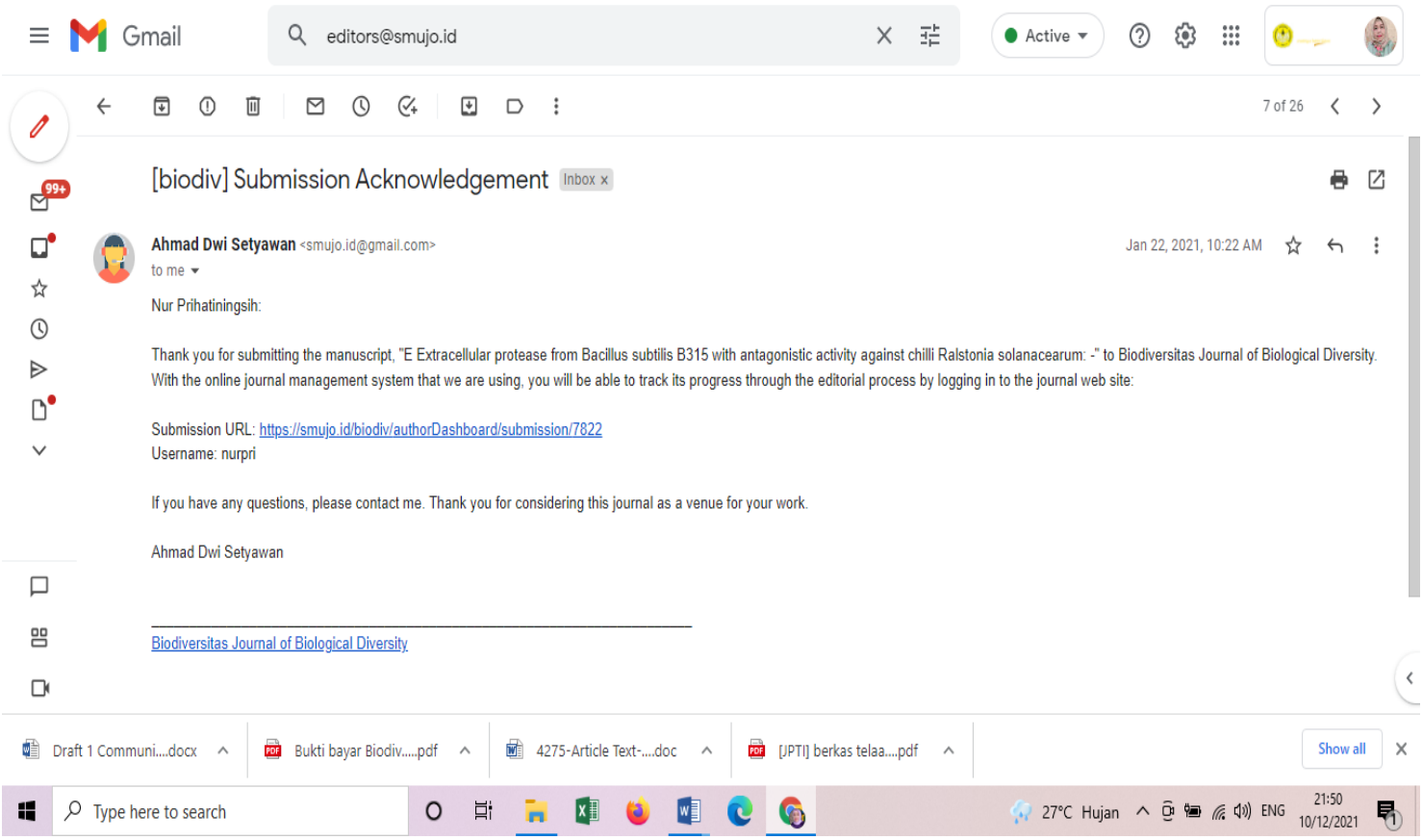
Bukti Korespondensi Jurnal Biodiversitas Vol 22(3) , March 2021

