Chitosan Synthesis and Optimization of Root Endophytic Fungi

Rida Oktorida Khastini1,2, Aris Munandar3, Indah Juwita Sari1
1Department of Biology Education, Faculty of Teacher Training and Education, University of Sultan Ageng Tirtayasa, Banten Province, Indonesia
2Indonesia Center of Excellence for Food Security (I-CIFORY), University of Sultan Ageng Tirtayasa, Banten Province, Indonesia
3Department of Fisheries, Faculty of Agriculture University of Sultan Ageng Tirtayasa Banten Province, Indonesia

Corresponding author: rida.khastini@untirta.ac.id

Abstract

The cell wall component of endophytic root fungi is the main source of chitosan synthesis. This research aimed to optimize chitosan synthesis from three different isolated species of root endophytic fungi in Pulau Dua Nature Reserve, Banten Indonesia. Three species of root endophytic fungi, namely, Aspergillus niger, Aureobasidium sp., Basipetospora sp., were cultured to produce chitosan. To optimize the production of fungal chitosan, cultures were treated with different conditions such as growth medium (Oat Meal broth, Potato Dextrose Broth, CMMY Broth), pH value (4, 5, 6, 7), and temperature (25, 30, 35, 40°C). Chitosan was extracted from dried mycelium after treated on NaOH 10% at 121°C for 2 min. The degree of deacetylation (DD) of chitosan was then examined and compared with the control (shrimp chitosan). The optimum fungal chitosan condition was higher in CMMY medium, pH 5, and 30°C. The DD value of three root endophytic fungi species, Aspergillus niger, Aureobasidium sp., Basipetospora sp. were 88.5, 83.3, 80.8, respectively, which was lower than DD value resulted from control. This study indicates that Aspergillus niger was a potential endophytic fungi for chitosan synthesis.

Keywords: chitosan; culture condition; endophytic root fungi; optimization

1. Introduction

Root endophytic fungi are a group of fungi that inhabit and colonize root intracellularly or intercellularly roots plant tissues without causing disease symptoms. The fungi’s ability to produce secondary metabolites sometimes related to a host plant growth-promoting effect and plant resistance to biotic and abiotic stress conditions (Pietro-Souza et al., 2019; Farhat et al., 2019). Root endophytic fungi are mainly belonging to the phylum of Ascomycota and Basidiomycota.

In previous research, Aspergillus niger, Aureobasidium sp., Basipetospora sp. are three fungal species that showed inhibition activity on bacterial and fungal growth. These 20 isolates of fungi from Pulau Dua nature reserve which is located on the North Coast of Banten Province. The area dominated with wetland areas which have been designated as conservation of many species of birds and migratory birds. Endophytic fungal cell walls comprise the complex structure of β glucans, chitin, chitosan, and other compounds formed by a network of interconnected molecules consisting of glycoproteins, lipids, and polyphosphates (Ruiz-Herrera & Ortiz-Castellanos, 2019). These components will maintain the shape, strength and structure integrity and will be
determined due to the environmental condition as a protection against high temperatures and cell inhibitors. The cell wall component of endophytic root fungi is the main source of chitosan synthesis. Chitosan is a polycationic polymer of polysaccharide comprised of β-(1-4)-2-acetamido-D-glucose (acetylated unit) and β-(1-4)-2-amino-D-glucosamine deacetylated unit) residues from chitin (Yang et al., 2019). Chitosan exhibits various potential biological activities such as antimicrobial properties antitumor, antioxidant and immune stimulatory (Tan et al., 2018; Younes et al., 2014; Moran et al., 2018). Therefore, chitosan is the potential to be applied in a wide variety of aspects whether the food and nonfood industry such as agriculture, pharmacy, and biotechnology.

Over the last decades, chitosan is mainly produced commercially from marine sources such as shellfish, and shrimp waste using potent alkali treatment with high temperatures for long periods. Many disadvantages found before the methods used (Dhillon et al. 2013). Besides, some inconsistencies influenced in Physico-chemical characteristics caused by protein contamination (Phuong et al., 2017), inconsistent deacetylation degree (Zhang et al., 2017), and high molecular weight (Boudouaia, Bengharez, & Jellali, 2019) also found in this commercial chitosan. Therefore, there has been an interest in better use of chitosan. Alternatively, chitosan can be produced from the fungi. According to Suntornsuk et al. (2002), there are several advantages to provide chitosan from fungal strains e.g. better uniformity in molecular weight, low polydispersity index and degree of deacetylation.

