

Examination of Chitinolytic Bacteria in Alginate-Chitosan Encapsulation on Chili Seed Against Damping off Caused by *Fusarium oxysporum*

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ABSTRACT

One alternative in biological control of plant pathogenic fungi is to provide biological agent inoculant in coated seed. This study was to evaluate potential control and viability of two chitinolytic bacteria, *Enterobacter* sp. BK15 and *Bacillus* sp. BK17 of alginate-chitosan encapsulation coated on chili seed against damping off caused by *Fusarium oxysporum*. Chili seed beads were stored at refrigerator and room temperature. Examination of the bacteria ability in reducing seedling off was conducted by growing and treating the seed beads with *F. oxysporum* inoculum. Bacterial cell viability was measured by growing grinded chili bead in minimum salt medium with chitin as sole C source. Re-isolation of infected seedling was done by growing it in potato dextrose agar. The result showed that the bacteria in alginate-chitosan coated seeds reduced chili damping off. Chili seedling height and dry-weight did not differ with those of (-) control, treatment with no bacterial and fungal inoculation and differed with (+) control, treatment of fungal inoculation only. Re-isolation of infected seedling showed similar fungal characterization of inoculated *F. oxysporum*. Bacterial cell viability decreased significantly in 30 days of storage both at refrigerator and room temperature. However, cell viability stored at refrigerator was higher rather than that of room temperature.

Keywords: *Bacillus* sp., Biological Control, Coated Seed, *Enterobacter* sp., Seedling Off

1. INTRODUCTION

In controlling plant pathogenic fungi, synthetic agrochemical fungicide has mainly been used. However, it caused pathogen resistance to the chemical and environmental problems and harm to non-target organism including human. Based on their nature, many biological agents have been evaluated in order to prevent and control diverse plant pathogens, given that these options are more ecofriendly (Compant *et al.*, 2005; Anitha and Rabeeth, 2010). Biological control agent has been applied through vegetative cells or spores, metabolites and cell immobilization/encapsulated matrix.

Encapsulating the living cells using biocompounds protect the microorganisms against many environmental stresses (Hernandez-Suarez *et al.*, 2011), so that they can

be stored dry at ambient temperatures for prolonged periods (Kasra-Kermanshahi *et al.*, 2010). Several experimental formulations based on polymers have been evaluated (Bashan, 1998). Rokka and Rantamaki (2010) reviewed the use of several polymer gels such as chitosan, carrageenan, gum arabic, gellan gum or mesquite seed gum, gelatin and other biopolymers to encapsulate probiotic bacteria. Among these materials alginate is the most common polymer material for the encapsulation of microorganisms (Rokka and Rantamaki, 2010). These inoculants can be amended with nutrients such as chitosan to improve the short-term survival of the bacteria upon inoculation (Anitha and Rabeeth, 2010).

One of the most devastating diseases in crop and other plants throughout the world is *Fusarium* wilt (Anitha and

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Rabeeth, 2010; Gupta *et al.*, 2011). It causes extensive commercial losses by reducing both quality and yield (Anitha and Rabeeth, 2010; Gangadara *et al.*, 2010). *Fusarium* is a common phytopathogenic fungus causing Fusarium wilt in chili, tomato, potato, tobacco and banana, infecting the plant seeds as soil-borne disease (Kavraulakis *et al.*, 2010; Gupta *et al.*, 2011; Sun *et al.*, 2011).

Based on the need of the use of antagonist microorganisms for biological control of pathogenic fungi like *F. oxysporum*, this study was carried out. This study was to know ability of chitinolytic bacteria of Gram-negative *Enterobacter* sp. BK15 and of Gram-positive *Bacillus* sp. BK17 to control Fusarium wilt in form of chili seed coated with bacterial alginate and with chitosan as amended nutrient encapsulation and to evaluate bacterial viability in the seed stored at refrigerator and room temperature.

2. MATERIALS AND METHODS

2.1. Microbial Isolates and Chili Seeds

Chitinolytic bacteria *Enterobacter* sp. BK15, *Bacillus* sp. BK17 and *Fusarium* sp. used in this study are collection of Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan Indonesia. The bacterial isolates were kept in a modified salt medium (0.7 g K₂HPO₄, 0.3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.001 g ZnSO₄ and 0.001 g MnCl₂ in 1.000 mL⁻¹) containing 2% (w/v) chitin colloidal (MSMC) agar, with a pH of 6.8, while *Fusarium* sp. was maintained in Potato Dextrose Agar (PDA). Certified chili seeds were obtained from agricultural market.

