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Control of Anthracnose Disease (*Colletotrichum gloeosporioides*) Using Nano Chitosan Hydrolyzed by Chitinase Derived from *Burkholderia cepacia* Isolate E76

(Pengendalian Penyakit Antraknosa [*Colletotrichum gloeosporioides*] Menggunakan Kitosan Nano Hasil Hidrolisis Kitinase Asal *Burkholderia cepacia* Isolat E76)

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ABSTRAK

Antraknosa (*Colletotrichum gloeosporioides*) merupakan salah satu penyakit penting pada buah yang perlu dikendalikan. Tujuan penelitian ini adalah memperoleh formula terbaik kitosan nano hasil hidrolisis dalam mengendalikan antraknosa. Hidrolisis kitosan dilakukan dengan enzim kitinase yang diektraksi dari *Burkholderia cepacia* isolat E76. Partikel kitosan nano dibuat dengan metode gelasi ionik dengan mereaksikan kitosan hasil hidrolisis (0,2%) dan agen penghubung silang Sodium tripolifosfat (STPP)(0,1%) dengan waktu pengadukan selama 30–60 menit. Aktivitas hayati formula kitosan nano diuji terhadap *C. gloeosporioides* secara *in vitro* dan *in vivo*. Aktivitas spesifik enzim kitinase hasil purifikasi lebih tinggi (0,19 U/mg) dibanding dengan ekstrak kasar enzim (supernatan) dengan nilai kemurnian meningkat sebesar 3,8 kali. Dari empat formula yang diuji, Formula A (rasio volume kitosan hasil hidrolisis terhadap STPP adalah 5 : 1, kondisi pengadukan 60 menit) menghasilkan karakteristik fisik partikel terbaik. Partikel formula kitosan nano berbentuk *spherical* dan memiliki ukuran partikel rerata 126,2±3,8 nm, indeks polidispersitas (IP) 0,4±0,02, dan zeta potensial (PZ) 27,8±0,2 mV. Kitosan nano memiliki aktivitas penghambatan terhadap *C. gloeosporioides* secara *in vitro* sebesar 85,7%. Selain itu, kitosan nano menghambat perkecambahan spora *C. gloeosporioides* sebesar 61,2%. Kitosan nano juga efektif dalam menekan keparahan penyakit antraknosa secara *in vivo* saat diaplikasikan secara preventif pada buah cabai dan pepaya. Hasil kajian dapat dijadikan sebagai acuan pada aplikasi menggunakan kitosan nano sebagai agen pengendali hayati pascapanen yang menjanjikan terhadap antraknosa.

Kata kunci: Antraknosa, *C. gloeosporioides*, kitinase, *B. cepacia* isolat E76, kitosan nano.

ABSTRACT

Anthracnose (*Colletotrichum gloeosporioides*) is one of the important diseases of fruit crops that need to be controlled. This study was aimed to obtain the best formula of hydrolyzed nano chitosan and its potensial in controlling anthracnose. The hydrolyzed chitosan was prepared using chitinase enzyme extracted from *Burkholderia cepacia* isolate E76. Chitosan nanoparticles were synthesized using ionic gelation method by reacting hydrolyzed chitosan (0.2%) with Sodium tripolyphosphate (STPP) (0.1%) as cross-linking agent using 30–60 minutes stirring condition. The bioactivity of the nano chitosan formula was tested to *C. gloeosporioides* under *in vitro* and *in vivo* assays. The specific enzymatic activity of the purified chitinase was higher (0.19 U/mg) than that of crude enzyme (supernatant) with the purity increased by 3.8 times. Of the four formula tested, Formula A (hydrolyzed chitosan to STPP volume ratio of 5 : 1 with 60 minutes stirring condition) was found good in terms of physical characteristic of the particle. The formula nano chitosan particle had the spherical-like shape with an average particle size of 126.2±3.8 nm, polydispersity index (PI) of 0.4±0.02, and zeta potential (ZP) value of 27.8±0.2 mV. Nano chitosan had an inhibitory activity to *C. gloeosporioides in vitro* up to 85.7%. Moreover, it could inhibit 61.2% of *C. gloeosporioides* spores germination. It was shown that nano chitosan was also effective to reduce anthracnose disease severity *in vivo* when applied as a preventive measure on chili and papaya fruits. This study could be used as a reference for further fruit coating application using nano chitosan as a promising postharvest biocontrol agent to *C. gloeosporioides*.

Keywords: Anthracnose, *C. gloeosporioides*, chitinase, *B. cepacia* isolate E76, nano chitosan.

INTRODUCTION

Cultivation of horticultural crops often encounters many obstacles due to biotic factors such as plant pest and diseases. The fungal pathogen *Colletotrichum gloeosporioides* (Penz.) Sacc., the causal agent of anthracnose disease, frequently infects many economically important plants and various types of vegetables, such as avocado, banana, passion fruit, citrus, guava, strawberry, mango, papaya, and chilli, causing a heavy yield loss, both in terms of fruit quality and quantity. In India, postharvest loss due to anthracnose was 29.6% (Sharma and Kulshrestha 2015). In Bangladesh, about 25% to 30% loses of total production due to anthracnose was reported, whilst the loss estimated due to anthracnose disease during the heavy rainy season was reported >60% (Chowdhury and Rahim 2010). According to Alvarez and Nishijima (1987), losses due to anthracnose postharvest diseases ranged from 1% to 93%, depending upon postharvest handling and packing procedure; for instance on papaya postharvest, losses during sea shipments ranged from 10% to 40%. The genus *Colletotrichum* which associated with chiliis classified into four major species, namely *C. gloeosporioides*, *C. acutatum*, *C. capsici*, and *C. coccodes* (Than et al. 2008). The most commonly found anthracnose pathogen that infects chili in Indonesia was *C. capsici* (Syd & Bisb), *C. gloeosporioides* (Penz.) Sacc., and *C. acutatum* (Syukur et al. 2009).

