Research Article

Biosynthesis of Copper Nanoparticles Using Chitosan Extracted from Prawn Shells, Characterization and Antimicrobial Activity

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Submission Date: 13-09-2022; Revision Date: 21-10-2022; Accepted Date: 18-11-2022.

ABSTRACT

Marine waste management has been one of the problems having the most significant impact on the environment. Food industrial wastes are an essential environmental contamination source. Research has been carried out to develop methods to convert these wastes into valuable products. Alternatively, this waste can be used to extract chitosan, which possess antimicrobial activity. Hence, an attempt has been made to extract chitosan from the marine waste-prawn shells, synthesize Copper Nanoparticles (CuNPs) using chitosan, and characterize the synthesized CuNPs using Ultra Violet -visible Spectroscopy (UV-Vis), Fourier Transform Infrared spectroscopy (FT-IR) and Scanning Electron Microscopic (SEM) analysis. The antimicrobial activity of the biosynthesized CuNPs against gram-positive and gram-negative bacterial strains: *Staphylococcus* sp and *Pseudomonas* sp, and fungal strains: *Aspergillus* sp and *Candida* sp were assessed in the present study using the agar well diffusion method. As per the current study's findings, the zone of inhibition increased with an increase in the sample concentration. It was also observed that the CuNPs showed maximum antibacterial activity towards *Pseudomonas* sp, followed by *Staphylococcus* sp, and antifungal activity against *Candida* sp.

Keywords: Antimicrobial Activity, Copper Nanoparticles, FT-IR, Prawn Shell, SEM, UV-Visible spectroscopy.

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INTRODUCTION

The shellfish sector is active and makes a significant contribution to the delicacies. Chitin, a polysaccharide with excellent intrinsic qualities like biocompatibility, biodegradability, antibacterial, anticancer, and anti-oxidant activity, is frequently present in these all coastal nations' fish offal in significant amounts.[1] When prawns and lobsters are processed, the meat is commonly taken, and the shell and head are thrown away. The upshot is that there is a huge amount of waste produced worldwide. The shellfish sector produces an estimated 60,000 to 80,000 tonnes of garbage annually. The accumulation of numerous waste items, such as skin, heads, tails, shells, scales, backbones, etc., results from modern ways of processing seafood. These waste products frequently include various valuable items that are unused as a result of improper management. This leads to massive waste production on a global scale. A crucial environmental concern has arisen from the disposal of such a large amount of garbage. Inadequate waste management also harms the environment and people's health. Although there is a huge amount of trash created by each processing activity, the rate of deterioration of these wastes is relatively slow. As a result, they produce unpleasant scents; that attract rats, flies, and other pests, which contaminate the environment and cause accumulation over time. Furthermore, improper waste management harms the environment and people's health.[2]

Chitin is a fundamental structural element found in the exoskeletons of crustaceans, such as crabs and

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DOI: 10.5530/ajbls.2022.11.98

shrimp, and in the cell walls of fungi.[3] Chitin is found naturally as organized macro fibrils. Chitosan (Cts) polymers are naturally occurring polymers created by deacetylating chitin and produced by semi-synthetic methods from complex amino polysaccharides. They are cationic polysaccharides made up of D-glucosamine and N-acetyl-D-glucosamine units. They are bioactive polymers with significant amine and hydroxyl group content that are non-toxic, biodegradable, bio-compatible, and bioactive. They are renowned for having various uses, including industrial, medicinal, and biomedical applications.[4]

Copper (Cu) is a transition metal with the atomic number 29, an atomic mass of 63.546, and a distinctive reddish-orange color. It is the eighth most prevalent metallic element in the crust of the Earth, making it a less expensive material to utilize. The unique characteristics of copper, including its high electrical conductivity, high thermal conductivity, high corrosion resistance, good ductility and malleability, and reasonable tensile strength, make it an essential component in the operation of society.[5]

