Biocontrol of Postharvest *Colletotrichum* Decay in Red Chili and Tomato with *Bacillus Subtilis* ATCC 21556

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Abstract. *Bacillus subtilis* ATCC 21556 produces the highest an antifungal of iturin A compared to the other three of *B. subtilis* strains ATCC 15841, ATCC 27505, and ATCC 21770. An Iturin A has a wide antifungal spectrum activity, therefore making it an ideal potential for controlling of the fungal diseases of crop not only for preharvest but also for postharvest control. This research work if very useful to overcome the problem of decreased post-harvest production of chili and tomato that caused by the fungal pathogen. Many of the fungi cause of postharvest disease in various fruits and vegetables. Biological control of postharvest diseases has emerged as an effective alternative control since the impact of chemical control of post-harvest disease cause serious consequences for human health and the environment. Twenty microliters of *B. subtilis* ATCC 21556 (approximately 1 x 10^7 cell mL^-1) were injected into the fruit of red chillies and tomatoes to control its postharvest diseases caused by fungal of *Colletotrichum scovillei* 244830. The lesion diameter of *C. scovillei* 244830 significantly (p<0.005) reduced about 52-64% in chili and 39-44% in tomato. An invitro test showed that percent growth inhibition of *B. subtilis* ATCC 21556 against *C. scovillei* 244830 was about 52.38% at 14 days of the incubation time. For successful infection of the fungal pathogens into the host plants, they produce the extracellular enzymes. Qualitative test of the extracellular enzymes showed that *C. scovillei* 244830 produced the enzymes of amylase, laccase, lipase, pectate lyase, and protease and it did not produce polygalacturonase.

1. Introduction
More than one-third of harvested fruit and vegetables are lost and do not reach customers mainly due to post-harvest decay [1]. Fungal pathogens are considered as one of the most serious losses in post-harvest production. The expansive of fungal pathogens postharvest losses are the main obstacle to solve in the chain of the food supply. Besides some of the fungal pathogens postharvest may also produce of the mycotoxin contamination.Cristina et al.[2] reported that pathogens caused post-harvest diseases in tomato were *Alternaria tenuissima, Botritis cinera, Cladosporium fulvum, Colletotrichum coccedodes, Fusarium oxysporum, Geotrichum candidum, Rhizopus stolonifer*, and *Stemphylium macrosporoideum*. *Colletotrichum* is a large genus involve a number of important species which are the most prevalent fungal pathogens causing diverse diseases in both subtropical and tropical fruits and vegetables. Ali et al. [3] revealed that *Colletotrichum* spp. is one of the main causes of postharvest decay of chili which it can develop on the field, during distant transport, cold storage and shelf-life.
The ability of fungal pathogen postharvest to infect agricultural products such as fruits and vegetables could be related to the production of fungal extracellular enzymes that degrade the plant cuticle and cell wall components. Therefore it is necessary for a qualitative test to determine the extracellular enzymes produced by *Colletotrichum scovillei* 244830. The synthesis of the enzymes depends on environmental conditions as well as the ability of each species and fungus strain. Ahmad et al. [4] reported that *Fusariumgraminearum*, *F. moniliforme*, *F. semitectum*, and *Rhizoctonia solani* have shown pectinase, cellulase, protease, and lipase enzymes activities.

During the past decade, several postharvest fungicides have been excluded from the market to reduce their residues. Therefore rising growing interest in eco-friendly and safe alternatives to synthetic fungicides. The impact of chemical control of post-harvest disease causes serious consequences for human health and the environment, which led to encourage to perform a research of Biocontrol of postharvest *Colletotrichum* decay of red chili and tomato with *Bacillus subtilis* ATCC 21556.

Biological control of postharvest diseases (BCPD) has emerged as effective alternative control. Because BCPD can be applied directly to the targeted area (fruit wounds) through existing application methods such as drenches, dips, sprayers, and biocontrol can significantly reduce fruit damages [5]. Several biological control agents have been reported to control post-harvest fruit and vegetable diseases such as *Pseudomonas flourescens* to suppress blue mold of apple caused by *Penicillium expansum* [6], *Bacillus megaterium* reduced peanut kernels of *Aspergillus flavus* [7], and *Stenotrophomonas* decreased anthracnose of mango caused by *Colletotrichum gloeosporoides* [8].

