Volume III, Issue 1, 2023
November 2023

E-ISSN: 2808-5361
http://e-journal.fkmumj.ac.id/

Proceeding The Third Muhammadiyah Internasional-Public Health and Medicine Conference

UTILIZATION OF MANGROVE ENDOPHYTIC FUNGI AS A SUSTAINABLE AND ENVIRONMENTALLY FRIENDLY SOURCE OF CHITOSAN

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ABSTRACT

Chitosan is an important biopolymer that is used as a raw material in several industries, but the manufacturing process has the potential to harm the environment. This study aimed to exploit chitosan in mangrove endophytic fungi and to characterize it. The four fungi isolates used were 20BB0501, 20CB07, 20BA04, and 20BA0502. Fungi were obtained from mangrove plants and identified macroscopically and microscopically using the coverslip method. After performing fungal maintenance on MEA media, fungi cultivation was done on rice media for 14 days. A chitosan extract was made by isolating and extracting the chitosan from the fungal biomass. FTIR was used to characterize the isolated chitosan extract. Macroscopic examinations revealed that each of the four strains had a delicate structure and was white. According to the extraction results, the yield of chitosan from the fungal biomass was 7.5%, 16.7%, 1.6%, and 11%, respectively. According to the FTIR data, showed the degree of deacetylation reached 85.02%, 97.78%, 88.7%, and 85.02%. It can be concluded that mangrove endophytic fungi can be used in the manufacture of chitosan, which produces a high degree of deacetylation using a more environmentally friendly process. The results of this study indicate that marine fungal strains can be used as a source of chitosan. This basis can be developed for the production of bio surfactants, enzymes, and the discovery of new bioactive compounds has pharmacological and biotechnological potential.

Keywords: *Chitosan, Degree of deacetylation, Endophytic fungi, Solid state fermentation.*

ABSTRAK

Kitosan merupakan biopolimer penting yang digunakan sebagai bahan baku di beberapa industri, namun proses pembuatannya berpotensi membahayakan lingkungan. Penelitian ini bertujuan untuk mengeksploitasi kitosan pada fungi endofit mangrove dan mengkarakterisasinya. Empat isolat fungi yang digunakan adalah 20BB0501, 20CB07, 20BA04, dan 20BA0502. Fungi diperoleh dari tanaman mangrove dan diidentifikasi secara makroskopis dan mikroskopis menggunakan metode coverslip. Setelah melakukan pemeliharaan fungi pada media MEA, dilakukan budidaya fungi pada media beras selama 14 hari. Ekstrak kitosan dibuat dengan mengisolasi dan mengekstraksi kitosan dari biomassa fungi. FTIR digunakan untuk mengkarakterisasi ekstrak kitosan hasil isolasi. Pemeriksaan makroskopis menunjukkan bahwa keempat strain tersebut memiliki struktur halus dan berwarna putih. Berdasarkan hasil ekstraksi, rendemen kitosan dari biomassa fungi masing-masing sebesar 7,5%, 16,7%, 1,6%, dan 11%. Berdasarkan data FTIR menunjukkan derajat deasetilasi mencapai 85,02%, 97,78%, 88,7%, dan 85,02%. Dapat disimpulkan bahwa fungi endofit mangrove dapat digunakan dalam pembuatan kitosan yang menghasilkan deasetilasi tingkat tinggi dengan proses yang lebih ramah lingkungan. Hasil penelitian ini menunjukkan bahwa strain fungi laut dapat dimanfaatkan sebagai sumber kitosan. Landasan ini dapat dikembangkan untuk produksi biosurfaktan, enzim, dan penemuan senyawa bioaktif baru yang berpotensi farmakologi dan bioteknologi.

Kata Kunci: *Kitosan, Derajat deasetilasi, Fungi endofit, Fermentasi solid state.*

INTRODUCTION

Chitosan is an environmentally friendly biopolymer and is needed as an industrial raw material. Chitosan is a chitin-derived compound that is widely distributed in nature as a supporting material for the exoskeleton of crustaceans, insect cuticles, fungal cell walls, and algae. Interest in chitosan depends on the myriad of biological and technological benefits demonstrated by this polymer, including antioxidant, antibacterial, body weight reduction, antihyperlipidemic, mineral absorption, anti-inflammatory, antitumor, antimicrobial, and antifungal. [1-2-3-4] In 2019, global market interest in chitosan reached USD 6.8 billion, with a compounded annual growth rate (CAGR) of 24,7% between 2020 and 2027. The increase in chitosan market demand is influenced by the many applications of polymers in several industries. high-value industries such as medical, cosmetic, pharmaceutical, and food. Due to the many benefits it produces, chitosan has been widely studied in several studies such as polymer chemistry, biology, biotechnology, and applications to broaden its applications, such as the study of the mechanisms involved in the activity of chitosan, chitosan derivatives, and in-depth studies of chitosan content in different microorganisms.