Copper is much more inexpensive than silver and gold, making it economically feasible to investigate CuNPs (both in metallic copper and copper oxide). Additionally extensively accessible in the market are CuNPs. Many methods have been developed, including those that are chemical, and physical) and biological synthesis (using bacteria, algae, fungi and plants). Studies have switched toward biological synthesis and its potential to manufacture CuNPs due to the numerous limitations of conventional methods, the novelty, and environmental concerns.[6]

The physicochemical properties of nanoparticles impact their behaviour, biodistribution, safety, and effectiveness. To evaluate the functionality of the produced particles, Copper Nanoparticle Characterization is essential. A number of methods characterises the samples. As a result, techniques including Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and UV-visible spectroscopy are employed.[7]

Hence based upon the above views, the present study was carried out to extract chitosan from marine waste (prawn shells), to synthesize CuNPs using chitosan extracted from prawn shells, to characterize CuNPs using Ultraviolet-Visible Spectroscopy, Fourier-Transform Infrared Spectroscopy, and Scanning Electron Microscopic Analysis, to determine the antibacterial activity of CuNPs against *Staphylococcus* sp and *Pseudomonas* sp and to determine the antifungal activity of CuNPs against *Aspergillus* sp and *Candida* sp.

MATERIALS AND METHODS

Sample Collection and Preparation for Chitosan Extraction

Prawn shells were procured from the local markets in Chennai, Tamilnadu, India. The collected prawn shells were washed with tap water to remove dust particles, and then the sample was dried in the sunlight for 1-2 days. The dried sample was blended and sieved. Approximately 20 grams of shell powders were used for further analysis.

Extraction of Chitin and Chitosan from Prawn Shells

Deproteinization

20 grams of prawn shell powder were added to 3.5% sodium hydroxide under stirring and left for 1 hr at 90°C. Subsequently, the solution was filtered, and the residue was washed with tap water until neutrality. The residue was then re-dried in a furnace at a temperature of 60°C for 4 hr. This deproteinized dried resultant powder was crude chitin.

Demineralization

Chitin powder obtained from deproteinization was then added with 2-N HCl in the ratio of 1:5(w/v) and allowed to stand for 1 hr at 90°C to separate the residue from the filtrate and the residue was washed with distilled water until neutral pH and then dried in a furnace at 60°C for 4 hr.

Deacetylation of chitin into chitosan

Deacetylation of chitin was carried out by adding acetone to the demineralized product in the ratio of 1:5 (w/v) for 4 hr at 80°C, and then the residue was washed with distilled water until neutral pH and dried in a furnace at 60°C for 4 hr. 5 grams of chitin was added to 50ml of 50% Sodium hydroxide, heated using a hot plate at 80°C for 40 min, then filtered and the residue was washed until neutral pH. It was then dried in a furnace at 60°C for 4 hr. The resultant powder obtained was chitosan powder.[8]

Synthesis of CuNPs using chitosan

0.5% Chitosan solution was prepared by dissolving 0.5 grams of chitosan in 100 ml of 2% acetic acid solution. 5ml of 0.5% chitosan solution was mixed with 5ml of 0.5% Copper sulfate solution. This mixture was kept in the autoclave at 15 psi pressure, at 120°C for 30 min. The final product obtained was CuNPs.[8]

Characterization of Synthesized CuNPs

The characterization of synthesized CuNPs was done using UV-visible Spectroscopy, FTIR, and SEM.

UV-visible Spectroscopic analysis of the synthesized CuNPs was carried out using a Digital Spectrometer (Model LT-38, Biotechnology Laboratory, Justice Basheer Ahmed Sayeed College for Women (Autonomous), Chennai – 600 018. The interactions of chitosan and CuNPs were analyzed with FTIR spectroscopy. For FTIR measurements, a sample in the pellet form was analyzed using an FTIR spectrophotometer (Model - FT/IR-6600typeA, Centre for Nanoscience and Technology, A.C.Tech Campus, Anna University, Chennai, Tamil Nadu, India.) in the range of 349.053 cm^{-1} to 7500.77 cm^{-1} at 4 cm^{-1} resolution. Morphological details of the synthesized CuNPs were analyzed using a Scanning Electron Microscope (Centre for Nanoscience and Technology, A.C.Tech Campus, Anna University, Chennai, Tamil Nadu, India.) at an accelerating voltage of 30 kV.