In the present study, we tested ability of a *Bacillus subtilis* ATCC 21556 in controlling of *Colletotrichum* of fresh of red chili and tomato fruits. *Bacillus subtilis* ATCC 21556 produces the highest antifungal of iturin A compared to the three of other *B. subtilis* strains ATCC 15841, ATCC 27 505, and ATCC 21770 as shown in Table 1. The *B. subtilis* strains are derived from American Type Culture Collection. An iturin A has a wide antifungal spectrum activity, making it an ideal potential for controlling of the fungal diseases of the crop not only preharvest but also for postharvest control [9].

| No. | *Bacillus subtilis* strains | Quantitative HPLC (iturin A; mg L⁻¹) |
|-----|---------------------------|-------------------------------------|
| 1.  | *B. subtilis* ATCC 6051    | -                                   |
| 2.  | *B. subtilis* ATCC 6633    | -                                   |
| 3.  | *B. subtilis* ATCC 7058    | -                                   |
| 4.  | *B. subtilis* ATCC 15841   | 9.3                                 |
| 5.  | *B. subtilis* ATCC 21228   | -                                   |
| 6.  | *B. subtilis* ATCC 21394   | 13.0                                |
| 7.  | *B. subtilis* ATCC 21556   | -                                   |
| 8.  | *B. subtilis* ATCC 21770   | 7.6                                 |
| 9.  | *B. subtilis* ATCC 21778   | -                                   |
| 10. | *B. subtilis* ATCC 27505   | 5.0                                 |
| 11. | *B. subtilis* ATCC 33677   | -                                   |

Note: The lowest limit for the detection of iturin A by the HPLC methods was 0.50 mg/L. Therefore, when the concentration of iturin A was below the limit, it was judged to be not detectable. Source: Hsieh et al. [9]

The objectives of this research were to assess (i) Efficacy of *B. subtilis* ATCC 21556 in suppression of diameter lesion *Colletotrichum scovillei* 244830 in red chilies and tomatoes, (ii) *in vitro* test of
inhibition growth of Colletotrichum scovillei 244830 by B. subtilis ATCC 21556 (iii) qualitative test of extracellular enzymes produced by Colletotrichum scoville 244830.

2. Materials and Methods

2.1. Efficacy of B. subtilis ATCC 21556 in the suppression of diameter rot lesion of Colletotrichum in chili and tomato

Eighteen fruits of red chili pepper and tomato were surface - sterilized with step washing in 5% sodium hypochloride for 3 minutes, followed by three rinses with sterile distilled water. The extra water on the fruits was removed with a sterilized tissue paper and they were put into 3 plastic boxes (Seal-ultra pack production Indonesia; 25 cm in length; 16.5 cm in width; 9 cm in height). The first of plastic box is for a healthy control treatment (chilies/tomatoes injected with a sterilized distilled water), the second of the box for disease control(chilies/tomatoes were injected with 20 µL of C. scovillei 244830 (approximately 27 x 10^6 cell mL^{-1}) and a third of the box is used for chillies/tomatoes that are injected with 20 µL of B. subtilis ATCC 21556 (approximately 1 x 10^7 cell mL^{-1}) and 20 µL of the fungal pathogen (approximately 27 x 10^6 cell mL^{-1}). Each chilli and tomato fruit were injected in three part in upperside, middle, and bottom side of the fruis. C. scovillei culture suspension was prepared as follows; Five mL of a SDW was pipetted onto a seventh days old of C. scovillei in PDA medium plate. The mycelia of a fungal pathogen were collected using a glass rod and pipetted into a plastic centrifuge tube (nominal volume 14 mL), and centrifugated at 2300 xg for 10 minutes. The cell precipitated was resuspended in a sterilized distilled water to a final density approximately 4 x 10^6 conidia ml^{-1}.

The boxes were kept at room temperature for 6 days. The diameter of the rot lesions was recorded till 6 days of incubation time and percentage of rot lesions inhibition were calculated by using the following formula

\[ \frac{\phi_{LC} - \phi_{LT}}{\phi_{LC}} \times 100\% \]  

\( \phi_{LC} \) is Average diameter of lesion in disease control (inoculated only with C. scovillei 244830)  
\( \phi_{LT} \) = Average diameter of lesion in the treatment (inoculated with B. subtilis ATCC 21556 and C. scovillei 244830)

2.2. Efficacy of B. subtilis ATCC 21556 on diameter colony reduction of C. scovillei 244830 in vitro condition

Assay for the suppression activity of the biocontrol agent was performed on PDA plates. The mycelial plugs of the fungal pathogen of C. scovillei 244830 sized 5 mm and one loop of biocontrol agent of B. subtilis ATCC 21556 was placed in the opposite side at 25 mm away from the edge of plates. PDA plates that were inoculated only with the fungal pathogen at the center of plates as control. The plates were incubated for 14 days at room temperature. The experiment was performed in triplicate and repeated twice for each treatment. The diameter growth of the fungal pathogen was recorded every two days. Percent inhibition of the fungal pathogen mycelia was calculated using the formula suggested by Pandey and Vishwakarma [10].

