Article

Marine fungi as biofactories for chitosan nanoparticles: Green synthesis, characterization and antimicrobial applications

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Abstract
The present study explores an eco-friendly and sustainable approach for synthesizing chitosan nanoparticles (CNPs) using marine fungi as a chitin source and *Bacillus* sp.-derived chitin deacetylase (CDA) for enzymatic deacetylation. Marine fungal isolates from coastal sediments of Nellore, Andhra Pradesh, were screened and optimized for biomass production and chitin extraction. Enzymatic conversion of chitosan from chitin was achieved using CDA-producing *Bacillus* sp., yielding chitosan with a high degree of deacetylation (78.5%). chitosan nanoparticles were synthesized via ionic gelation with tripolyphosphate (TPP) and characterized using UV-Vis spectroscopy, DLS, SEM-EDX, XRD, FTIR, and zeta potential analysis. The resulting CNPs were spherical, well-dispersed, and exhibited a particle size of ~138.6 nm with a zeta potential of +32.5 mV, indicating good stability. The nanoparticles demonstrated strong antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, and antifungal activity against *Candida albicans* and *Aspergillus niger* in a concentration-dependent manner. MIC, MBC, and MFC values confirmed the potent antimicrobial nature of the nanoparticles, with *S. aureus* being the most susceptible. The antimicrobial mechanism involved membrane disruption, reactive oxygen species generation, and interference with microbial DNA and metabolism. This study highlights the potential of marine fungal-derived chitosan nanoparticles as efficient biogenic agents for antimicrobial applications and wastewater remediation, particularly in aquaculture settings.

Keywords Chitin, Chitosan nanoparticles; Antibacterial activity; Antifungal activity; FTIR; Uv-visible spectrophotometer; SEM-EDX.

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1 Introduction

Chitosan is a linear polysaccharide derived from the partial deacetylation of chitin, which ranks as the second most abundant natural polymer following cellulose (Aranaz et al., 2009, Rinaudo, 2006). Chitin is commonly

found in nature, particularly in the exoskeletons of marine crustaceans like crabs, shrimps, and lobsters, as well as in the cell walls of fungi and the cuticles of insects. It plays a structural role in these organisms, providing them with rigidity and mechanical strength (Kumar, 2000, Vinusha et al., 2022). In its physical form, chitin appears as a hard, white, and brittle material that resists dissolution in water and most organic solvents due to its crystalline nature and extensive hydrogen bonding (Muzzarelli et al., 2012, Vinusha et al., 2020). Chemically, it is a polymer composed of repeating N-acetylglucosamine units and exists in three polymorphic forms: α-chitin, β-chitin, and γ-chitin. The α-form, which is the most common and stable, consists of tightly packed anti-parallel chains (Brugnerotto et al., 2001, Vinusha & Vijaya, 2019), whereas the β-form found in squid pens is characterized by a more loosely packed, parallel chain arrangement. The γ-form is a blend of both α and β structures.

Chitin possesses significant economic worth because it can be utilized in numerous industries, including biomedicine, agriculture, wastewater treatment, and food preservation (Kurita, 2006, Vinusha et al., 2023). Its derivative, chitosan, exhibits improved functionality due to the presence of free amino groups, rendering it highly reactive and amenable to modifications (kumar, 2000, Dutta et al., 2004). The worldwide seafood industry produces millions of tons of shellfish waste each year, providing a plentiful and sustainable source of chitin (Arbia et al., 2013, Vinusha et al., 2017). The process of valorizing this waste not only tackles environmental issues but also generates economic value by transforming it into valuable biomaterials. In the field of agriculture, chitosan is employed as a natural biopesticide and plant growth promoter (Badawy and Rabea, 2011, Vinusha et al., 2015). In the medical field, it has various applications in wound healing, drug delivery, and tissue engineering due to its biocompatibility, biodegradability, and antimicrobial properties (Dash et al., 2011, Islam et al., 2017). Additionally, its application in water purification as an adsorbent for dyes and heavy metals plays a crucial role in promoting environmental sustainability (Wan ngah et al., 2011, Vounes & Rinaudo, 2015). In the past, extracting chitin from crustacean shells required chemical processes like acid demineralization and alkali deproteinization (Synowiecki & Al-khateeb, 2003). Nevertheless, these approaches present environmental concerns as they result in the production of harmful waste and the potential deterioration of the biopolymer (No et al., 1989, Vinusha et al., 2016). To address these limitations, scientists have created biological extraction methods that utilize proteolytic enzymes or microbial fermentation, specifically lactic acid bacteria, to effectively eliminate proteins and minerals (Sini et al., 2007, Hajji et al., 2014). These sustainable methods not only maintain the structural integrity of chitin but also enhance its purity and yield.

Characterization of chitin and chitosan is critical to determine their physicochemical properties and suitability for specific applications. Techniques such as Uv-visible spectrophotometer, x-ray diffraction (XRD), dynamic light scattering (DLS), thermogravimetric analysis (TGA), fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) are commonly employed to analyze functional groups, crystallinity, thermal stability, and surface morphology (Kasaai, 2008, Paulino et al., 2006). The conversion of chitin to chitosan is commonly achieved through chemical deacetylation using concentrated sodium hydroxide at elevated temperatures (Kumar, 2000). However, enzymatic methods using chitin deacetylases have emerged as an efficient and environmentally benign alternative (Li et al., 2011). These enzymes catalyze the hydrolysis of n-acetyl groups from chitin in a controlled manner, allowing for the production of chitosan with a defined degree of deacetylation and improved molecular characteristics (Vounes and Rinaudo, 2015). Enzymatic deacetylation is particularly advantageous for applications requiring high-purity chitosan, such as in pharmaceuticals and biomedical engineering (Zhu et al., 2020).

In recent times, there has been a growing focus on the nanoformulation of chitosan, due to its large surface area, bioactivity, and nanoscale characteristics (Shah et al., 2020). Various approaches have been

investigated for the creation of CNPs, which can be categorized into physical, chemical, and biological methods. Physical techniques, like ultrasonication and high-pressure homogenization, utilize mechanical forces to decrease particle size and ensure even distribution of chitosan nanoparticles without the need for harsh chemicals (Kasaai, 2010). In contrast, chemical methods utilize ionic gelation, emulsification solvent evaporation, and reverse micellar techniques. Among these, ionic gelation using sodium -TPP as a cross-linker is the most widely employed due to its simplicity and mild reaction conditions (Calvo et al., 1997, Qi et al., 2004). Utilizing plant extracts in green synthesis methods provides a sustainable and environmentally friendly approach for producing chitosan nanoparticles. The bioactive compounds found in plant extracts function as reducing and stabilizing agents, facilitating the creation of stable CNPs with antimicrobial and antioxidant properties (Vivekanandhan et al., 2022, Ahmed et al., 2020). Similarly, microbial synthesis using bacteria, fungi, and algae has become a biocompatible and scalable method. Marine-derived fungi and cyanobacteria have shown promising results in the production of nanoparticles, resulting in enhanced stability and improved functionality (Sathiyabama and Manikandan, 2018, Shanmuganathan et al., 2022).

The primary objective of the present study is to explore the potential of marine fungi as a sustainable and rich source of chitin, which can be enzymatically converted into chitosan using CDA enzymes produced by selected bacterial cultures. The objective of this approach is to establish an environmentally friendly and biologically driven process for chitosan production, reducing the reliance on harsh chemicals commonly used in conventional methods. The extracted chitosan is then employed in the green synthesis of CNPs utilizing mild and environmentally friendly methods. The study also aims to characterize the synthesized nanoparticles using various analytical tools such as Uv-vis spectroscopy, FTIR, XRD, SEM, and energy dispersive x-ray spectroscopy (EDX/EDS) to determine their structural, morphological, and functional properties. A comparative analysis of the physical and chemical properties of chitosan and CNPs is also conducted.