Anthracnose is considered as a fruit postharvest disease, but the pathogen may also infect crops during pre-harvesting stage. At initial infection process, the pathogen may occur on plant in a dormant phase, then the fungi become active when entering the ripening stage as induced by ethylene. In addition, the disease is triggered by relatively moist conditions and high temperatures. The infected fruits show typical symptoms such as concave spots with light brown or black on its edge. Severely diseased fruits produced light orange to pink spots, causing the infected fruit cannot be consumed. To date, the management of this disease is somewhat difficult and heavily relies on chemicals. The use of environmentally-safe agent such as chitosan is considered as an alternative strategy for anthracnose management on postharvest disease of fruits. The successful chitosan application has been reported for control of anthracnose disease on banana fruit (Pamekaset et al. 2009), papaya (Rahman et al. 2012), and dragon fruit (Zahid et al. 2013). Chitosan is a natural nontoxic cationic polysaccharide, consisting of glucosamine and N-acetyl glucosamine, and it can be obtained from the deacetylation of chitin. Biological activity of chitosan is affected by several factors, such as temperature, molecular weight, degree of deacetylation, and pH. Chitosan with low molecular weight (LMW) has significant difference in its activity as an antimicrobial, antioxidant, antitumor, and other usage compared with high molecular weight (HMW) of chitosan (Agnihotriet et al. 2004; Goyet et al. 2009).

In previous study, it was reported that *Burkholderia cepacia* isolate E76 could be a promising biocontrol agent since it was able to inhibit the growth of pathogenic fungi (Suryadi et al. 2013). Moreover, the bacteria also showed chitinase and glucanase activities (Manzila et al. 2015; Suryadi et al. 2014). This isolate had strong chitinase activity and potential in controlling anthracnose disease. It was also reported that the hydrolyzed chitosan using chitinase produced by *B. cepacia* isolate E76 at concentration of 2% showed as the most effective treatment in inhibiting the growth of *Colletotrichum* sp. (94.22%) than that of non-hydrolyzed chitosan (Suryadi et al. 2016).

The hydrolyzation of chitosan with chitinase derived from *B. cepacia* isolate E76 is important because this enzymatic treatment could affect LMW of chitosan. In addition, this process also may low cost effective instead of long chemical preparation using acids such as HCl. In relation to the present study, this approach is still potential to further develop microbial-assisted hydrolysis of chitosan to optimize its reduction activity against the phytopathogen.

Antifungal mechanism of chitosan may occur at extracellular and intracellular level (Hernández-Lauzardo et al. 2010). Chitosan nanoparticle formulation has many advantages, i.e. nontoxic, stable, and can be used as matrices for different types of food, medicinal and plant extracts (Agnihotriet et al. 2004; Mominet et al. 2013). Nanoparticles may improve stability of the active compound against environmental degradation, such as oxidation, hydrolysis, and enzymatic decomposition. In addition, modification of particle size and surface properties is useful for achieving cell-specific actions in order to optimize the therapeutic effects of drugs (Patel et al. 2014; Singh and Lillard 2009). Nanoparticle and its particle size distribution are the most important characteristic because they can determine the distribution, toxicity, and delivery ability to the target. Several studies have been conducted on nano chitosan preparation, including preparation of nano chitosan as a matrix retinol, as stocks of nasal absorption, and as enhancer for estradiol to the brain

target-organ (Kim et al. 2012; Wang et al. 2011). However, research on nano scale chitosan formulations and its application for biocontrol of post harvest anthracnose disease on fruit crops has not extensively been done.

This study was aimed to obtain the best formula of hydrolyzed nano chitosan and its potensial in controlling anthracnose. The findings in this study may provide information on the optimal nano chitosan formula that can be used to control anthracnose on horticultural crops, especially chili, and papaya fruits.

MATERIALS AND METHODS

Chitinase Activities of Crude and Purified Enzyme Derived from *B. cepacia* Isolate E76

The bacterial isolate of *B. cepacia* E76 (BiogenCC-E76) was cultured on Luria-Bertani (LB) broth medium for 18 h incubation on a stirrer plate (Hitachi), then 1 ml microbial culture was mixed into the colloidal chitin liquid medium. The culture was incubated at room temperature for 48 h while shaken at 75 rpm speed using an orbital shaker (K260, IKA). Chitin liquid medium containing bacterial culture was centrifuged (D-78532, Andreas Hettich) at 8,400 × *g* for 20 minutes at 4°C, and the pellet was discarded. Supernatant containing crude extract (enzyme) was precipitated by mixing them with the same volume of saturated 70% Ammonium sulfate and stirred at 10°C. Further, the mixture was centrifuged (D-78532, Andreas Hettich) with a speed of 8,400 × *g* for 30 minutes at 4°C. The collected pellets (purified enzyme) was dissolved in Phosphate-buffered saline (PBS) pH 6.8 to maintain the stability and stored in a freezer for further assay (Vishu et al. 2007).

