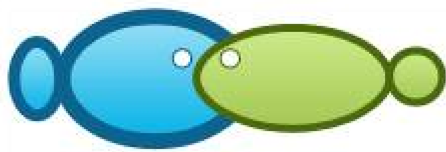

Antibacterial activity of nano chitosan derived from mangrove fungus endophyte, *Fusarium* sp. 20CB07

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Abstract. This study aimed to evaluate antibactereal activity and characterize nano chitosan obtained from mangrove fungal endophytes, exploiting the chitin components in fungal cell walls. Four fungal isolates were collected from various parts of mangrove plants. Chitosan extraction from fungi involved a 14-day solid-state fermentation process in rice media, followed by deacetylation. The resulting chitosan extract underwent characterization through infrared spectroscopy to determine the degree of deacetylation and differential scanning calorimeter analysis to characterize chitosan. Nano chitosan synthesis used the ionic gelation method with sodium tripolyphosphate. The particle size of nano chitosan was analyzed using a particle size analyzer, and its antibacterial bioactivity was tested against clinically resistant bacterial pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Among the four isolates, isolate 20CB07, identified as *Fusarium* sp., demonstrated superior chitosan production. This isolate yielded chitosan with a percentage yield of 16.7% and a deacetylation degree of 97.77%. The differential scanning calorimeter analysis of chitosan exhibited a thermogram with broad endothermic peaks around 72.9°C and between 278.6 and 314°C due to the decomposition of water and chitosan, respectively. Nano chitosan characterization revealed a nanoparticle size of 127.2 nm. The compound demonstrates antibacterial activity against both *P. aeruginosa* and *S. aureus* at a concentration level of 50 μg mL^{-1}, exhibiting greater selectivity towards inhibiting *P. aeruginosa* over *S. aureus*. These findings highlight the potential of isolate 20CB07 as a chitosan source, providing foundational information for future studies on the bioactivity applications of nano chitosan.

Key Words: antibacterial agent, clinical bacterial resistance, *Fusarium* sp., mangrove, nano chitosan.

Introduction. Chitin, the second-largest polysaccharide on earth after cellulose, finds widespread use in various industries today (Sady et al 2021). Chitosan, a derivative of chitin, serves as a multifunctional raw material for synthesizing diverse products in medical, pharmaceutical, food, healthcare, and agricultural fields (Morin-Crini et al 2019). Chitosan and its derivatives are known for their non-toxic properties (García-Carrasco et al 2023). However, the conventional production process, often sourced from crustaceans, generates waste that poses environmental pollution risks (Kaya et al 2015). Moreover, crustacean-derived chitosan exhibits inconsistent properties, including a low deacetylation degree and a high molecular weight, resulting in limited solubility (Carrera et al 2023).

To address these challenges, numerous studies focus on fungi as an alternative source for chitin and chitosan (Sebastian et al 2020). Fungi represent renewable natural resources with versatile applications in human life (Elkhateeb & Daba 2019). Fungal cell walls play a crucial role in their life cycle. Ongoing research on fungal chitosan highlights its potential to surpass crustacean chitosan in terms of physicochemical properties. Chitosan derived from fungus produces a material with higher fiber content, five times lower viscosity, medium-low molecular weight, and a high degree of deacetylation. These

properties enhance fungal chitosan’s solubility, making it well-suited for applications in biomedicine (Ghormade et al 2017).

Various fungi, including *Mucor rouxii*, *Aspergillus niger*, *Absidia blakesleeana*, *Absidia coerulea*, *Absidia glauca*, *Phycomyces blakesleeanus*, *Trichoderma reesei*, *Colletotrichum lindemuthianum*, and *Gongronella butleri*, have been studied for chitin and chitosan production (Crognale et al 2022). However, no study reported the production of chitosan from *Fusarium* sp. Therefore, this research focuses on chitosan production from *Fusarium* sp. obtained from mangrove endophytes. Endophytic fungi are microorganisms that live within the internal tissues of plants without causing any damage. In mangrove ecosystems, these fungi form symbiotic relationships with mangrove trees and other plants. They can colonize various parts of the plant, including the roots, stems, and leaves (Hamzah et al 2020). Some endophytic fungi associated with mangrove plants have the ability to produce chitosan. These fungi contain enzymes capable of deacetylating chitin, converting it into chitosan. This process involves removing acetyl groups from chitin, resulting in the formation of chitosan (Iber et al 2022).

The presence of endophytic fungi that produce chitosan within mangrove plants can have ecological implications. Chitosan possesses various properties, such as antimicrobial, antifungal, and plant growth-promoting characteristics (Román-Doval et al 2023). When produced by endophytic fungi in mangroves, this can contribute to the health and resilience of the mangrove ecosystem by potentially enhancing the plant's resistance to threats from living organisms and environmental factors (Palit et al 2022). Understanding the relationship between chitosan production by endophytic fungi and mangrove forests contributes to the sustainable utilization of natural resources. It also emphasizes the interconnectedness of biodiversity within mangrove ecosystems and the potential benefits that this natural relationship can offer in various fields (Rahmadi et al 2023).