Antimicrobial Assays

Antibacterial Activity of biosynthesized CuNPs

The antibacterial activity of synthesized CuNPs against two isolates of bacteria, *Staphylococcus* sp, and *Pseudomonas* sp,, followed the Agar well diffusion method.

Antifungal Activity of biosynthesized CuNPs

The antifungal activity of synthesized CuNPs against two isolates of fungi, *Aspergillus* sp and *Candida* sp, was carried out by following the Agar well diffusion method.

Preparation of Culture Inoculum

The test organisms (*Staphylococcus* sp, *Pseudomonas* sp, *Aspergillus* sp, and *Candida* sp) were procured from the University of Madras, IBMS Campus, Taramani, Chennai, Tamil Nadu, India and then inoculated by transferring a loop full of culture from the stock culture to the tube containing nutrient broth which was incubated at 37°C for 24 hr in case of bacterial strains and kept at room temperature for 3-4 days for fungal strains.

Agar Well Diffusion Method

The effect of the synthesized CuNPs on the test organisms was assayed by the agar well diffusion method of Magaldi *et al.* (2004). Nutrient agar was poured into the Petri plates aseptically and was allowed to solidify. The lawns of the bacterial test strain were done with the help of a sterile cotton swab. Wells were made with the help of sterile micropipette tips, and the cut agar discs were removed aseptically with sterile needles. Four different concentrations (300µl, 150µl, 75µl, and 30µl) of CuNPs were added to the well. The test plates were incubated at 37°C for 24 hr for the bacterial species and kept at room temperature for 3-5 days for the fungal species. After incubation, the results were recorded based on the presence or absence of an inhibition zone. The antibacterial and antifungal activity was assayed by measuring the diameter of the inhibition zone formed around the species.[9]

RESULTS

Biosynthesis of CuNPs using chitosan

The prawn shells containing chitin were dried in the sunlight to yield chitosan, as shown in Figure 1. Chitosan that was extracted by the process of deproteinization and demineralization is shown in Figure 2. The result of the synthesis of CuNPs using chitosan extracted from prawn shells is depicted in Figure 3. The development of the study revealed that the CuNPs synthesized are light blue in color, indicating the formation of a Chitosan copper complex.

Figure 1: Prawn shells dried in the sunlight.

Figure 2: Chitosan extracted from Chitin.

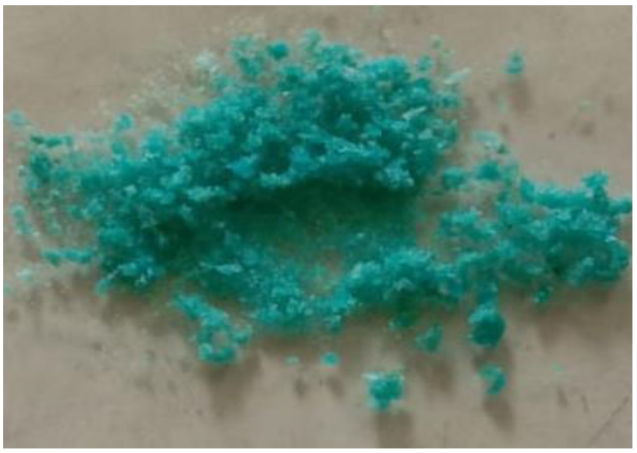

Figure 3: CuNPs biosynthesized using chitosan.