Chitinolytic activity of enzyme solution derived from *B. Cepacia* isolate E76 was quantitatively measured for its N-acetyl-D-glucosamine (GlcNAc) concentration, following the procedure of (Toharisman et al. 2005) using commercial GlcNAc (Sigma) as a standard. The assay was carried out *in duplo* by mixing 150 ml of sample solution with 150 ml of PBS pH 7 and 300 ml of colloidal chitin, then it was mixed using a vortex and incubated at 37°C for 30 minutes. The mixture was centrifuged at 4,200 × *g* for 5 minutes and then the resulting pellet was removed from clear supernatant. Supernatant was taken up to 500 ml, added with 500 ml of distilled water and 1 ml of Schales' reagent, and then heated at 100°C for 10 minutes. After cooling down, the sample-protein content was measured at λ = 420 nm using a spectrophotometer (U2800, Hitachi). One unit of enzyme activity is expressed as the amount of enzyme that produces 1 mol GlcNAc per minute (Greenet et al. 2005).

Protein content was measured using albumin as a standard (Bradford 1976). Sample solution consisted of 0.1 ml chitinolytic enzyme, 5 ml Bradford reagent. Distilled water was used as a blank. The mixture was vortexed and allowed at room temperature for 15 minutes, then it was measured at λ = 595 nm (U2800, Hitachi).

Reducing sugar was measured using glucose as a standard. The chitosan supernatant was added with 1 ml of dinitrosalicylic (DNS) reagent, and incubated at 37°C for 60 minutes. Afterwards, it was boiled at 100°C for 10 minutes and left to cool at room temperature. The absorbance of sample was measured at λ = 540 nm. The specific activity of the enzyme was estimated as number of enzyme units per mg protein.

Nano Chitosan Formulation by Ionic Gelation Method

Preparation of chitosan nanoparticle formulation was done according to the method of Agnihotriet et al. (2004) with slight modification. A total of 2 g of biological grade chitosan (MW = 600 kDa; Sigma Aldrich) was dissolved in 100 ml of 1% acetic acid pH 3.5 and stirred for 24 h in order to completely dissolve chitosan. Soluble chitosan solution was then filtered and adjusted to pH 5.3 by addition of NaOH (Vishuet al. 2007). About 20 ml of chitosan solutions were hydrolyzed using 0.2 ml of purified chitinase enzyme from *B. cepacia* isolate E76 and incubated at 37°C for 2 h. The hydrolysis process was stopped by heating at 100°C for 5 minutes. The hydrolyzed chitosan was precipitated by centrifugation (D78352, Hitachi) at 8,400 × *g* for 10 minutes. The pellet was washed several times with distilled water until neutral pH achieved and stored in a refrigerator for further use.

The hydrolyzed chitosan pellet was dissolved in 1% to 0.2% acetic acid solution and stirred with a magnetic stirrer at medium speed. The tested volume ratios of hydrolyzed chitosan to STPP were 4 : 1 and 5 : 1 (v/v). After adding with 80 ml of 0.2% Tween 80 and stirring in slow agitation at room temperature for 30 minutes and 60 minutes, the mixture solution was added with 0.1% STPP as cross-linking agent, then the suspension was stored in a jar and refrigerated prior to testing.

Physical Characterization of Nano Chitosan Formula

The treatments were consisted of Formula A (5 : 1, 60 minutes), Formula B (5 : 1, 30 minutes), Formula C (4 : 1, 60 minutes), and Formula D (4 : 1, 30 minutes). Physical characteristic was determined based on particle size analyzer (PSA) to measure the uniformity of particle size, polydispersity index (PI) to measure size distribution, and zeta potential (ZP) to measure stability. A total of 1 ml suspension of each nano chitosan formula was incorporated into the cuvette of PSA, then analyzed under Zetasizer Nano (Malvern Instruments). The treatment of formula assay was done in triplicate.

Analysis by scanning electron microscopy (SEM) was done to characterize the surface structure of nano chitosan. SEM sample preparation was carried out by dropping the sample into the specimen holder (specimen stub) and dried for 24 h. The samples were then coated with gold (Au) using ion coater (Q150R ES, Quorum) and subsequently observed using SEM (Zeiss). The analysis was carried out in Laboratory of Nanotechnology, Indonesian Center for Agricultural Postharvest Research and Development (ICAPOSTRD), IAARD, Bogor.

Effect of Formulated Nano Chitosan on *C. gloeosporioides In Vitro*

Fungi *C. gloeosporioides* were isolated from anthracnose infected-papaya fruit from the papaya field garden collection of Sumani Experimental Station, Solok, West Sumatra. *C. gloeosporioides* was isolated from lesions associated with papaya skin. The tissue of skin papaya fruit (±1 cm × 1 cm in size) was cut from the edge of the healthy symptomatic fruit, then it was grown on potato dextrose agar (PDA) medium (Difco) with addition of 0.2% Streptomycin sulfate. The characteristics of fungus *Colletotrichum* sp. has been purified using a single spore method and identified according to the guidelines of Barnett and Hunter (1998).

The experiment was carried out following the procedure of Palma-Guerrero et al. (2008). The treatment was arranged in a completely randomized design with three replications. A lukewarm PDA medium in a petri dish (9 cm in diameter) was mixed with 1 ml of the best nano chitosan formula based upon physical characteristic (Formula A). The control treatment was prepared using PDA without chitosan. At the center of petri dish containing mixture of PDA and chitosan, a hole was created aseptically using by a corkborer and subsequently a 5 day-old mycelia culture disk of *C. gloeosporioides* isolate was placed into the hole. The petri dish was incubated at room temperature for 6 days. The effectiveness of growth inhibition was determined by the formula as follows:

$$\text{RDI} = \frac{(\text{Gc}–\text{Gt})}{\text{Gc}} \text{ x } 100\%$$

RDI = relative degree of inhibition, Gc = growth of pathogen on control, Gt = growth of pathogen on treatment.