\[ PI = \frac{D_{c} - D_{t}}{D_{c}} \times 100\% \]  

\( PI \) = Percent of inhibition  
\( D_{c} \) = Average diameter of fungal pathogen growth (mm) in control  
\( D_{t} \) = Average diameter of fungal growth (mm) in treatment

2.3. Qualitative test of extracellular enzymes produced by C. scovillei 244830

2.3.1. Amylase. C. scovillei 244830 was grown in nutrient agar medium supplemented with 0.2% of soluble starch (pH 6.0). Five days after incubation of the plates at room temperature, then they were
flooded with 1 mL of iodine solution. Amylase activity was judged by a white zone formed around the colony as described by Hankin and Anagnostakis [11].

2.3.2. Laccase. C. scovillei 244830 was grown in nutrient agar plate (NA: Himedia, India) supplemented with 0.04% of guaiacol (Sigma, USA), then the plates were incubated 4-5 days at room temperature. The formation of dark brown colour around the fungal colonies was a positive reaction, resulting from the oxidation of guaiacol [12].

2.3.3. Lipase. Tween 20 was used as a lipid substrate. The composition of a medium per liter was as follows; 10 g of peptone, 5 g of NaCl, 0.1 g of CaCl₂.2H₂O, 20 g of agar, and pH 6.0. The tween 20 was sterilized separately by autoclaving, and 1 mL of its was added to 100 mL of the medium.

The fungus was grown in the medium and then incubated for 5 days. The lipolytic activity was judged by the formation of a visible precipitate around the fungal colony due to the degradation of the salt of fatty acid [11].

2.3.4. Pectate lyase. Fungus was grown in medium plates had following composition (per liter of distilled water); 1 g of yeast extract, 15 g of agar, 5 g of pectin apple, and added with 2 g of (NH₄)₂SO₄, 4 g of KH₂PO₄, 6 g of Na₂HPO₄, 0.2 g of Fe₂SO₄.7H₂O, 1 mg of H₂BO₃, 10µg of H₃BO₃, 10µg of MnSO₄, 70 µg of ZnSO₄, 50 µg of CuSO₄, 10 µg of MoO₃, and pH=7.0.

The plates were incubated at room temperature for 3-5 days and then flooded with a 1% aqueous solution of hexadecyltrimethylammonium bromide. This reagent precipitates pectin in the plates incubated at room temperature. The formation of dark brown colour around the fungal colonies was judged by a visible reaction [11].

2.3.5. Polygalacturonase. The ability to degrade pectin was also used for determining the ability to produce the enzyme of polygalacturonase. The medium composition is the same as the medium for the determination of pectate lyase activity, pH 5.0 instead of pH 7.0. The enzyme activity was judged by a clear zone around the fungal colony [11].

2.3.6. Protease. The fungus was grown in nutrient agar plates (NA: Himedia, India) medium supplemented with 0.4% gelatin, pH 6.0. Then the plates were incubated at room temperature for 3-5 days and then flooded with an aqueous saturated solution of ammonium sulfate, when a precipitate formed which made the agar more opaque and enhanced the clear zones around the fungal colonies was judged it had proteolytic activity [11].

2.4. Data analysis

Data were statistically tested to an analysis of variance (ANOVA) using Minitab 16 software with the significance of mean differences was determined using Duncan’s test. The responses were judged significant at 5% level.

3. Results and Discussion

3.1. Efficacy of B. subtilis ATCC21556 in the suppression of diameter C. scovillei 244830 rot lesion on chili and tomato

Efficacy of B. subtilis ATCC 21556 was determined by its ability to reduce diameter rot lesion on chilies and tomatoes caused by the fungal pathogen. At an in vivo test, the results showed that the Bacillus strain significantly (P <0.05) reduced the diameter rot lesion of C. scovillei on chilies (Figure 1) and tomatoes (Figure 2). At day 2 of the incubation time, B. subtilis reduced the diameter of chili rot lesion significantly from 2.6 to 1.2 mm (PI=52%) (Figure 1(A)). The highest ability of the biocontrol agent of Bacillus to reduce diameter rot lesion on chilies was observed on day 3, it was reduced from 4.0 to 1.4 mm (PI=64). Until the last day of the observation (day 8) B. subtilis still able to reduce diameter of rot lesion 7.7 to 3.9 mm (PI=49%). On tomatoes fruit still showed that the highest ability of B. subtilis to decrease diameter rot lesion was observed at day 3, its reduced from 4.3 to 2.4 mm (PI=44%). At day 8 of in vivo test on tomatoes, the bacterial biocontrol agent still can...
decrease the diameter of its rot lesion from 6.1 to 3.9 mm (PI=36%), although there was not significantly different.