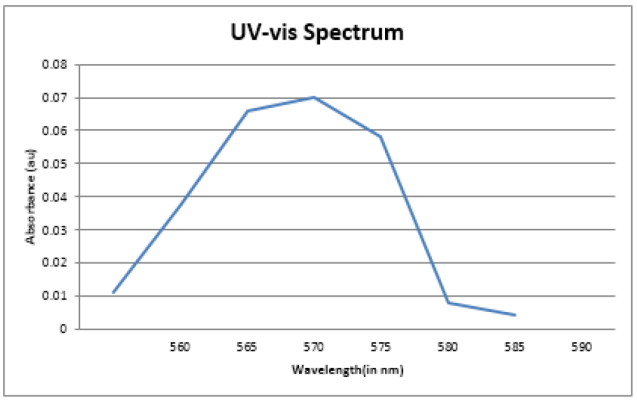

Figure 4: UV–visible spectral analysis of Biosynthesized CuNPs using chitosan.

Characterization of Biosynthesized CUNPS

UV–Visible Spectral Analysis of Biosynthesized CuNPs Using Chitosan

The result of UV visible absorption spectra of biosynthesized CuNPs is shown in Figure 4. The biosynthesized CuNPs were confirmed by UV-Visible spectrophotometry with wavelengths ranging between 560-585 nm and the maximum absorption spectrum was determined. Based on the result of this study, the lambda max for the chitosan CuNPs showed a maximum peak at 574 nm.

FTIR Spectrum of Biosynthesized CuNPs

The result of the FTIR spectrum of Biosynthesized CuNPs is depicted in Figure 5. The molecular interactions between chitosan and the biosynthesized Nanoparticles were determined using FTIR analysis. The result of FTIR analysis revealed the existence of bands at 3336 cm^{-1} which may be due to O-H and C-H overlapping, 1017cm^{-1} probably due to C=O stretching, 1659 cm^{-1} may be due to NH_2 bending,

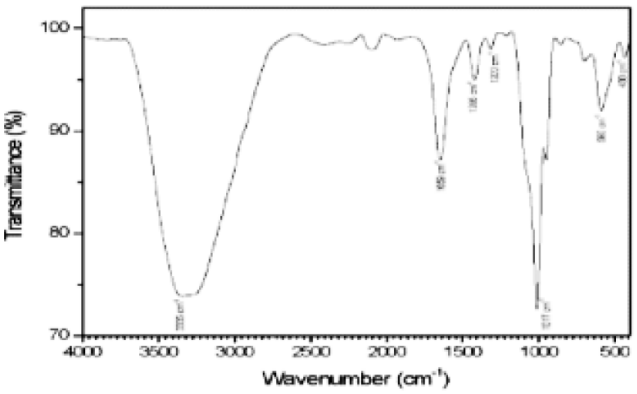

Figure 5: FTIR spectrum of Biosynthesized CuNPs.

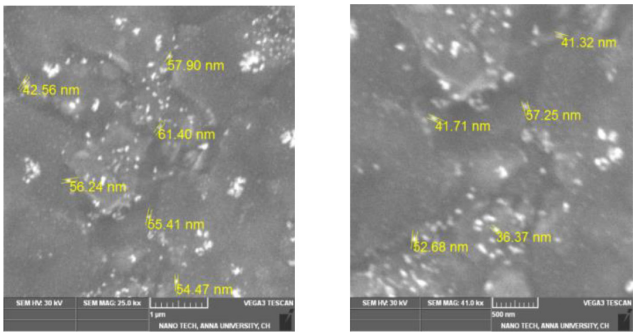

Figure 6: SEM images of Biosynthesized CuNPs.

1396 cm^{-1} and 1322 cm^{-1} due to bending of C-H group, 1017 cm^{-1} may be due to bending of NH_2 group and C-O-C stretching. The signal at 560 cm^{-1} demonstrates the interaction of CuNPs and chitosan, showing that the polymer encapsulated the nanoparticles.