Bukti Submit



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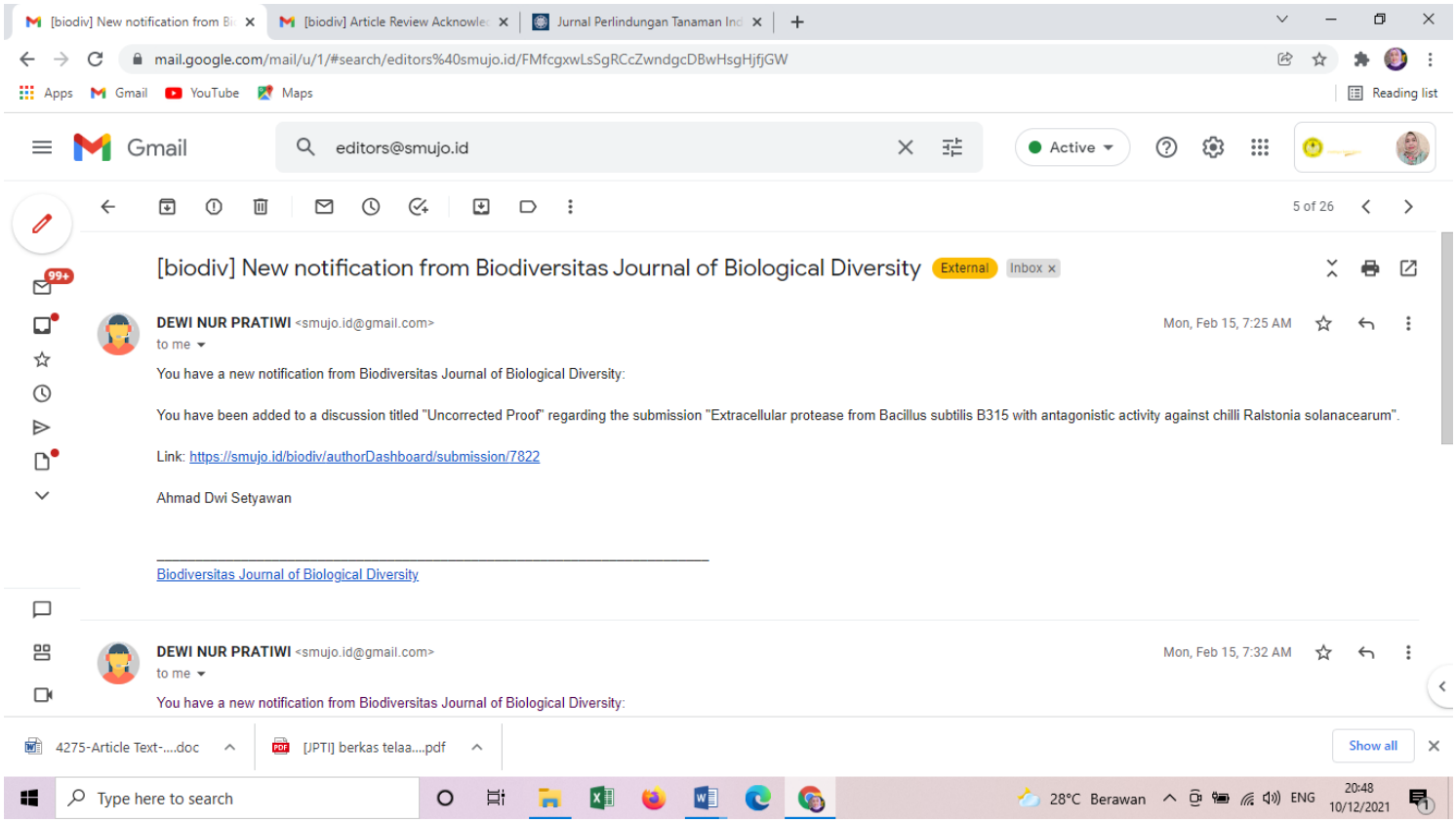

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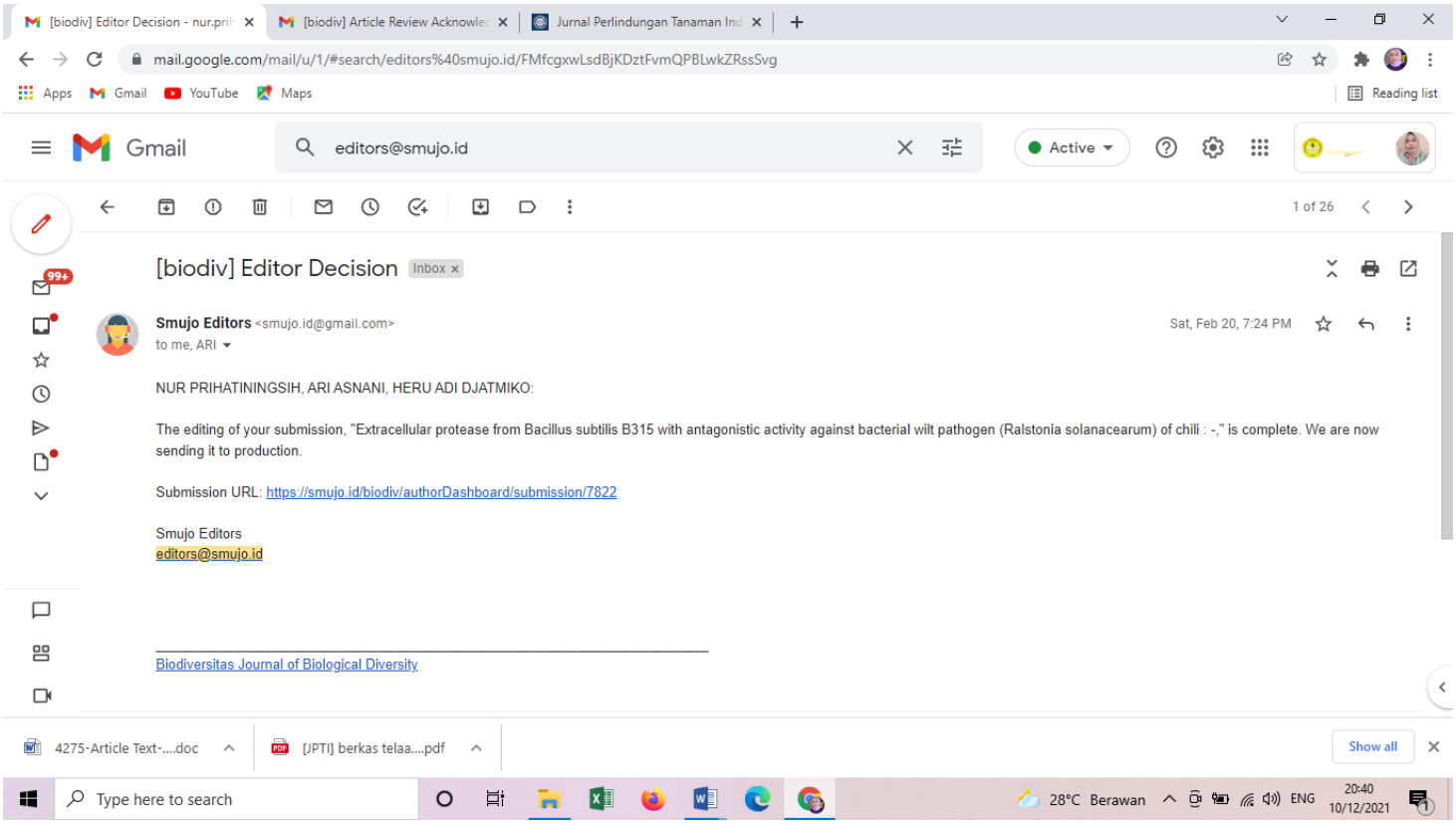