Chitosan finds extensive use in nanotechnology, particularly in the pharmaceutical field, where it serves as a polymer forming nanoparticles (Baharlouei & Rahman 2022). The application of chitosan in nanotechnology offers several advantages, such as the ability of nanoparticles to penetrate spaces between cells, higher cell wall penetration through diffusion or opsonification, and flexibility to combine with various technologies, providing vast potential for diverse needs and targets (Zajdel et al 2023). Nanoparticles also exhibit increased affinity within systems due to their enhanced contact surface area, even when using the same quantity (Forest & Pourchez 2017). Nanoparticles, including chitosan nanoparticles, can be formed through various techniques, with the ionic gelation method being one of them (Van Bavel et al 2023). Chitosan nanoparticles, obtained through cross-linkage via ionic gelation, especially with sodium tripolyphosphate (Na-TPP), rely on electrostatic interactions between ions with different charges. This interaction occurs between chitosan as a polycation compound and Na-TPP as a polyanion compound. Upon contact, chitosan forms a gel, leading to the formation of electrostatic interactions between the opposite charges of the components (Patiño-Ruiz et al 2020). This research focused on creating and analyzing nano-sized chitosan derived from endophytic fungi found in mangroves. The aim was to evaluate antibacterial activity and characterize nano chitosan obtained from mangrove fungal endophytes, exploiting the chitin components in fungal cell walls.

Clinical bacterial resistance is a significant and urgent issue due to the increasing number of bacteria becoming resistant to antibiotics (Mancuso et al 2021). This resistance limits treatment options, prolongs illnesses, raises healthcare costs, and poses challenges in developing new effective antibiotics. Overuse and misuse of antibiotics, limited treatment choices, and the economic and health impacts make bacterial resistance a pressing global concern requiring immediate attention and multifaceted solutions (Ardal et al 2020). Nanochitosan exhibits promising potential in combating clinical bacterial resistance. Its nano-sized particles enhance interactions with bacterial cells, disrupting their membranes and inhibiting growth. Nanochitosan's ability to penetrate biofilms and interfere with bacterial processes makes it effective against resistant strains. Nanochitosan's antimicrobial properties offer hope in addressing

bacterial resistance, potentially serving as an alternative or complementary approach to existing antibiotics (El-Zehery et al 2022).

The present study was carried out in several stages involving cultivation of mangrove endophytic fungi, isolation of chitosan from selected fungi, synthesis of nano chitosan using ionic gelation method, and characterization. The aim is to evaluate and characterize nano chitosan obtained from mangrove fungus endophytes utilizing the chitin component in fungal cell walls. Four fungal isolates were collected from various parts of the mangrove plant. Extraction of chitosan from mushrooms involves a 14-day solid-state fermentation process in rice medium, followed by deacetylation, and synthesis of nano chitosan using ionic gelation method. The antibacterial activity of nanochitosan was evaluated against the clinical bacterial resistance, specifically targeting *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Material and Method

Isolated fungi and maintenance. Four fungal isolates, namely 20BB0501, 20CB07, 20BA04, and 20BA0502 were obtained from fungal deposits at the Integrated Laboratory Technical Services Unit at the Center for Innovation and Technology (UPT-LTSIT), Lampung University. These isolates were collected in August 2023 from mangrove plants growing in the mangrove forest area of Sriminosari village, East Lampung Province, Indonesia; fungal endophytes were isolated from the roots and stems of *Rhizophora* sp. plants (Figure 1) using dilution techniques and sub-cultured on 1% colloidal chitin agar media and artificial seawater media (ASW). The isolates were maintained on malt extract agar (MEA) media in ASW (Setiawan et al 2022).

Figure 1. Mangrove plant ecosystem: (a) mangrove tree at Sriminosari; (b) *Rhizophora* sp.

Morphology analysis. To analyze the morphology of the endophytic mangrove strain, it was cultured on MEA for 6 days at 27°C. Subsequently, the microscope slide was sterilized using 70% alcohol and gently heated over a Bunsen burner. The slide was positioned adjacent to the area where the isolated fungus was growing, and it was examined using a microscope (Sangeetha et al 2020).

Solid-state fermentation. Each strain was fermented in a 1000-mL Erlenmeyer flask containing 50 g of rice media in 200 mL of seawater and left at 27°C for 14 days. Then, the biomass was separated by centrifugation (4000 rpm, 20 min) (Hitachi CF16RXII). The supernatant was washed with distilled water, dried in the oven (60°C for 24 h), separated from rice media, and stored for chitosan extraction (Amer & Ibrahim 2019).

Chitosan extraction. The fungal mycelia were collected and treated with 1N NaOH at the ratio 1:40 (w/v) and homogenized. Alkali-insoluble materials were collected by centrifugation (6000 × g for 30 min), and the solid fraction was rinsed thrice with distilled water until pH reached 7. The solid paste was dried in an oven at 40°C. The dried samples were subsequently treated with 3% acetic acid (1:40 w/v) and mixed for 2 h at

room temperature. The acid-soluble fraction was separated by centrifugation at 6000 × g for 15 min. The chitosan-represented supernatant was neutralized with 2N NaOH until pH reached 10 to precipitate the chitosan. The flocculated chitosan was centrifuged at 6000 × g for 15 min. The separated chitosan was washed thrice with distilled water to neutralize it. Then, the chitosan underwent consecutive rinses with ethanol and acetone (Elsoud et al 2023).