Based on previous studies, chitosan was isolated from crustaceans with four main stages: deproteination, demineralization, depigmentation, and deacetylation. However, the acquisition of chitosan from these crustaceans encountered several problems, such as the limited availability of crustaceans, and could only be obtained at certain seasons; the production process took a long time and was high-cost; and chemically, it has the potential to pollute the environment due to the use of large amounts of chemicals such as acids and alkalis at high temperatures (generally the concentration of alkali used ranges from 30–50% at 100˚C). In addition, the resulting chitosan has a high molecular weight, thus limiting its biomedical applications. To overcome these problems, recently, studies on the production of chitosan from fungi have begun to be of interest.

Fungi have cell walls consisting of glycoproteins as structural components (chitin and glucans) and polysaccharides as interstitial components of fungal cell walls (galactose, glucuronoprotein, mannoprotein, and xylomannoprotein). *Aspergillus niger* and *Mucor rouxii* cell walls are composed of 45% chitin. Chitin through the deacetylation process will produce chitosan. The production of chitosan from fungi can produce chitosan with a medium-low molecular weight which is suitable for biomedical applications. Fungal preparations are not limited to certain seasons because they can be obtained from the pharmaceutical and biotechnology industries, and the use of substrates for growth is very economical. In addition, chitosan from fungi is more controlled in terms of low viscosity and shows a high degree of deacetylation.

Early studies of chitosan from fungi mostly used *Mucor rouxii*, a Zygomycota species. Meanwhile, recently, the fungal species that have received much attention as a source of chitosan are Ascomycota (*Aspergillus niger*), Deuteromycota, and Basidiomycota (*Lentinus edodes*), and also from

other Zygomycota species (*Absidia* sp. and *Rhizopus oryzae*).[5] The content of chitin/chitosan in fungal cell walls varies, influenced by species, physical conditions, and growth time. In addition, the key factor that affects the quality and quantity of chitosan is the fermentation condition. Fungal biomass production can be carried out by submerged fermentation (SMF) and solid-state fermentation (SSF). Fungal fermentation directly using the SMF technique can occur more quickly. However, the amount of biomass produced is greater in SSF than in SMF, therefore the production of chitosan with SSF will be higher.[6]

Based on the description above, the isolation of chitosan can be done in a more environmentally friendly way by utilizing marine fungi as a source of chitosan. This study aims to obtain chitosan from fungi through a fermentation process using rice media and to characterize it using Fourier Transform Infrared Spectroscopy (FTIR).

METHOD

In this study, fungi samples were taken from the mangrove area of Sriminosari Village, East Lampung, Lampung Province, Indonesia. Samples of endophytic fungi were taken from the stems and roots of mangrove plants. Sampling was carried out randomly at coordinates 5° 19' 01.0" S and 105° 49'19.8" E.

To identify the morphology of the endophytic mangrove isolate, the isolate was grown on malt extract agar/MEA for 4-6 days at 27°C. The object glass was cleaned with 70% alcohol and heated on a Bunsen flame. Place the deck glass where the isolated fungus grows, then observe it under a microscope lens.[7]

Each strain was cultivated in a 1000 mL Erlenmeyer flask containing 250 g of rice medium and incubated at 27˚C for 14 days. The mycelium is separated by centrifugation (4000 rpm, 20 min). The supernatant was washed with distilled water, dried at 60˚C for 24 hours, separated with rice media, and stored for chitosan extraction.[8]

The dry mycelium was treated with a 5% (v/v) acetic acid solution overnight (1:40 w/v). The filtrate containing chitosan was centrifuged and collected for the next procedure. Furthermore, the chitosan in the filtrate was precipitated using absolute ethanol (1:3, v/v) and neutralized with distilled water. The isolated chitosan was treated with NaOH solution until pH 14 was reached, then rinsed with distilled water to neutralize it before being dried with nitrogen gas for further analysis.