[biodiv] Editor Decision

Smujo Editors <smujo.id@gmail.com> Sat, Feb 20, 7:24 PM
to me, ARI

NUR PRIHATININGSIH, ARI ASNANI, HERU ADI DJATMIKO:

The editing of your submission, "Extracellular protease from Bacillus subtilis B315 with antagonistic activity against bacterial wilt pathogen (Ralstonia solanacearum) of chili : -," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/7822

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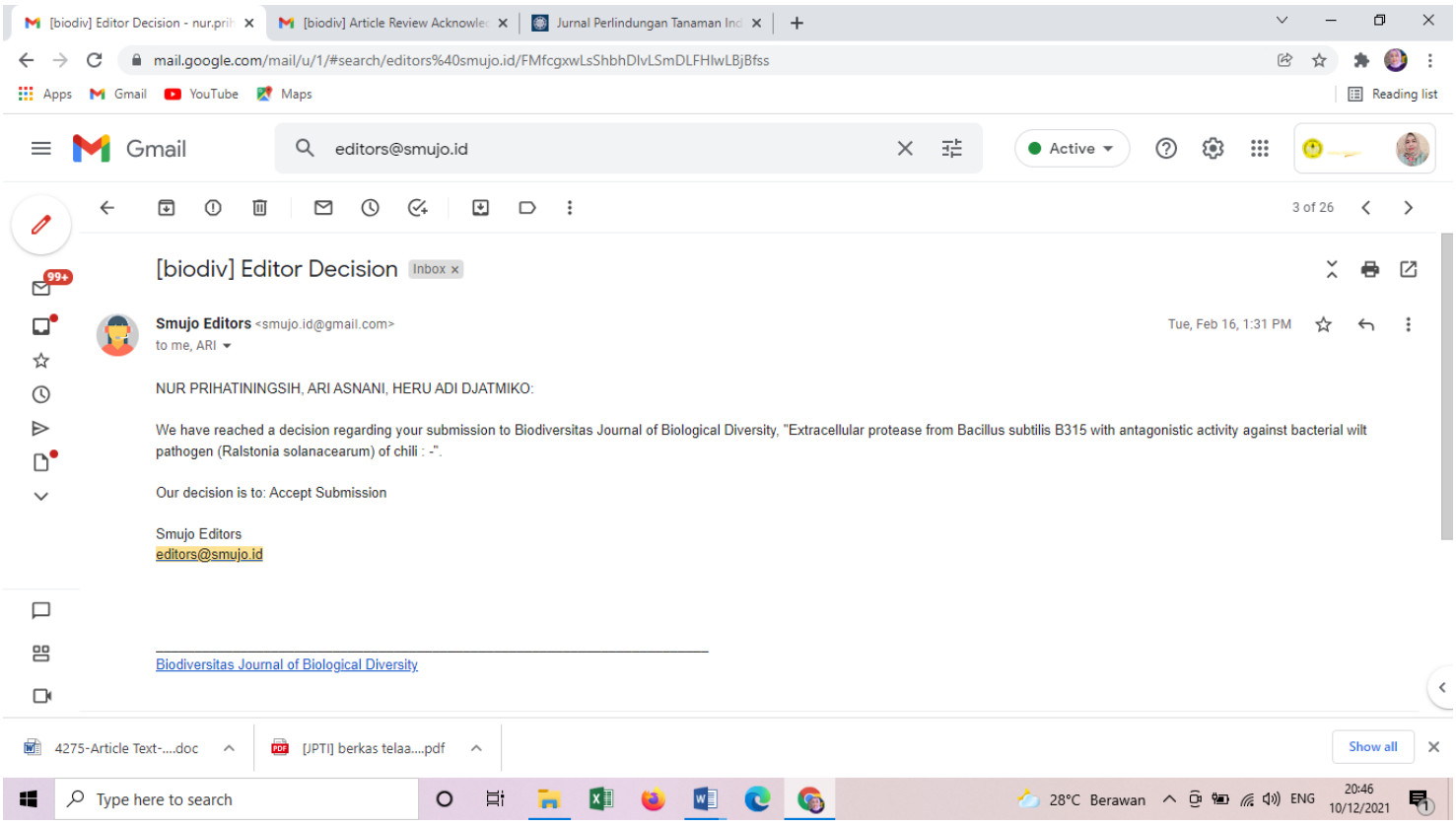