For spore germination test, a 14-day old of *C. gloeosporioides* spore culture was harvested and suspended in sterile distilled water. The spore suspension was filtered through two layers of sterile gauze and its concentration was adjusted to a density of 10^6 spores/ml. Subsequently, spore suspension was mixed with nano chitosan Formula A. A total of 10 ml of spore suspension was dropped on potato dextrose (PD) liquid media in a sterile microscope slides, incubated for 24 h, and observed using a microscope at 40× magnification. Control treatment was done using similar procedure, but spore was not treated with distilled water. The degree of inhibition to spore germination was calculated by the following formula:

$$\text{RDI} = \frac{(\text{TS}–\text{GS})}{\text{TS}} \text{ x } 100\%$$

RDI = relative degree of inhibition, TS = total spore, GS = germination spore.

Effect of Formulated Nano Chitosan on *C. gloeosporioides In Vivo*

Red chili pepper (*Capsicum annuum* L. 'Hot Pepper') and papaya (*Carica papaya* L. 'California') fruits were used for *in vivo* assay. Fruits were washed with sterile water and allowed to dry at room temperature. This test was performed using two methods, i.e. preventive and curative methods followed the procedure of Bautista-Banos et al. (2006).

In the preventive method, fruits were initially soaked with chitosan nanoparticle solution (2 ml/l suspension) for 60 minutes and air-dried, then fruits was wounded using sterile needle, and inoculated by means of fruit-immersion using spore suspension of *C. gloeosporioides* (10^6–10^7 spores per ml suspension) for 15 minutes and then air-dried.

In curative method, fruits were spore-immersed by *C. gloeosporioides* (10^6–10^7 spores per ml suspension) for 15 minutes and air-dried, then the fruits were soaked in a solution of nano chitosan (2 ml/l suspension) for 60 minutes and air-dried. The fruits were stored on a sterile tray and wrapped to maintain their moisture.

Control treatment was conducted using the similar method, but nano chitosan was replaced with sterile distilled water. The experiment was arranged in a completely randomized design using three replication (consist of 10 fruits in each treatment). Disease severity (DS) was calculated with the following formula:

$$DS\ (\%) = \frac{\Sigma\ (\text{number of samples in each category attack x severity score})}{(\text{number of observed samples x the highest scare})} \text{ x } 100\%$$

The severity of anthracnose disease was scored according to the guidelines presented by (James 1971) with slight modifications, i.e. score 0 = 0≤x≤1%, 1 = 2≤x≤20%, 2 = 21≤x≤40%, 3 = 41≤x≤60%, 4 = 61≤x≤80%, and 5 = 81≤x≤100%. The effectiveness of nano chitosan inhibition was measured as relative degree of fungal growth inhibition as follows:

$$RDI = \frac{(DSc–DSt)}{DSc} \text{ x } 100\%$$

RDI = relative degree of inhibition, DSc = disease severity in control, DSt = disease severity in treatment.

RESULTS AND DISCUSSION

Chitinase Activities of Crude and Purified Enzyme Derived from *B. cepacia* Isolate E76

Chitinase activity was present in a medium containing colloidal chitin inoculated with *B. cepacia* isolate E76. Media supplemented with colloidal chitin is a valuable source of carbon and nitrogen for the growth of *B. cepacia* isolate E76. These components are in general strongly support the speed of metabolic changes in the bacteria to produce chitinolytic enzymes. The different fractions of chitinase obtained from partial purification in this study showed different degree of activity (Table 1).

After precipitation with Ammonium sulfate the specific enzyme activity of purified chitinase was higher (0.19 U/mg) than that of crude enzyme (supernatant), with the purity increased by 3.8 times (Table 1). This study demonstrated that chitinase is an inducible enzyme excreted during bacterial culture containing chitin as the sole carbon source, which is in a good agreement with other reports (Kim and Ji 2001; Vega and Kalkum 2012).

The hydrolyzed chitosan with LMW can be obtained by depolymerization of HMW chitosan using physical, chemical, or enzymatic methods (Vishuet et al. 2007). The HMW chitosan was more sensitive to depolymerization (Mao et al. 2004). In our previous study, a LMW of the depolymerized HMW chitosan with a high degree polymerization was successfully done using chitinase extracted from *B. cepacia* isolate E76 (Suryadi et al. 2016). This depolymerization was based on the enzymatic hydrolysis process by breaking the chitosan bonds at 1,4-β-glycosidic site (2009).

Chitosan was soluble at a pH of less than 6.5 in most organic acid such as formic, acetic, tartaric, and citric acid, but insoluble in phosphoric acid and sulfuric acid (Tiyaboonchai 2003). At the optimal pH conditions, as demonstrated in this study, chitosan was adjusted at pH 5.3, then the enzymes can work well. The solubility of different MW chitosan was affected by pH and it decreased parallel with the reduction of MW (Mao et al. 2004; Yinet et al. 2009). The enzymatic hydrolysis process to produce chitosan needed certain optimal temperature, suggesting that decomposition of MW was also temperature dependent (Tian et al. 2015).