Additionally, the effectiveness of the synthesized CNPs is evaluated against various pathogenic bacterial and fungal strains, including those that are resistant to antibiotics. The study involves determining the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) to assess the effectiveness of the nanoparticles as a therapeutic agent. Lastly, the study aims to explore the mechanism of antimicrobial action of the chitosan nanoparticles synthesized from marine fungal-derived chitosan, thereby contributing to the development of novel biogenic antimicrobial agents.

2 Related Works

Chitosan, a biopolymer obtained through the deacetylation of chitin, has emerged as a versatile and biodegradable material for numerous biomedical and environmental applications. Recent developments in nanotechnology have highlighted the utility of CNPs, particularly due to their biocompatibility, mucoadhesiveness, and ability to encapsulate a wide range of bioactive molecules (Iswanti et al., 2019). These features make chitosan nanoparticles promising candidates for drug delivery, vaccine transport, and antimicrobial treatments. Among various synthesis methods, ionic gelation stands out as a simple, mild, and eco-friendly approach for fabricating CNPs. This technique relies on the electrostatic interaction between the positively charged amino groups of chitosan and the negatively charged groups of cross-linkers like sodium TPP, forming nanoparticles spontaneously under ambient conditions (Kunjachan and Jose, 2010). Parameters such as polymer concentration, pH, TPP-to-chitosan ratio, and stirring speed have been found to significantly influence nanoparticle size, polydispersity, and stability (Sreekumar et al., 2018; Sawtarie et al., 2017). Proper optimization of these variables is essential to obtain monodisperse nanoparticles suitable for targeted applications.

Studies have also demonstrated that size-controlled nanoparticles can be synthesized using ionotropic gelation, and such precision tuning of nanoparticle characteristics is vital for controlled release and surface interaction in biomedical and environmental applications (Budi et al., 2020). In comparative evaluations, chitosan nanoparticles were found to perform favorably in terms of drug encapsulation and release profiles compared to PLGA and silica-based systems, reinforcing their potential for oral vaccine and drug delivery platforms (Amin & Boateng, 2019). Apart from their carrier potential, chitosan nanoparticles exhibit inherent antimicrobial and antibiofilm properties. For example, CNPs have been shown to disrupt biofilm formation and enhance the eradication of *Staphylococcus* species, a common pathogen associated with chronic infections (Felipe et al., 2019). This bioactivity is attributed to the positive surface charge of CNPs, which allows them to interact with negatively charged microbial membranes, leading to structural disintegration and inhibition of microbial growth.

The functional performance of chitosan nanoparticles can also be improved through surface modification or by integrating other functional groups. For instance, magnetic Fe O /chitosan nanocomposites have been synthesized via reduction-precipitation methods and demonstrated effective adsorption properties for removing toxic dyes from wastewater (Cao et al., 2014). This dual functionality adsorption and antimicrobial activity makes chitosan nanoparticles attractive for environmental remediation, particularly in aquaculture effluent treatment where organic pollutants and pathogenic microbes coexist. The current literature, while rich in applications of chemically synthesized CNPs, indicates a growing interest in biologically inspired or green synthesis routes, including those using marine fungi. These microbial sources provide a sustainable and environmentally responsible alternative to conventional crustacean-derived chitosan, eliminating allergen risks and improving scalability. However, studies on fungal-derived chitosan nanoparticles, especially with emphasis on marine isolates and their antimicrobial efficacy, remain limited. The existing body of work supports the wide-ranging potential of chitosan nanoparticles for biomedical and environmental use. Yet, there is a significant gap in the exploration of marine fungal chitosan as a green source for nanoparticle synthesis, particularly for dual-functional applications such as antimicrobial action and aquaculture wastewater treatment. This study aims to bridge this gap by leveraging marine fungi for the eco-friendly synthesis of CNPs and evaluating their structural, functional, and antimicrobial characteristics.

3 Materials and Methods

3.1 sample collection and isolation of marine fungi

Marine fungal samples were collected from coastal sediments and decaying organic matter along the shoreline of Nellore district, Andhra Pradesh (Latitude: 14.45°N, Longitude: 79.99°E). Samples were collected in sterile containers and transported to the laboratory under cold chain conditions. For fungal isolation, 1 g of sediment was suspended in 9 mL sterile seawater, serially diluted, and spread onto Potato Dextrose Agar (PDA) supplemented with 2% NaCl and streptomycin (50 µg/mL) to inhibit bacterial growth (Vidya sagar & Vijaya, 2018). Plates were incubated at 28 ± 2°C for 5–7 days. Distinct fungal colonies were subcultured and purified.

3.2 Identification and characterization of marine fungal isolates

Morphological features of purified fungal strains were recorded using lactophenol cotton blue staining under a compound microscope (Vidya sagar and Vijaya, 2020).

3.3 Culture conditions and optimization of fungal biomass

Identified fungal strains were cultured in Sabouraud Dextrose Broth (SDB) enriched with 2% NaCl and incubated in a shaking incubator at 28 ± 2°C and 120 rpm for 7–10 days. Optimization of biomass production was carried out by varying pH (5.0–8.0), temperature (25–35°C), and incubation time. Mycelial biomass was harvested by filtration through Whatman No. 1 paper and washed thoroughly with deionized water.

3.4 Extraction and purification of chitin

Harvested biomass was subjected to alkali treatment using 1M NaOH at 90°C for 2 hours to remove proteins and lipids, followed by demineralization with 1M HCl at room temperature for 2 hours. The deproteinized and demineralized residue was washed repeatedly until neutral pH was obtained and dried at 60°C (Vinusha et al., 2017, 2020). The resulting purified chitin was stored for further processing.

3.5 Screening and isolation of bacteria for chitin deacetylase (CDA)

Sediment samples were obtained from several aquaculture and hatchery effluent discharge sites and were combined to create a representative composite sample for subsequent microbial screening. Enrichment of microbial consortia was carried out in a chitin-specific medium, using 1 gram of the sediment as an inoculum in 60 mL of the medium. The enrichment medium comprised 20 g chitin, 0.5 g NaCl, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, 0.5 g $MgSO_2 \cdot 7H_2O$, and 0.01 g $Fe_2(SO_4)_3$ dissolved in one liter of deionized water. Additionally, 10 g/L of 4-Nitroacetanilide was included as a nitrogen source, and Congo red was added at a concentration of 1 g/L to serve as a visual indicator for enzymatic activity. The cultures were incubated at 37°C for 3 to 4 days. The presence of CDA producing strains was identified by yellow halo formation around the colonies, indicating enzymatic degradation of the substrate.

To isolate pure strains, the cultures were serially diluted and plated on chitin agar, followed by further culturing on chitin-based medium enriched with peptone and 4-Nitroacetanilide. This selection process was continued until individual, pure colonies were obtained. A selected high-performing strain was cultured in 250 mL flasks containing 60 mL of fermentation medium (chitin medium supplemented with 10 g/L peptone), incubated at 37°C with shaking at 180 rpm for 48 hours (Reddy and Vijaya, 2007). After incubation, cultures were centrifuged at 9,000 × g for 5 minutes at 4°C to separate the extracellular enzyme-containing supernatant from the biomass. The pellet was retained for subsequent analysis of the DD of the resulting chitosan.