2.2. Preparation of Alginate-Chitosan Coated Chili Seed

Seed coating was conducted with modification method as described by Kasra-Kermanshahi *et al.* (2010) and Hernandez-Suarez *et al.* (2011). Ten mL of 1% chitosan was dissolved in 10 mL⁻¹ of 1% acetic acid and 2% calcium chloride. The pH was then adjusted to ±7 by adding 0.1 N calcium hydroxide. Chitinolytic bacteria were sub-cultured in nutrient agar and incubated at 30°C for 2 days. Sub-culture was diluted with Sterile Distilled Water (SDW) to get bacterial suspension of ≈ 10⁸ CFU/mL. Chili seeds were soaked in 10 mL⁻¹ of bacterial suspension for 30 minutes. The suspension was added with 3 mL glycerol and finally mixed with 3% sodium alginate to have 100 mL⁻¹ suspension. This mixture was then dropped into chitosan-calcium chloride suspension using small glass tube. Seed alginate-chitosan beads were formed as soon as the drop fell into chitosan-

calcium chloride solution. The beads were put into 0.9% sodium chloride solution to make the beads more compact and then removed and agitate in SDW using stirrer to remove sodium chloride residue. The beads were air-dried and put into sterile plastic bag. Coated seeds (Fig. 1) were kept in refrigerator (ref) (±4°C) and at room temperature (rt) (±26-30°C) for further study.

2.3. Control of Fusarium wilt in Alginate-Chitosan Coated Chili Seed

Fusarium oxysporum was sub-cultured in 100 mL⁻¹ of potato dextrose broth at 30°C for 10 days. Fungal sub-culture was blended with 500 g sterilized soil mixed with sterilized compost (3:1) in a 30×22×10 cm tray. Coated chili seeds were planted for 30 days in the trays covered with plastic wrap. (-) control coated seeds were treated similarly but in soil without fungal inoculation, while (+) control were seed planted in fungal inoculated soil. (-) control was placed separately with others. Thirty seeds were used for each treatment. Direct observation was conducted to know number of infected seedlings.

2.4. Measurement of Seedling Damping off, Damping off Relative Reduction, Seedling Height and Dry-Weight

Percentage of chili seedling damping off was observed as [number of seedling damping off/number of treated seeds] ×100%. Damping off relative reduction (%) was measured as [100-(percentage of damping off of treatment/percentage of damping off of (+) control)] ×100%. Plant height and dry-weight of the plant left was measured at end of study.

2.5. Fungal re-Isolation from Infected Seedling

Stem of infected seedling was cut by 1 cm. The stem was surface disinfected for few seconds in 2% NaClO and then rinsed thoroughly with SDW. Surface-disinfected leaves were grown on PDA for 72 hours at room temperature. Growing fungal colonies were then purified. Identification morphological characteristics such as colony, mycelia as well as shape and size of conidia under light microscopy observation were carried out.

2.6. Assay of Bacterial Viability of Alginate-Chitosan Encapsulation

A coated chili seed was grinded and SDW was added to make 10 mL⁻¹ suspension. A 0.1 mL⁻¹ of suspension was streaked on MSMC agar and incubated at room temperature. The Colony Forming Units (CFU) of microorganisms were counted to evaluate cell viability. Colony was counted for 0, 15 and 30 days of seed stored at refrigerator and room temperature.

3. RESULTS

3.1. The Fusarium manifestation in chili seedling

Fusarium wilt in chili seedling was manifested as leaf and seedling wilted with yellowing leaf followed by

seedling slanted (**Fig. 1**). Fusarium enters through root wound and proliferates within the vascular tissue, leading to the typical wilt symptoms (Sun *et al.*, 2011). Eventually, the whole plant wilts and dies as the disease progresses (Sun *et al.*, 2011). Severe seedling disease resulted in stand failure as seen in this study.

