTIR spectrum. In this study the DA values for both chitins in this investigation were close to 100%, indicating that the extracted chitin was nearly pure. DA values for chitin extracted from various species have previously been determined to be 102% for cicada sloughs or 87% for bumblebees.[15] Our findings also suggest that a few protein residues remained in the *Tenebrio Molitor* beetles' chitins. These findings, on the other hand, show that the extracted chitins from the first procedure were purer than the chitins obtained from the second method.

The observed FTIR peaks for chitin and chitosan in this study were in accordance with previous studies and are the characteristic peaks of chitosan and alpha chitin.[27,28]

The degree of deacetylation of chitosan from adult mealworm beetles was calculated according to a formula and their FTIR spectrum. The results showed a DD of 75.84%, and 72.6% which was relatively high and in accordance with the previous study by Shin CS.[10] The commercial chitosan DD range between 66-95%.In a study by Hardani *et al*.[19] the extracted chitin from crab was 52.63% and from shrimp, water was 45% which was lower than our study.

Finally, our results showed that chitosan had antimicrobial activity against *pseudomonas aeruginosa* which is in the same line as the Shin CS study in which the 8% chitosan showed a 2 mm inhibitory zone against *Staphylococcus aureus*.[10]

Conclusion

Our study demonstrated that Chitin and Chitosan extracted from adult mealworm beetles could be a replacement for commercial chitosan and could be considered an alternative source for shrimp waste. Furthermore, the results of this study were in accordance with the previous study by Shin CS and suggest two improved chitin extraction methods. The results of this study could be further investigated for utilization of this chitin in many properties such as wound healing bands.

Ethics approval

This study was approved by Police Headquarter with the ethical code of (IR.BMSU.REC.1401.031).

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Nil.

Conflicts of interest

There are no conflicts of interest.

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