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# Preparation of Chitosan Material and its Antifungal Activity for Bamboo

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**Abstract:** ***The main aim of present study was to prepare chitosan nanoparticle, chitosan-ZnO nanoparticle and chitosan montmorilonite to evaluate their in vitro antifungal activities.The antifungal properties of nanoparticles against phytopathogenic fungi Trametes versicolor and Tyromyces palustris were investigated at various concentrations 0.01 and 0.1%. Among the various formulations of nanoparticles, chitosan-ZnO nanoparticles were found most effective at 0.1% concentration and showed 89.5 and 63.0 % growth inhibition of Trametes versicolor and Tyromyces palustris, respectively in in vitro model. At the same concentration, chitosan-ZnO nanoparticles also showed maximum of 87.4% inhibition rate of spore Trametes versicolor. Chitosan nanoparticles showed the maximum growth inhibitory effects 84.3% on in vitro mycelial growth of Trametes versicolor at 0.1% concentration, and 85.8% growth inhibition of Tyromyces palustris at 0.1%. From our study it is evident that chitosan material particularly chitosan nanoparticle and chitosan-ZnO nanoparticles have tremendous potential for further field screening towards crop protection.***

**Keywords:** chitosan materials, antifungal,Trametes versicolor, Tyromyces palustris

## 1. Introduction

Chitosan has emerged not only as a promising and economic source for efficient and versatile antimicrobial material but also as a biocompatible and biodegradable polymer with various applications(Geisberger et al, 2013). It has been extensively used as edible coatings to preserve the quality of many foods (Huang et al, 2012) and as a bioactive fungicide(EI Ghaouth et al, 1992). Studies have concluded that chitosan possesses antifungal activity via affinity of its cationic amino groups to cellular components.

The antifungal activity of chitosan has been reported and developed in several studies both in invitro and invivo, although chitosan activity against fungus has been shown to be less efficient as compared with its activity against bacteria (Tsai et al., 2000). The inhibitorefficiency of chitosan has been related to chitosan properties such as deacetylation degree (DD) and molecular weight (Mw). Islem et al(2014) prepared fifteen homogeneous chitosans with different acetylation degrees (DA) and molecular weights (MW). They were tested at different pH values for their antifungi activities against three fungi (Aspergillus niger, Fusarium oxysporum and Alternaria solani). Chitosans markedly inhibited growth of fungi tested, although the inhibitory effect depends on the type of microorganism and on the chitosan characteristics (DA and MW) with minimum inhibitory concentrations in the range of 0.001 to 0.1 w%.For that purpose, it was examined antimicrobial activity of fifteen different chitosans perfectly soluble in acidic medium and prepared under homogeneous conditions against four Gram-negative bacteria, four Gram positive bacteria and three fungi.

Nevertheless, the bulk chitosan biopolymer has not been widely applied as antifungal agent mainly because of its insolubility in aqueous media and lower antifungal activity. To improve the antifungi activity of chitosan, researchers have prepared many derivatives which showed higher antifungi activities than chitosan. Rongchun et al (2010) describe here the preparation of three novel derivatives of chitosan including the above active groups, 2-pyridylacetyl chitosan chloride (PACS), 2-[4-(5-chloro-2-hydroxybenzylideneamino)-pyridyl] acetyl chitosan chloride(CHPACS), and 2-[4-(5-bromo-2hydroxybenzylideneamino-pyridylacetyl chitosan chloride (BHPACS), as well as their antifungal activity against four plant threatening pathogenic fungi Cladosporium cucumerinum, Monilinia fructicola, Colletotrichum lagenarium, and Fusarium oxysporum.. Miao et al (2014) reported that chitosan derivative coating was effective in reducing the decay of green asparagus caused by F. concentricum. As tested in vitro, L-chitosan and H-chitosan inhibited the radial growth of F. concentricum, with a remarkable effect at a concentration of 4 mg/ml, and totally inhibited spore germination at a concentration of 0.05 mg/ml, indicating that chitosan derivatives were either fungistatic or fungicidal.