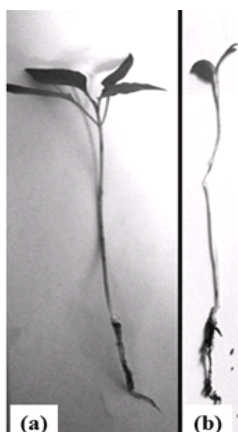


Fig. 1. (a) Healthy chili seedling and (b) Fusarium infected ones

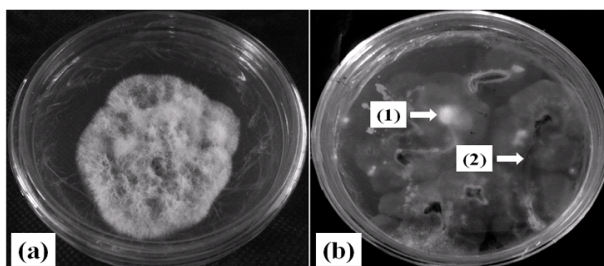


Fig. 2. *F. oxysporum* used as (a) Inoculant and (b) From infected seedlings: (1) white cottony hyphae and (2) infected stem of chili seedling

Re-isolation of seedling with fusarium with symptom showed that the fungus had white cottony colony, purple at the base of medium and smooth at the surface similar to that of the fungus used as inoculant (**Fig. 2**). This confirmed that *Fusarium* used was pathogen to chili.

3.2. Chili Damping off Suppression

Alginate-chitosan coated seeds were planted in soil inoculated with *F. oxysporum*. Thirty days after planting seedling damping off, seedling height and dry-weight were evaluated. The seeds planted in Fusarium-inoculated soil were susceptible to Fusarium wilt showed by (+) control. (+) control showed that the fungi using in this study was pathogen to chili by 56.7% of total seedling wilted, implicating a partial death of chili seedlings. On the other hand, chili seeds coated with

alginate-chitosan and chitinolytic bacteria obviously decreased chili damping off to some extent (**Fig. 3**). *Enterobacter* sp. BK15 seemed more active to suppress fungal growth as described by less chili damping off. Damping off relative reduction of this isolate was relatively high (70.6%). Contrary with our study using these bacteria in controlling *Ganoderma boninense* of oil palm seedling *Enterobacter* sp. BK15 reduced less disease incidence compared to that of *Bacillus* sp. BK17 (Suryanto *et al.*, 2012).

In addition to number of damping off observation, chili seedling height and dry-weight as possible manifestation of Fusarium infection were also observed (**Fig. 4**). Coated seed with *Enterobacter* sp. BK15 produced more vigorous seedling showed by its higher seedling height to 9.4 cm and dry-weight to 4.2 g compared to other treatments even with (-) control. In addition, direct observation of the seedling showed that the seedling was more stout and fresh.

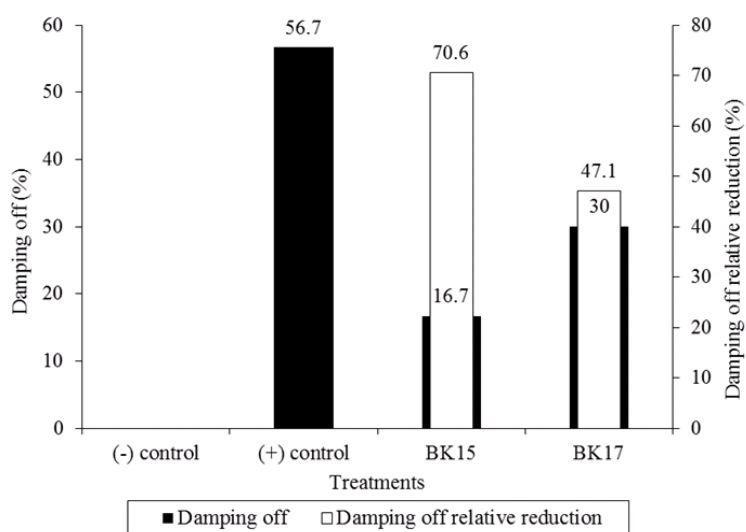


Fig. 3. Effect of chitinolytic bacterial treatment in alginate-chitosan encapsulation on percentage of seedling damping off caused by Fusarium wilt and damping off relative reduction