***Degree of deacetylation**.* Degree of deacetylation (DD) was estimated according to the infrared spectroscopy (IR) spectrum using the ATR method (Carry 630, Agilent Technologies) by measuring the ratio wave number of A_{1655} cm^{-1}, which represent the amine ($-NH_2$) group band, and A_{3450} cm^{-1}, which represent the hydroxyl (-OH) group band. The equation proposed for the determination of DD is as follows (Tan et al 2020):

$$DD\% = 100 - \frac{A_{1655}}{A_{3450}} \times \frac{100}{1.33}$$

***Differential scanning calorimeter**.* Differential scanning calorimeter (DSC) analysis was conducted to record temperature and heat flow changes associated with material transitions over time and temperature, within a controlled atmosphere. This was achieved using a DSC instrument (Model 822, Mettler Toledo, Switzerland) with a gradual temperature increase of 10^oC per min, covering the range of 30 to 500^oC, in the presence of a nitrogen flow at a rate of 25 mL per min (Eulalio et al 2019).

***Synthesis of nano chitosan**.* Chitosan nanoparticles were made using the ionic gelation method by preparing a solution of chitosan and Na-TPP (mg mL^{-1}). The chitosan solution was made from 6 mg of chitosan dissolved in 1 mL of acetic acid (1%, v/v) and 5 mL of sterile distilled water, while the Na-TPP solution was made by dissolving 4 mg of Na-TPP powder in 4 mL of sterile distilled water. The chitosan nanoparticles formed after adding Na-TPP to the chitosan solution were spontaneously homogenized under magnetic stirring. Then, the solution was stored at room temperature for further analysis (Rajan et al 2019).

***Characterization of nano chitosan using particle size analysis (PSA)**.* Particle size analysis was performed using the dynamic light scattering (DLS) principle reffering to Rosyada et al (2019). Briefly, the nano chitosan sample solution of 1 mL was diluted with dH_2O until it reach pH 7. After that, the sample solution was put into a 10 mL cuvette, and then the photon correlation was analyzed at 25°C using Zetasizer nano ZSP Malvern.

***Assays for antibacterial activity**.* The antimicrobial activity of fungal nano chitosan was assessed using the disc diffusion method against clinical resistant bacterial pathogens obtained from UPT LTSIT Lampung University. *P. aeruginosa* and *S. aureus* were individually sub-cultured on tryptic soy agar. Each bacterial pathogen was evenly spread on plates using a sterile cotton swab. A nano chitosan solution was prepared in 0.2% acetic acid at concentrations of 50 μg mL^{-1}, 100 μg mL^{-1}, and 200 μg mL^{-1}, and acetic acid was used as negative control. Paper discs soaked in chitosan solution for 24 h were added to the plates. Each plate was incubated for 18 h at 37°C, and the inhibition zone of growth was measured in millimeters (Guarnieri et al 2022).

Results and Discussion. Mangroves constitute unique ecosystems thriving in tropical and subtropical coastal regions, notable for their resilience in saline water and flood-prone environments. These areas harbor diverse flora and fauna, including a variety of fungi (Wijayawardene et al 2022). Fungi inhabiting aquatic environments wield the potential to shape food web dynamics and influence biogeochemical element cycling. Their presence can augment resources for higher-level consumers and facilitate the efficient transfer of nutrients across aquatic ecosystems, as elucidated by Grossart et al (2019).

Over the last two decades, there has been a noticeable surge in literature publications, reflecting a significant emphasis on exploring bacteria and fungi associated with mangroves. This focus is propelled by the pursuit of bioactive compounds and potential chemicals, leveraging the unique ecological characteristics of mangroves and their abundance in distinctive bioactive secondary metabolites, as highlighted by Ancheeva et al (2018). Numerous studies have investigated endophytic fungi extracted from mangrove plants, primarily for the production of biosurfactants and enzymes, as emphasized by Martinho et al (2019). Additionally, there has been a dedicated effort to unearth new bioactive compounds with potential applications in pharmacology and biotechnology, as indicated by Cadamuro et al (2021). Nonetheless, research on the extraction of chitosan from mangrove endophytic fungi remains relatively limited.

In this study, four strains of endophytic fungi were isolated from *Rhizophora* sp. Two fungal strains named 20BB0501 and 20CB07 were isolated from the stem, while the other two fungal strains named 20BA04 and 20BA0502 were isolated from the roots. The biodiversity of endophytic fungi in mangroves is not yet fully understood. Nonetheless, studies indicate a broad diversity of these fungi, with some estimates suggesting that there could be over 1000 species of endophytic fungi in mangroves. The diversity of endophytic fungi is likely attributed to the unique environmental conditions of the mangrove ecosystem, as well as the diversity of plant species inhabiting mangroves (Sridhar 2019). The study of endophytic fungi in mangroves is a relatively new research field. However, it is rapidly expanding as scientists increasingly recognize the importance of these fungi to the health and functioning of the mangrove ecosystem.