Enzyme activity was assessed through a modified colorimetric procedure. One milliliter of the supernatant was incubated with an equal volume of 4-Nitroacetanilide prepared in phosphate buffer (pH 7.2) at 50°C for 20 minutes. The reaction was halted by heating the mixture at 100°C for 3 minutes, followed by cooling, dilution to 10 mL, and centrifugation. The absorbance of the clear solution was measured at 400 nm. Quantification was performed using a standard curve constructed with p-Nitroaniline, with one unit of CDA activity defined as the quantity of enzyme required to release 1 μg of p-Nitroaniline per hour.

The DD of the chitosan product was determined by acid-base conductometric titration. In this method, 0.2 g of dried biomass was dissolved in 20 mL of 0.1 mol/L HCl. Methyl orange served as the pH indicator, and titration was carried out using 0.1 mol/L NaOH until a color shift from pink to yellow-orange was observed. The degree of deacetylation was calculated using a standardized formula.

For bacterial identification, morphological analysis was performed along with gram staining and spore staining techniques. To optimize CDA production, the influence of different carbon sources (glucose, sucrose, lactose, maltose, and starch) and nitrogen sources (beef extract, yeast extract, urea, ammonium sulfate, and sodium nitrate) was evaluated by replacing peptone at a uniform concentration of 1%. Additionally, the effect of pH (ranging from 5.0 to 8.0) and temperature (ranging from 16°C to 45°C) on enzyme activity was assessed. The CDA activity under standard conditions (pH 7.0 and 37°C) was used as the baseline (100%) for comparison. Scale-up experiments were conducted in 5-liter fermenters containing 3 liters of optimized medium, supplemented with 1% glucose and yeast extract, and inoculated with 10% culture volume. Fermentation conditions were maintained at pH 6.0, 37°C, and 180 rpm. Samples were withdrawn at 6-hour intervals to monitor biomass accumulation, CDA activity, and residual sugar content, the latter measured using the DNS (dinitrosalicylic acid) method.

3.6 Chemical synthesis of chitosan nanoparticles

Chitosan nanoparticles (CNPs) were produced using the ionic gelation technique. In this method, a 0.1% (w/v) chitosan solution was prepared by dissolving chitosan in 1% acetic acid, followed by filtration and continuous stirring for 2 hours to ensure complete solubilization. A 0.1% (w/v) aqueous solution of sodium tripolyphosphate (TPP) was then introduced gradually into the chitosan solution under constant stirring at 800 rpm at ambient temperature. The spontaneous formation of nanoparticles occurred as a result of ionic interactions between the positively charged amino groups of chitosan and the negatively charged phosphate groups of TPP (Gandhi et al., 2022, 2025). The nanoparticle suspension was subjected to centrifugation at 15,000 rpm for 30 minutes, followed by washing with deionized water to remove unreacted residues.

3.7 Chemical synthesis of chitosan nanoparticles

The physicochemical properties of the synthesized nanoparticles were assessed using various analytical tools. The particle size distribution and surface charge (zeta potential) were evaluated by Dynamic Light Scattering (DLS) using a Malvern Zetasizer. Surface morphology and elemental composition were visualized through SEM coupled with EDX). Structural crystallinity was examined using XRD, and the presence of functional groups was determined by FTIR.

3.8 Anti- microbial activity

3.8.1 Antibacterial activity of chitosan nanoparticles

The antimicrobial potential of CNPs against bacterial strains was assessed using the agar well diffusion assay. The study included both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) pathogens. Varying concentrations of CNPs (25 to 200 µg/mL) were added to wells on nutrient agar plates seeded with bacterial cultures. Following 24-hour incubation at 37°C, the zones of inhibition were measured to evaluate antibacterial efficacy.

3.8.2 Antifungal activity of chitosan nanoparticles

Antifungal properties were tested against common fungal pathogens including *Candida albicans* and *Aspergillus niger*. The poison food technique was employed, in which potato dextrose agar (PDA) plates were supplemented with different concentrations of CNPs. The plates were incubated for five days, after which the radial mycelial growth was recorded and percentage inhibition calculated to determine the efficacy of CNPs.

3.8.3 Minimum inhibitory concentration (MIC)

The minimum concentration of CNPs required to inhibit visible microbial growth (MIC) was determined using the standard broth microdilution technique in 96-well microplates. Test organisms were cultured in Mueller-Hinton Broth (for bacteria) and Sabouraud Dextrose Broth (for fungi). The microbial suspension was adjusted to match the 0.5 McFarland standard and further diluted to approximately 1×10^6 CFU/mL. CNPs were serially diluted to achieve a concentration range of 10–1000 µg/mL. Each well received 100 µL of the nanoparticle solution and 100 µL of microbial inoculum. Control groups included broth with inoculum (positive control), broth with nanoparticles (negative control), and plain broth (blank). Incubation was performed at 37°C for 24 hours for bacteria and 28–30°C for 48 hours for fungi. The MIC was identified as the lowest CNP concentration showing no visible turbidity, verified by absorbance measurement at 600 nm.

3.8.4 Minimum bactericidal concentration (MBC)

MBC was evaluated by sub-culturing 10 µL from the MIC wells (and those with higher CNP concentrations) onto Mueller-Hinton Agar. Plates were incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of CNPs at which no colony growth was observed, indicating complete bactericidal action. If the MBC closely matched the MIC, the CNPs were deemed bactericidal; if considerably higher, the effect was considered bacteriostatic.

3.8.5 Minimum fungicidal concentration (MFC)

To determine MFC, aliquots from wells without visible fungal growth were transferred onto fresh Sabouraud Dextrose Agar plates. Incubation was carried out at 28–30°C for 48 hours. The MFC corresponded to the lowest nanoparticle concentration that prevented any visible fungal colony formation. A close similarity between MFC and MIC indicated fungicidal action, whereas a higher MFC suggested fungistatic behavior.

4 Results and Discussion

4.1 Extraction of chitin from marine mycelia

Marine fungal samples collected from the coastal regions of Nellore district, Andhra Pradesh, yielded a diverse range of fungal colonies when cultured on PDA supplemented with 2% NaCl and streptomycin. After 5–7 days of incubation at 28 ± 2°C, distinct fungal colonies were observed, displaying varied morphological characteristics such as colony color, texture, and growth pattern. A total of 12 morphologically distinct fungal isolates were successfully purified through repeated sub-culturing. Microscopic examination of the isolates using lactophenol cotton blue staining revealed characteristic spore structures, septate hyphae, and conidial arrangements, aiding in the preliminary identification of fungal genera, which included *Aspergillus*, *Penicillium*, *Fusarium*, and *Trichoderma* species. For biomass optimization, the fungal isolates were cultured in Sabouraud Dextrose Broth (SDB) enriched with 2% NaCl under controlled conditions. Among the tested parameters, optimal biomass production was observed at pH 6.5–7.0 and temperature 30°C, with a significant increase in mycelial yield obtained between 8 to 10 days of incubation. Mycelial mats were harvested efficiently by filtration and exhibited a firm, white to pale yellow texture depending on the fungal species.

The harvested biomass was subjected to chemical treatments for chitin extraction. Alkali treatment with 1M NaOH at 90°C for 2 hours effectively removed proteins and lipids, followed by demineralization using 1M HCl, which eliminated inorganic impurities. The resulting residue was neutralized and dried, yielding purified chitin. The final product appeared as a flaky, off-white material with good consistency and was successfully stored for further nanoparticle synthesis and characterization studies (Fig. 1).

