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Research Article

Chitosan Synthesis and Optimization of Root Endophytic Fungi

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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^{o}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^{0}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^{0}C under 125 rpm rotary shaker. After incubation time, mycelia were harvested by filtration using filter paper Whatman No.4, washed by distillated 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^{o}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^{o}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).

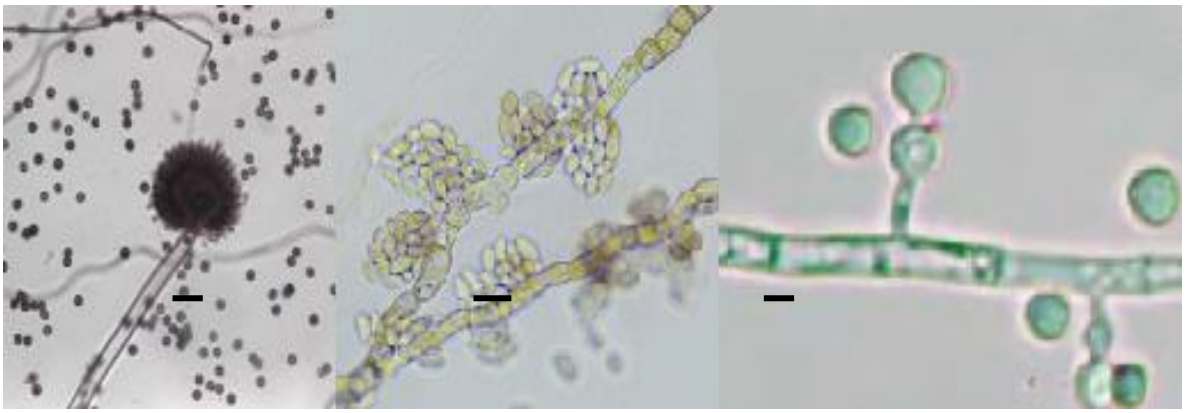

FIGURE 1. The morphological charachterization of root endophtic fungi from Pulau Dua nature reserves. a. *Aspergilus niger* b. *Aureobasidium* sp. c. *Basipetospora* sp. scale bar = 10 µm

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 (Elsbaey 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.

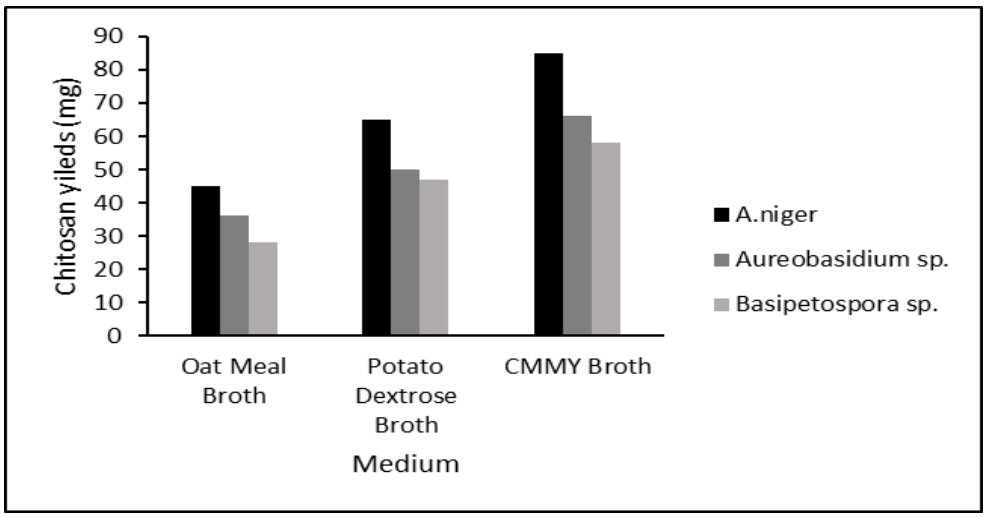

FIGURE 2. Effect of different growth media on chitosan yields by the three root endophytic fungi

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.