SEM Analysis of Biosynthesized CuNPs

The result of SEM images of Biosynthesized CuNPs is presented in Figure 6. SEM imaging was carried out to determine the morphology and size of biosynthesized CuNPs by Scanning Electron Microscope, and the maximum magnification employed was 41.0 Kx. The particle size of the CuNPs obtained was around 50 nm, and they were spherical and agglomerates.

Antimicrobial Assays

Antibacterial Activity of Biosynthesized CuNPs

The antibacterial activity of Biosynthesized CuNPs is presented in Figures 7, 8, and Table 1. A recent study revealed that bio-synthesized CuNPs have prominent bactericidal activity against *Staphylococcus* sp. and *Pseudomonas* sp. The bio-synthesized CuNPs showed a maximum zone of inhibition of 3.25 mm ± 0.08 at 300 µl with *Pseudomonas* sp, whereas with *staphylococcus* sp, it showed a maximum area of inhibition of 2.75 mm ± 0.08 at 300 µl. The bactericidal property of CuNPs

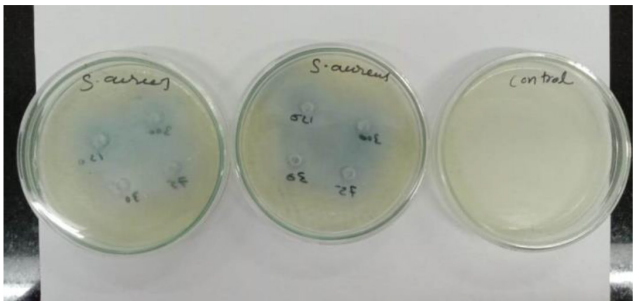

Figure 7: Antibacterial activity of Biosynthesized CuNPs against *Staphylococcus* sp.

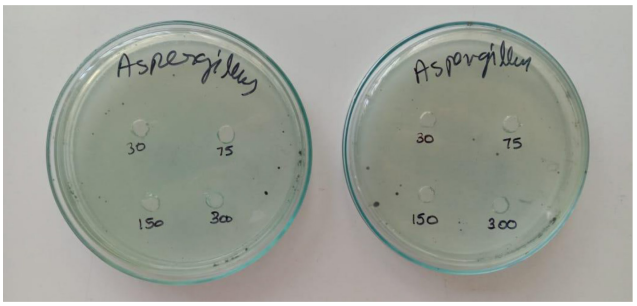

Figure 9: Antifungal activity of Biosynthesized CuNPs against *Aspergillus* sp.

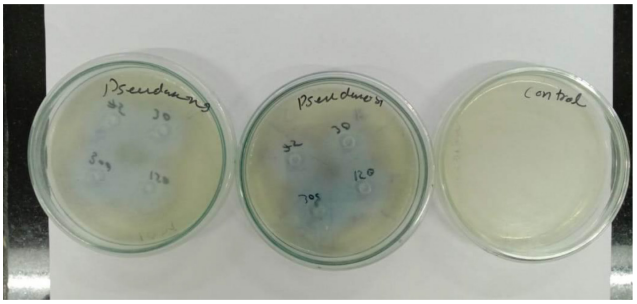

Figure 8: Antibacterial activity of Biosynthesized CuNPs against *Pseudomonas* sp.

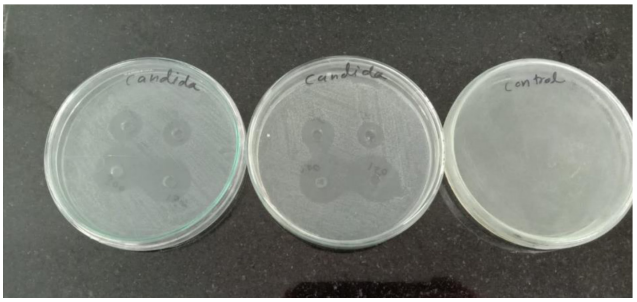

Figure 10: Antifungal activity of Biosynthesized CuNPs against *Candida* sp.