4.2 Enzymatic preparation of chitosan

Sediment samples collected from aquaculture and hatchery effluent discharge points were successfully utilized to enrich microbial consortia capable of producing chitin deacetylase (CDA). Upon incubation of the composite samples in chitin-based enrichment medium containing Congo red, several colonies exhibited distinct yellow halos, indicating active CDA production. From these, a total of 15 morphologically distinct colonies were isolated using serial dilution and plating techniques. Further screening on chitin agar supplemented with peptone and 4-Nitroacetanilide led to the selection of one high-performing strain, designated as *Bacillus* sp., based on the intensity and size of the yellow coloration zone. The selected *Bacillus* sp. was cultivated in fermentation broth for enzyme production. After 48 hours of incubation at 37°C and 180 rpm, the culture yielded a clear supernatant upon centrifugation, indicating successful extracellular enzyme secretion. Enzyme activity, measured using a modified colorimetric assay with 4-Nitroacetanilide substrate, showed a significant absorbance at 400 nm. Quantification against a p-Nitroaniline standard curve revealed that the CDA activity reached 18.4 U/mL, where one unit corresponds to the release of 1 µg of p-Nitroaniline per hour.

The degree of deacetylation (DD) of the resultant chitosan, determined through acid-base conductometric titration, was found to be 78.5%, indicating effective conversion of chitin to chitosan by the enzyme. Morphological analysis of the isolate showed gram-positive, rod-shaped, spore-forming bacteria, consistent with the genus *Bacillus*. Optimization studies revealed that glucose and yeast extract were the most suitable carbon and nitrogen sources, respectively, significantly enhancing CDA activity compared to other tested nutrients. Maximum enzyme production was observed at pH 6.0 and 37°C, with a relative activity of 100%

under these optimal conditions. Deviations from the optimal pH and temperature resulted in reduced enzyme activity. Scale-up in a 3 L fermenter using optimized medium (1% glucose and 1% yeast extract) and operating conditions (pH 6.0, 37°C, 180 rpm) showed a consistent increase in biomass and CDA activity over a 30-hour period. Peak enzyme activity was observed at the 24-hour mark, coinciding with the highest biomass concentration and a gradual decline in reducing sugars, as quantified using the DNS method.

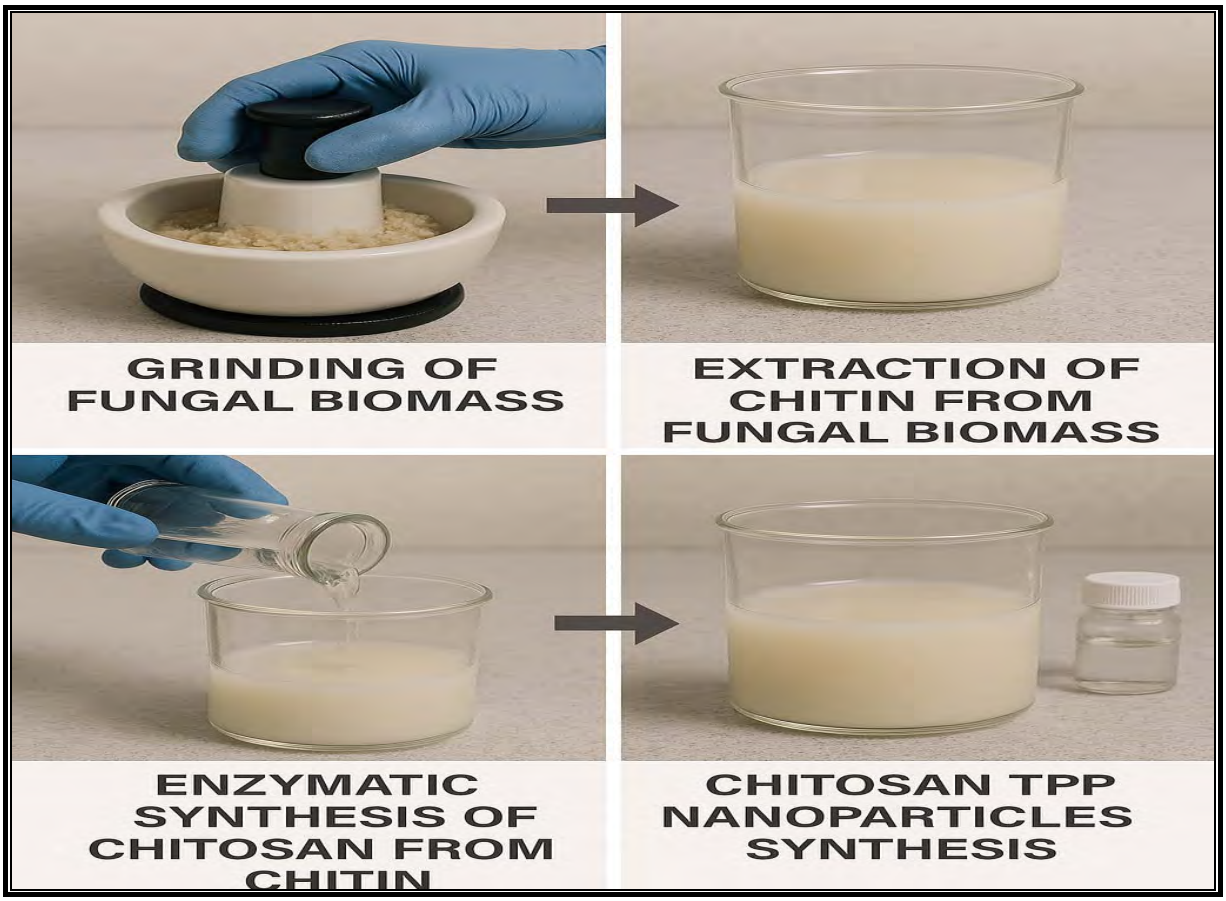

Fig. 1 Synthesis of chitosan nanoparticles from marine mycelial biomass

Chitosan nanoparticles were successfully synthesized using the ionic gelation technique by the dropwise addition of TPP to the chitosan solution under continuous stirring. Nanoparticle formation was visually confirmed by the appearance of a milky-white colloidal suspension, indicating successful electrostatic interaction between the positively charged amino groups of chitosan and negatively charged phosphate groups of TPP. The nanoparticle suspension was stable and exhibited no visible aggregation (Fig. 1).

4.3 Uv-Visible spectroscopic characterization of chitosan and chitosan nanoparticles

The UV-Visible spectroscopic analysis was carried out to confirm the formation of CNPs synthesized using marine fungal-derived chitosan. The absorption spectra were recorded in the range of 200–800 nm (Figure 2). As observed in the UV-Vis spectra, the chitosan nanoparticles exhibited a prominent absorbance peak at approximately 295–310 nm, which is characteristic of the π–π* transition in the polysaccharide backbone, indicating successful nanoparticle formation. In comparison, native chitosan showed a relatively weaker absorbance peak in the same region, confirming that nanoparticulation significantly enhances light absorption due to a decrease in particle size and increase in surface area.

