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Bacillus sp. effectivity test as a growth supressor agent from antrachnose disease caused by *Colletotrichum* sp. on cayenne pepper plant (*Capsicum frutescens* L.)

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Abstract

cayenne pepper plant (Capsicum frutescens L.) is one of the popular vegetables comodity in Indonesia. cayenne pepper plant used as main ingridients for kitchen's need, sauce industry, chili powder, instant noodle, and pharmaceutical industry. The demand for cayenne pepper plant in Indonesia is considerably high, about 4kg/capita/year. Antrachnose diseease caused by Colletotrichum sp is on of the major problem in cayenne pepper plant cultivation. Colletotrichum sp is from Nectrioidaceae family that has plenty of aservulus beneath the cuticule or on the surface. Bacillus sp has antagonist feature is consider to treat the Antrachnose disease. Bacillus sp is a marine bactreia that can produce antibiotic to fight pathogen. This test uses the antagonist test method where bacteria and fungi are grown in one medium, namely PDA. However, from this study there are two different antagonist activites between two isolate due to different chytinolytic activitiy between the two isolate.

Keywords*: antagonistic activity, colletotrichum sp., bacillus sp., chitinolytic activity, heterotrophic bacteria.*

INTRODUCTION

Cayenne pepper plant (*Capsicum frutescens L.*) is one of the popular vegetables comodity in Indonesia. In general, . cayenne pepper plant used as main ingridients for kitchen's need, sauce industry, chili powder, instant noodle, and pharmaceutical industry. The need for cayenne pepper in Indonesia is very high, namely: reach 4 kg/capita/year (Saraswati et al., 2002). There are several problems on cayenne pepper plant cultivation such as ; limited area, weahter, and disease from pathogen. Antrachnose diseease causes brown-black colored spots then spread into rot on stem, branch, leaf, and fruit. This diseae causes 50 % market loss. Infection occurs during implantation to harvesting with the result decrease in quality and quantity of crop (Nurjasmi & Suryani, 2020).

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Colletotrichum sp is from Nectrioidaceae family that has plenty of aservulus beneath the cuticule or on the surface, the diameter is up to 10 nm, black colored and many setae. The setae has dark brown colored, rigid, and sharp. This fungus created a lot of sclerotium on a sick crop or medium (Firdausyi, 2005).

Massive Pesticidizing is the most common way to control the spread of the disease. Over a 60 types of pesticides used by farmers to the central production with 2-3 times a day for a week. Nevertheless, pesticides chemically damage the environtment and also reduce the quality of crop. Hence, an alternative solution is needed to control the disease without bad impact. One of the possible way to be an alternative solution is with the utilization probiotic bacteria (Nurjasmi & Suryani, 2020). Several bacteria has antagonist feature either around the root, or endophytes with the roots. This bacteria physiologically able to produce extrcelullar enymes (chytinase, protease, cellulase), HCN, phospat solvent, and flourescence activity. *Bacillus* sp is one of the probiotic bacteria with the capability to infect the fungi pathogen and produce mannitol and salisin (Sriyanti et al., 2015).

Probiotic bacteria inhibits the growth of pathogen with decomposing chyitin from fungus. Chitinase activity from the bacteria breaks the β-1,4 bond between N-acetylglukosamin from chytin polymer (Wang et al., 2005), Another mechanism is to produce siderophores under conditions that are limited in iron, by binding to ferum ions needed by pathogenic fungi (Schulz et al, 2006). so that supress the hifa development of fungus. The other way for bacteria to inhibit the fungus is bind ferrum ion needed for fungus by produce limited ferrum siderophore (Triyanto et al., 2009).

Bacillus sp is a uniseluller heterotrophic bacteria and act as decomposer. *Bacillus* sp is a marine bactreia that can produce antibiotic to fight pathogen. The bacteria is able to produce several enzymes such as ; alanine and forniat, α-amilase, isoamilase, β amilase, glukoamilase, chitinase, dan cholesterol oxydase (Hatmanti, 2000). Therefore, a research of effectifity of bacillus sp to inhibits the growth of *Colletotrichum* sp is needed to reduce the use of massive pesticides.