Besides, the application of chitosan is limited for its poor solubility in water or high pH region. Therefore, taking low molecular weight chitosan with high solubility and low viscosity in water at physiologically acceptable pH values as starting material can enlarge the scope of application. Sudipta et al, (2014) study was to evaluate antifungal effect of water-soluble chitosan (s-chitosan) on Macrophomina phaseolina (M. phaseolina) causing jute seedling infection and monitor the change in activity of released enzymes during infection . It was observed thats chitosan enhanced the activity of defense related enzymes like chitosanase and peroxidase in the seedlings during infection by M. phaseolina.

Nanotechnology offers great opportunities for new antifungal additives with enhanced properties compared to conventional ones. Specific characteristics of nano-metals include high surface to volume ratio, homogeneous particles size distribution possibility of facile surface medication, good stability and the ease of preparation. These unique properties offer nano-metals great application in many fields. For example, nano-metals (e.g. silver, copper and zinc) has been added to improve fungal(*Aspergillus*

*brasiliensis* or Penicillium funiculosum) growth resistance of building materials(Hsiao et al, 2015). Sathiyanarayanan and Muthukrishnan(2014) studied glucan nanoparticles inhibited the growth of P. aphanidermatum and suggest the use of glucan nanoparticles in prevention of diseases caused by this phytopathogenic fungus. Fabrice et al (2013) studied effect between chitosan nanogels and copper in inhibiting Fusarium graminearum growth.

Vinod et al (2015) were synthesized and evaluated Cu–chitosan nanoparticles for their growth promotory and antifungal efficacy in tomato (*Solanum lycopersicum Mill*). In pot experiments, 0.12% concentration of Cu–chitosan nanoparticles was found most effective in percentage efficiancy of disease control (PEDC) in tomato plants with the values of 87.7% in early blight and 61.1% in Fusarium wilt.

The objective of this work investigation, chitosan materials viz chitosan nanoparticle, and chitosan-ZnO nanoparticle and chitosan-montmorilonite which were further examined against phytopathogenic fungi viz.

## 2. Materials and Methods

### 2.1 Chemicals

Sodium tripolyphosphate, sodium hydroxide, was supllied by Sigma Co and used as received. Glacial acetic acid and hydrochloric acid were of analytical grade, Chitosan DD 90 was purchased from Material Science Research Centre, BATAN, Jakarta and montmorilonite from Laboratory Petrologi, Trisakti University chemistry laboratory, State University of Jakarta).

Fungal strains Trametes versicolor and Tyromyces palustris Were obtained from Laboratory of Micro- biology,State University of Jakarta,Indonesia and used for antimicrobialassays.

### 2.2 Preparation Chitosan Nanoparticle(ChNp)

ChNp was prepared by the ionic gelation process based on the report by Calvo et al (1997), with severalmodifications. The chitosan solutions with mass concentrations 2.5 g/L were prepared by dissolving purified chitosanin 1% (w/w) acetic acid 2% solution with sonication at 800 W for 15 min.The pH of chitosan solutions was adjusted to 4.5 with NaOH 1.0 M solution . The chitosan NP suspension was spontaneously formed by adding dropwise the 50 mL sodium tripolyphosphate 1.5 g/L solution into the 150 mL chitosan solution under a magnetic stir of 250 rpm at room temperature for 60 min.centrifuged at 10,000 × gfor 30 min at room temperature in order to separate the nanoparticles from large particles or aggregates or freeze dried to obtain powder samples.. After centrifugation sediment and supernatantwere separated by carefully removing the supernatant layer.

### 2.3 Preparation chitosan/ZnO nanoparticle

The appropriate amount of 1.0 g ZnO powder was dissolved in 100 mL of 1% (v/v) acetic acid and changed to zinc cations. Then 1.0 g of chitosan was added to the above solution. The mixture was sonicated for 30 min after magnetic stirring and then the acidity adjusted by 0.1 M NaOH solution (pH 4.5). The clear sol was obtained after keeping overnight at room temperature. Afterwards, the resulting sol was added to 0.1 M NaOH solution,stirrer for 12 hr at room temperature. Then the color became light white. Chitosan/ZnO nanoparticles were obtained through sufficiently washing with deionized water and dried in an infrared oven.