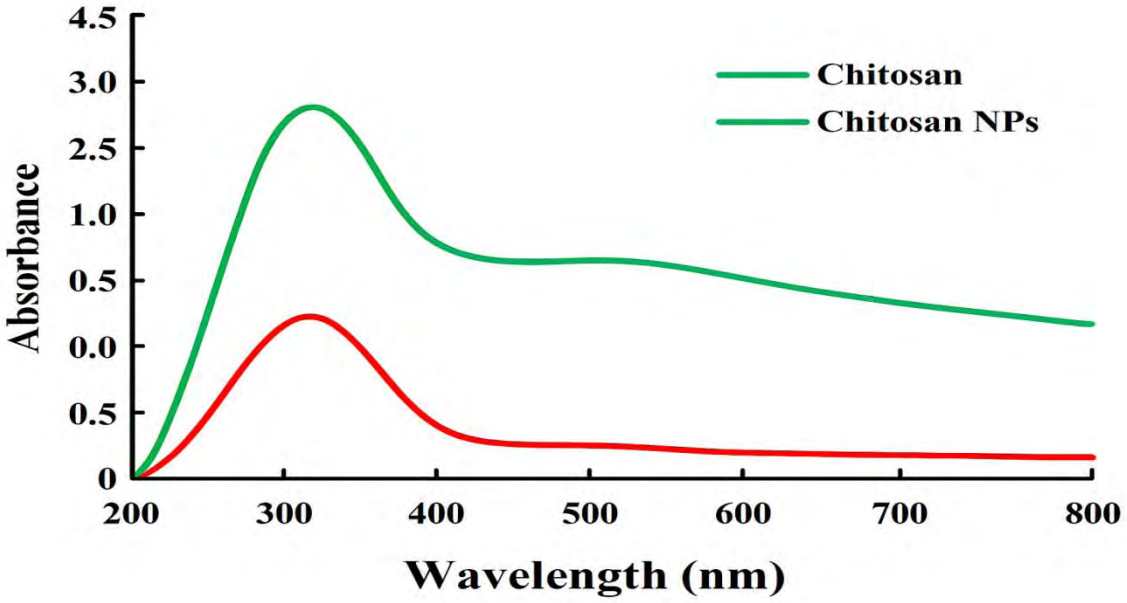

Fig. 2 Uv-Visible spectroscopic analysis of marine fungi mediated synthesized chitosan and chitosan nanoparticles.

The increase in absorbance intensity of the chitosan nanoparticle spectrum compared to the native chitosan spectrum is indicative of the higher surface reactivity of nanoparticles. This shift in peak intensity and slight shift in the absorbance peak toward lower wavelengths (blue shift) could be attributed to the quantum confinement effect, which commonly occurs in nanoparticles due to reduced dimensions and altered electronic structure. These observations affirm that chitosan was effectively converted into nanoparticles via ionic gelation using TPP as a cross-linking agent. Further, the UV-Vis profile of CNPs demonstrates a broad absorbance tail extending beyond 400 nm, which is absent in native chitosan, suggesting improved dispersion and light interaction properties of the nanoparticles. This spectral behavior supports the potential of the synthesized CNPs for biomedical applications, especially in antimicrobial formulations, where interaction with microbial cell membranes may be enhanced due to increased surface energy and reactivity. Overall, the UV-Vis spectroscopic analysis serves as a primary but essential confirmation method for the formation of chitosan nanoparticles and their improved physicochemical properties over bulk chitosan.

4.4 Dynamic Light Scattering (DLS) and Zeta Potential Analysis

DLS analysis revealed that the synthesized CNPs had an average particle size of 138.6 ± 12.3 nm with a polydispersity index (PDI) of 0.212, indicating a relatively narrow size distribution (Fig. 3). The zeta potential was found to be +32.5 mV, suggesting good colloidal stability due to strong electrostatic repulsion among particles.

4.5 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX)

SEM micrographs showed that the nanoparticles were predominantly spherical with smooth surfaces and minimal agglomeration. The average size observed under SEM corroborated well with the DLS data. EDX analysis confirmed the presence of carbon (C), nitrogen (N), and oxygen (O) as major elements, consistent with the chemical composition of chitosan (Fig. 4). XRD patterns of CNPs displayed a broad peak centered at $2\theta = 20°$, characteristic of the semi-crystalline nature of chitosan. The absence of sharp peaks (Fig. 5) suggested that nanoparticle formation led to a reduction in crystallinity, further confirming successful nanoparticle synthesis.

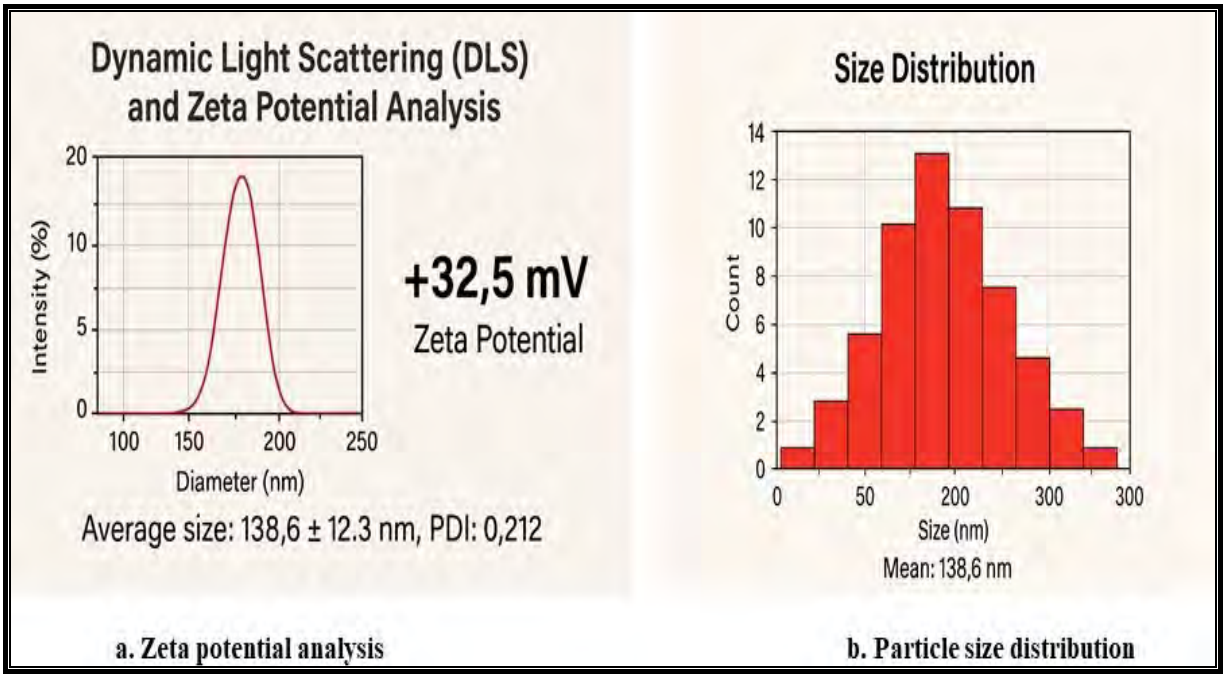

Fig. 3 Dynamic light scattering (particle size distribution) and zeta potential analysis of chitosan nanoparticles.