Macroscopic and microscopic features of isolated fungi. The cultural, microscopic, and morphological traits of the isolated fungi were evaluated. Figures 2 and 3 illustrate the identifications of the four isolated fungal species. The colony morphology of 20BB0501, 20CB07, and 20BA0502 reveals white-colored colonies, smooth and cottony (Figure 2), while 20BA04 reveals colonies on the surface that appear flat and oval in shape, exhibiting a rough, fibrous texture with smooth edges. In the center, they display dark green coloring with distinct boundaries, while the outer edge resembles a white, cotton-like texture, as depicted in Figure 2.

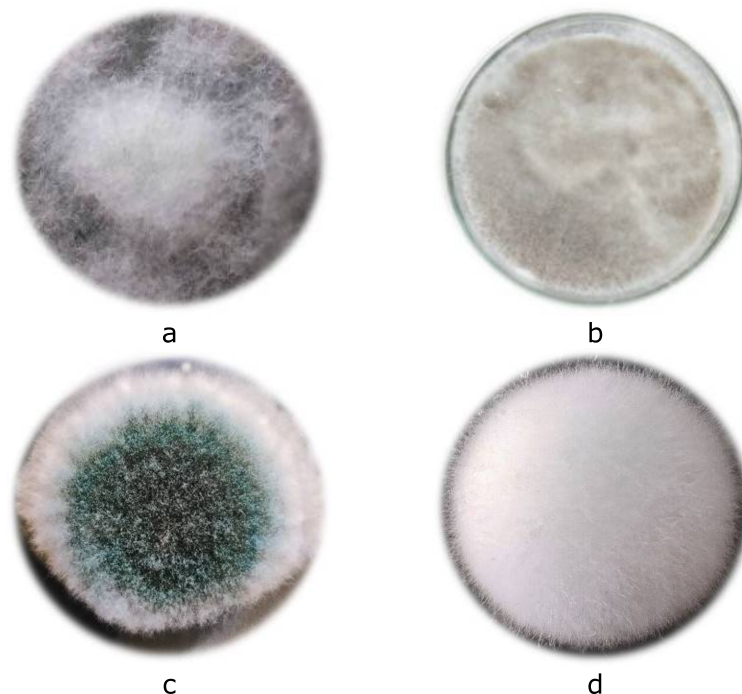


Figure 2. Macroscopic characteristics of isolated mangrove fungi endophytes: (a) 20BB0501; (b) 20CB07; (c) 20BA04; (d) 20BA0502.

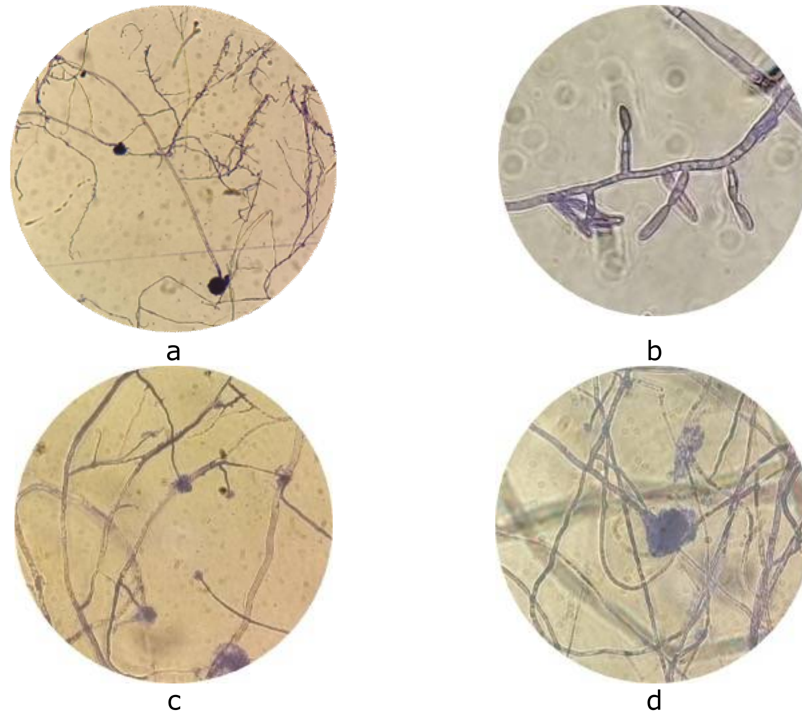


Figure 3. Microscopic characteristics isolated mangrove fungi endophytes: (a) 20BB0501; (b) 20CB07; (c) 20BA04; (d) 20BA0502.

The microscopic photograph of 20BB0501 shows hyaline, unbranched hyphae, and irregular conidia that are cylindrical, short, and wide, characteristic features of *Paecilomyces* sp., as documented by Paul et al (2013). Additionally, isolate 20CB07 displayed macroconidia that were cylindrical, slightly curved or straight, along with fiber-like, unbranched hyaline hyphae, consistent with the characteristics of *Fusarium* sp. When inspecting isolate 20BA04, branching hyphae forming flat and short conidiophores were observed, and its conidia possessed a two-layer wall comprising an outer electron layer (epispore) and an innermost electron layer. These traits indicated a possible association with *Trichoderma* sp. (Mukhopadhyay & Kumar 2020). As for isolate 20BA0502, it exhibited hyaline with conidia having round, semi-round, or oval shapes, attached to phialides positioned at the tip of the conidiophore. The swollen conidiophores, referred to as vesicles, are distinctive features of the genus *Aspergillus* sp. (Salvamani & Nawawi 2014).