[biodiv] Editor Decision

Smujo Editors <smujo.id@gmail.com> Tue, Feb 16, 1:31 PM
to me, ARI

NUR PRIHATININGSIH, ARI ASNANI, HERU ADI DJATMIKO:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Extracellular protease from Bacillus subtilis B315 with antagonistic activity against bacterial wilt pathogen (Ralstonia solanacearum) of chili : -".

Our decision is to: Accept Submission

Smujo Editors
editors@smujo.id

Biodiversitas Journal of Biological Diversity

COVERING LETTER

Dear **Editor-in-Chief,**

I herewith enclosed a research article,

Title:

Protease from *Bacillus subtilis* B315 with antagonistic activity against chilli *Ralstonia solanacearum*

Author(s) name:

NUR PRIHATININGSIH 1), ARI ASNANI 2), HERU ADI DJATMIKO 3)

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Novelty:

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Protease of *Bacillus subtilis* B315 was potential to control bacterial wilt mechanism on chili and could be produce by *B.subtilis* B315 at optimum incubation period, incubation pH and concentration of extract protease.

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Purwokerto, 22-1-2021

Sincerely yours,

(fill in your name, no need scanned autograph)

Nur Prihatiningsih

Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against chilli *Ralstonia solanacearum*

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Manuscript received: DD MM (Date of abstract/manuscript submission). Revision accepted: ..... . (8 pt)

Abstract. Antagonistic bacteria isolated from a healthy potato is potential as a biocontrol agent for bacterial pathogens. The capability of antagonistic bacteria to produce extracellular protease could be considered as an indicator of its antagonic capacity. The objective of this research was to study the optimum activity of protease produced by *Bacillus subtilis* B315 to control chili *Ralstonia solanacearum.* In this research optimum incubation time, pH and crude extract protease concentration of *B. subtilis* B315 was examined for maximum production of extracellular protease. The results showed that *B. subtilis* B315 was capable of producing protease at optimum incubation time of 30 min, pH 9 and crude extract protease concentration 0.50% with an activity of 1.3185 U/mL. Protease-producing capability of *B. subtilis* B315 was shown by the formation of a clear zone around the colony of *B. subtilis* B315 grown on skim-milk agar medium. *B. subtilis* B315 was capable of inhibiting the growth of *R. solanacearum* in vitro as indicated by 32 mm clear zone. Furthermore, *in planta* application of *B subtilis* showed that this bacteria was capable of controlling chili bacterial wilt caused by *R. solanacearum* with an effectiveness of 60,89%. The prospectically of *B. subtilis* B315 could formulated as biopesticide.

Key words: *Bacillus subtilis* B315, bacterial wilt, biocontrol, chilli, protease activity

INTRODUCTION (10 PT)

Bacterial wilt caused by *R. solanacearum* is an important disease in plants that may includes solanaceae, bananas, potatoes, ginger and mulberries. In chili and tomato, bacterial wilt could reduce yields up to 80% (Vanitha et al. 2009). Curently, biological control using antagonistic bacteria has been widely studied, as it is more environmentally friendly and supports sustainable agriculture. Antagonistic bacteria could be explored from habitats of which the pathogen naturally lives.

B. subtilis B315 isolated from a healthy potato rhizosphere has been reported to be capable of controlling bacterial wilt in potatoes with an effectiveness of 64.9% (Prihatiningsih 2013), and 74.6% in tomato, chili and eggplant (Prihatiningsih and Djatmiko 2016). The potential of bacteria to be antagonist is shown by its ability to produce secondary metabolites (antibiotics and other compounds) and enzymes. Protease is a product of microbes which could functions to control plant pathogens, either fungi or bacteria. *Bacillus* sp. and other bacteria such as: *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Shumi and Anwar 2004) secretes extracellular enzymes including proteases. Saha et al. (2012) found two new strains of *B. subtilis* from eggplant rhizosphere that have the potential to be biocontrol agent which is shown by their capabilities of producing secondary metabolites, such as siderophore, and hydrolytic enzymes, such as chitinase, protease, lipase and amylase. *B subtilis* was also reported to be able to produce IAA (*indole acetic acid*) ranging from 57.56-79.33 ppm (Prihatiningsih et al. 2020). *B. subtilis* has ability to secrete amylase, protease, pullunase, chitinase, xylanase and lipase (Morikawa 2006; Lestari et al. 2017).