The gap of this research is a little information previously reported regarding chitosan synthesis from fungi, especially from endophytic root fungi. Consequently, information regarding the fungal culture condition and chitosan synthesis methods are valuable. This research aimed to determine and carried out the optimization of chitosan synthesis produce from endophytic fungi species which isolated from Pulau Dua nature reserve. Different types of carbon source, pH and temperature were used as a variable in fungal culture. To our knowledge, this research represents the first report to establish a relationship between culture condition and chitosan yield produced by endophytic root fungi.

2. Material and Method
2.1. Microorganism used

Three endophytic root fungi isolated from Pulau Dua Nature Reserve: Aspergillus niger, Aureobasidium sp., and Basipetospora sp. used for chitosan production. They obtained from the culture collection of the Laboratory of Biology Education, Faculty of Teacher Training and Education, University of Sultan Ageng Tirtayasa. To prepare inoculum, the DSE fungi were grown on Potato Dextrose Agar (PDA) and incubated in 28°C.
2.2. Morphological characterization of root endophytic fungi

Root endophytic fungi were observed through the colony growth on PDA medium macroscopically and microscopically by microscope (Leica DM 500). The macroscopic character found were colony morphology, color, surface, texture, growing area, radial, and concentric lines and the edge of the colony formed. Microscopic observation characters were the presence or absence of septa in hyphae, hypha pigmentation, clamp connection, and asexual spore characteristics.

2.3. Culture condition and optimization

The endophytic root fungi were grown and produce spores in PDA for 7-day in 28°C. The spores were harvested and prepared to 10^6 spores/ml using a hemocytometer. About 10 ml inoculum was inoculated into 240 ml each of Oat Meal broth (grams per liter: oatmeal, 10), Potato Dextrose Broth and CMMY Broth (grams per liter: cornmeal 8.5; malt extract, 10; yeast extract, 2). The cultures then incubated in 28°C under 125 rpm rotary shaker. After incubation time, mycelia were harvested by filtration using filter paper Whatman No.4, washed by distilled water twice. The medium that gives the best yields of chitosan used for pH and temperature treatment. To observe the temperature effect on the chitosan yields on root endophytic fungi, the fungal culture was performed as described previously then incubated at 25, 30, 35, and 40 °C for one week while for pH, the pH of the medium was adjusted into 4, 5, 6 and 7.

2.4. Chitosan extraction and Determination of Deacetylation degree (DD)

Chitosan extraction was carried out by the modification method (Tajdini et al., 2010). Chitosan was extracted from dried mycelium after treated on NaOH 10% at 121°C for 2 min. Alkali insoluble fraction was centrifuged at 10,000 rpm, and then the precipitate was suspended in acetic acid 5% at 100°C for 8h. The centrifugation at 10,000 rpm for 30 min removed the insoluble acid components. The supernatant contained chitosan separated and precipitated by adjusting the pH up to 10 with 2M NaOH, then filtered, washed with distilled water and acetone and dried at room temperature. The degree of deacetylation (DD) of chitosan was determined according to (Yuan, Chesnutt, Haggard, & Bumgardner, 2011). The DD value was compared with the control, chitosan derived from shrimp (Merck)

3. Results and Discussion

Based on observation through the colony form, all three root endophytic fungi isolated from Pulau dua nature reserves showed a similar color, the dark colony. Aspergillus niger showed the fast growth colony compared with the two others. The colonies were round, soft, with flat edges. The colonial growth was almost reaching the edge of the medium plate after 1-week incubation. Under microscope observation, the A. niger showed hyaline hyphae, elongated and unbranched. Conidium was globose with uniseriate conidiophore and ellipsoidal vesicles. Colonies of Aureobasidium sp. was light
brown smooth, spreading, smooth with aerial mycelium often covered with slimy masses of conidia Basipetospora sp. formed flat and brownish colonies. Microscopically the hyphae were hyaline colonies of has branched, and septate hyphae conidiophores have very short branches and solitary conidia (Figure 1).

**FIGURE 1.** The morphological characterization of root endophytic fungi from Pulau Dua nature reserves. a. Aspergillus niger b. Aureobasidium sp. c. Basipetospora sp. scale bar = 10 µm

Aspergillus niger, Aureobasidium sp, Basipetospora sp are confirmed as endophytic root fungi isolated from the root of mangrove plant in Pulau Dua nature reserves. Fungal endophytes are ubiquitous in nature. They can be found colonized every part of the host plant root, leaves, stem, flower, and even the fruit. Species of Aspergillus is considered as endophytic fungi. Recently (Elbaey et al., 2019) successfully isolated this fungus from the mangrove Avicennia marina from the Red Sea, Egypt possessed a new secondary metabolite. ( Parsa et al., 2016) stated that species of Aureobasidium were the dominant endophyte which colonized in the first true leaves of the seedlings of common bean cultivars. Basipetospora sp. has been reported by Greiner et al. (2014), as a tolerate highly unfavorable environmental conditions fungi such as extreme temperatures or osmotic pressures.