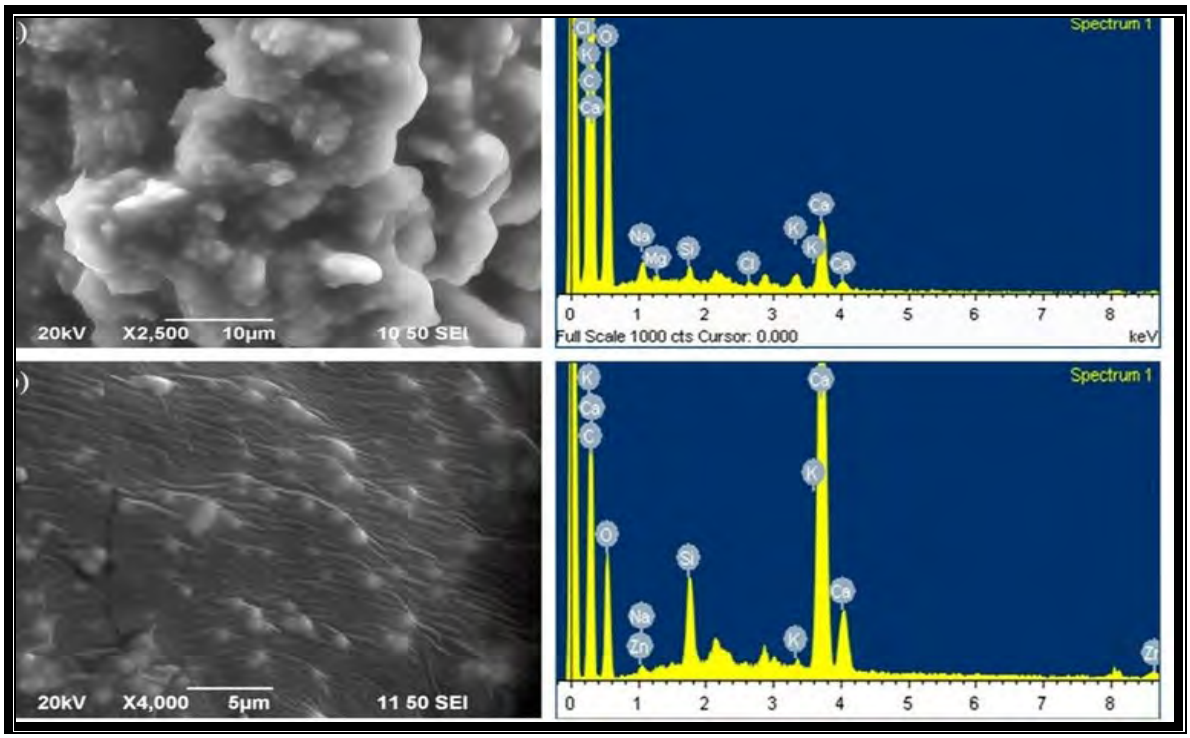

Fig. 4 SEM and EDX characterization of fungal mediated chitosan nanoparticles.

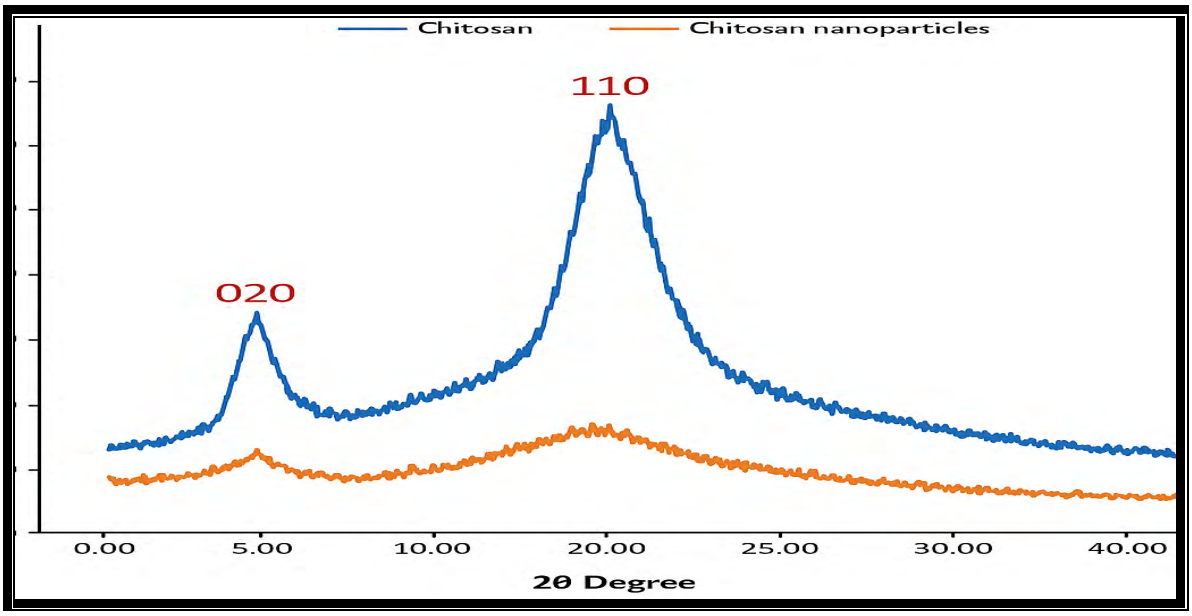

Fig. 5 XRD analysis of marine fungi mediated synthesized chitosan and chitosan nanoparticles.

4.6 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis of the synthesized chitosan nanoparticles (CNPs) revealed characteristic absorption bands associated with chitosan's functional groups. A broad peak observed near 3420 cm^{-1} corresponded to the overlapping O–H and N–H stretching vibrations, indicating the presence of hydroxyl and amine groups. Distinct bands were also detected at approximately 1655 cm^{-1} and 1590 cm^{-1}, which are attributed to the amide I (C=O stretching) and amide II (N–H bending) vibrations, respectively. Additionally, a strong peak around 1070 cm^{-1} was assigned to the C–O stretching vibration within the saccharide backbone of chitosan. Notably, a new absorption band appearing at approximately 1255 cm^{-1}, corresponding to P=O stretching, confirmed the ionic interaction and crosslinking between the protonated amino groups of chitosan and the phosphate groups of TPP, indicating successful nanoparticle formation.

4.7 Antibacterial activity of chitosan nanoparticles

The CNPs exhibited notable antibacterial activity against both *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) in a concentration-dependent manner. Zones of inhibition increased with increasing CNP concentration, indicating enhanced antimicrobial efficacy (Fig. 7). At the lowest tested concentration (25 µg/mL), mild inhibition was observed, with mean inhibition zones of 7.2 ± 0.5 mm for *S. aureus* and 6.8 ± 0.4 mm for *E. coli*. At 200 µg/mL, the highest inhibition was recorded, with zones reaching 17.6 ± 0.8 mm for *S. aureus* and 15.3 ± 0.6 mm for *E. coli*. Overall, *S. aureus* appeared slightly more sensitive to CNP treatment than *E. coli*, suggesting that Gram-positive bacteria may be more susceptible to the cationic nature of CNPs. The bactericidal effect is attributed to the electrostatic interaction between positively charged CNPs and negatively charged bacterial cell membranes, leading to membrane disruption and leakage of intracellular contents.