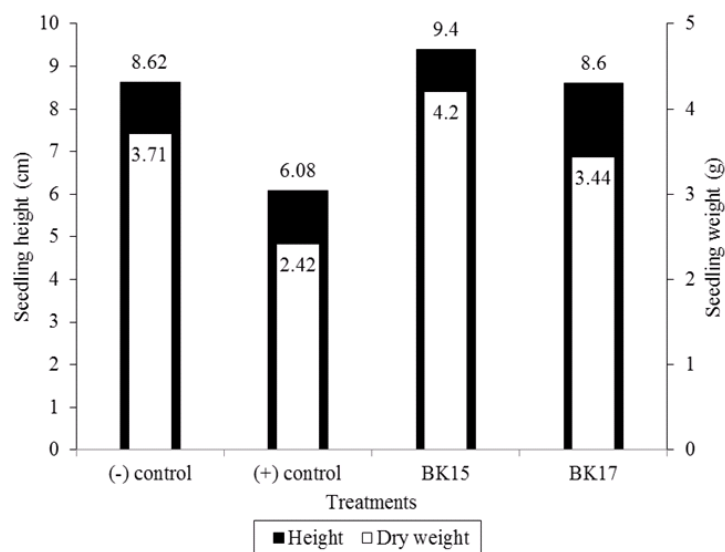


Fig. 4. Effect of chitinolytic bacterial treatment in alginate-chitosan encapsulation on chili seedling height and dry-weight

Some bacterial control agent produce plant growth regulator to increase plant performance (Forchetti *et al.*, 2010; Hernandez-Suarez *et al.*, 2011). Fusarium wilt caused unhealthy seedlings by reducing seedling height to 6.08 cm and dry-weight to 2.42 g.

3.3. Enumeration of Viable Bacterial Cell of Coated Chili Seed

In order to determine cell viability of both BK15 and BK17 in alginate-chitosan encapsulation this

study was conducted at different temperature up to 30 days. Cell number of both BK15 and BK17 was extremely decreased after production (**Fig. 5**). Decreasing pattern of both bacteria whether at refrigerator and room temperature was similar. However, the coated seed stored at refrigerator contained more viable bacterial log number of viable cell both BK15 and BK17, respectively compared to that of room temperature after 15 and 30 days of storage.

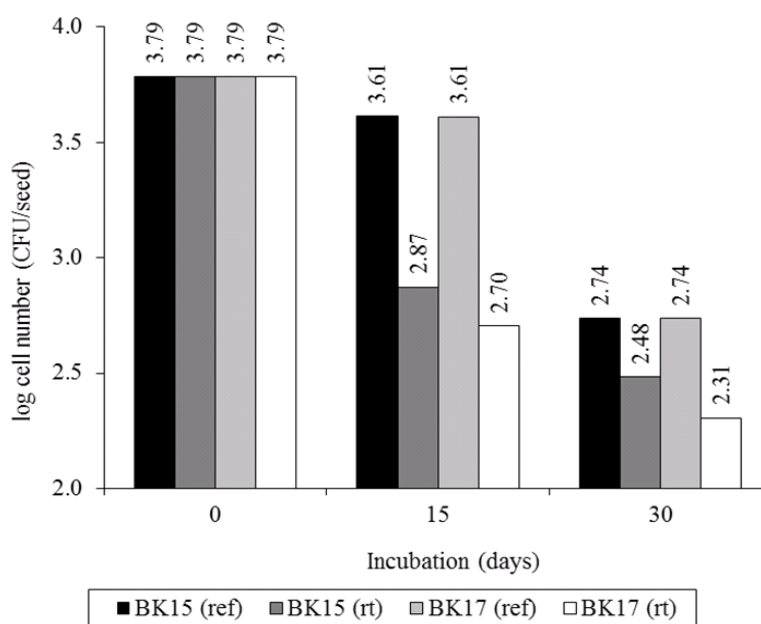


Fig. 5. Viability of chitinolytic bacterial cell of coated chili

4. DISCUSSION

Reisolation of *Fusarium* from infected seedling were to determine that the fungal was the only causal agent of the Fusarium wilt in the chili seedlings. All samples of seedling with Fusarium wilt symptoms showed that the pathogen was in the seedling. Fusarium is the most common fungi that cause such soil-borne diseases (Kucharek, 2003). These pathogens can survive long periods in the soil without a host by producing some thick-walled spores called chlamydo spores which are formed within the hyphae and occur in or out of plant debris within the soil (Kucharek, 2003; Sun *et al.*, 2011).

Enterobacter sp. BK15 and *Bacillus* sp. BK17 encapsulated with alginate and chitosan showed to reduce chili damping off incidence, however, BK15 was reduce more. Different effect in controlling fungal growth might be caused by different lytic enzyme produced by the isolates. Enzymes can vary both within and between microbes (Kobayashi *et al.*, 2002).