In this research, chitosan produced through several steps. The first step is deproteinization for chemical bonds disruption among chitin and protein, carried out using an alkaline reagent, NaOH. It is followed by demineralization using acetic acid. Not all minerals efficiently to be removed. Therefore a larger volume and more concentrated of the acid solution are needed (Younes & Rinaudo, 2015). Endophytic fungi show the distinct feature of a complex growth morphology which can grow as a biomass granule or as a dispersed mycelia granule in the culture medium and this condition will influence the chitosan yield. According to Basu et al. (2015), the preparation of suitable culture media is one of the prerequisites to study on fungal growth since different microorganisms required different environments such as nutrients, pH, and temperature. Based on research result showed that the highest yields of chitosan produced by endophytic root fungi cultured in CMMY medium. The chitosan yields in the different culture medium can be seen in Figure 2. A. niger produced 85 mg. g⁻¹ dry
weight followed by *Aureobasidium* sp. was 66 mg g⁻¹ dry weight and *Basipetospora* sp. was 58 mg g⁻¹ dry weight. Yeast extract is one of the components in CMMY medium supply nitrogen content which increases the synthesis of enzymes involved in chitin biosynthesis (Hamid, R et al., 2013). Root endophytic fungi which were cultured in Oatmeal broth, give the lowest chitosan yields. The oatmeal medium was chosen as a production medium because it is used for long term cultivation and deficiency of essential elements.

![FIGURE 2. Effect of different growth media on chitosan yields by the three root endophytic fungi](image)

The pH of culture influences the transport of the component across the cell membrane and the physiology processes which chitin deacetylases as an enzyme for chitosan production. According to the reported results Zhao et al. (2010), and Grifoll-Romero et al. (2018), the optimum pH of most extracellular chitin deacetylases is neutral or in the alkaline range from 7–12 while most intracellular chitin deacetylases have optimal pH values in the 4.5–6 range depending on the microorganism. In this research, pH ranges from 4-8, and pH 5 gave the maximum yields of fungal chitosan (Figure 3). The result showed on pH 5 provide the highest yields compared to 4, 6, 7 and 8.

![FIGURE 3. Effect of pH on chitosan yields by A. niger and root endophytic fungi](image)
FIGURE 3. Effect of different pH on chitosan yields by the three root endophytic fungi

Temperature is one of the parameters that have to be controlled for maximum fungal cell growth in line with chitosan production. Different temperatures 25°C, 30°C, 35°C, and 40°C gave a different response to the production of chitosan. The yield increased with increasing the temperature from 25°C until 30°C and further decreased at 45°C (Figure 4). The chitosan produced by A. niger showed the highest yield in the 30°C. This in agreement with Mannaa & Kim. (2018), that increasing temperature resulted in more significant effects on fungal populations growth of Aspergillus and showed the optimum condition in 30°C. Higher temperature reducing the hyphal growth of all species which has correlation with the less chitosan yields. High temperature will influence the growth of fungi as it is affects the synthesis and assembly of cell walls (Qiu et al., 2018). Generally, this condition will disrupt the cell wall integrity and for long periods, eliminate the fungi.

FIGURE 4. Effect of different temperature on chitosan yields by the three root endophytic fungi

The degree of deacetylation (DD) is one of the essential parameters for producing chitosan. The degree of deacetylation is an important parameter affecting the physicochemical properties of chitosan. The high DD value of chitosan indicated on excellent quality to be applied in the industry as a coagulating or chelating agent, a clarifying agent or an antimicrobial agent Table 1 showed the DD on fungal chitosan produced from root endophytic fungi compared with the control, chitosan produced from shrimp.
In general, the N-deacetylation degree of chitosan between 55% and 70% is called a low degree of deacetylation of chitosan; the 70%–85% is the medium; the 85%–95% is high, and the 95%–100% is the ultra-high chitosan (He et al., 2016). Based on the result, the DD value of those three species was lower than the control and categorized as medium-high. Even though the DD value lower from the control, the chitosan produced from three species of endophytic root fungi isolated from Pulau Dua Nature still have DD value above 80%. Related with the solubility factor, chitosan with lower than 80% DD does not completely solubilize in weak organic acid, an essential characteristic of chitosan (Yusharani et al., 2019). The degree of Deacetylation result from this research was slightly different from the reported percentage degrees of deacetylation of chitosan from several species of fungi fungal mycelia of 84–90% conducted by (Pochanavanich & Suntornsuk, 2002)