The dried chitosan was characterized using an Agilent Cary 630. The FTIR spectrum of chitosan was scanned in the range of 4000-650 cm^{-1} (resolution 16, 1/cm).

RESULTS AND DISCUSSION

In this study, four strains of fungal were isolated from the roots and stems of mangrove *Avicennia* sp. in Sriminosari Village, which are shown in Table 1. Four fungal strains were studied to

determine their ability to produce chitosan. Fungi are an important part of human life. Fungi can grow in the spaces between plant cells on stems, leaves, roots, and petioles that do not cause disease symptoms in their host plants. These fungi are known as endophytic fungi. The mangrove endophytic fungi have been widely used for the production of bio surfactants, enzymatic potential, and the discovery of new bioactive compounds with potential pharmacological and biotechnological interest.[9-10] Meanwhile, studies on the acquisition of chitosan from endophytic mangrove fungi have not been much studied.

Figure 1. Mangrove (*Avicennia* sp.) of Sriminosari, Lampung, Indonesia

Table 1. Isolate Fungi

No	Sample code	Color	Description
1	20BB0501	white	stem
2	20CB07	white	stem
3	20BA04	white	root
4	20BA0502	white	root

Morphological Identification

Identification of mangrove endophytic fungi was carried out macroscopically and microscopically. Macroscopic identification is carried out with the naked eye or with the help of a magnifying glass on various organs of living things. Macroscopic identity is based on shape, size, color, and surface characteristics. Meanwhile, microscopic identification was carried out by using a microscope to observe the mycelium (hyphae), structure, spores, and morphology. The results of macroscopic observations showed that each fungal isolate had a fine structure and was white (Figure 2), while microscopic observations showed the character of each isolate (Figure 1). Microscopic observation of isolate 20BB0501 revealed hyaline and unbranched hyphae, irregular conidia, cylindrical in size, short, and wide. These characteristics are the characteristics of *Paecilomyces* sp.

reported by Paul et al.[11] Furthermore, isolate 20CB07 showed that macroconidia were cylindrical, slightly curved or straight, and hyaline hyphae were fiber-like and unbranched, which was characteristic of *Fusarium* sp.[12]

Microscopic visualization of isolate 20BA04 showed the presence of branching hyphae forming flat and short conidiophores. Conidia have a two-layer wall; outer electron layer (epispore) and innermost electron layer (conidia). From these observations, it was suspected that isolate 20BA04 was *Trichoderma* sp.[13] Then, isolate 20BA0502 was observed to have hyaline hyphae, having a round, semi-round, or oval shape in its conidia structure. The conidia are attached to the phialides, which are attached to the tip of the conidiophore. The conidiophores are swollen, known as vesicles. Vesicles are characteristic of the genus *Aspergillus* sp.[12] The four strains identified were fungi from the phylum Ascomycota. These results are from the study conducted by Chen et al., which found fungi species Ascomycota from mangrove endophytic fungi.[14]

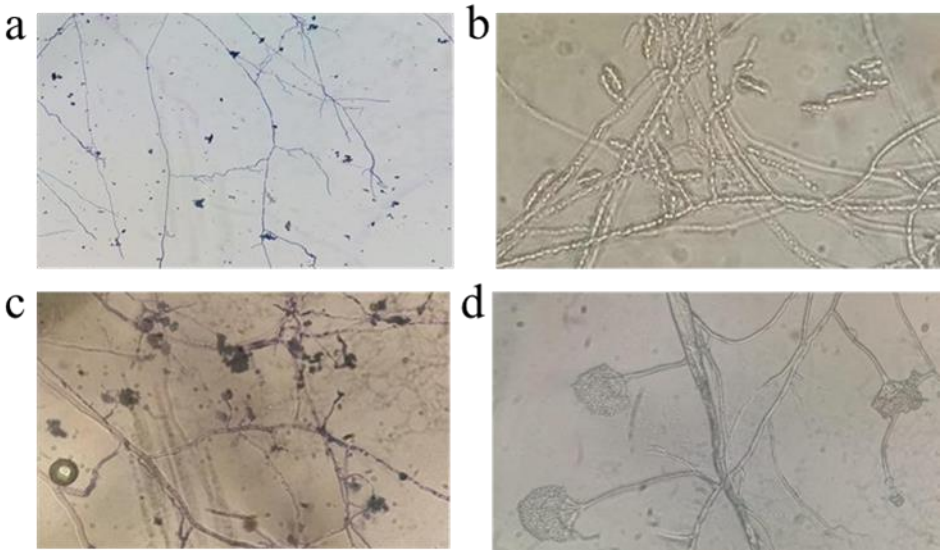

Figure. 2 Visualization of fungi, with light microscopic scale 400×
(a) 20BB0501; (b) 20CB07; (c) 20BA04; (d) 20BA0502