Protease is a hydrolytic enzyme that hydrolyses extracellular proteins such that increases the ability of cells to absorb useful hydrolysis products. *B. subtilis* has important bacteria because its capability to secretes several hydrolytic enzymes, including protease, into the culture medium (Almas et al. 2009). Antagonistic *Bacillus* sp. significantly colonized rice plants and secreted various biocontrol determinants like protease of 1.1±5.5 U/mg of soil or U/mL of hydroponic solution. *Bacillus* spp. strains KFP-5 and KFP-17 produced variable quantities of siderophore, protease and glucanase in absence as well as in presence of rice blast pathogen *Pyricularia oryzae*. The protease production is ranging about 20.9-29.3 U/mL and 11.6-24 U/mL in KFP-5 and KFP-17, respectively (Rais et al. 2017).

B. subtilis B315 has important to search as protease production because this enzym supported to crops for enhanched resistance to disease. Nevertheles enzymatic treatment was secreted by *B. subtilis* B315 as antagonist bacteria resulted in degradation of the EPS (*extracellular polymeric substances*) components and significant eradication of the biofilm of bacteria pathogens. Biofilm bacteria pathogens were role on allow cell-to-cell communication, and responsible for adhesion to surface, its advantage to able survive in unfavorable environment conditions and it's much less susceptible to the antimicrobial agents (Mitrofanova et al. 2017). Most antibiotics cannot penetrate to biofilm but use the protease could

degradation the biofilm of bacterial pathogens, so the optimum of protease production by *B. subtilis* B315 should be important to detection.

The objective of this research was to study the optimum activity of protease produced by extracellular *B. subtilis* B315 to control chilli *R. solanacearum*. In this research optimum incubation time, pH and concentration of crude extract protease *B. subtilis* B315 was examined for maximum production of extracellular protease.

MATERIALS AND METHODS

Preparation of *B. subtilis* B315 dan *R. solanacearum* isolates

Culture stock of *B. subtilis* B315 in 20% glycerol was used in this study. This isolate was previously reported to be capable of controlling against *R. solanacearum* of potato (Prihatiningsih et al. 2006; Prihatiningsih 2013). *R. solanacearum* was isolated from the roots of bacterial wilt chilli and that was grown on CPG-TTC medium (Denny and Hayward 2001). *R. solanacearum* was characterized by creamy white fluidal colony with pink-red colour in the middle, irregular or regular shape, flat surface with uneven edges (Singh et al. 2010).

Protease Assay of *B. subtilis* B315 on skim milk agar medium

The qualitative test of protease producing by *B. subtilis* B315 was prepared it's bacteria grown on skim milk agar medium (casein 5g, yeast extract 2,5g, glucose 1g, agar 15g, destilled water 1000 mL, skim milk 7% was added as inducer (Majumdar and Chakraborty 2017). Protease assay of *B. subtilis* B315 was carried out through protease screening using a skim milk agar medium with scratched inoculated method of one loopfull (Vijayaraghavan and Vincent 2013; Majumdar and Chakraborty 2017). Clear zone appeared around scratched was protease secreted by *B. subtilis* B315.

Protease producing assay of *B. subtilis* B315

Protease activity was measured using a modified Kunitz method (Majumdar and Chakraborty 2017). For the assay, a total of 0.5 ml Casein substrate (0.6 w/v) was added in 0.1 M Tris-HCl buffer at pH 8.0. Enzymatic reaction was initiated by adding 0.1 ml of protease solution to the Tris-HCl buffer at 45ºC and incubation time of 30 minutes. The protease was prepared using ethyl acetate extraction method of Alnahdi (2012; Abu-Mejdad et al. 2013). The reaction was stopped by adding 0.5 ml cold TCA (*Trichloroacetic Acid*) followed by centrifugation at 4000 rpm at 4ºC for 15 minutes. The dissolved peptide in the supernatant was further measured using spectrophotometer at ƛ 275 nm. The control solution is made with the same treatment but the substrate and the cold TCA were first dissolved and then protease solution was added. The standard solution used to measure proteolytic activity was tyrosine 1-120 µg /mL. One unit of protease activity (U) is defined as the amount of enzyme needed to produce 1 µg of tyrosine/minute ml of the enzyme solution from the Casein substrate at certain pH and temperature conditions, calculated by the formula

$$\text{Activity} = \frac{(a) - (b)}{30 \text{ min.} \times \text{mL enzyme}} \times \text{dilution factor}$$

Note: (a) = µg tyrosine/mL sample, (b) = µg tyrosine/mL control, dilution factor = total volume /(enzyme volume + filtrate sample volume).

In this research 4 factors, namely: incubation periods, incubation pH, and crude extract protease concentration were studied to know the optimum condition for protease activity of *B subtilis* B315. The incubation period were 15, 30, 45 and 60 min. The incubation pH: 7 of Phosphate buffer, pH 8 and 9 of Tris-HCl buffer, pH 10 of NaHCO, and pH 11 of NaOH buffer. While the crude extract protease concentration were 0,1, 0,3, 0,5 and 0,7%. For determination of optimum temperature for enzyme activity, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time and 1 ml protease extract of *B. subtilis* B315). For determination of optimum pH, incubation condition was set following standard protocol (30 minutes incubation time, 45ºC and 1 ml protease extract of *B. subtilis*) except the pH was varied at pH 7, 8, 9, 10 and 11. For determination of optimum enzyme concentration, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time at 45ºC) except the protease extract concentration was varied at 0.1, 0.3, 0.5, and 0.7%.

