Volume 7 Issue 2 2021

ISSN 2454-3055

**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

***Forum for Biological and
Environmental Sciences***

Published by Saran Publications, India

Antifungal Activity of Chitosan Based Biosynthesized Gold Nanoparticles Isolated from *Portunus sanguinolentus* Crab Shell

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Received: 4th November, 2021; **Accepted:** 6th December, 2021; **Published online:** 11th December, 2021

<https://doi.org/10.33745/ijzi.2021.v07i02.075>

Abstract: Chitosan has become a prominent polymer attracting a lot of interest. The fact that this precious polymer may be recovered from shell waste created by the food industry adds to its worth. Because of its biological and physicochemical characteristics, chitosan has become a popular choice for biomedical applications. The different processes involved in the production of chitosan and chitosan Nano forms are briefly discussed in this article. We bring together for the first time, studies on diverse initiatives at green chitosan production, chitosan nanoparticles, and chitosan Nano composites. Chitosan aids in the production of more stable nanoparticles. The formation of nanoparticles was confirmed by UV-Visible spectroscopy, which detected a peak at 529 nm. Based on the interpretation of the spectroscopy in this study, the interaction between chitosan and generated gold nanoparticles (AuNPs) was studied and assessed, and it can be concluded that FT-IR spectroscopy was utilized to analyze all functional groups. XRD examination confirmed the presence of gold nanoparticles with crystalline nature, which were discovered to have an FCC lattice structure. Atomic force microscopy (AFM) is a technique for analyzing the surface of a rigid material all the way down to the level of the atom. AFM uses a mechanical probe to magnify surface features up to 100,000,000 times, and it produces 3-D images of the surface. The potency of the synthesized AuNPs against the selected fungi increases with increase in concentration of AuNPs. Therefore, the antifungal investigation revealed that the synthesized AuNPs exhibited good antimicrobial activity.

Keywords: Chitosan AuNPs, UV-Vis, FT-IR, XRD, AFM, Antifungal activity, Atomic force microscopy

Citation: Sangeetha B., Indra V., Ramya B. and Sheela D.: Antifungal activity of chitosan based biosynthesized gold nanoparticles isolated from *Portunus sanguinolentus* crab shell. Intern. J. Zool. Invest. 7(2): 866-876, 2021.

<https://doi.org/10.33745/ijzi.2021.v07i02.075>

Introduction

Marine bio-nanotechnology is a new subject in biological study that has a lot of potential. The maritime environment is diverse, with a diverse range of micro- and macro-species, making nanoscience and nanotechnology a viable option (Dhillon *et al.*, 2013). A key component of nanotechnology research is the advancement of a dependable and environmentally acceptable

process for the production of gold nanoparticles (AuNPs) (Manivasagan and Oh, 2016). Bigger particles with particular significant characteristics factors such as size, distribution, and shape of NPs with sizes up to 100 nm display totally new or enhanced qualities (Murugan *et al.*, 2017). The capacity to regulate the size of NPs is extremely desirable for most nanotechnology applications

(Affes *et al.*, 2020). NPs offer a wide variety of potential applications in catalysis, optical, photo-electrochemical, and antibacterial activities, as well as biomedicine and health care, due to their unique properties (Chen *et al.*, 2020). Different physicochemical techniques may be used to make NPs; however, these processes are extremely reactive and pose a significant environmental risk. Biomimetic techniques offer the benefit of employing nontoxic chemicals and ecologically benign solvents, as opposed to physicochemical approaches (Kalaivani *et al.*, 2020; Begum *et al.*, 2021; Bunyatova *et al.*, 2021; Emam *et al.*, 2021; Hajji *et al.*, 2021).

Chitosan (CHS) (Fig. 1), sometimes known as deacetylated chitin, is a natural polycationic linear polysaccharide derived from partial deacetylation of chitin (Bunyatova *et al.*, 2021). Chitin is the structural element in the exoskeleton of insects, crustaceans (mainly shrimps and crabs) and cell walls of fungi, and the second most abundant natural polysaccharide after cellulose. The complexity of the chitin structure, difficulty in its extraction and insolubility in aqueous solution limited the research on this polymer until 1980s. Chitosan is composed of β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine randomly distributed within the polymer. The cationic nature of chitosan is rather special, as the majority of polysaccharides are usually either neutral or negatively charged in an acidic environment. This property allows it to form electrostatic complexes or multilayer structures with other negatively charged synthetic or natural polymers (Novais *et al.*, 2021; Salomatina *et al.*, 2021).