The differences among isolated and identified fungal genera are linked to variations in plant types and environmental conditions. In this research, fungal isolates were initially identified at the genus level by assessing colony colors through morphological examination. Subsequent identification involved examining the shape of spore-producing structures through microscopy. These morphological examination and identification methods are valuable for determining isolates up to the family or genus level (Maharachchikumbura et al 2021). All four strains identified belonged to the fungal phylum Ascomycota. These findings align with the research conducted by Chen et al (2018), which identified Ascomycota fungi among mangrove endophytic fungi. The extensive dispersal of Ascomycota spores, along with their rapid multiplication, contributes to a widespread presence of species within this phylum (Gautam & Avasthi 2019).

Solid-state fermentation*.* The results of cultivating the four strains using solid-state fermentation with a medium containing 50 g of rice in 200 mL of ASW are shown in Figure 4. Strain 20CB07 exhibited the highest yield (31.1 g) in terms of the weight of the dry crude extract, followed by 20BA0502 at 26.5 g, 20BB0501 at 23.7 g, and 20BA04 at

15.6 g. The investigation of maximum extract yield was conducted over a 14-day incubation period, and observations revealed a decrease in mass within the rice medium.

Figure 4. Solid-state fermentation: (a) 20BB0501; (b) 20CB07; (c) 20BA04; (d) 20BA0502.

The correlation between rice media and fungal biomass production is influenced by factors such as the nutrient composition, moisture content, sterilization, cultivation techniques, and the specific fungal strain used (Kumar et al 2021). Optimizing these factors in rice media can enhance fungal growth and biomass production.

Chitosan extraction. The extraction outcomes for chitosan from four distinct fungal isolates showed the highest yield in the case of isolate 20CB07, with a recovery rate of 16.7% (Figure 5). Following this, 20BA0502 achieved an 11.1% yield, 20BB0501 had a yield of 7.6%, and the lowest recovery rate was observed for 20BA04 at 1.7%. Variations in the percentage of yield can be attributed to the specific fungal isolate used. Each fungus is composed of unique components comprising its cell wall.

According to research by Sebastian et al (2020), natural chitosan is typically present in Zygomycota species, while Basidiomycota, Deuteromycota, and Ascomycota have not been reported to naturally contain chitosan as part of their cell walls. In the context of this study, the four fungal isolates were tentatively identified through microscopy and macroscopy as *Fusarium* sp. (20CB07), *Aspergillus* sp. (20BA0502), *Paecylomices* sp. (20BB0501), and *Trichoderma* sp. (20BA04), which belong to the Ascomycota family. This confirms the success of the study, highlighting that Ascomycota species do indeed include chitosan as a constituent of their cell walls.

Figure 5. Chitosan powder of 20CB07.

The outcomes of chitosan production from the four isolates reveal that isolate 20CB07, likely a *Fusarium* sp. based on its macroscopic and microscopic characteristics, shows a strong potential for sustainable chitosan production due to its exceptional performance in producing the highest biomass and chitosan extract. Consequently, isolate 20CB07 was identified as the most promising and was selected for further research.

Fourier transform infrared spectroscopy (FTIR). Based on the fungal chitosan extraction results, isolate 20CB07 was selected for further study. In this study, chitosan characterization and determination of the degree of chitosan deacetylation were carried out using FTIR (Figure 6). The FTIR spectrum of chitosan shows a characteristic band at 3722 cm^{-1} caused by O-H stretching vibrations and N-H stretching (1° amide). Chitosan contains O-H groups, and this area represents the stretching vibration of these groups. This wide band is caused by the presence of various O-H groups, including groups on the glucosamine unit. Aliphatic C-H stretching at 2962 and 2840 cm^{-1}, band is associated with the stretching vibration of C-H bonds in the chitosan aliphatic chain. The two absorptions at 1558.5 cm^{-1} are associated with N-H bending vibrations and C-N stretching in the amide group, thus providing further information regarding the amide bond in chitosan. Concerning glycosidic absorption at 1013 cm^{-1}, this region contains various vibrations related to the chitosan framework structure, including bending and stretching of C-C and C-O bonds (Drabczyk et al 2020).