Biocontrol potency test against *R. solanacearum in vitro*

Potency of *B. subtilis* B315 as a biocontrol of *R. solanacearum in vitro* was done by the two-layer medium method as stated by Ghosh et al. (2007). *B. subtilis* B315 inoculated a point on the YPGA medium (*yeast peptone glucosa agar*), after 24 hours the Petri dish was turned over and droped with 500 µl chloroform, incubated for 3 hours until the chloroform evaporated. Pathogenic bacteria of *R. solanacearum* chilies isolates that have been grown on YPGA medium are harvested

with 10 ml of sterile water, then 200 µl are put into 4 mL of 0.6% water agar solution which slowly poured into the Petri dish. After 48 hours of incubation an inhibition zone was formed.

Plant Resistance of bacterial wilt on chilli by *B. subtilis* B315

Control bacterial wilt of chilli with *B. subtilis* B315 is carried out by soaking chilli seeds before seedling in *B. subtilis* B315 suspension with a population density of 10^8 cfu/ml, the next treatment is watering with *B. subtilis* B315 suspension at the same concentration when moving planting age 10, 20 and 30 HST (days after planting) at a dose of 100 ml/plant. The observation of the effectiveness of wilt disease control is done by assessing the intensity of the disease using the formula DI = a / b x 100%, with a: number of wilted plant; b: total number of plants observed (Aslam et al. 2015). Then, calculation of control effectiveness is to compare the intensity of control disease with the intensity of treatment disease according to Pawaskar et al. (2014) E = (control DI - treatment DI)/control DI x 100%.

RESULTS AND DISCUSSION

The qualitative test of protease

The qualitative protease was produced by *B. subtilis* B315 showed that clear zone around scratched was protease secreted by *B. subtilis* B315 (Fig. 1). The role of this protease in biocontrol plant pathogens and enhanced plant resistant to pathogens infection (Jha and Bhattacharyya 2012; Mitrofanova et al. 2017).

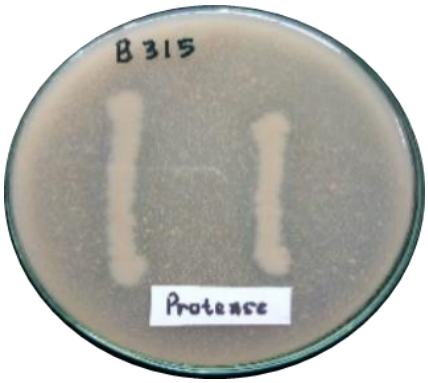

Figure 1. Clear zone around the scratches of *B. subtilis* B315 on skim milk medium to show its potency to produce protease

The clear zone in the protease-producing test of *B. subtilis* B315 means that *Bacillus* is able to produce proteases indicated by a zone width ranging from 12-26 mm. In qualitative plate assay, isolate S1 exhibited the largest clear zone (30±1.13 mm) in skim milk agar and isolate S5 exhibited the lowest (18±1.41 mm), which S1 and S5 isolates are Gram positive bacteria (Bhowmik et al. 2015; Joshi and Ghike 2019).

The protease activity of *B. subtilis* B315

The protease activity of *B. subtilis* B315 was calculated based on the regression equation obtained from the absorbance value of the tyrosine standard at ƛ 275 nm, as follows: Y = 0.0045x + 0.0016 (Fig. 2), the protease activity of *B. subtilis* B315 at various incubation period, pH and crude extract protease concentration are shown in Fig. 3, 4 and 5. The activity of protease *B. subtilis* B315 showed at 30 minutes incubation period was the highest by 1.259 U/ml (Fig 3) and at pH incubation 9 was maximum protease activity by 1.422 U/ml (Fig 4).