RESEARCH METHODS

1. Tools and materials

Tools used for this study are; petridish glass, erlenmeyer tube, reaction tube, measuring cup, micropipete, autoclave, laminar air flow, vortex, gas stove, bunsen lamp, pan, ose, microscope, digital measurer, allumunium foil, pinset, tissue, and oven.

The materials used for this study are; cayenne pepper plant infected by *Colletotrichum* sp., *Bacillus* sp. isolate, PDA (*potato dextrose agar*) medium, ciprofloxacin. Lipolytic medium, celulolytic medium, amilolytic medium, pikovskaya's medium, proteolytic medium, aquades.

2. *Colletotrichum* sp. isolation

The method followed (Sriyanti et al., 2015) with modification. Infected fruits were taken from farm at P.T Biotek Cipta Kreasi. The fruit later fully washed then dried with tissue. The fruit were cut in into 0,5 x 0,5 size then dipped on to sodium hipoklorit

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(NaClO) for 3 minutes. Then fruit were grew in PDA medium which contain ciprofloxacin to prevent contaminant. Medium were incubated for 3 days then subcultured until pure isolate shown.

3. *Bacillus* sp. isolation

Graded diluitoin were used for *Bacillus* sp. isolation. Soil were taken around the cayenne pepper plant root rizosphere at 5 cm deep and then added to sterilizied water. Soil were heated at 80^{o}C for 10 minutes to gain bacillus sp isolate. Soil suspension were homogenated then 1 ml soil added to reaction tube with 9 ml sterilizied water, dilution were repeated until 10^{-4} (Sriyanti et al., 2015).

4. Enzymatic Test

- Lipolytic test

The lipolytic test followed a modified (Simamora and Sukmawati, 2020) method. Lipolytic media in 50 ml consisted of peptone 0.01 g, NaNO3 0.05 g, CaCl2 0.05 g, Tween-80 0.5 g, K2HPO4 0.05 g, MgSO4 0.025 g, KCl 0.025 g, Agar 0.75 g. The mixed material was then sterilized in an autoclave at 121℃ for 15 minutes. Bacillus megaterium bacteria were inoculated at four points of the petri dish as a repeat. Then incubated for 24 hours at room temperature. If Bacillus megaterium bacteria can grow in this medium, it can be concluded that these bacteria can dissolve lipase, this is because they are able to survive in various extreme environments, with carbohydrate content that only comes from Tween-80 (Simamora and Sukmawati, 2020).
Lipolytic, celulolytic, amilolytic, proteolytic medium was used for the enzymatic test. Pure culture of *Bacillus* sp. were used for the test. In lipolytic media was using tween-80 solution, cellulolytic media using 0.4% CMC (Carboxyl Methyl Cellulose) material, while in amylolytic was using 1% soluble starch material. In the proteolytic test were using NA media mixed with skim milk that has been oven-dried at 80°C for 10 minutes (Simamora & Sukmawati, 2020).

- Cellulolytic test

The cellulolytic assay followed a modified method (Rosalia et al, 2021). Carboxy Methyl Cellulose (CMC) media was prepared in 50 ml with a composition of 0.1 g CMC; 0.025 g MgSO4.7H2O; 0.05 g K2HPO4; 0.75 g agar; 0.05 g NaNO3; 0.025 KCl; 0.01 peptone). The mixed material was then sterilized in an autoclave at 121℃ for 15 minutes. Bacillus megaterium bacteria were inoculated at four points of the petri dish as a repeat. Then incubated for 24 hours at room temperature. Then lugol's iodine was added to see the clear zone. The clear zone around the bacterial colony indicates that the bacteria can dissolve cellulase (+) (Rosalia et al., 2021).

- Amylolytic test

The amylolytic assay followed the modified method (Putra et al, 2019). The amylolytic medium in 50 ml consisted of 0.5 g of starch; 0.025 g MgSO4.7H2O; 0.05 g K2HPO4; 0.75 g agar; 0.05 g NaNO3; 0.025 KCl; 0.01 peptone. The mixed material was then sterilized in an autoclave at 121℃ for 15 minutes. Bacillus megaterium bacteria were inoculated at four points of the petri dish as a repeat. Then incubated for 24 hours at room temperature. Then lugol's iodine was added to see the clear zone. The clear zone around the bacterial colony indicates that the bacteria can dissolve amylase (+) (Putra et al., 2019).