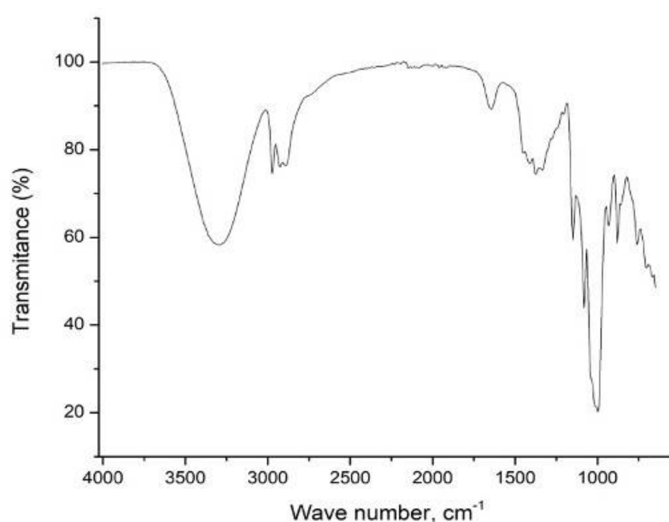


Figure 6. FTIR spectrum of 20CB07.

Based on the results of the DD calculation for 20CB07, a value of 97.8% was obtained, which shows that almost all of the chitin was converted into chitosan. The DD is an important parameter that influences the physicochemical properties of chitosan (Carrera et al 2023). It is related to the number of functional groups, impacting the solubility and positive charge. A higher solubility and positive charge can increase the application of chitosan in various fields, including the food industry, as an antimicrobial agent, antioxidant, and chelating agent (Yang et al 2023).

Differential scanning calorimeter (DSC). The DSC thermogram of fungal chitosan showed broad endothermic peaks around 72.9°C and between 278.6°C and 314.0°C (Figure 7). The first peak is related to the release of residual water or solvent from chitosan, with an enthalpy of 202 mJ mg^{-1} required for this component's release. The second endothermic peak indicates the chitosan thermal decomposition process (depolymerization, saccharide ring dehydration, deacetylation decomposition, and acetylated chitosan units) (El-Naggar et al 2022).

In the solid state, chitosan polysaccharide exhibits an irregular structure and has a strong affinity for water, making it easily hydrated. The first peak indicates that the chitosan has not completely dried, and there is still bound water that is not lost during drying. The second peak indicates the thermal degradation of chitosan (monomer dehydration, glycosidic bond breaking, acetyl unit decomposition, and deacetylation) (Dey et al 2016).

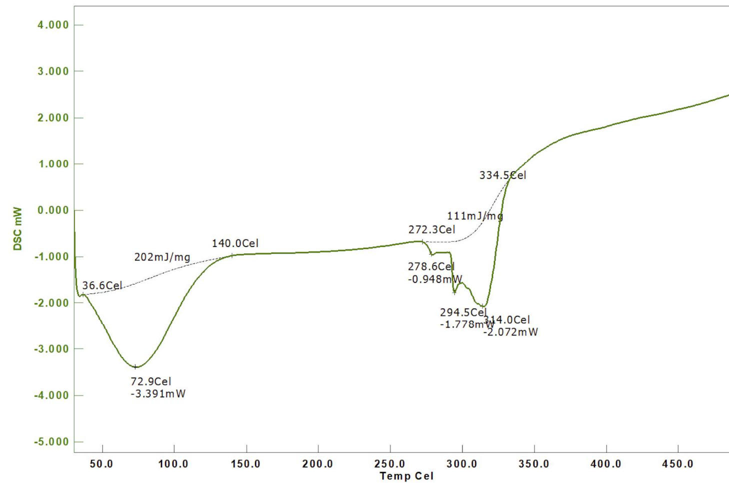


Figure 7. DSC thermogram of chitosan 20CB07.

Nanoparticles synthesis. Nano chitosan was produced using the ionic gelation method. The chitosan solution was prepared by dissolving chitosan in an acetic acid solution, followed by stirring with a magnetic stirrer at room temperature overnight to ensure complete dissolution. This is necessary because chitosan can only dissolve in acidic solvents. The chitosan solution was slowly added to Na-TPP as a cross-linker for ionic gelation until a nano chitosan suspension was formed.

The characterization of nano chitosan was conducted using a PSA to determine the size of the nanoparticles formed. PSA measures the particle size based on their Brownian motion in a liquid medium. Based on the PSA characterization results, the average intensity of the nano chitosan size was 127 nm (Figure 8). According to Ishkeh et al (2021), chitosan nanoparticles typically have a size range of 15-150 nm.