### 2.4 Preparation of chitosan-montmorilonite (Chi-MMT)

Chitosan solution was prepared by dissolving 4 g of chitosan in 196 mL of 2% v/v acetic acid. The pH of chitosan solution was adjusted to 4.9 with 1 M NaOH. The solution was left overnightbefore was slowly added into a suspension bath containing100ml montmorilonite 2.50% (w/v).The mixture was treated at $60^0$C for 24 hr. The chitosan intercalated MMT (Chi-MMT)was washed with distilled water until the pH of the washed water became neutral. Chi-MMT was separated from water by centrifugation at 3500 rpm for 10 min and then dried at 60°C for 48 hr.

### 2.5 Antifungal activities

Different concentrations (0.01 and0.1%, w/v) of various nanoparticles in aqueous solution were used in antifungal activity test against twofungus species Trametes versicolor (L: Fr.), Fomitopsis palustris.Potato dextrose agar medium was prepared and poured in Petri dishes(90 mm × 15 mm), with above mentioned percentages of various nanoparticles, separately. Mycelial bit fromperipheral end of uniform size (diameter, 5.0 mm) was taken from7 days old culture of test pathogens and placed in the centre of test Petri dishes. All the Petri dishes were incubated at 28 ± 1◦C for 7days and the observation of radial mycelial growth was recorded when controlled Petri dish cover full growth (90 mm). All the treat-ments consisted of three replications and experiment was repeated twice. The inoculated plates were compared with control (without nanoparticles) to calculate the % inhibition rate of mycelia of the pathogen by using the formula given by Vincent.

$$\% \text{ Inhibition rate} = \frac{Mc - Mt}{Mc} \times 100\%$$

where Mc is the mycelial growth in control, Mt is the mycelial growth in treatment

### 2.6 Spore germination method

The antifungal activities of nanoparticles (0.001, 0.005, 0.01,0.02, 0.06 and 0.1%, w/v) on spore germination of Trametes versicolor were tested. Spore suspension ($1.0x10^3$ spores/ml) of Trametes versicolor prepared aseptically from 7 days old pure culture; 50 µl of spore suspension and 50 µl of nanoparticle at above mentioned concentrations in aqueous were taken on glass slides in 10 replicates. All the treatments were maintainedat 28 ± 1◦C for 8 h and the observations
were made under microscope to calculate the % inhibition rate by counting the number of spore germinated compared to control.

$$\% \text{ Inhibition rate} = \frac{Gc - Gt}{Gc} \times 100\%$$

where Gc is the germination in control and Gt is the germination intreatment.

### 2.7 Decay test procedure for bamboo

The treated bamboo specimens, beech (Fagus orientalis L.) was tested for decay resistance according to Indonesian National standard (SNI). Brown-rot fungi, Trametes versicolor (L: Fr.),and white-rot fungi, Fomitopsis palustris, were grown in 250 ml glass bottles to establish an active mycelium. The treated and untreated bamboo were sterilised in an autoclave for 20 min, and then bamboo blocks were placed in bottles containing actively growing cultures of either Fomitopsis palustris or Trametes versicolor. Five repetitions were collected for each test group. The bottles were incubated for 12 weeks at $25^0$ C and 70% relative humidity.

After incubation, the test blocks were withdrawn from the culture bottles. The mycelium was carefully brushed off of the blocks. The bamboo blocks were then dried for 24 h at room temperature and stored at $105^0$ C overnight. After oven-drying, the final weight of each bamboo block was acquired. The weight loss due to decay was calculated as a percentage determined using the dry weight loss of each bamboo block before and after incubation.

## 3. Results and Discussion

### 3.1 FTIR analysis of ChNp

Fig. 1 shows the FTIR spectra of ChNP, in the wave number range of 4000–600 $cm^{-1}$. For pure chitosan. the characteristic bands due to the stretching vibration of –NH2 and –OH groups were observed at 3356 $cm^{-1}$. The feature peak at 1547 $cm^{-1}$ for amide II (N–H bending vibration) and the small shoulder peak at 1647 $cm^{-1}$ for amide I (–CO stretching vibration) indicate the high degree of deacetylation of chitosan. The flattening of the amine peak at 3356 $cm^{-1}$ in Fig1. indicates that majority of amino group of chitosan participated in the electrostatic interaction with TPP(data not show).