The reducing sugar analysis of the biological grade chitosan and hydrolyzed chitosan was estimated as high as 0.5 mg/ml and 1.3 mg/ml, respectively. The reducing sugar is inversed proportionally with the degree of polymerization which affected to the lowering of MW (Handayani et al. 2013). It was shown from this study value of reducing sugar of hydrolyzed chitosan was increased, thus causing lower MW of chitosan solution. Therefore, the hydrolized chitosan with LMW resulted in this study may be potential for further utilization.

Table 1. Chitinase activities of *Burkholderia cepacia* isolate E76.

Fraction	Activity[1] (U/ml)	Protein[2] (mg/ml)	Volume (ml)	Total activity[3] (U)	Total protein (mg)	Specific activity[4] (U/mg)	Purity[5]
Crude extract	0.040	0.773	250	10.00	193.20	0.05	1.0
Purification, 70% $(NH_4)_2SO_4$	0.042	0.212	5	0.21	1.05	0.19	3.8

[1]Chitinase activity = activity (U/ml) × volume (ml).
[2]Protein was determined using Bradford assay with BSA as standard (mg/ml).
[3]Total activity = volume chitinase × protein.
[4]Specific activity = total activity (U/mg) per total protein (mg).
[5]Purity = specific activity after purification/specific activity before purification.

Physical Characterization of Nano Chitosan Formula

Physical characteristics of nano chitosan formula were presented in Table 2 and Figure 1. The corresponding nanoparticles products are obtained spontaneously using ionic gelation method. The mechanism of nano chitosan formation was resulted from spontaneously ionic gelation as a result of mechanical stirring using magnetic stirrer at room temperature, based on the electrostatic interaction between the chitosan amine group (-NH2) and a negative charge of polyanion such as tripolyphosphate (TPP) (Chattopadhyay and Inamdar 2012); chitosan (2%) dissolved in acid (1% acetic acid) released hydrogen atoms to produce protons and a solution will be charged positively. The STPP (0.1%) was alkaline so that the solution will be negatively charged (Mohanraj and Chen 2006). The complexity of the interaction between different charges have resulted chitosan to undergo ionic gelation and precipitate to form particles (Xu and Du 2003).

Based on visual observation to the formula formations, the suspension formula had somewhat yellowish-color; however, the color of each formula was not significantly different (data not shown). The good physical appearance of nano chitosan formula has been optimized based upon the volume ratio of hydrolyzed chitosan to STPP of 5 : 1 with gently stirring at room temperature for 60 minutes. The particle size of Formula A had the smallest average particle size of 126.2±3.8 nm with a PI value of 0.4±0.02, and ZP value of 27.8±0.2 mV (Table 2). The particle size of nano chitosan was inversely proportional with the greater volume ratio of hydrolyzed chitosan-STPP and longer stirring period.

The nanoparticle sizes that obtained in this study (126.2±3.8 nm) was not similar to that obtained by (Chen et al. 2010), although still in the range of their results. The antibacterial activities of LMW chitosan products (6.9–22.4 kDa) had an average nanoparticle sizes ranged from 117 nm to 965 nm (Chen et al. 2010).

The particle size can be determined by comparing the size of nano-scale particles in the micrograph image. However, the average size of the particles was more accurately calculated using PSA. The greater ratio of hydrolyzed chitosan with STPP and the longer stirring period, the smaller particle size was obtained. SEM analysis was in in good agreement with the PSA result analysis.

The structural analysis of nano hydrolyzed chitosan using SEM revealed that particle had the spherical-like shape (Figure 1). In contrast with previous result, chitosan nanoparticles showed crystal-like, or cubic grains with sharp edges (Chookhongkha et al. 2013).

The size, polydispersity, and the stability of the particles were strongly influenced by the volume ratio of chitosan and STPP. Based on PSA measurement to the formula nano chitosan, it was shown that the volume ratio of hydrolyzed chitosan with STPP solvent and stirring period affected the average size, uniformity, and stability of the particles. The particle size increased with the raising volume ratio hydrolyzed chitosan and STPP (Rismana et al. 2014), however, when the concentrations of chitosan are high, the electrostatic reaction between chitosan and STPP are very much dense, which affected formation of nano chitosan in a colloidal system was less stable and tend to agglomerate into the nanometer size (submicron-sized).

The size and stability of nano chitosan was also influenced by the addition of stabilizer. The modifications to the ratio composition of hydrolyzed chitosan with STPP while stirring was the use of Tween 80 (as a surfactant) to stabilize the particles in solution and to prevent suspension from clotting (agglomeration) between the particles. The uniformity of the particle size was expressed in the value of PI (Avadi et al. 2010). The PI value close to indicated the uniformity of the particle size or the size of the homogeneous dispersion formula, while the PI values >0.5 to 1 indicated a high heterogeneity. The uniformity of the particle size of Formula A in this study was quite good, with the smallest PI value of 0.4, followed by the Formulas B, C, and D. The ZP

Table 2. Physical characteristics of nano chitosan formula.