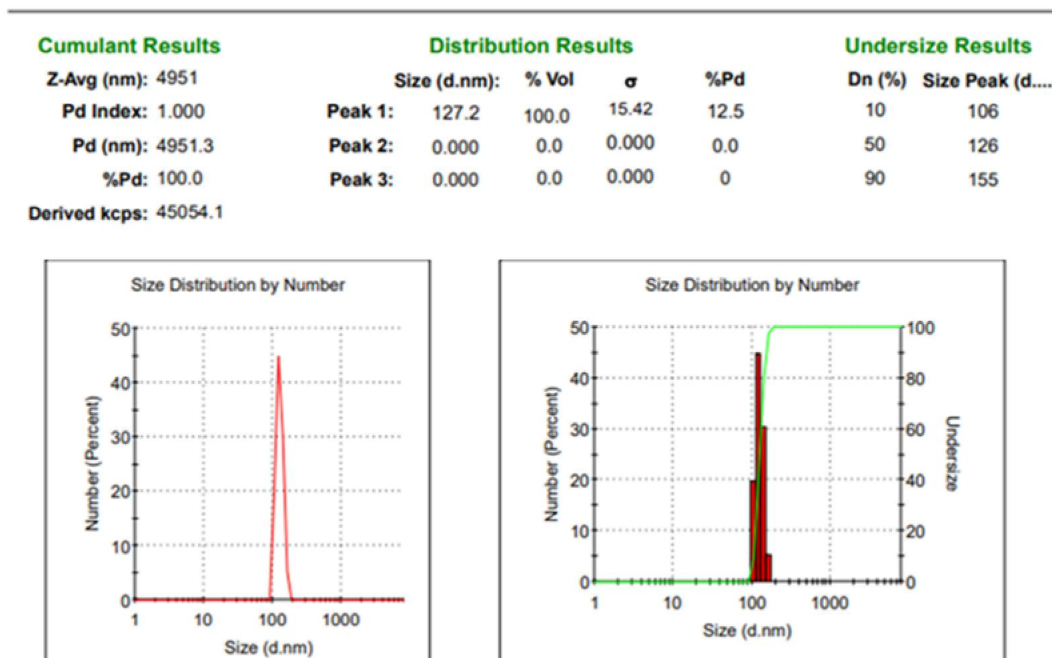


Figure 8. PSA spectrum of nano chitosan 20CB07.

The most commonly used method to produce nano sized chitosan is the ionic gelation method, which is a chemical synthesis of microparticles or nanoparticles based on electrostatic interactions between ions with different charges (Gonçalves et al 2023). This ionic interaction occurs between chitosan as a polycation compound and Na-TPP as a polyanion compound. When contact occurs between them, chitosan can form a gel and cause of the formation of electrostatic interactions between the opposite charges of the components (Patiño-Ruiz et al 2020). There are parameters that influence the formation mechanism of nano chitosan, both surface topology and internal particle structure, which include chitosan molecular weight, DD, concentration, intrinsic viscosity, and -NH_3^+/TPP molar ratio. The properties of nanoparticles can also be influenced by other factors, such as stirring speed, temperature, and flow rate of the added Na-TPP, affecting polydispersity and particle size. The ionic gelation method is a suitable method for synthesizing nano chitosan in an aqueous medium, as it produces particles in the nano scale. This method also requires simple materials and equipment, which are readily available in most laboratories.

Antibacterial activity*.* The results of the antibacterial activity test using the agar diffusion method for nano chitosan against *P. aeruginosa* and *S. aureus* at concentrations of 200, 100, and 50 μg mL^{-1} showed varying levels of effectiveness against these bacterial strains.

The antibacterial assay of fungal nano chitosan against *P. aeruginosa* demonstrated the ability to hinder the growth of these microbes at all tested concentrations (Figure 9). The measured zones of inhibition were each consistent at 20 mm, while the negative control had an inhibition zone of 12 mm (Santoso et al 2020). Chitosan produced a 20-mm inhibition zone. These results indicate that nano chitosan significantly inhibits *P. aeruginosa*. All concentrations showed better antibacterial activities compared to the negative control, suggesting the effectiveness of nano chitosan against *P. aeruginosa*. Furthermore, comparison with chitosan showed similar effectiveness, implying that as a surface coating agent, chitosan can retain its antibacterial properties. The restricted capacity of chitosan macromolecules to spread within the agar hydrogel structure explains this, as chitosan molecules exhibit their effects on microorganisms solely upon direct contact.

For bacterium *S. aureus*, a consistent 8-mm inhibition zone was observed at all tested concentrations (Figure 9). The negative control showed no inhibition (0 mm). Comparison with chitosan displayed a similar 8-mm inhibition zone. These indicate that all tested concentrations of nano chitosan have an inhibitory effect on *S. aureus* growth. The nonappearance of an inhibition zone in the negative control confirms the specific antibacterial activity of nano chitosan against this strain. Comparison with chitosan demonstrates a similar efficacy between the nano and chitosan formulations against both bacterial strains.