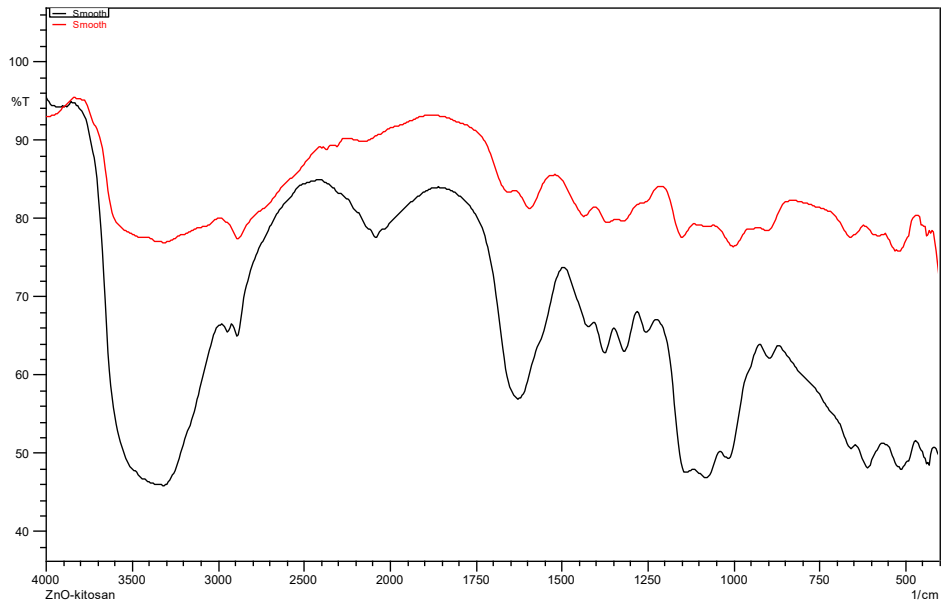
### 3.2 Characterization of chitosan/ZnO nanoparticle

FTIR analysis was performed to confirm the interaction of chitosan, ZnO-chitosan.Bare chitosan was characterizedwith some specific peaks located at 1643, 903 and 3308,8 $cm^{-1}$which related to amide ( $CONH_2$), anhydro glucosidic ring and primary amine ( $NH_2$), respectively.

The main characteristic peaks of CS–ZnO at 3333,14 $cm^{-1}$ signify the stretching modes of $NH_2$ and OH group. The peaks at 2978 and 2885 $cm^{-1}$ denote the stretching modes of asymmetric stretching of ($CH_3$) and ($CH_2$) in the CS network while those at 1625.10 and 1317.44 $cm^{-1}$ were attributed to CQO stretching vibration and scissoring vibration of –NH2. In addition, a broad absorption peak at the range of 580–400 $cm^{-1}$ was ascribed to the vibration of the O–Zn–O group.While the spectrum of chitosan(Fig.3) showed peaks at 3000-3750 $cm^{-1}$ due to the overlapping of O–H and N–H stretching bands, 2920 $cm^{-1}$ for aliphatic C–H stretching, 1634 and 1594 $cm^{-1}$ for N–H bending,1420 and 1382 $cm^{-1}$ for C=O bending, 1151 and 1079 $cm^{-1}$ for C–O stretching.

### 3.3 FTIR analysis of ChMmt

The spectrum of the Chi-MMT shows the combination of characteristic absorptions due to the chitosan and MMT groups. The peak at 1594 $cm^{-1}$ of the $–NH_2$ group in the starting chitosan was shifted to 1520 $cm^{-1.}$ in the Chi-MMT spectrum, corresponding to the deformation vibration of the protonated amine group ($–NH_3^+$) of chitosan. This ($–NH_3^+$) group interactswith the negatively charged sites of MMT.