Conclusion

This present study concluded that among the root endophytic fungi isolated from Pulau Dua Nature reserves, A. niger was considered as a potential candidate for the chitosan production. the optimum condition to produce a high yield of chitosan was using CMMY medium, pH 5 and temperature 30\(^\circ\)C

ACKNOWLEDGMENTS

The authors are thankful to the Indonesian Ministry of Higher Education, Research, and Technology for the financial grant (No. B/19/UN43.9/PT1.3/2019) to carry out this research. The authors would also like to acknowledge all of the students of Biology Education Department of Sultan Ageng Tirtayasa University who contributed to the study in various ways

References

Basu, S., Bose, C., Ojha, N., Das, N., Das, J., Pal, M., & Khurana, S. (2015). Evolution of bacterial and fungal growth media. Bioinformation, 11(4), 182-184. https://doi.org/10.6026/97320630011182
Boudouaia, N., Bengharez, Z., & Jellali, S. (2019). Preparation and characterization of chitosan extracted from shrimp shells waste and chitosan film: application for Eriochrome black T removal from aqueous solutions. *Applied Water Science, 9*(4), 1–12. https://doi.org/10.1007/s13201-019-0967-z

Dhillon, G. S., Kaur, S., Brar, S. K., & Verma, M. (2013). Green synthesis approach: Extraction of chitosan from fungus mycelia. *Critical Reviews in Biotechnology, 33*(4), 379–403. https://doi.org/10.3109/07388551.2012.717217

Elsbaey, M., Tanaka, C., & Miyamoto, T. (2019). New secondary metabolites from the mangrove endophytic fungus Aspergillus versicolor. *Phytochemistry Letters, 32*(February), 70–76. https://doi.org/10.1016/j.phytol.2019.04.023

Farhat, H., Urooj, F., Tariq, A., Sultana, V., Ansari, M., Ahmad, V. U., & Ehteshamul-Haque, S. (2019). Evaluation of antimicrobial potential of endophytic fungi associated with healthy plants and characterization of compounds produced by endophytic Cephalosporium and Fusarium solani. *Biocatalysis and Agricultural Biotechnology, 18*(November 2018), 101043. https://doi.org/10.1016/j.bcab.2019.101043

Greiner, K., Peršoh, D., Weig, A., & Rambold, G. (2014). *Phialosimplex salinarum*, a new species of Eurotiomycetes from a hypersaline habitat. *IMA Fungus, 5*(2), 161–172. https://doi.org/10.5598/imafungus.2014.05.02.01

Grifoll-Romero, L., Pascual, S., Aragunde, H., Biarnés, X., & Planas, A. (2018). Chitin deacetylases: Structures, specificities, and biotech applications. *Polymers, 10*(4), 1–29. https://doi.org/10.3390/polym10040352

Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., & Javed, S. (2013). Chitinases: An update. *Journal of pharmacy & bioallied sciences, 5*(1), 21–29. https://doi.org/10.4103/0975-7406.106559

He, X., Li, K., Xing, R., Liu, S., Hu, L., & Li, P. (2016). The production of fully deacetylated chitosan by compression method. *Egyptian Journal of Aquatic Research, 42*(1), 75–81. https://doi.org/10.1016/j.ejar.2015.09.003

Mannaa, M., & Kim, K. D. (2018). Effect of temperature and relative humidity on growth of Aspergillus and Penicillium spp. and biocontrol activity of Pseudomonas protegens AS15 against Aflatoxicgenic Aspergillus flavus in stored rice grains. *Mycobiology, 46*(3), 287–295. https://doi.org/10.1080/12298093.2018.1505247

Moran, H. B. T., Turley, J. L., Andersson, M., & Lavelle, E. C. (2018). Immunomodulatory properties of chitosan polymers. *Biomaterials, 184*(5), 1–9. https://doi.org/10.1016/j.biomaterials.2018.08.054
Parsa, S., García-Lemos, A. M., Castillo, K., Ortiz, V., López-Lavalle, L. A. B., Braun, J., & Vega, F. E. (2016). Fungal endophytes in germinated seeds of the common bean, Phaseolus vulgaris. *Fungal Biology, 120*(5), 783–790. https://doi.org/10.1016/j.funbio.2016.01.017