Table 1: Antibacterial activity of Biosynthesized CuNPs.

Sl. No	Bacterial Culture	Concentrations of the sample(µl)	Zone of Inhibition (ml)	One Way ANOVA
1.	*Staphylococcus* sp	Control	0	0.01%
		30	2.25± 0.08	
		75	2.45± 0.08	
		150	2.65± 0.08	
		300	2.75± 0.08	
2.	*Pseudomonas* sp	Control	0	
		30	2.55± 0.08	
		75	2.75± 0.08	
		150	2.9± 0.08	
		300	3.25± 0.08	

± = Standard deviation, Significant at 0.01%

Table 2: Antifungal Activity of Biosynthesised CuNPs.

Sl. No	Fungal Culture	Concentrations of the sample(µl)	Zone of Inhibition (ml)	One Way ANOVA
1.	*Aspergillus* sp	Control	0	0.01%
		30	0	
		75	0	
		150	0	
		300	0	
2.	*Candida* sp	Control	0	
		30	1.5± 0.08	
		75	1.8± 0.08	
		150	2.55± 0.08	
		300	2.6± 0.08	

± = standard deviation, Significant at 0.01%

is mainly due to the release of copper cations (Cu+) attached to the bacterial cell wall due to electrostatic attraction. Moreover, the metal ions interact not only with the surface of a membrane but can also penetrate inside the bacteria.

Antifungal Activity of Biosynthesized CuNPs

The antifungal activity of Biosynthesized CuNPs is presented in Figures 9, 10, and Table 2. Among the fungal organisms tested, biosynthesized CuNPs exhibited pronounced fungicidal activity against *Candida* sp with a zone of inhibition 2.6 mm ± 0.08 at 300 µl of biosynthesized CuNPs. In contrast, there was no antifungal activity against *Aspergillus* sp.

Statistical Analysis

The one-way ANOVA of antimicrobial activity of biosynthesized CuNPs showed significant variation in the zone of inhibition produced by the biosynthesized CuNPs against test organisms of about 0.01%. (*p*-value is 0.0125).

DISCUSSION

As their name suggests, nanoparticles (NPs) are solid particles with all three exterior dimensions and a size range of 1–100 nm. The physical and chemical properties of NPs are drastically different from those of their bigger material counterparts. Depending on the chemical composition, biological actions, size, and shape, it can explicate actions,.[10,11]

Copper-based nanoparticles are of significant interest due to their low cost, increased availability and properties that are similar to those of other metallic nanoparticles. CuNPs (Cu-NPs) have recently gained a great deal of recognition from scientific researchers due to their applications in heat transfer systems as super-strong materials, high-temperature superconductors, solar cells, potential modern utilizations, sensors such as gas sensors, catalytic processes, wound dressings and biocidal properties, antibacterial and bactericidal agents used to coat medical equipment and as catalysts.[12,13]

Various physical, chemical, and biological methods have been used to synthesize CuNPs. CuNPs were biologically synthesized using *Morganella* bacteria under aqueous physiological conditions.[14] A rapid physical method was employed to synthesize CuNPs from a non-pathogenic bacterium *Pseudomonas stutzeri*.[15] *Aspergillus* species was used for the synthesis of CuNPs, as reported by Pavani *et al.* (2013).[16]

Based upon the above literature cited, the present study was carried out to synthesize CuNPs from chitosan obtained from prawn shell wastes to characterize the biosynthesized CuNPs using UV–visible spectroscopy, Fourier Transform Infrared Spectrum Analysis [FT-IR] and Scanning Electron Microscopy [SEM] and to determine antibacterial and antifungal activities of the biosynthesized CuNPs.