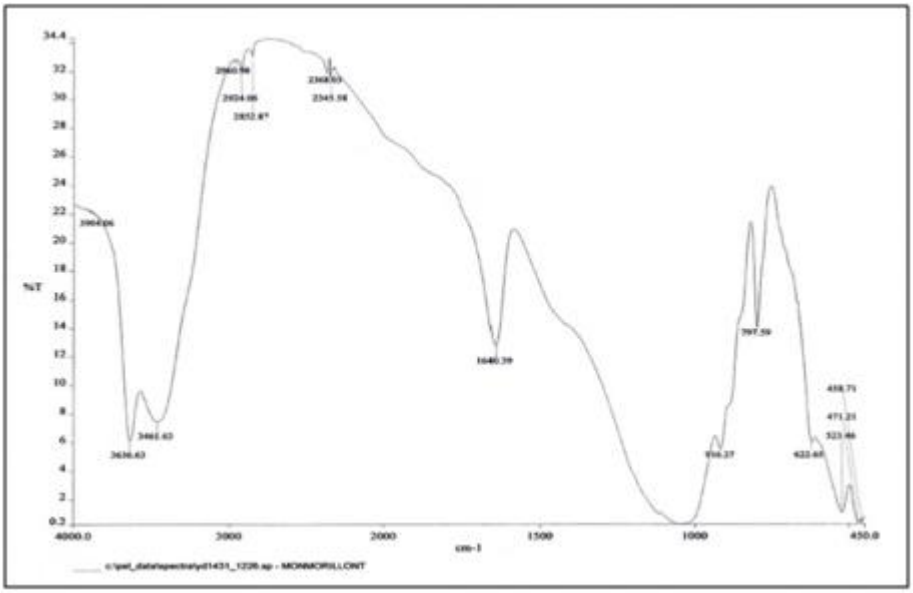


**Figure 1:** FTIR of (a) chitosan and (b) chitosan/ZnO nanoparticle

Invitro mycelial growth of Trametes versicolor was comprehensively controlled by 0.01 and 0.1% concentrations of all the chitosan. A maximum 89.5% inhibition rate was recorded at 0.1% concentration of chitosan-ZnO nanoparticle followed by 84.3% at 0.1% chitosan nanoparticle. In the case of chitosan montmorilonite, 81.6% of mycelial growth was inhibited at 0.1% concentration

A maximum 87.4% spore germination was inhibited by 0.1% concentration of chitosan-ZnO nanoparticles followed by chitosan (86.4%) at 0.1%. Taken as a whole, all the examined nanoparticles were found effective in controlling spore germination ofTrametes versicolor. Bulk chitosan nanoparticle, chitosan-ZnO nanoparticle at 0.1% level were found less effective for inhibition of mycelial growth and spore germination