Phuong, P. T. D., Minh, N. C., Cuong, H. N., Van Minh, N., Han, N. T., Van Hoa, N., Trung, T. S. (2017). Recovery of protein hydrolysate and chitosan from black tiger shrimp (Penaeus monodon) heads: approaching a zero waste process. *Journal of Food Science and Technology, 54*(7), 1850–1856. https://doi.org/10.1007/s13197-017-2616-6

Pietro-Souza, William & Pereira, Felipe & Mello, Ivani & Stachack, Fernando & Terezo, Ailton & Cunha, Cátia & White, James & Soares, Marcos. (2019). Mercury resistance and bioremediation mediated by endophytic fungi. *Chemosphere, 240*. 124874. https://doi.org/10.1016/j.chemosphere.2019.124874

Pochanavanich, P., & Suntornsuk, W. (2002). Fungal chitosan production and its characterization. *Letters in Applied Microbiology, 35*(1), 17–21. https://doi.org/10.1046/j.1472-765X.2002.01118.x

Qiu, Z., Wu, X., Gao, W., Zhang, J., & Huang, C. (2018). High temperature induced disruption of the cell wall integrity and structure in Pleurotus ostreatus mycelia. *Applied Microbiology and Biotechnology, 102*(15), 6627–6636. https://doi.org/10.1007/s00253-018-9090-6

Ruiz-Herrera, J., & Ortiz-Castellanos, L. (2019). Cell wall glucans of fungi. A review. *The Cell Surface, 5*, 100022. https://doi.org/10.1016/j.tcsw.2019.100022

Suntornsuk, W., Pochanavanich, P., & Suntornsuk, L. (2002). Fungal chitosan production on food processing by-products. *Process Biochemistry, 37*(7), 727–729. https://doi.org/10.1016/S0032-9592(01)00265-5

Tajdini, F., Amini, M. A., Nafissi-Varcheh, N., & Faramarzi, M. A. (2010). Production, physiochemical and antimicrobial properties of fungal chitosan from Rhizomucor miehei and Mucor racemosus. *International Journal of Biological Macromolecules, 47*(2), 180–183. https://doi.org/10.1016/j.ijbiomac.2010.05.002

Tan, Y., Leonhard, M., Ma, S., Moser, D., & Schneider-Stickler, B. (2018). Efficacy of carboxymethyl chitosan against Candida tropicalis and Staphylococcus epidermidis monomicrobial and polymicrobial biofilms. *International Journal of Biological Macromolecules, 110*, 150–156. https://doi.org/10.1016/j.ijbiomac.2017.08.094

Yang, Y., Xing, R., Liu, S., Qin, Y., Li, K., Yu, H., & Li, P. (2019). of. *Carbohydrate Polymers, 115*423. https://doi.org/10.1016/j.carbpol.2019.115423
Younes, I., Hajji, S., Frachet, V., Rinaudo, M., Jellouli, K., & Nasri, M. (2014). Chitin extraction from shrimp shell using enzymatic treatment. Antitumor, antioxidant and antimicrobial activities of chitosan. *International Journal of Biological Macromolecules, 69*, 489–498. https://doi.org/10.1016/j.ijbiomac.2014.06.013

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs, 13*(3), 1133–1174. https://doi.org/10.3390/md13031133

Yuan, Y., Chesnutt, B. M., Haggard, W. O., & Bumgardner, J. D. (2011). Deacetylation of chitosan: Material characterization and in vitro evaluation via albumin adsorption and pre-osteoblastic cell cultures. *Materials, 4*(8), 1399–1416. https://doi.org/10.3390/ma4081399

Yusharani, M. S., Stenley, Harmami, Ulfin, I., Suprapto, S., & Ni’mah, Y. L. (2019). Synthesis of water-soluble chitosan from squid pens waste for capsule shell materials. *Journal of Renewable Materials, 7*(7), 643–653. https://doi.org/10.32604/jrm.2019.04185

Zhang, H., Yun, S., Song, L., Zhang, Y., & Zhao, Y. (2017). The preparation and characterization of chitin and chitosan under large-scale submerged fermentation level using shrimp by-products as substrate. *International Journal of Biological Macromolecules, 96*(10), 334–339. https://doi.org/10.1016/j.ijbiomac.2016.12.017

Zhao, Y., Park, R. D., & Muzzarelli, R. A. (2010). Chitin deacetylases: properties and applications. *Marine drugs, 8*(1), 24–46. https://doi.org/10.3390/md8010024