In UV-Visible Spectroscopy Analysis, the study's results revealed that maximum absorption of 0.07 was recorded at 575 nm. CuNPs showed characteristic absorption peaks in the 200-800 nm, thus confirming biosynthesized CuNPs.[17] Nanoparticle production is indicated by a steady increase in the characteristic peak with increasing reaction time and concentration of biological extracts with salt ions. The peak characteristics of the surface plasmon resonance of nanosized particles can be seen in the UV-vis absorption spectrum. The result of this study is supported by Swarnkar *et al.* (2011) and Abboud *et al.* (2014).[18,19]

A scanning Electron Microscope [SEM] was used to determine the morphology of biosynthesized CuNPs. SEM micrographs of biosynthesized CuNPs revealed spherical and irregular shapes of biosynthesized CuNPs, much less agglomeration, and the size of the nanoparticles ranged from 36.16 - 61.40 nm.

The spherical shape of the synthesized CuNPs can be substantiated by Bali *et al.* (2006), who worked on the characterization of CuNPs using TEM to assess the size and morphology of the CuNPs.[20] The CuNPs synthesized by Subhankari and Nayak (2013) and Bogdanović *et al.* (2014) were also spherical.[21,22]

Nanoparticles that have been stabilized can form clusters and get close to each other. Individual nanoparticles, however, were enclosed by the stabilizing reagent and can be re-dispersed. Stabilizers are essential in controlling particle size distribution and preventing clustering and flocculation.[23] This research shows that starch can be used as a capping agent to create tiny nanoparticles. The structure of nanoparticles is determined by competition between processes, such as growth, nucleation, aggregation, and impurity adsorption.[24]

Fourier Transform Infrared (FTIR) spectroscopic analysis was done to determine the molecular interaction between the chitosan and the copper nanoparticles. The report of the study revealed that the absorption peaks located mainly at 3336 cm^{-1} are generally attributed to C–H and O–H overlapping, 1017 cm^{-1} usually are assigned to the alkyl C=O stretching, C–O–C trying and NH_2 bending. In contrast, peaks at 1659 and 1332 cm^{-1} are due to NH_2 bending and C-H bending respectively. The result of this study is by the reports of Kaur *et al.* (2016), showing the interaction between CuNPs and chitosan.[25]

For decades, copper has been utilized as an antimicrobial agent. Copper has been accepted for registration as an antimicrobial agent by the US Environmental Protection Agency [EPA], which can lower specific dangerous bacteria linked to potentially fatal microbial diseases.[26] CuNPs have been shown to have antibacterial action against various gram-positive and gram-negative bacteria, as well as fungal species.

The antimicrobial activity of the biosynthesized CuNPs against gram-positive and gram-negative bacterial strains: *Staphylococcus* sp. and *Pseudomonas* sp., and fungal strains: *Aspergillus* sp. and *Candida* sp. were assessed in the present study using the agar well diffusion method.

As per the current study's findings, the zone of inhibition increased with an increase in the sample concentration. It was also observed that the CuNPs showed increased antibacterial activity towards *Pseudomonas* sp., followed by *Staphylococcus* sp., and antifungal activity against *Candida* sp.

The precise mechanism behind the antimicrobial properties of CuNPs is unknown; however, it is linked

to ions emitted by nanoparticles. Its compact size and high surface area to volume ratio allow them to communicate extensively with microbial membranes, further enhancing its activity.[26] The antimicrobial activity of CuNPs is because of their ability to alternate between cuprous - Cu [I] and cupric - Cu [II] oxidation states, When Cu is separated from other trace metals, hydroxyl radicals are produced, which bind to DNA molecules and cause disorder of the helical structure by cross-linking within and between the nucleic acid strands as well as to damage essential proteins by binding to the sulfhydryl amino and carboxyl groups of amino acids. This causes the protein to denature, rendering the enzymes ineffective.[27] It affects membrane integrity and lipids by inactivating cell surface proteins required for material transport across cell membranes. Copper ions inside bacterial cells also disrupt biochemical activities.[28] According to all of these analyses, the antimicrobial effect of CuNPs is linked to the ion's denaturing effect on proteins and enzymes in microbes.