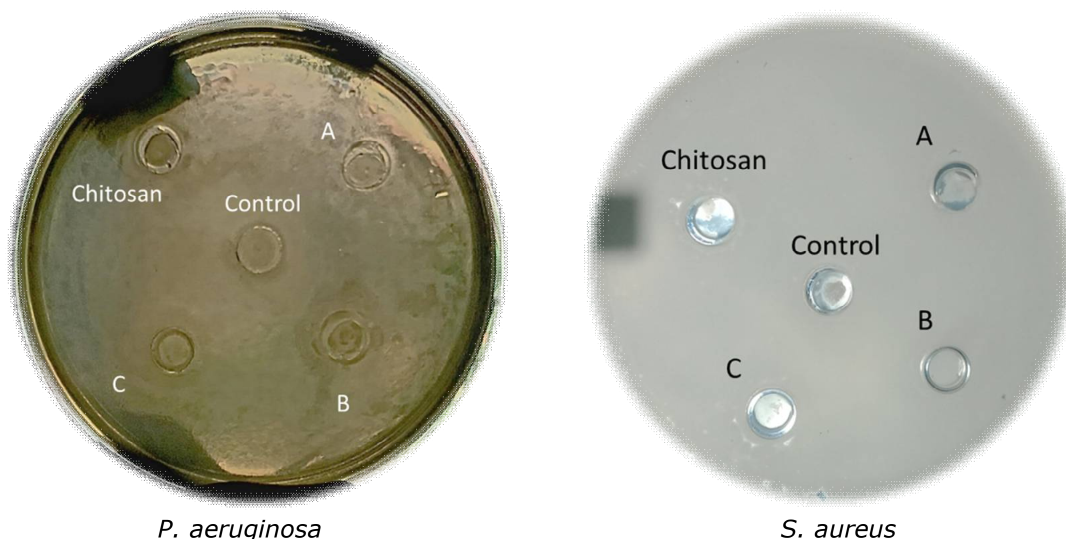
The antibacterial activity of fungal nano chitosan against *P. aeruginosa* and *S. aureus* is attributed to interactions between fungal nano chitosan and bacterial cell components. Chitosan, being a cationic polymer, interacts positively with the microbial surface, causing changes in membrane permeability, inhibiting microbial growth, and leading to microbial death (increasing vulnerability to death) (Frank et al 2020). The interaction between microbial factors and chitosan also depends on the cell wall characteristics, such as hydrophilicity, explaining the differences in microbial susceptibility and the low toxicity of chitosan toward mammalian cells (Ardean et al 2021). The greater antibacterial activity of fungal nano chitosan against *P. aeruginosa* compared to *S. aureus* is likely due to contrasts in their cell wall structures, with the cell wall of *P. aeruginosa* (Gram-negative bacteria) being more susceptible to degradation by fungal nano chitosan compared to that of *S. aureus* (Gram-positive bacteria).

This illustrates that fungal nano-chitosan has a specific ability to target and hinder the growth or eliminate bacteria possessing a Gram-negative cell wall structure. This specificity arises from fundamental disparities in the composition of cell walls between Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess a more intricate cell wall makeup in contrast to Gram-positive ones. Their cell walls comprise a

thin peptidoglycan layer enveloped by an outer membrane containing lipopolysaccharides (LPS) and other intricate molecules. This outer membrane functions as an added barrier, rendering Gram-negative bacteria less susceptible to certain antibiotics and other substances (Tavares et al 2020).

The specificity in combating Gram-negative bacteria yields multiple benefits, notably in precise targeting by specifically addressing unique elements within Gram-negative cell walls, thereby minimizing harm to beneficial bacteria. It lessens additional damage, safeguards Gram-positive bacteria, and maintains the body's normal microbiota, fostering equilibrium and reducing secondary infections (Yao et al 2023). It proves effective against resistant strains by combating Gram-negative bacteria that are resistant to multiple drugs, tackling the challenge posed by treatment-resistant infections. Moreover, it effectively manages particular pathogens, tailored to address infections caused by specific Gram-negative bacteria such as *P. aeruginosa* or *Escherichia coli* (Morris & Cerceo 2020). There's potential for restricting resistance development, focused actions on particular targets might impede the emergence of resistance compared to broad-spectrum antibiotics (Cook & Wright 2022). Additionally, it may result in fewer side effects and lower toxicity due to its precise targeting mechanism.

The specificity towards Gram-negative bacteria holds promise for treating infections caused by these specific pathogens without significantly impacting beneficial or commensal Gram-positive bacteria within the body.



P. aeruginosa *S. aureus*

Figure 9. Results of the antibacterial assays nano chitosan: (A) 50 μg mL^{-1}; (B) 100 μg mL^{-1}; (C) 200 μg mL^{-1}.

Overall, the results of the antibacterial assays nano chitosan against *P. aeruginosa* and *S. aureus* indicate that nano chitosan has the potential as an effective antibacterial agent.

Conclusions. The endophyte of the mangrove fungus *Fusarium* sp. offers an environmentally friendly and sustainable alternative source of chitosan. The results of synthesizing and characterizing nano chitosan from chitosan extracted from *Fusarium* sp. 20CB07 biomass demonstrated antibacterial properties, inhibiting the growth of resistant pathogenic bacteria *P. aeruginosa* and *S. aureus*. This preliminary information serves as a foundational basis for further studies on the development of nano chitosan applications.

Acknowledgements. The authors would like to extend their sincere appreciation to Lampung University for the Postgraduate research funding, grand number no. 846/UN26.21/PN/2023. The authors also thank the Head of the Technical Services Unit, Integrated Laboratory of Innovation and Technology for providing the facilities of the Laboratory. The authors would also like to thank Wiji Rahayu from PT DKSH Indonesia who facilitated the particle size analysis using Mavern Zetasizer Nano ZSP.

Conflict of interest. The authors declare that there is no conflict of interest.

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Received: 16 January 2024. Accepted: 10 February 2024. Published online: 28 February 2024.
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How to cite this article:
Aisah S., Nahrowi R., Setiawan W. A., Setiawan F., Mulyono M., Juliasih N. L. G. R., Hendri J., Yuwono S. D., Setiawan A., 2024 Antibacterial activity of nano chitosan derived from mangrove fungus endophyte, *Fusarium* sp. 20CB07. AACL Bioflux 17(1):449-463.