Formula (hydrolyzed chitosan [0.2%] to STPP [0.1%] volume ratio, stirring period)	Mean±SD		
	Z (nm)	PI	ZP (mV)
A (5 : 1, 60 minutes)	126.2±3.8	0.44±0.02	27.8±0.2
B (5 : 1, 30 minutes)	167.1±2.7	0.47±0.03	25.4±0.4
C (4 : 1, 60 minutes)	493.3±69.2	0.69±0.02	22.9±0.4
D (4 : 1, 30 minutes)	573.1±135.1	0.54±0.01	20.8±0.3

Z = particle size, PI = polidispersity index, ZP = zeta potential, SD = standard deviation.

value indicated the stability of the colloidal system. The ZP value of Formula A was found the highest, whereas the smallest ZP value was observed in Formula D. A nanoparticle with a ZP value more than +/- 30 mV has a good stability in terms of surface charge that prevents suspension aggregation (Mohanraj and Chen 2006). It was reported that the antimicrobial activity of chitosan nanoparticles depends on its zeta potential, which plays a significant role in binding with negatively charged microbial membrane (Chen et al. 2010). The ZP value resulting from this study was still less than +/- 30 mV, hence the agglomeration of the particle may still occur and reduce its activity.

Effect of Formulated Nano Chitosan on *C. gloeosporioides In Vitro*

Formula A was chosen this test test (*in vitro* assay) due to the uniformity of the particle size of with the smallest PI value. On the control treatment, *C. gloeosporioides* hyphae were brown, smooth, and septate. Conidia were cylindrical (10–15 μm × 3–5 μm in size). Conidia which were round to irregular in shape were formed on the conidiophores in the acervuli. The colony color appeared white and gradually turned grayish as the culture grew older on the PDA. The mean of fungal colony growth on PDA containing nano chitosan Formula A was significantly different from that on the petri dish containing no nano chitosan formula ($P<5\%$). The fungal growth on non treated petri dishes were 63.6 ± 0.0 cm^2, while on PDAs with nano chitosan was only 9.8 ± 5.3 cm^2 (Figure 2A).

The fungal growth reached the perimeters of the non treated petri dish at 6 days after inoculation, while on nano chitosan-treated petri dish the diameter of fungal growth did not develop further. The relative degree of inhibition by nano chitosan was 85.7%.

The ability of nano chitosan to the inhibition growth of *C. gloeosporioides* was suspected because of the fungi toxic effect by antifungal compounds (Vasyukova et al. 2001). Chitosan with a concentration of 0.02 g/ml may reduce the decay process, maintain quality, and improve shelf-life of papaya fruit stored at 5°C (Gonzalez-Aguilar et al. 2008). The degree of inhibition of nano chitosan in

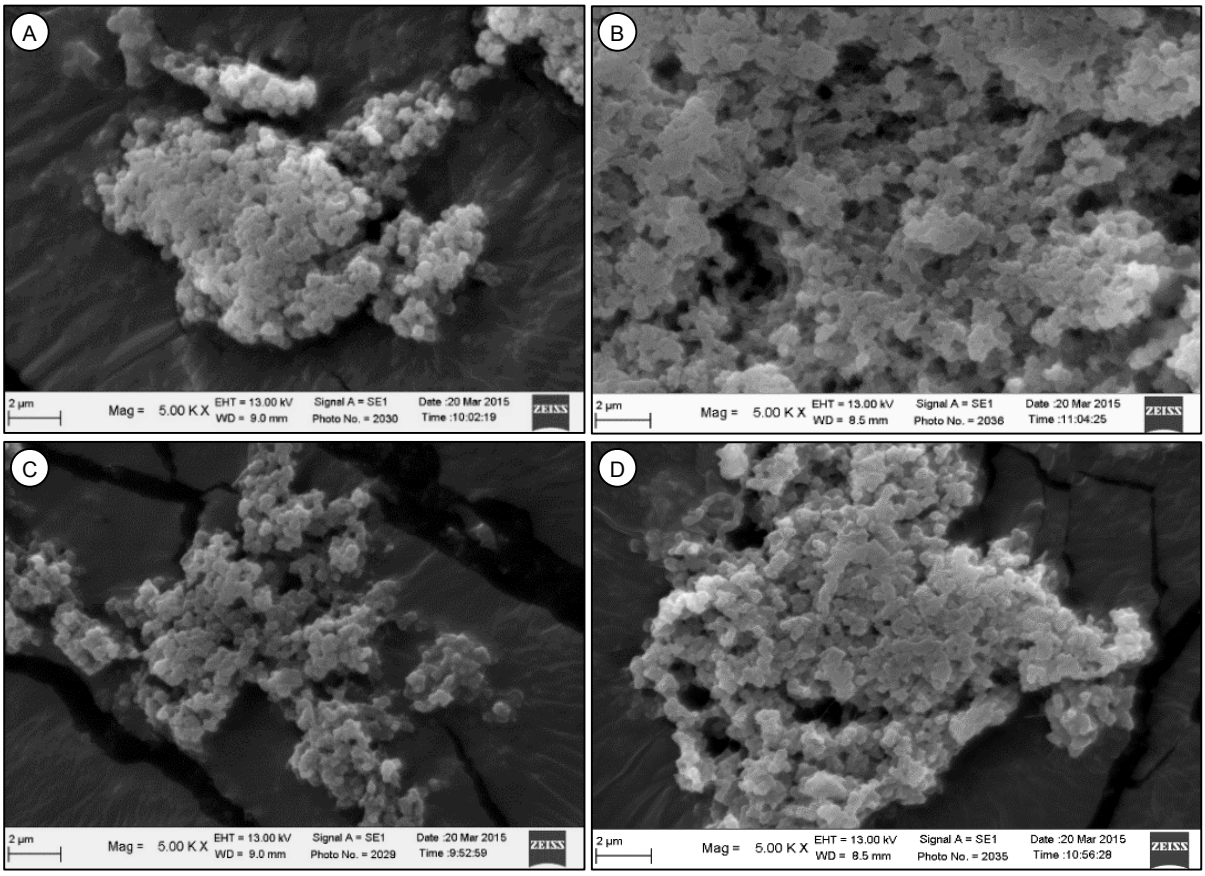

Figure 1. SEM analysis of nano chitosan. A = Formula A (5 : 1, 60 minutes), B = Formula B (5 : 1, 30 minutes), C = Formula C (4 : 1, 60 minutes), D = Formula D (4:1, 30 minutes).

this study was relatively high (85.7%) compared with other study.