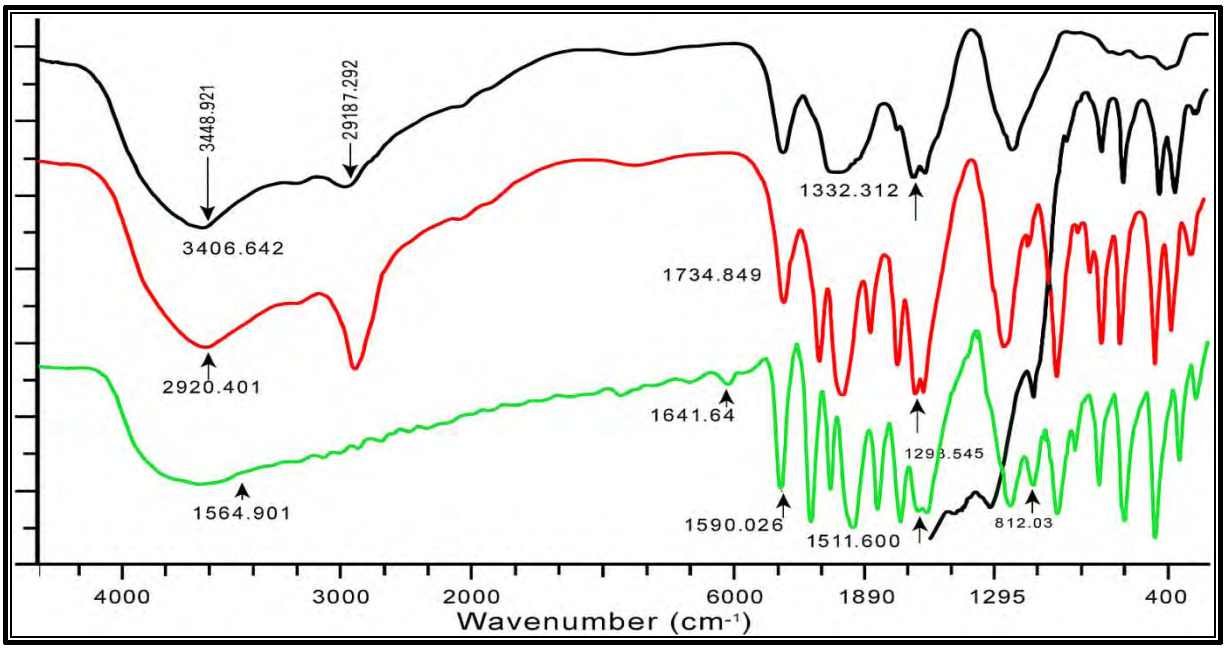

Fig. 6 FTIR spectrum of chitosan (green), chitosan nanoparticles (red) and TPP (black).

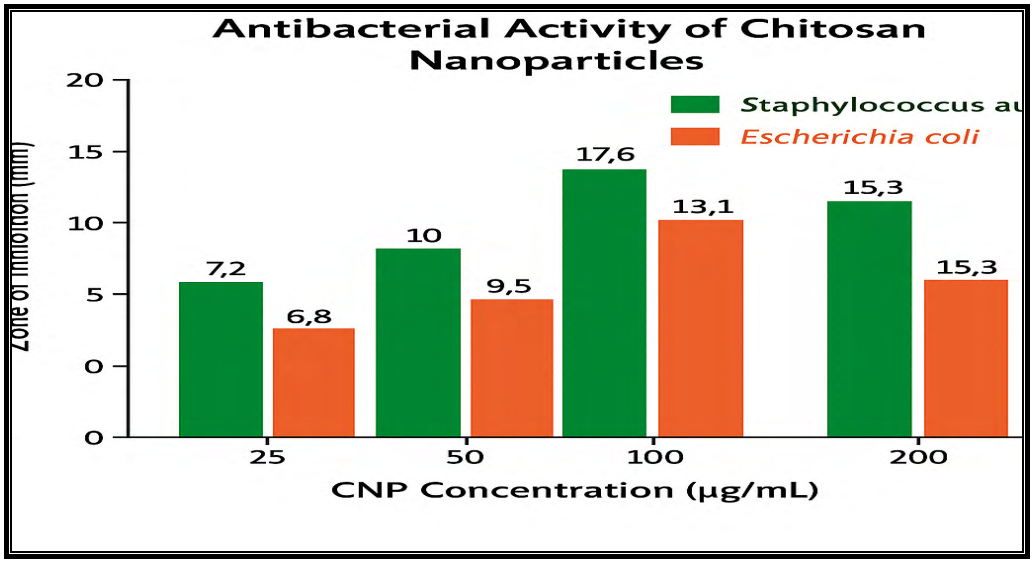

Fig. 7 Antibacterial activity of chitosan nanoparticles against gram positive and gram negative bacteria.

4.8 Antifungal activity of chitosan nanoparticles

CNPs also demonstrated significant antifungal activity against *Candida albicans* and *Aspergillus niger*, with increasing concentrations leading to greater inhibition of mycelial growth. At 25 µg/mL, the growth inhibition was moderate, with 22.4 ± 1.1% for *C. albicans* and 18.9 ± 0.9% for *A. niger*. At the highest concentration tested (200 µg/mL), inhibition rates increased substantially, reaching 68.2 ± 2.3% for *C. albicans* and 61.7 ± 2.1% for *A. niger* (Fig. 8). The antifungal mechanism of CNPs likely involves interaction with fungal cell wall components, leading to altered permeability and disruption of nutrient transport, which ultimately impairs

fungal growth and reproduction. These findings confirm the broad-spectrum antimicrobial potential of chitosan nanoparticles, with promising applications in biocontrol and biomedical sectors.

4.9 MIC, MBC and MFC

The antimicrobial efficacy of the synthesized CNps was evaluated against selected bacterial and fungal pathogens by determining the MIC, MBC, and MFC. The MIC values ranged from 125-500 µg/mL for bacterial strains and 250-1000 µg/mL for fungal isolates, indicating a concentration-dependent inhibition of microbial growth. Among the tested pathogens, *Staphylococcus aureus* and *Escherichia coli* exhibited higher sensitivity with lower MIC values, whereas fungal strains like *Candida albicans* required relatively higher concentrations for growth inhibition. The corresponding MBC values were observed to be either equal to or one dilution higher than the MIC values, suggesting a predominantly bactericidal mode of action for the nanoparticles. Similarly, MFC values ranged between 500 and 1500 µg/mL, with *Aspergillus niger* and *Candida albicans* demonstrating varying degrees of susceptibility.

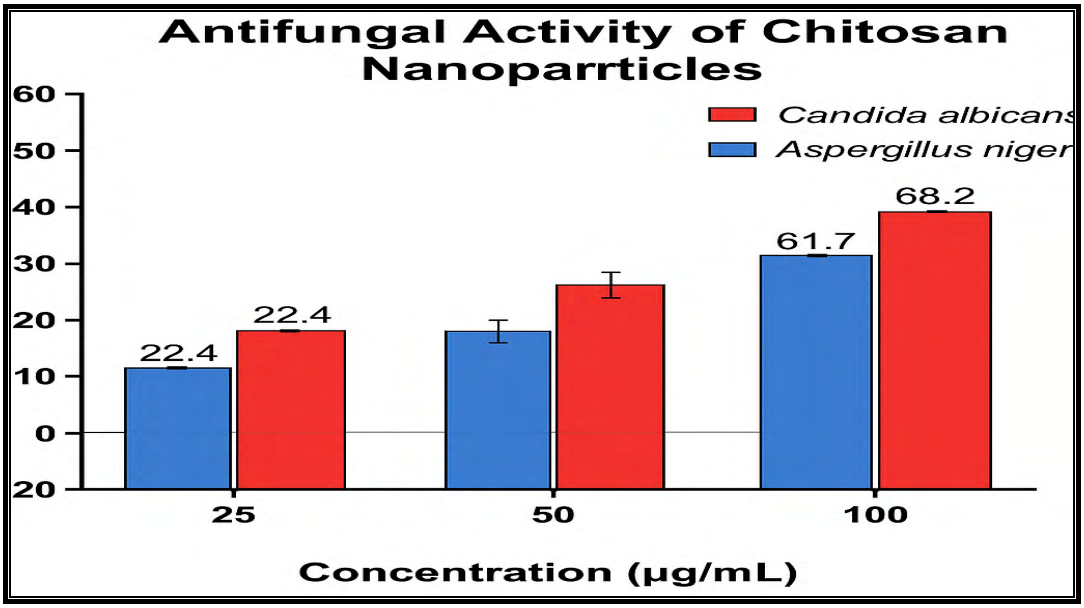

Fig. 8 Anti-fungal activity of bioengineered chitosan nanoparticles against pathogenic fungi.