CONCLUSION

The results of the present study conclude that the biosynthesized CuNPs reveal extraordinary performance as an antimicrobial agent. Because of their high surface area to volume ratio compared to their bulk counterparts, CuNPs provide a large active surface with many coordinate atoms available to interact with microbial membranes or release metal ions. Although more extensive studies are necessary, the present work results showed that biosynthesized CuNPs exhibited a more significant antimicrobial effect against bacterial species than fungal species. CuNPs are also cheap and easily accessible. Thus they can be utilized as an alternative to other metal nanoparticles as an antimicrobial agent in various fields.

ACKNOWLEDGEMENT

We are grateful to the Management of Justice Basheer Ahmed Sayeed College for Women (Autonomous), Chennai – 600 018, for providing financial assistance under the MRCC-PG students scheme. We express our gratitude to the Principal and Coordinator, Multidisciplinary Research and Consultancy Center, Justice Basheer Ahmed Sayeed College for Women (Autonomous), Chennai – 600 018 for selecting this project.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CuNPs: Copper Nanoparticles; **FT-IR:** Fourier Transform Infrared spectroscopy; **SEM:** Scanning Electron Microscope; **UV-Vis:** Ultra Violet -visible Spectroscopy.

SUMMARY

Marine waste management has been recognized as one of the most serious environmental issues. The head, tails, shells, scales, backbones, etc. are discarded when processing seafood. The upshot is that there is a tremendous amount of waste produced worldwide. The development of strategies to transform these wastes into usable products has been studied. The aim of the present study was to synthesize Copper Nanoparticles from chitosan extracted from prawn shell waste. Chitosan powder was extracted from prawn shell waste by the process of deproteinization, demineralization and deacetylation. 5ml of 0.5 % chitosan solution was mixed with 5ml of 0.5% copper sulphate solution. This mixture was autoclaved at 15 psi pressure, at 120°C for 30 min. Blue colored CuNPs were obtained as the final product. To confirm the synthesis of the biosynthesized CuNPs, characterization was done using UV-Visible spectroscopy, Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Analysis (FT-IR). The results of UV-visible spectroscopy analysis revealed maximum absorption of 0.07 recorded at 575 nm. The scanning electron micrographs showed that the biosynthesized CuNPs were spherical and also in agglomerates. Size ranged from 36.16- 61.40 nm. Fourier Transform Infrared (FTIR) spectroscopic analysis revealed that the absorption peaks located mainly at 3336 cm^{-1} are generally attributed to C–H and O–H overlapping, 1017 cm^{-1} are generally assigned to the alkyl C=O stretching, C–O–C stretching and NH2 bending, whereas peaks at 1659 and 1332 cm^{-1} are due to NH_2 bending and C-H bending respectively. The antimicrobial activities of the biosynthesized CuNPs were studied using the agar well diffusion method against two bacterial strains: *Staphylococcus* sp. and Pseudomonas sp. and two fungal strains: Aspergillus sp. and Candida sp. According to the present study's findings, the zone of inhibition increased with an increase in the sample concentration. CuNPs showed significant antimicrobial activity towards *Pseudomonas* sp. followed by *Staphylococcus* sp. and *Candida* sp. The results of the present study reveal that CuNPs exhibit higher inhibitory activity against bacteria than in fungi. The exact mechanism behind this antimicrobial effect of biosynthesized CuNPs is unknown however it

is often linked to the ions emitted from the nanoparticles which denature the proteins and enzymes in microbes.

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Cite this article: Poonguzhali G, Noorjahan CM, Keerthana S. Biosynthesis of Copper Nanoparticles using Chitosan Extracted from Prawn Shells, Characterization and Antimicrobial Activity. Asian J Biol Life Sci. 2022;11(3):737-44.