Effect of Nano Chitosan on *C. gloesporioides* Spore Germination

The relative degree of inhibition (RDI) by nano chitosan against *C. gloeosporioides* spore germination is depicted in Figure 2B. The amount of germination spore differed significantly between the control and nano chitosan Formula A (P = 5%) treatment. Spore can germinate well in control up to 62.3%±1.2, while less than half of spores treated with nano chitosan germinated (30.8±7.4%) (Figure 2B). The RDI of *C. gloeosporioides* by nano chitosan was calculated as 61.2% compared with that on control. The complete inhibition by composite chitosan containing silver nanoparticle (chitosan-AgNp) to the conidial germination of *C. gloeosporioides* was observed at concentration 100 μg/ml (Chowdappa and Gowda 2013). The mechanism by which chitosan blocks the growth of *C. musae* was due to inhibition of germ tube length, hyphal growth, hyphal length and width, as well as hyphal lyses (Pamekaset et al. 2009).

The decrease of the germination degree was suspected because of antifungal activity in nano chitosan. The interaction between the positively charged amino group of nano chitosan with the negatively charged-cell wall of *C. gloeosporioides* affected in changes of the cell structure, lowering cell permeability and lead to leakage of intracellular substances such as electrolytes, proteins, amino acids, and glucose (Fouda 2005). The leakage of *C. gloeosporioides* disturbed metabolism and caused cell death. Relevant to previous report, stated that minerals electrolyte leakage, i.e. calcium ions was required for the metabolism of *C. gloeosporioides*, consequently no cell spore was capable of germination.

Effect of Formulated Nano Chitosan on *C. gloeosporioides In Vivo*

The average disease severity on fruits treated with nano chitosan (2 ml/l suspension) either applied preventively or curatively was significantly differed from those on untreated fruits (P<5%). The severity of anthracnose caused by *C. gloeosporioides*, on fruits was greater (80–100%) on control treatment than that of fruits treated with nano chitosan Formula A. The disease severity on papaya was higher than that of chili fruit, as depicted by the graph (Figure 3). Inhibition rate on chili preventive and curative methods was 87.5% and 75.0%, respectively (Figure 3A).

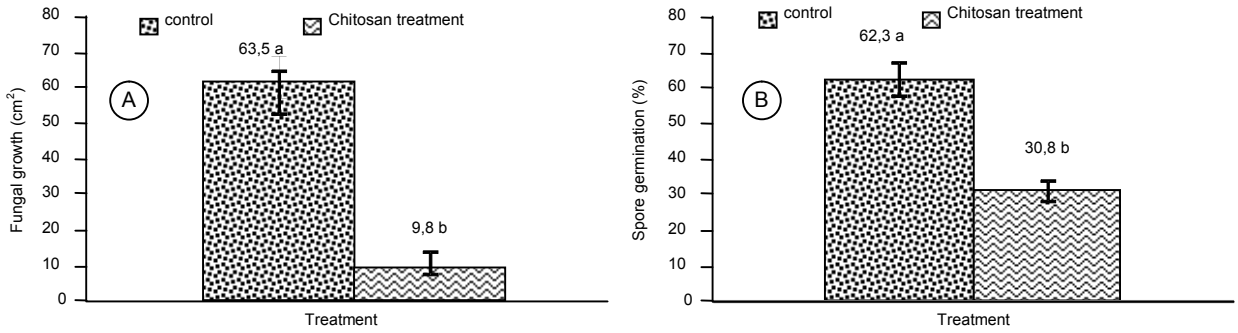

Figure 2. Effect of nano chitosan Formula A (5 : 1, 60 minutes) *in vitro*. A = effect on fungal colony growth, B = effect on spore germination of *C. gloeosporioides*. Means followed by the same letter are not significantly different by DMRT at P = 5%.

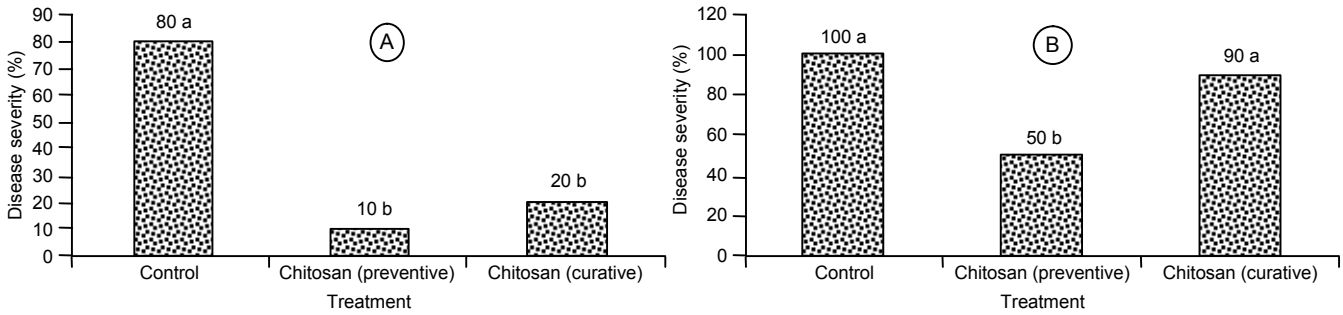

Figure 3. Effect of nano chitosan Formula A (5 : 1, 60 minutes) against anthracnose disease severity *in vivo*. A = effect of nano chitosan on chili, B = effect of nano chitosan on papaya. Means followed by the same letter are not significantly different by DMRT (P<5%).