The efficacy of *B. subtilis* ATCC 21556 in suppression of postharvest disease of *C. scovilei* 244830 rot lesion in chili and tomato also has been proved by the other strains of *B. subtilis* such as efficacy of *B. subtilis* B106 reduced anthracnose of *C. musaeon* banana by 72% [13], *B. subtilis* APEC170 reduced anthracnose of *C. gloeosporiodes* on apples by 51% [14] and *B. subtilis* EXWB1 reduced rot lesion of *Alternaria alternata* on melon [15].

The mechanisms of *Bacillus subtilis* in controlling postharvest disease among others are the bacterial strains produced antibiotic substances and lytic enzymes [13, 14], and reduced the production of ethylene that may cause to prolong the storageduration of fruit and vegetables [15]. In the case of *B. subtilis* ATCC 21556 it was supposed that it produced an antifungal of iturin A as stated by Hsieh et al. [9].
**Figure 1.** Efficacy of *B. subtilis* ATCC 21556 on diameter rot lesion of chili fruits caused by *Colletotrichum scovillei* 244830.

**Figure 2.** Efficacy of *B. Subtilis* ATCC 21556 on diameter rot lesion of tomato fruits caused by *Colletotrichum scovillei* 244830.

### 3.2. Efficacy of *B. subtilis* ATCC 21556 on percent inhibition of diameter colony of *C. scovillei* 244830 in vitro condition

The results in Figure 3 showed that the percentage growth inhibition of *C. scovillei* colony diameter by *B. subtilis* was seen starting on the second day of incubation time. By increasing the incubation time, the percentage inhibition of the colony growth diameter also tends to increase. The highest percentage
of colony growth inhibition of the fungal pathogen was observed at day 14 as high as 52%. The efficacy of *B. subtilis* to suppress the fungal pathogen growth was demonstrated by its ability to inhibit the growth of the colony diameter of *C. scovilei*.

The ability of *B. subtilis* ATCC 21556 to inhibit *Colletotrichum* growth due to its ability to produce an antifungal of iturin. Hsieh et al. [9] reported that *B. subtilis* ATCC 21556 produced an antifungal of iturin A 13.0 mg L⁻¹ in potato sucrose broth medium.

Figure 3. Efficacy of *B. subtilis* ATCC 21556 on percent inhibition of colony diameter of *C. scovilei* 244830.

### 3.3. Qualitative test of extracellular enzymes produced by *C. scovilei* 244830

Results of the qualitative enzymes test indicated that *C. scovilei* 244830 produced amylase, laccase, lipase, pectate lyase, and protease, and it did not produce polygalacturonase (Table 2). The successful establishment of the fungal pathogen of *C. scovilei* and its ability to degrade the chilli and tomato fruit depend on its ability to produce the extracellular enzymes. Specific conditions in the host plants tissue, such as pH and temperature are also important for the production of the enzymes [16].

The function of amylase, lipase, and protease production for the fungal pathogen as a degrader of plasma-membrane components, providing nutrients to helps its spreading through plant tissue [17]. In addition, these enzymes act facilitating hyphal penetration, release carbon source, or modify chemical signals of the host plants [18].

Table 2. Extracellular enzymes produced by *C. scovilei* 244830.

| Name of enzymes        | Production |
|------------------------|-----------|
| Amylase                | +         |
| Laccase                | +         |
| Lipase                 | +         |
| Pectate lyase          | +         |
| Polygalacturonase      | -         |
| Protease               | +         |

Not: + = produced, - = did not produce
Pectate lyase (PL) and polygalacturonase (PG) are the main pectinases secreted by *Colletotrichum*. PL is responsible to degrade pectin polymerase directly by β-elimination mechanism, while PG cleaves α-1,4 glycosidic bonds between two galacturonic acid residues[19], these enzymes play an important role in the pathogenicity [16,20]. Whereas laccase is an extracellular enzyme widely found in fungi and it has an important role in pathogenicity by detoxifying capsaicin chili pepper in infected by *C. gloesporoides*[17].

4. Conclusions

*B. subtilis* ATCC 21556 controled post-harvest rot lesion of *C. scovillei* 244830 in chili pepper and tomato fruits effectively with the reduction of the rot lesion diameter were in the range of 52-64% in chili and 39-44 in tomato. Both of *in vitro* and *in vivo* test of *B. subtilis* ATCC21556 inhibited *C. scovillei* growth. Furthermore extracellular enzymes involved in the infection of *C. scovillei* 244830 in chili and tomatowere amylase, laccase, lipase, pectate lyase, protease. Conversely, polygalacturonase did not involve in the infection of the fungal pathogen.

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5. References

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