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- Proteolytic test

The proteolytic assay followed a modified (Manik and Simanjuntak, 2020) method. The proteolytic medium in 50 ml consisted of 0.75 g agar and 0.5 g skim milk. Making proteolytic media by using 2 Erlenmeyer containing 25 ml of RO water each. The first erlenmeyer contains 25 ml of RO water and 0.75 g of agar, while the second erlenmeyer only contains 25 ml of RO water. Then autoclaved at 121℃ for 15 minutes. 0.5 g of skimmed milk in UV in LAF for 15 minutes, this is done to minimize contamination and to avoid damage to the protein in the skim milk. After the RO water in the second erlenmeyer cools, the skim milk is added and then shaken until evenly distributed. Then it was poured into the first erlenmeyer containing 25 ml of RO water and 0.75 g of agar in a lukewarm condition. Bacillus megaterium bacteria were inoculated at four points of the petri dish as a repeat. Then incubated for 24 hours at room temperature. After 24 hours a clear zone will appear around the bacterial colony which indicates that the bacteria can dissolve proteases (+) (Manik & Simanjuntak, 2020).

- Phosphate solvent test

The phosphate solvent test followed the modified method (Walida et al, 2019). Phosphate solvent medium in 50 ml consisted of 1,565 g pikov'skaya, 0.25 g Ca3(PO4)2, and 50 ml RO water. The media was autoclaved at 121℃ for 15 minutes. *Bacillus megaterium* bacteria were inoculated at four points of the petri dish as a repeat. Then incubated until a clear zone appears at room temperature. If a clear zone appears around the bacterial colony, it indicates that the bacteria can dissolve phosphate (+) (Walida et al., 2019).

5. Antagonists Test

The antagonist test followed the modified method (Keliat and Iftari, 2017). The antagonist test was carried out on PDA media (Potato Dextrose Agar) consisting of 0.2 g yeast extract, 0.2 g malt extract, and 1 g agar in 50 ml RO water (Keliat & Iftari, 2017).

RESULTS AND DISCUSSION

1. *Bacillus* sp. isolation

Soil was taken around the cayenne pepper plant root rizosphere at 5 cm deep and then added to sterilizied water. Soil were heated at 80oC for 10 minutes to gain *Bacillus* sp. isolate. *Bacillus* sp. were taken out from rizosphere due to the bacteria around the root are able to control the pathogen by producing antibiotic substances, and nutrient space compete with pathogen (Shulz et al, 2006). The media used in this isolate was lipolytic media with the main component is Tween-80. This medium used to determine lipase activity in *Bacillus* sp.. The presence of lipolytic activity in *Bacillus* can be used as an agent to degrade the body structure of insect pests and diseases containing lipid substrates. Lipolytic test using tween 80 solution which able to detect lipase due to oleic acid ester content (Hatmanti, 2000). *Bacillus* sp. isolates. a clear zone is formed with the initial marking of the media from cloudy to clear. This shows that *Bacillus* sp are able to degrade lipid substence. *Bacillus* sp isolation result are shown in figure 1. Isolate has round shape and beige colored colony. in accordance

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with the study from saputri et al (2020) that bacillus sp has small round shaped, dull white colored, and not slimy (Agustiansyah et al., 2013) [10].

Figure 1. *Bacillus* sp. Isolate

2. *Bacillus* sp. enzmatic test and phospate solvent result

Enzymatic test are consist of lipolytic test, proteolytic test, amilolytic test and amilolytic test. This test used to determine enzymatic activity from bacillus sp. table 1. Enzymatic test result. Table 1 shows that bacillus sp close-related isolate has lipolytic activity characterized by the formation of clear zone around the colony in lipolytic medium.

Table 1. Enzmatic Test and Phospate Solvent Result

No.	Perlakuan	Indeks (%)
1.	Lipolytic test	+
2.	Amylolytic test	62,5%
3.	Cellulolytic test	48,825%
4.	Proteolytic test	32,125%
5.	Phosphat esolvent test	53,75%