Bautista-Banos (2006) reported that chitosan has a preventive rather than curative effect. In the present study, the inhibition on papaya preventive treatment was also higher (50.0%) than that of the curative treatment (10.0%) (Figure 3B). Notably, the preventive showed greater in degree of inhibition than that of curative method. The results also indicated that a preventive effort gave better results in controlling anthracnose on chili.

The inhibition mechanism of nano chitosan is more likely due to fungitoxic properties involving the interaction of chitosan with the pathogen cell membrane. The polycationchitosan can bind to the negatively charge of the pathogen cell membrane via electrostatic interactions, thus affecting the permeability of cell membranes and causing leakage of intracellular materials, such as enzymes, proteins, genetic material, etc. In addition, chitosan that binds to DNA inhibits mRNA in protein synthesis (Ing et al. 2012; Singh et al. 2008; Vellingiri et al. 2013). Nano chitosan is capable of damaging the genetic material of microbes, where the chitosan as a chelating metal capable of binding metal ions in solution, which is crucial for intracellular survival of microbial cells. The bioactivity of nano chitosan on the growth of *C. gloeosporioides* in this study is thought to occur through the antifungal properties of a broad spectrum of amino groups in the form of acetyl amino ($HCOCH_3$) and glucosamine ($C_6H_9NH_2$).

In lines with this study, it was reported that application of chitosan solutions at concentrations of 10 ml/l suspension were effective in decreasing the severity of anthracnose disease caused by *C. gloeosporioides* on manggo in the field test (Singburaudom and Dethaub 2011). Chitosan coating has many advantages to reduce the incidence and severity of postharvest fruit pathogens; however, in the long-term use it still demonstrates limited ability to inhibit some microorganisms that lead fruit to decay. Hence, to improve antimicrobial activity, application of chitosan alone can be enriched with some bioagents to improve its efficacy (Jovanovic et al. 2016). The coating formulation with 55% chitosan-thyme oil nanoparticle could reduced the incidence of *C. gloeosporioides* up to 60% until 8-day storage period on avocado cv. Hass (Correa-Pacheco et al.2017). The chitosan-Ag nanoparticle (1%) could inhibited conidial germination of *C. gloeosporioides* and also reduce anthracnose incidence on manggo in the field (Chowdappa and Gowda 2013; Chowdappa et al. 2014). Rahman et al. (2012) reported that papaya fruit treated with the combination of *B. cepacia* B23-chitosan-$CaCl_2$ affected ethylene and respiration rate, and reduced fruit weight loss 25% than control treatment. It was reported that the most significant inhibition of chitosan nanoparticle combine with silver (Ag) to plant pathogenic fungi was observed at concentration of 100 ppm at *in vitro* study on PDA plates (Kim et al. 2012).

The long-term effect of the chitosan coating to the fruits was reported by Pamekaset et al. (2009). They reported that chitosan is able to inhibit the rate of respiration and ethylene levels of fruit, extending the shelf life of fruit, induces the formation calose lignifications on fruit skin epidermis, and increased induces levels of total phenolic compounds and total crude protein content of fruits.

Since physical barier do not exist on fruit surface, the fruit inoculation method used in the present study by wounded method using pathogen inoculums may provide the fungus ease to penetrate into fruit surface; however, this artificial wounding caused rapid loss of mechanical defenses in fruit; therefore, the assay with direct chitosan application on fresh fruit surface may be done ideally under natural infection (without pathogen inoculation) to minimize fruit lead to decay as well as prolong shelf life of fruits.

It is still needed to add more variables in the optimization of nano chitosan formula to attain better in particle size and stability. Further analysis such as viscosity test to determine the molecular weight of nano chitosan as well as FTIR analysis to confirm structural change of the molecule need to be done. Further studies should be directed to research activities for improving the activities of nano chitosan formula, such as inoculation method or formulation ingredients combination. Combination of chitosan with other materials such as antifungal secondary metabolites produced by microbial antagonist may also improve its efficacy.

CONCLUSIONS

The enzymatic activity of purified chitinase enzyme derived from *B. cepacia* isolate E76 using Ammonium sulfate was higher (0.19 U/mg) than that of crude enzyme (supernatant) with the purity increased by 3.8 times. The best physical characters of nano chitosan formulation was obtained by hydolyzed chitosan to STPP volume ratio of 5 : 1 and stirring period for 60 minutes, with the average particle size was 126.2 ± 3.8 nm, PI of 0.4 ± 0.02, and ZP value of 27.8 ± 0.2 mV. The bioactivities of nano chitosan formula was capable to inhibit the growth of *C. gloeosporioides in vitro* of 85.7%. Nano chitosan

was also able to inhibit the spore germination of *C. gloeosporioides* up to 61.2%. Bioactivity of nano chitosan was a promising as postharvest biocontrol agent of *C. gloeosporioides in vivo* when applied as a preventive measure on chili and papaya fruits. Further study on formulation ingredients, inoculation method, as well as combination of with other materials (i.e. antifungal secondary metabolites) are required to improve the activities of nano chitosan formula.

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