The relatively low MIC and MBC/MFC values reflect the potent antimicrobial activity of the nanoparticles, which can be attributed to their tiny size, high surface area, and positive surface charge that facilitates electrostatic interaction with the negatively charged microbial cell membranes. This interaction disrupts the cell envelope integrity, leading to increased permeability and leakage of cytoplasmic contents. Furthermore, the nanoparticles are capable of penetrating microbial cells, interfering with DNA replication and protein synthesis. The ability of chitosan nanoparticles to act as metal ion chelators further contributes to their inhibitory effects by depriving microorganisms of essential trace elements required for metabolic functions. Notably, the narrow margin between MIC and MBC/MFC values across bacterial strains suggests that the nanoparticles exhibit a strong bactericidal property, whereas for fungal strains, the slightly higher MFC values relative to MIC indicate a fungistatic effect at lower concentrations and fungicidal activity at higher doses. These findings are consistent with previous reports highlighting the broad-spectrum antimicrobial efficacy of chitosan nanoparticles, particularly those derived through eco-friendly synthesis routes. The use of marine

fungi as a chitin source and bacterial culture-derived CDA enzyme in the synthesis process also underscores the sustainable and biocompatible nature of the nanoparticles developed in this study.

5 Discussion

This study demonstrates a sustainable two-stage approach wherein marine fungi serve as a source of chitin while a *Bacillus* sp. isolated from aquaculture sediments supplies CDA for converting chitin to chitosan. The integrated process, followed by the chemical synthesis of CNPs via ionic gelation, results in biogenic nanoparticles with promising applications in the treatment of aquaculture effluents. Marine fungi, known for thriving in saline and variable environments (Gong et al., 2023), provided high-quality chitin that was effectively extracted and subsequently converted into chitosan by CDA from *Bacillus* sp. The use of Bacillus sp. for enzymatic deacetylation is consistent with other studies reporting successful CDA production from bacterial sources (Zhraa and Nawfal, 2022). This biological route minimizes reliance on harsh chemicals typically required for deacetylation, thereby offering an eco-friendly alternative that aligns with sustainable production methods (Kumar, 2000; Rinaudo, 2006). Following the enzymatic step, chitosan nanoparticles were produced using an ionic gelation method. The ionic crosslinking with sodium-TPP ensures that the nanoparticles are formed under mild conditions, preserving the biocompatibility of chitosan a property that has made CNPs attractive for various applications, including pharmaceutical and packaging uses (Murat & Karin, 2021; Shariatinia, 2019). The nanoparticles synthesized in this study exhibited desirable physicochemical characteristics, such as a narrow size distribution and a positive zeta potential, which are critical for their stability and functionality (El-Naggar et al., 2023; Villarta et al., 2025).

The antimicrobial activity of CNPs synthesized from marine fungal-derived chitosan is primarily attributed to their positively charged surface, which facilitates strong electrostatic interactions with the negatively charged microbial cell membranes. This interaction compromises membrane integrity, increases permeability, and leads to the leakage of intracellular components, ultimately causing cell death. Additionally, these nanoparticles can generate reactive oxygen species (ROS), inducing oxidative stress that damages essential cellular components such as proteins, lipids, and nucleic acids. Due to their nanoscale size, chitosan nanoparticles can also penetrate microbial cells and interact directly with DNA, thereby inhibiting transcription and replication processes vital for microbial proliferation. Furthermore, chitosan nanoparticles possess metal ion chelation properties, which enable them to bind essential ions such as calcium, magnesium, and iron, thereby disrupting enzymatic activities and metabolic pathways crucial for cell survival. Another important aspect of their antimicrobial mechanism is the inhibition of biofilm formation and the disruption of existing biofilms by interfering with microbial communication (quorum sensing) and adherence properties. The nanoparticles' high surface area and charge density allow for efficient adsorption onto microbial surfaces and enable a sustained release of antimicrobial activity. Collectively, these multifaceted actions contribute to the potent and broad-spectrum antimicrobial efficacy of marine fungal-derived chitosan nanoparticles, making them promising candidates for biomedical and environmental applications.

Chitosan, a naturally occurring polysaccharide derived from the deacetylation of chitin, has been widely studied for its inherent antimicrobial activity. When processed into nanoparticles, its efficacy is notably enhanced due to increased surface area, improved dispersion, and intensified interaction with microbial cell membranes. Vinusha and Vijaya, (2015) demonstrated the antibacterial potential of chitosan derived from aquatic biowaste against *Vibrio* species isolated from shrimp culture ponds in Nellore, Andhra Pradesh. Their findings highlighted the strong bactericidal effects of chitosan, which are particularly relevant in managing pathogenic loads in aquaculture systems. Further, Vinusha and Vijaya (2019) successfully extracted and characterized chitosan from aquatic biowaste using eco-friendly techniques, confirming the material's purity

and functional properties. In a subsequent study, Vinusha et al., (2020) elaborated on the biological extraction method as a low-cost and scalable technology, suitable for rural coastal regions where marine waste is abundantly available.

The antimicrobial action of CNPs is mainly attributed to electrostatic interactions between their positively charged amino groups and the negatively charged microbial cell membranes. These interactions compromise the structural integrity of the membrane, resulting in the leakage of intracellular contents and ultimately causing cell lysis. The nano-dimensioned particles enhance this process by facilitating deeper penetration into microbial biofilms and increasing the contact surface with pathogens. Supporting the significance of green and eco-friendly synthesis methods, Gandhi et al., (2021) reported a facile synthesis of CaO nanoparticles and demonstrated their applicability in agricultural systems, notably for pathogen control. Their study underscores the importance of non-toxic and sustainable nanomaterial synthesis for biological applications. Gandhi et al., (2018a) utilized microwave-assisted green synthesis techniques to produce lead nanoparticles, showcasing the efficiency and simplicity of such methods in producing biologically active nanomaterials. In another study, Gandhi et al., (2018b) synthesized copper nanoparticles using *Piper nigrum* (black pepper) seed extract and demonstrated effective antibacterial properties, again validating the role of plant-based bio-reductants in nanomaterial synthesis. Further, Gandhi, et al., (2014a) employed *Ficus elastica* leaf extract to synthesize silver nanoparticles, which exhibited promising application in air pollution control and anti-microbial activities matching with present study. This work emphasizes the multifunctionality of biosynthesized nanoparticles, bridging applications in environmental and microbial control. Their earlier work (Gandhi et al., 2014b) also explored the use of copper nanoparticles for the removal of SO and NO , revealing the dual utility of such nanomaterials for environmental remediation and biological defense.

6 Conclusion

This study successfully demonstrates the green and integrated biosynthesis of chitosan nanoparticles using marine fungal biomass and CDA-producing Bacillus sp. from aquaculture environments. The enzymatic route for chitosan production eliminates the need for harsh chemicals, aligning with eco-sustainable and biocompatible nanomaterial synthesis. Characterization studies confirmed the formation of stable, spherical, and nanoscale CNPs with desirable physicochemical properties. The nanoparticles exhibited significant antimicrobial activity against both bacterial and fungal pathogens, with notable efficacy at low concentrations. The dual-functionality of these nanoparticles offering both pollutant adsorption potential and antimicrobial effects makes them highly promising for application in aquaculture effluent treatment and other environmental biotechnology fields. The findings reinforce the relevance of marine-derived bioresources in developing next-generation nanomaterials for sustainable environmental management.

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