Lytic activity of bacteria is one of a number of mechanisms that has been implicated in biocontrol for several years. Members of chitinolytic bacteria genera *Bacillus*, *Lysinibacillus*, *Enterobacter*, *Serratia* and *Alcaligenes* have been reported and played important role as biological control agents of plant pathogenic fungi including *Fusarium oxysporum* f.sp. *radicis-lycopersici*, *F. oxysporum* f.sp. *raphani*, *Phytophthora cinnamomi*, *P. nicotianae* and *Rhizoctonia solani*

(Kavraulakis *et al.*, 2010). Our previous studies also showed that chitinolytic bacterial isolates reduce disease incidence of *G. boninense* in oil palm seedling (Suryanto *et al.*, 2012) and inhibited some plant pathogenic fungal growth (Suryanto *et al.*, 2011).

Fungal cell wall usually is composed not only with chitin but also with other sugar such as β -1,3 glucan which binds to chitin in amorph structure. Chitinase and β -glucanase activity was detected in all bacterial strains exhibiting in vitro antagonistic activity against soil-borne plant pathogens (Kavraulakis *et al.*, 2010). Therefore, chitinase and β -glucanase produced by the isolates should be considered as enzyme responsible in lysing chitin polymer of *Fusarium* hyphae.

Many reported that antagonistic bacteria contribute to plant performance and vigor. Our study showed that *Enterobacter* sp. BK15 application produced higher seedling height and dry-weight. *Enterobacter cancerogenus* MSA2 showed overall plant growth promotion of *Jatropha curcas* by producing metabolites such as 1-aminocyclopropane-1-carboxylic acid deaminase, phytase, indole acetic acid and siderophore (Jha *et al.*, 2011). *Enterobacter* has also been reported to control pathogenic fungi (Okamoto *et al.*, 2000; Slininger *et al.*, 2004). The use of antagonist rhizobacteria belonging to *Bacillus* genus showed to stimulate plant growth and to increase crop yield of tomato (Hernandez-Suarez *et al.*, 2011). Endophytic

bacterial strains SF2 (99.9% homology with *Achromobacter xylosoxidans*) and SF3 and SF4 (99.9% homology with *B. pumilus*) enhanced growth of sunflower seedlings under water stress and inhibited growth of pathogenic fungi (Forchetti *et al.*, 2010). Tariq *et al.* (2009) reported the dual role of *Pseudomonas* spp. in plant-growth promotion and disease control. *P. aeruginosa* inhibited the radial growth of root rotting fungi *Macrophomina phaseolina*, *Rhizoctonia solani*, *F. solani* and *F. oxysporum* and caused significant suppressive effect on root rotting fungi and root knot nematode infecting chili roots. Some *P. aeruginosa* strains also showed to increase the plant height and fresh shoot weight by producing indole acetic acid.

Encapsulation maintains bacterial viability and stability for prolong term storage (Rokka and Rantamaki, 2010). Our study showed that the bacterial population in chili beads dropped rapidly after production both storage at refrigerator and room temperature. However, inactivation of cell during refrigerator storage prolonged self-live cells. In contrast with our study, encapsulation of isolated bacterium with alginate and chitosan showed higher stability during 1 month of storage (Kasra-Kermanshahi *et al.*, 2010).

Although our initial bacterial culture was taken from 2-days old culture, physiological status of both bacteria was not confirmed before producing alginate-chitosan encapsulation. Vegetative cells are more susceptible to environmental stress compare to endospores. However, Chumthong *et al.* (2008) observed that application of an equivalent number of un-formulated endospores resulted in much loss of the bacterial endospores even 1 day after application. Furthermore chili bead producing by method described seemed not to uniform. Next study needs to be conducted to know the effect of encapsulation on bacteria of varied physiological status i.e., log phase, lag phase and endospore, to examine consistency of the bacteria in suppressing damping off after given storage time and to produce more uniform bead.

5. CONCLUSION

Chitinolytic bacterial isolates, *Enterobacter* sp. BK15 and *Bacillus* sp. BK17, in alginate-chitosan coated seeds suppressed chili damping off caused by *F. oxysporum*. No different effect was observed on chili seedling height and dry-weight treated with bacterial isolates to treatment with no bacterial and fungal inoculation, but differed with those of fungal inoculation only. Cell viability of the isolates decreased significantly in 30 days of storage both at refrigerator and room temperature. However, cell stored at refrigerator was more viable rather than that of room temperature.

6. ACKNOWLEDGEMENT

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