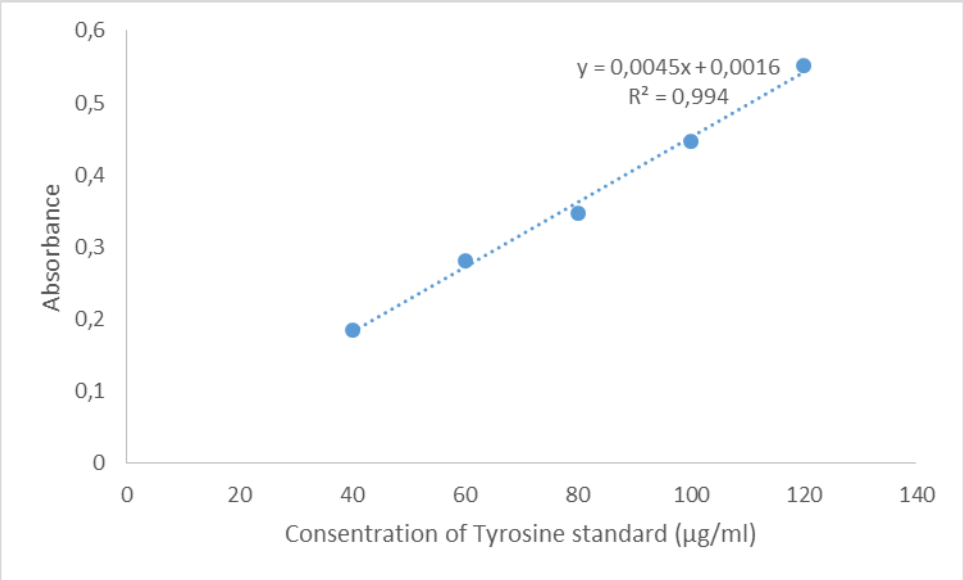

Figure 2. Absorbance of tyrosine standard at ƛ 275 nm

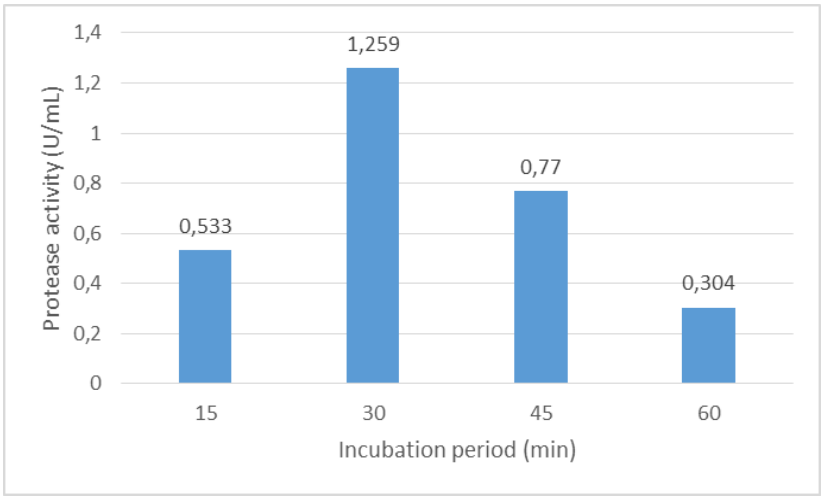

Figure 3. Protease activity of *B. subtilis* B315 at variation incubation periods

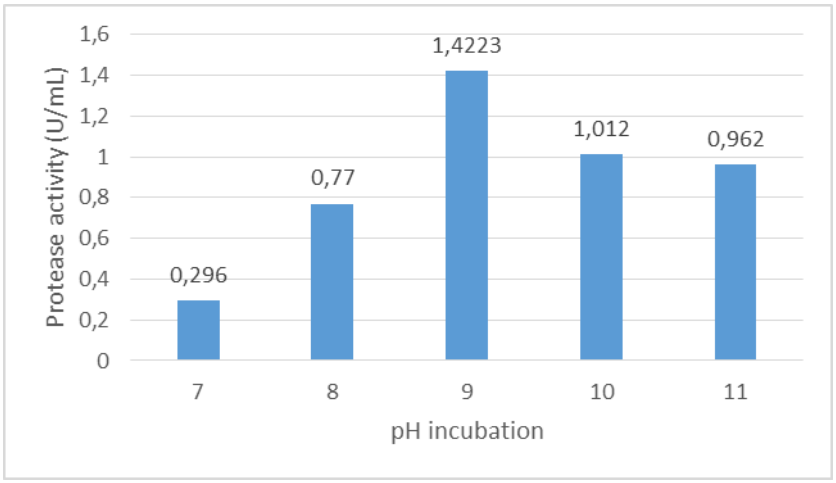

Figure 4. Protease activity of *B. subtilis* B315 at variation pH

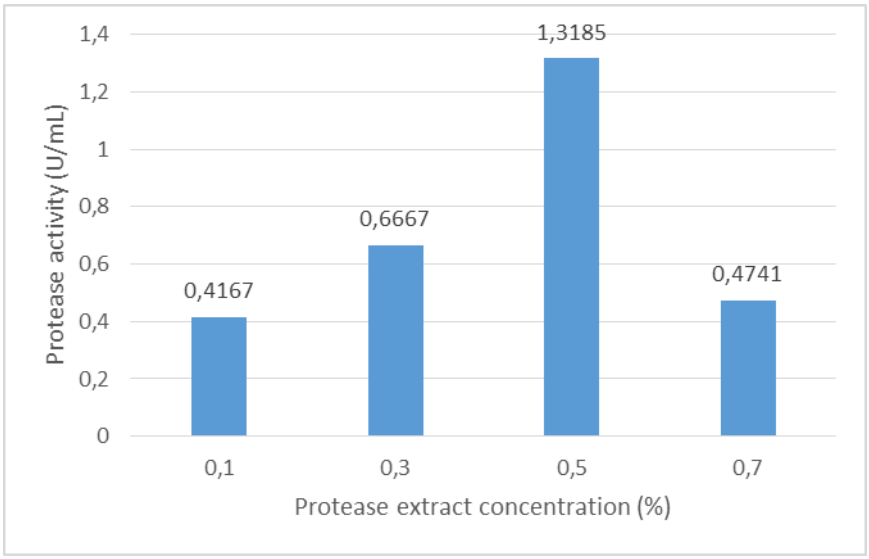

Figure 5. Protease activity of *B. subtilis* B315 at variation crude extract protease concentration