Fig. 1: Chemical structure of Chitosan.

Chitosan is widely recognized for its nontoxicity and great biocompatibility. Stabilizers, such as Chitosan, can aid in the modification of the surface's physical absorption, special recognition, and electrostatic interactions in the creation of NPs, which is critical in assessing the potential therapeutic use of metal NPs (Novais *et al.*, 2021). Chitosan was utilized in the production of gold nanoparticles as a stabilizer and reducing agent. The concentration of Chitosan influences the size and stability of NPs (Salomatina *et al.*, 2021). Electrostatic attraction between metal anions and protonated amine groups is caused by the interaction of chitosan with anionic Gold (III) chloride hydrate ions (Sivanesan *et al.*, 2021). The biosynthesis of metallic NPs is gaining prominence owing to its eco-friendliness and low cost. Chemical methods, on the other hand, are associated with biological hazards and environmental impact (Sun v 2021). Metallic NPs derived from biological agents such as microorganism, fauna and flora are extremely stable (Lv *et al.*, 2021). Many researches have been conducted to study the uses of gold nanoparticles (AuNPs) in the health sciences; moreover, the predicted NPs sizes based on spectroscopic measurements have been investigated. Furthermore, this study will help in the exploration of the medicinal benefits of Chitosan, opening the path for the therapeutic application of *Portunus sanguinolentus* crab shell. The particle size, shape, and Nanocrystallinity of CHS/AuNPs were investigated and we have identified the antifungal efficiency against Chitosan/AuNPs.

Materials and Methods

Chemicals:

The required chemicals were purchased from the following sources: Gold (III) chloride hydrate from Sigma-Aldrich. Distilled water of resistivity and 6.4-6.5 pH was used for all the experiments. All the analytical grade chemicals were purchased from Merck, Bangalore, India. Culture media were purchased from Himedia Mumbai, India.

Collection of Crab Shell:

Crab shells were collected from the fish landing centre at Kasimedu, Tamil Nadu, India (Fig. 2).

Fig. 2: Study area showing the Kasimedu fish landing centre.

Authentic identification:

Dr. C.P. Balasubramanian, Principal Scientist, Crustacean Culture Division, Central Institute of Brackish Water Aquaculture, Chennai, Tamil Nadu, authenticated the crabs and identified as *Portunus sanguinolentus*, a member of the Portunidae family (Fig. 3).

Fig. 3: *P. sanguinolentus* anterior region of the crab shell.

Extraction of Chitosan:

The experimental procedure the following standard chemical methods were to isolate chitin from chitosan such as demineralization, deproteinization, decolourization and

deacetylation (Fig. 4). The detailed illustrations are as follows:

Crab Shells → Deproteinization → Demineralization → Decolourization → Deacetylation

Demineralization:

Demineralization is removal of minerals, primarily calcium carbonate. Demineralization is easily achieved because it involves the decomposition of calcium carbonate into the water-soluble calcium salts with the release of carbon dioxide as shown in the following equation: Then, salts can be easily separated by filtration of the chitin solid phase followed by washing using deionized water.

Demineralization needs two molecules of HCl to convert one molecule of calcium carbonate into calcium chloride. In order to have a complete reaction, acid intake should be equal to the stoichiometric amount of minerals, or even greater. Since, it is difficult to remove all minerals (due to the heterogeneity of the solid), larger volume or more concentrated acid solution is used. Demineralization can be followed by acidimetric titration: the evolution of pH towards neutrality corresponds to acid consumption but the persistence of acidity in the medium indicates the end of the reaction, Demineralization treatment by different concentration (up to 10 % w/v) was completed at room temperature in one hour. After one hour, the processed shells were washed thoroughly in running tap water until it reaches neutral pH.

Fig. 4: Sequential steps for the isolation of Chitosan from crab shell.