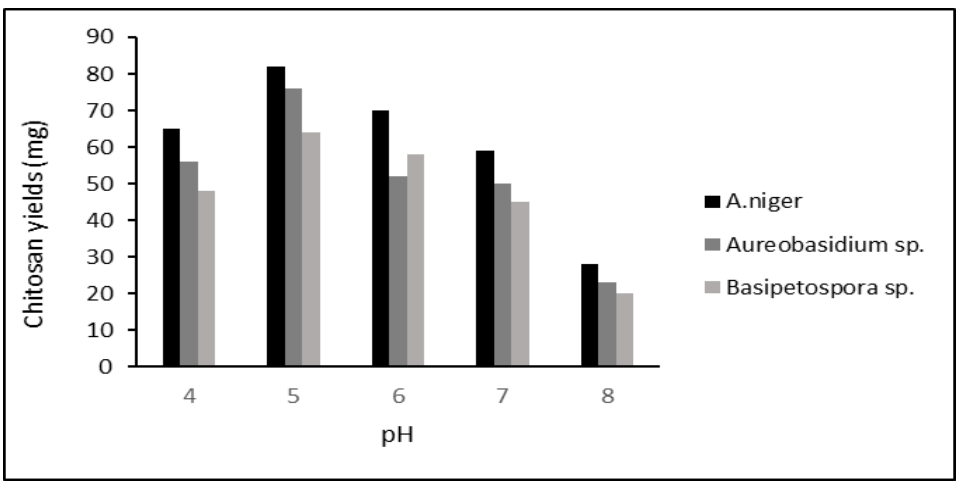

FIGURE 3. Effect of different pH on chitosan yields by the three root endophytic fungi

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 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.

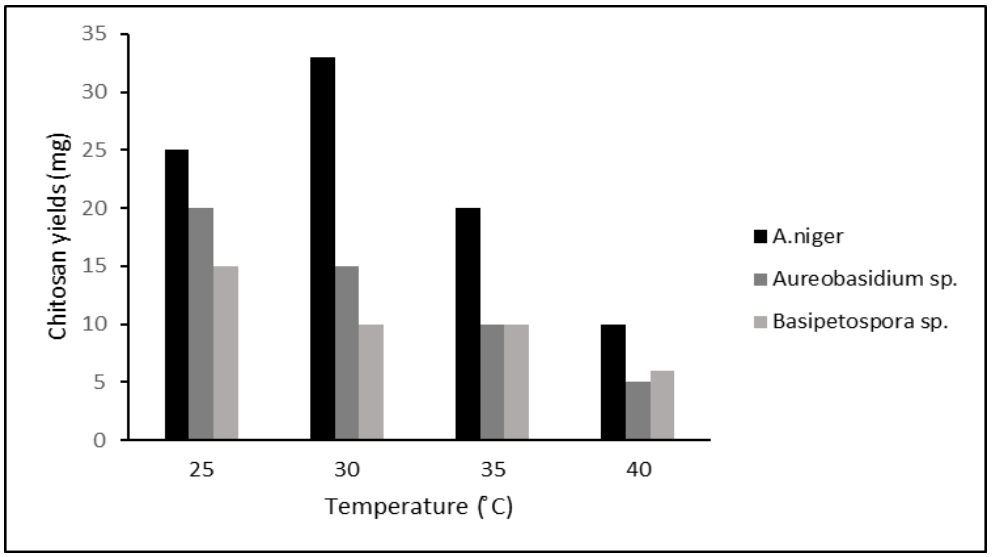

FIGURE 4. Effect of different temperature on chitosan yields by the three root endophytic fungi

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.

TABLE 1. Degree of Deacetylation of fungal chitosan's

Source of Chitosan	Degree of Deacetylation (%)
Shrimp	90
A. niger	88,5
Aureobasidium	83,3
Basipetospora	80,8

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^{o}C

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