Solid State Fermentation

The results of the cultivation of the four strains by submerged fermentation using rice residue containing 50 g of rice in 200 mL of seawater are shown in Figure 2. The weight of dry crude extract with the highest yield is shown by the 20CB07 strain, which reached 31.3 g, then 20BA0502 at 26.6 g, 20BB0501 at 23.8 g, and the lowest yield was found in 20BA04 (15.7 g). The maximum extract yield was studied after 14 days of incubation, but the observations showed a decrease in mass. This decrease can be caused by the consumption by microorganisms as nutrients and an increase in the concentration of biomass due to the use of enzymes, such as their use in the hydrolysis process; polymer hydrolysis by hydrolytic enzymes.

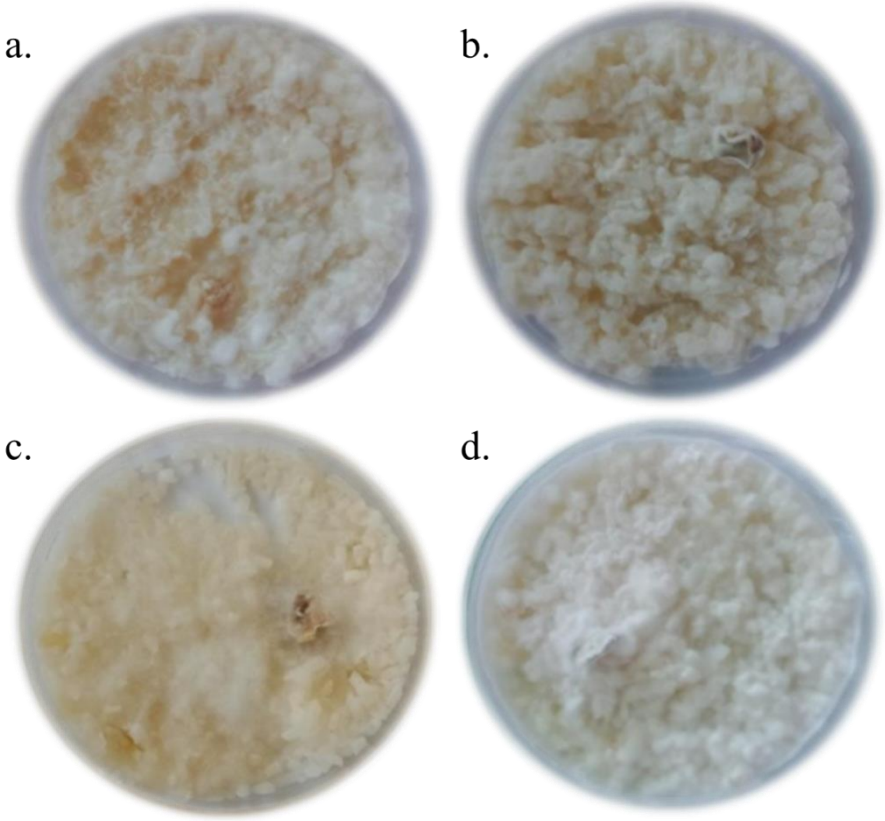

Figure. 3 Isolated fungi submerged cultivation
(a) 20BB0501; (b) 20CB07; (c) 20BA04; (d) 20BA0502

Extraction of Chitin/Chitosan

The results of chitosan extraction from the four strains of fungi showed the highest yield was produced by the 20CB07 strain, with a yield percentage of 16.7%, followed by 20BA0502, which reached 11%, and 20BB0501 had 7.5%. The 20BA04 strain had the lowest yield at 1.6%. The difference in yield percentage results can be caused by the type of fungal isolate. Each fungus has different components that make up its cell wall. According to a study conducted by Sebastian et al., natural chitosan is mostly found in Zygomycota species, while the Basidiomycota, Deuteromycota, and Ascomycota species have not reported the presence of natural chitosan as a component of their cell walls.[15] The four strains of fungi used in this study (20CB07, 20BA0502, 20BB0501, 20BA04), which are suspected to be fungi of the type *Fusarium* sp., *Aspergillus* sp., *Paecilomyces* sp., and *Trichoderma* sp., are fungi of the Ascomycota species. It shows that this study succeeded in finding that Ascomycota species contain chitosan as a component of their cell walls.