Deproteinization:

The deproteinization step is difficult due to disruption of chemical bonds between chitin and proteins. This is performed heterogeneously using chemicals which also depolymerize the biopolymer and complete removal of protein. The deproteinization reagents include NaOH, Na₂CO₃, NaHCO₃, KOH and K₂CO₃. It is used in NaOH 5% (w/v) in ratio 1:10 and kept at 100 C one hour in water bath. After 1 h it was cooled to room temperature and washed with running water continuously, then check the pH until neutral. The end product is chitin.

Decolouration:

The identical samples were collected in the glass beaker with potassium permanganate (KMnO₄) and kept in the water for 3 h. Samples were washed 5 to 7 times with double distilled water and pH was maintained to neutral.

Deacetylation:

One of the main reactions carried out on chitin is deacetylation. Although amides may in principle dehydrolyzed under the acidic or base conditions the use of acid hydrolysis is precluded because of the glycosidic links in chitin to acid hydrolysis. Severe treatment is required to bring about deacetylation. Deacetylation is used in NaOH (1% w/v) and maintain at three hours at 80 in water bath then cooled to room temperature and pH was maintained to neutral.

Synthesis of AuNPs using Chitosan:

The technique used by Villa-Garca *et al.* (2021) to synthesize AuNPs was followed. In a 400 ml beaker, 10 g of chitosan powder was combined with 100 ml distilled water to make a chitosan aqueous extract for the reduction of Au⁺ ions to Au⁰. The mixture was then centrifuged for 10 min in a 10,000-rpm magnetic stirrer. The resulting chitosan aqueous extraction was filtered using Whatmann No. 1 filter paper to achieve a pure extract in a typical experiment. In a 1 mM aqueous Gold (III) chloride hydrate solution (45

ml), 5 ml of chitosan aqueous extract was added. Excitation of Surface Plasmon vibration caused the colour shift, as evidenced by the decrease of Au⁺ ions to Au⁰ (Fig. 5). AuNPs were spun at 15000 rpm for 30 min after the reaction was completed. The supernatant was discarded, and the fine sediment pellet was centrifuged once more with distilled water to remove any biomaterials that may interfere with Au⁺ binding to biomass or AuNP formation.

Fig. 5: (A) Chitosan aqueous extract (pale half white colour), (B) 1 mM aqueous Gold (III) chloride hydrate solution (light yellowish colour), (C) elevation of dark colour denotes the synthesis of Au⁺, and (D) indicates the dark coloured pellet formation of AuNPs.

Characterization of CHS/AuNPs:

Particle measurement was carried out by UV-VIS spectroscopy (UV-2600, SHIMADZU), FTIR (IR Tracer-100, SHIMADZU) and X-ray Diffraction (XRD) (Malvern Pananalytic) and Atomic Force Microscopy (AFM) (Nano surf Easy scan -2).

Antifungal activity of CHS/AuNPs:

Antifungal activities were carried out according to the methods of Vijilvania *et al.* (2020). Fungal strains *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* and *Rhizopus* were prepared by agar well diffusion method. SDA plates were prepared by sterile cork borer. Aliquots of 20 µl from CHS aqueous extracts and its AuNPs (100 mg/ml) was added into each well on the seeded medium and culture plates allowed to stand on the bench for 1 h for proper diffusion and there after incubated at 37 C for 48 h. for fungal strains. The resulting

inhibition zones were measured in millimetres (mm). Fluconazole was used as positive control.

Results and Discussion

UV-VIS Spectral analysis:

Due to the excitation of electrons contained in the Chitosan for reducing Au^{3+} to Au^0 , AuNPs were produced utilising Chitosan aqueous extract with 20 min due to containing less active chemicals at room conditions. The colour shifts from yellow to pink at a wavelength of 510–580 nm revealed the production of AuNPs. After 7 days, the stability observations revealed a greater max shift and a substantial drop in absorbance, indicating agglomeration. Under ambient temperature, oxidation of functional active chemicals in aqueous extract may yield keto forms, which act as a reducing agent for Au^{3+} to Au^0 . The existence of bioactive functional compounds in Chitosan aqueous extract caused unassigned peaks, which may be attributed to the presence of bioactive functional compounds in it. As a result, we hypothesised that the Chitosan aqueous extract employed in this work may not only decrease gold ions but also function as effective capping agents, stabilising AuNPs by electrostatic and/or steric repulsion at 529 nm, as demonstrated in Figures 6 and 7.