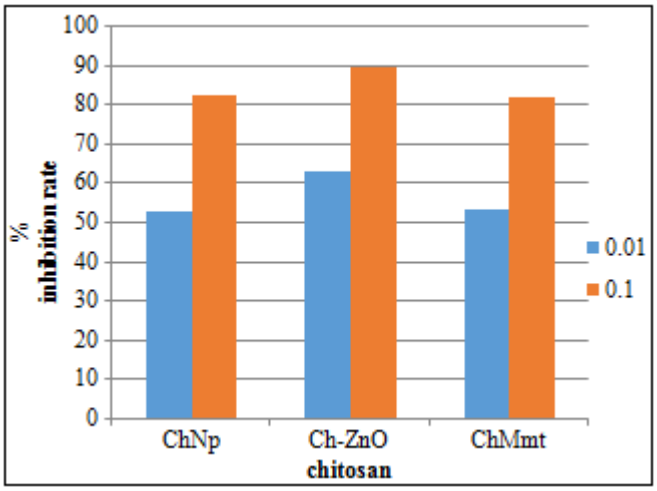



**Figure 4:** Effect of various chitosan and concentration on in vitro mycelial growth of Trametes versicolor

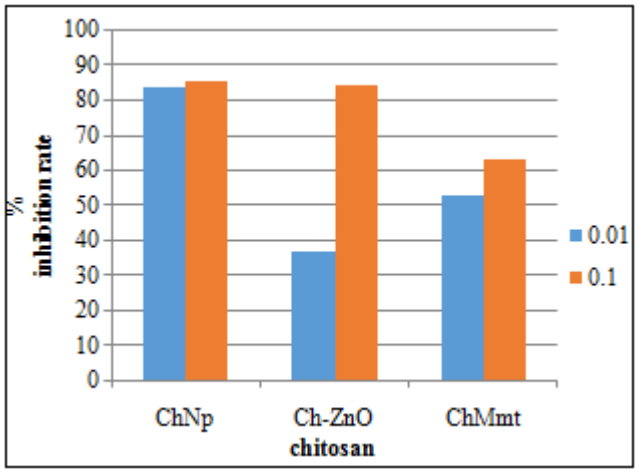



**Figure 5:** Effect of various chitosan and concntration on in vitro mycelial growth of Fomitopsis palustris

Chitosan nanoparticle displayed strong inhibition of mycelial growth of Fomitopsis palustris. A maximum 85.8% inhibition rate was recorded at 0.1% of chitosan nanoparticle. However, 0.01 and 0.1% concentrations showed similar growth inhibition rate as per the statistical analysis. Chitosan montmorilonite,showed a dose dependent effect on mycelial growth. The highest inhibition rate (64.4%) was recorded at 0.1% concentration while the other concentrations 0.01 gave inhibition rate from 36.8. Chitosan ZnO nanoparticle at 0.01 % concentrations inhibited growth from 53.0% whereas 0.1% formulation inhibited 63.0% of mycelia growth.

The mechanism, which chitosan affects the growth and survival of fungi, may be related to its ability to interfere with the negatively charged residues of macromolecules exposed on fungal surfaces forming polyelectrolytic complexes, thereby affecting membrane permeability and causing leakage of intracellular electrolytes and proteinaceous constituents . Additionally, it has been proposed that the inhibitory efficacy of chitosan against pathogenic fungi in bamboo could also be related to its ability to increase the production of defense-related enzymes (e.g., chitinase and β-1,3-glucanase) and antioxidant activity (e.g., polyphenol oxidase and peroxidase) in chitosan coated bamboo.

### 3.5 Weight loss of bamboo

The weight loss of un-aged (control) beech specimens was found to 39.5 % for Trametes versicolor, and 32.1 for Fomitopsis palustris attack, respectively. These weight loss results verified that the decay test was valid according to SNI. Weight loss caused by Trametes versicolor attack was found to be 10.2 % and 6.9 for aged and control specimens with node sections, and 5.4 and 7.9 % for aged and control specimens without node sections, respectively. Weight loss after Fomitopsis palustris attack of aged specimens with nodes, control specimens with nodes, aged specimens without nodes, and control specimens without nodes were in the ranges 3.4 -10.7 %, respectively. Expectancy of service life of bamboo specimens varied from 2 to 3 yr to 8 yr, while the expectancy of service life of wood specimens was service life against Trametes versicolor and Fomitopsis palustris.

## 4. Conclusions

Chitosan nanoparticles, chitosan-ZnO nanoparticles and Chitosan montmorilonite were prepared and their structures were well characterized by FTIR.. The antifungal capacity of chitosan is real but complicated to control. The effectiveness of chitosan does not only depend on the chitosan formulation but also on the fungus type. The antifungal activity of three chitosan materials were effective

Chitosan ZnO nanoparticles contribute to their higher antifungal activity against Trametes versicolor in in vitro studies.Chitosan nanoparticles also showed maximum inhibition rate of spore germination of Trametes versicolor. The effiancy of chitosan based nanoparticles mainly chitosan and ZnO-chitosan could further be tested on other plant pathogenic fungi in in vitro and in vivo models. The present studyis a effort to explore potential of chitosan based nanoparticles andthe findings of present study could be utilized for development ofnano fungicide for crop protection in future.

## 5. Acknowledgements

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