Chitosan extraction is divided into several important steps, i.e. treatment with acetic acid, precipitation with ethanol, and the addition of NaOH. The use of acetic acid aims to extract chitosan contained in fungal biomass. In this study, the concentration of acetic acid used was 5%. According to a study conducted by Santoso et al., 5% acetic acid can hydrolyze chitosan to reduce its molecular

weight, thereby increasing the solubility of chitosan in water.[16] This has intensified the application of chitosan as an important biopolymer in pharmacology and biotechnology. The acetic acid-treated chitosan extract was then mixed with absolute ethanol (1:3) to precipitate the extracted chitosan in the filtrate. Chitosan is insoluble in some organic solvents such as alcohol, acetone, dimethyl formamide, and dimethyl sulfoxide, so when chitosan is dissolved in these solvents, precipitation will occur. Next, the chitosan residue was added with NaOH for the deacetylation process. Deacetylation is a process for the removal of acetyl groups. In fungi, there is free chitosan and chitosan obtained from chitin deacetylation. Therefore, deacetylation is very necessary.

Characterization of Chitosan

In this study, the characterization of chitosan and the determination of the degree of deacetylation of chitosan were carried out using FTIR (Figure 4).

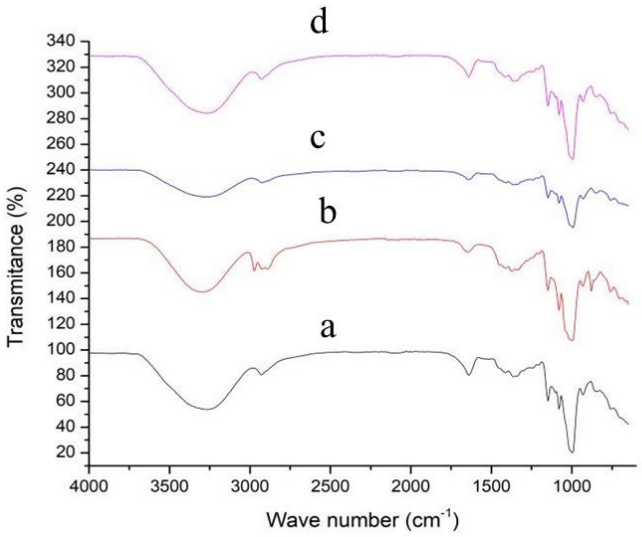

Figure. 4 FTIR Spectrum from different strains (a) 20BB0501; (b) 20CB07; (c) 20BA04; (d) 20BA0502

The spectrum peaks of each sample were compared with the FTIR spectrum of commercial chitosan reported by Drabczyk et al.[17] The results of the comparison show that the chitosan isolated from the four strains showed significant similarity with commercial chitosan. Furthermore, the degree of deacetylation of chitosan was determined by the following formula: [18]

$$DD\% = 100 - \left(\frac{A\ 1655}{A\ 3450} * \frac{100}{1{,}33}\right)$$

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The results of the calculation of the degree of deacetylation of each fungal isolate 20BB0501, 20CB07, 20BA04, and 20BA0502 were 85.02%, 97.78%, 88.7%, and 85.02%. As previously stated, the degree of deacetylation is an important parameter that affects the physicochemical properties of chitosan. The degree of deacetylation is related to the number of pronation groups, thus having an impact on solubility and positive charge. The high solubility and positive charge can increase the

application of chitosan in several fields, such as in the food industry, as an antimicrobial agent, antioxidant, and chelating agent.

CONCLUSION AND SUGGESTIONS

In this study, it was shown that mangrove endophytic fungi can be used in the manufacture of chitosan with a high degree of deacetylation. Furthermore, the manufacture of chitosan from fungi can be carried out with a simple process because the extraction process does not require demineralization treatment, as in the acquisition of chitosan from crustaceans. This can reduce the use of chemicals that have an impact on environmental pollution. Then, the results showed that among the strains isolated from mangrove endophytes, the 20CB07 strain, which is suspected to be a *Fusarium* sp. based on microscopic identification, had the most potential for sustainable chitosan production because it produces the highest biomass, chitosan extract, and degree of deacetylation.

ACKNOWLEDGMENT

The authors would like to thank the 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.

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