FT-IR spectral analysis:

FT-IR in the scan range of 600–3800 cm^{-1} wavenumbers were carried out to identify the possible functional groups of the bioactive molecules taking part in the biosynthesis process. The FTIR spectra of chitosan aqueous extract and synthesized nanoparticles (Fig. 8) showed strong and broad bonds between 921.6 cm^{-1} , 1180 cm^{-1} , 1603 cm^{-1} and 2999 cm^{-1} corresponding to stretching vibrations of surface hydroxyl group (-OH) and can also be attributed to N-H stretching of the C-OH stretch (strong) and -C-H aldehydic (variable) in Chitosan aqueous extract. The sharp peak at 1128 and 1492 cm^{-1} in AuNPs is attributed to -CH₂ band (medium) and -C-OH (strong) molecular stretching responsible for AuNPs

formation in the present study.

X-ray Powder Diffraction (XRD):

The XRD was used to validate crystallinity and determine the structural phase of the material. Figure 9 shows the diffraction pattern at CuK-Alpha with the wavelength of the light source ($=0.154056$ Angstrom). For the face-centred cubic (FCC) metallic gold, Bragg reflections were detected at $2\theta=38.17^\circ$, 43.62° , and 64.60° , corresponding to the lattice planes of 111, 200, and 220 (JCPDS 03-1174). The intensity ratio between the planes verifies the articles preferred orientation in the 111 plane, which is typical of the FCC crystalline structure. A number of unassigned peaks were detected in addition to these reflection peaks, which can be attributed to the presence of salt residues and bio-organic phases on the surface of the AuNPs. The crystal structure of AuNPs was shown in the FCC lattice structure as an atom at each cube corner and an atom in the centre of each cube face. It is a densely packed plane in which atoms are supposed to contact along face diagonals on each face of the cube, and each edge of the cube is 4.07 in length.

UV-Viz, FT-IR and XRD were used to characterise gold (III) chloride hydrate salts, which were found to be less than 100 nm. Because biological uses of AuNPs have received little attention in the past, we decided to investigate their catalytic activity. The catalytic activity of both chemical and biological AuNPs was therefore investigated. At low and definite concentrations, biosynthesized AuNPs were shown to be superior catalysts than chemical AuNPs. In today's science, such comparative studies are necessary to distinguish the biological process from the chemical processes of AuNPs. Furthermore, crystallography investigations can be simplified based on the concentration of AuNPs utilised here. The XRD pictures show that a AuNPs have formed a homogenous distribution as FCC lattice.

All samples are expected to have high stability based on the given data. These findings indicated that chitosan has effectively coated and stabilised

Fig. 6: UV spectral analysis of Chitosan.

Fig.7: UV –Spectral of Chitosan based AuNPs.

AuNPs, resulting in free amino groups and a net positive charge (Maldonado-Ortega *et al.*, 2021; Maliszewska *et al.*, 2021). However, particle size distribution and scanning electron microscopy studies indicated that some of the particles in this sample have a diameter of less than 100 nm, raising the danger of particle sedimentation (Mehravai *et al.*, 2021; Ronavari *et al.*, 2021). The size distribution of chitosan-based formulations shows a bimodal distribution with a tiny fraction of substantially smaller particles (less than 100 nm). The development of tiny agglomerates, as shown in the XRD picture of this sample, is most likely the cause of the broad dispersion. In the

formulation of AuNPs, the lowest diameter (100 nm) and the narrowest particle size distribution were found. The use of nanoparticle systems, particularly interactions with biological entities, necessitates their stability. Because the interaction of unstable systems may rapidly alter due to particle agglomeration, they are unreliable.