Based on Table 1, it shows that the isolates close to *Bacillus* sp. has amylolytic activity characterized by the formation of a clear zone around the colony where the amylolytic index is 62.5%. Amylolytic test is an enzymatic test that aims to determine the activity of amylase in bacteria. Amylolytic bacteria are the bacteria that can produce amylase enzymes that can break down starch into glucose. In the amylolytic test used 1% soluble starch which able to produce turbidity in the medium so that if the bacteria has amylase activity it will form a clear zone around the colony. The amylolytic test produced a clear zone with a percentage of 62.5%. This clear zone appears after dropping 1% iodine, the iodine solution will be absorbed by the selective media around the clear zone so that it will produce a dark blue color (Ervina et al., 2020). Based on the research of Tampangallo (2013), the clear zone produced in the amylolytic test was 62.8%. In the amylolytic test the size of the clear zone produced is smaller than the previous study. So it can be concluded that the size of the resulting diameter depends on the ability of the bacteria to hydrolyze amylase (Saputri et al., 2020).

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Cellulolytic test is an enzymatic test that aims to determine cellulase activity in bacteria. Cellulolytic bacteria are bacteria that able to produce cellulase enzymes in response to the presence of cellulose in their environment, and have the ability to hydrolyze cellulose into glucose. In the cellulolytic test, 0.4% CMC was used to produce turbidity in the media so that if the bacteria had cellulase activity, a clear zone would be formed around the colony. The cellulolytic test produces a clear zone with a percentage of 48.825%. Hydrolysis by the extracellular enzyme cellulase excreted by each bacterial isolate to form a clear zone. The size of the clear zone depends on the ability of each isolate to hydrolyze cellulos (Ervina et al., 2020).

Proteolytic test used to determine the activity of protease enzyme in bacteria. Proteolytic test using skim milk. Skim milk able to produce turbidity in the media so that if the bacteria have protease activity, a clear zone will be formed around the colony. The proteolytic test produced a clear zone with a percentage of 32.125%, so *Bacillus* sp. It has very high protease activity. The clear zone formed is the result of the protein hydrolysis process, which is a white colloid suspension into derivative compounds that are more soluble and transparent (Zubaidah et al., 2019).

Phosphate solvent test aims to determine the activity of bacteria to dissolve phosphorus. Based on the phosphate solvent test, it produces a clear zone with a percentage of 53.75%. The ability to dissolve phosphate is indicated by the presence of a clear zone around the bacterial colonies. This occurs due to the presence of organic acids excreted by bacteria and then binds to Ca ions from Ca3(PO4)2 in Pikovskaya media and release H2PO4 then form a clear colored area (Oksana et al., 2020).

3. *Colletotrichum* sp. isolation result

Sampling was carried out by cutting the parts of fruit that were affected by the fungus Colletotrichum sp. Then the samples were taken to the laboratory to be washed and soaked in sodium hypochlorite (NaClO) to select the growth of fungi in the media. Image of cayenne pepper affected by Colletotrichum sp. The cause of anthracnose disease shown in Figure 2.

Figure 2. *Colletotrichum* sp. Isolate

4. Antagonists test result

Antagonist test aims to determine the ablity of bacillus sp to inhibits Colletotrichum sp. growth in PDA medium. From the antagoists test, There are several bacteria with antagonists properties and several bacteria without the antagonist properties as seen in table 2.

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Table 2. Hasil Pengamatan Uji Antagonis

Tipe *Bacillus* sp.	Gambar	Aktivitas
Bacillus 1		-
Bacillus A2		+

The *Bacillus* 1 isolate do not have antagonistic activity due to the lack of activity of *Bacillus* sp. in hydrolyzing chitin in fungi. Meanwhile, the *Bacillus* A2 isolate belonging to PT Biotek Cipta Kreasi has antagonistic activity against fungi. This is because *Bacillus* A2 has the ability to hydrolyze chitin in fungi.

The inhibition zone around the bacterial colonies was thought to be due to the hydrolysis of chitin released by the bacteria on the cell wall of *Colletotrichum* sp. This causes the mycelium *Colletotrichum* sp. unable to grow close to the bacterial colony and appear to be damaged (Wibowo et al., 2017).

CONCLUSION

Based on intern research at PT.Biotek Cipta Kreasi, we conclude that *Bacillus* A2 bacteria has antagonis activity against the *Colletotrichum* sp. so that the bacteria able to inhibits the fungi growth. However, *Bacillus* sp. isolate do not have antagonists activity so that the bacteria are unable to supress *Colletotrichum* sp. growth that causes antrachnose on cayenne pepper plant (*Capsicum frutescens* L.).

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