Atomic Force microscopic (AFM):

The AFM 3D images indicate the formation of homogeneous distribution of gold nanoparticles and no agglomeration was observed. Figure 10 shows the AFM images for 1 mM of gold trichloride hydrate concentration where the

Fig. 8: FT-IR spectral image; (A) Chitosan aqueous extract, and (B) Chitosan AuNPs.

particles shape and size was indicated. Some agglomeration was observed at 8nm and 10 nm with large nanoparticles diameter. AFM shows the Granularity Accumulation Distribution (GAD), which gives the particles size distribution of gold nanoparticles. The average particle diameter of the synthesized gold nanoparticles is between 8-10 nm. Yellow coloured encircling representing the ovoid shaped gold nanoparticles as shown in Figure 10. The large diameter could be due to sedimentation of the particles resulting in a decrease in absorbance. The AFM analysis confirmed the Nano sphere shapes of the gold nanoparticles which correlate with the TEM image.

Antifungal activity of Chitosan/AuNPs extract against the tested fungal species:

The efficacy of the gold nanoparticle against the test fungal increases as the concentration of

CHS/AuNPs increases (Table 1; Figs. 11, 12). The increasing order of potency of the synthesized CHS/AuNPs against the selected pathogenic human fungi was *Rhizopus*>*A. flavus*>*Penicillium*>*A. niger*.

The antifungal investigation of the synthesized CHS/AuNPs in this study was in line with previous study on antifungal activities of gold nanoparticle from different studied plants against different pathogenic human fungi where notable activity were observed (Sivakavinesan *et al.*, 2021; Ahmad *et al.*, 2021; Ajaz *et al.*, 2021;; Farias *et al.*, 2021; Li *et al.*, 2021; Marathe *et al.*, 2021). To date, chitosan has demonstrated minimal or no toxicity in animal models and no reports of serious complications have been reported in healthy subjects; nevertheless, clinical evidence is insufficient. Despite the fact that chitosan is permitted for use in food, topical ointments, and cartilage

Fig. 9: XRD spectral image; (A) Chitosan aqueous extract, and (B) Chitosan AuNPs.

Fig. 10: Synthesized chitosan based gold nanoparticles.

Table 1: Inhibition induced by Chitosan/AuNPs extract against the tested fungal species

S. No.	Name of the organisms	Zone of inhibition in mm				
		Fluconazole	10 mg	20 mg	30 mg	40 mg
1	<i>A. niger</i>	19	9	10	11	14
2	<i>Penicillium</i>	22	9	9	10	15
3	<i>Rhizopus</i>	29	9	10	10	13
4	<i>A. flavus</i>	24	11	12	12	14

Fig. 11: Zone of inhibition induced by Chitosan/AuNPs extract against the tested fungal species.

Fig. 12: Antifungal activity of AuNPs, F denotes Fluconazole positive control represented as yellow dotted circle, C1- 10 mg/ml, C2- 20 mg/ml, C3- 30 mg/ml and C4- 40 mg/ml denoted by red dotted circle.

formulations, chitosan-based medicine formulations have failed to receive FDA approval for aggressive marketing (Yassin *et al.*, 2021). We found that medication delivery applications of ChitosanAuNPs are the most explored and examined of all the numerous biological applications. ChitosanAuNPs should be investigated further for cancer theranostics, dermatological applications, and tailored parenteral drug delivery systems. We anticipate seeing more chitosan research effort in the near future, especially in nasal and pulmonary drug administration, as novel techniques for overcoming chitosan's limitations emerge. Even though this study reveals, there are just a few scattered studies on the green synthesis of ChitosanAuNPs; given that this is the most recent trend in nanomaterial synthesis, it is surprising that little attention is dedicated to this area. According to this study, bio-produced ChitosanAuNPs will see an increase in research investigations. Furthermore, there is little research on the usage of produced ChitosanAuNPs for biological purposes; this is an interesting topic that ought to be explored further. What are we waiting for if a substance collected from marine debris can be extracted using environmentally friendly procedures and used for beneficial biomedical purposes?

Conclusion

Crustacean debris, such as crab shell, is commonly utilised for the low-cost and environmentally acceptable synthesis of AuNPs. The use of crustacean waste helps to reduce significant environmental pollution in coastal areas while also enhancing the social economic condition of the population. Biomedical applications may be possible in the future with these environmentally friendly AuNPs. The synthesized AuNPs from Chitosan show broad inhibition against test fungal thereby making the synthesized AuNPs a good antimicrobial agent. The information obtained from this study can be adopted by drug agencies in production of antibiotics drugs which will help in

combating microbes that have developed resistance against existing antibiotic drugs.

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