The incubation time variations showed that 30 minutes was the best treatment with the protease activity was 1.259 U/ml. The treatment of variations in incubation pH showed the highest protease activity was at pH 9 of 1.422 U/ml. Protease activity at various concentrations at pH 9 with 30 min. incubation time of 1.318 U/ml was at a concentration of 0.5% solution. The effect of extract enzym concentration on protease activity is shown in Figure 5 based on the best pH and incubation time (9 and 30 min.). *B. subtilis* is the bacteria resistance to growth at a high temperature. The activity crude protease at 40ºC, pH 8 by 255 U/ml (Jadhav et al. 2014). The protease activity was 236.37 U/ml and 175,083 U/ml with carbon source and nitrogen source at pH 7.4 and incubation temperature 50ºC respectively (Pant et al. 2015). The activity of protease depends on the Bacillus strain , medium, physical and chemical factor (Queiroga et al. 2013; Tebyanian et al. 2018). *Bacillus* spp. strains KFP-5, KFP-7 and KFP-17 produced variable quantities of siderophore, protease and glucanase in absence as well as presence of rice blast pathogen *Pyricularia oryzae*, protease (20.9±29.3U/mL) presence *P oryzae*, and protease (11.6±24 U/mL) if absent *P oryzae* (Rais et al. 2017).

Biocontrol potency test against *R. solanacearum in vitro*

The potency of *B. subtilis* B315 as a biocontrol of *R. solanacearum in vitro*, is shown that the largest inhibition zone for *R. solanacearum in vitro* of chilli by 32 mm (Fig. 6.). *In vitro* inhibition test results of *R. solanacearum* isolat chilli was smaler than its suppress for potato isolates of *R. solanacearum* because *B. subtilis* B315 is isolated from healthy potato plants as there habitat, the bacterial have adaptif to the condition. *B. subtilis* B315 can suppressed the *R. solanacearum* potato was 36 mm. However *B. subtilis* B315 capable control against *R. solanacearum* family of solanaceae.

Figure 6. The inhibition zone of *B. subtilis* B315 against chilli *R. solanacearum*

Protease secreted by *B. subtilis* B315 has effect for the virulence of bacterial pathogens with combating biofilms formation, and induce stress tolerance in plants. *Bacillus* sp. which the genomics, proteomics and metabolics were required to elucidate the mechanism of *Bacillus*-plant interaction for biotic and abiotic stress management in crops

Radhakrishnan et al. (2017). *Bacillus* is also known to produce an array of extracelullar proteolytic enzymes. *Bacillus* proteases have high stability and low pathogenicity and can be easily purified and obtained in industrial quantities. These features make them promising matrix-degrading agents for combating bacterial biofilms (Mitrofanova et al. 2017)**.** The effect of *B. subtilis* B315 treatment to inhibition of *R. solanacerum in vitro* and *in planta* showed at Table 1 with plant resistance factor to bacterial wilt showed by the effectiveness.

Table 1. Inhibition effect of *B. subtilis* B315 *in vitro, in planta*, and the effectiveness

Treatment *in vitro*	Inhibition (mm)	Disease intensity (%)	Effectiveness (%)
Control	0	7.34	-
B. subtilis B315	36	2.87	60,89
Bactericide	24	3.06	58.31

Note: Control (without *B. subtilis* B315, without bactericide)

Protease from *Bacillus* sp. can promote of plant resistance to pathogen, showed the decreased of disease intensity (Table 1). The plant resistance indicated by phenol compound as an indicator of increase of plant resistance to pathogens and decrease of disease intensity with the effectiveness more than 50%. The induce plant resistance by *B. subtilis* strain FZB24 could improve plant strength and furthermore avoid to pathogens infection (Jha and Bhattacharyya 2012). *B. subtilis* B298 in microencapsulated formula that applied to chilli can reduced the bacterial wilt caused by *R. solanacearum* and induced systemic resistance by increased phenol compound up to 6.27% (Prihatiningsih et al. 2019).

In conclusion, the bacteria of potato rhizosphere *B. subtilis* B315 was capable to produce protease with an activity of 1.3185 U/mL. *B. subtilis* B315 was an effective control of bacterial wilt by 60,89% in chilli. Protease from *B. subtilis* B315 could indicates of potency for control bacterial wilt because this enzyme serves to increase *B. subtilis* B315 activity as biological control agents and inductical resistance of plant varieties. The future *B. subtilis* B315 formulated as biopesticide to supported sustainable agriculture, and protease from *B. subtilis* B315 can produce as well as inductrically to control bacterial wilt of solanaceae.

ACKNOWLEDGEMENT

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CONFLIC OF INTEREST

The authors declare no Conflic of interest

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Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chilli ~~*Ralstonia solanacearum*~~

Abstract. Antagonistic bacteria isolated from a healthy potato ~~is~~ are potential ~~as a~~ biocontrol agent ~~for~~ against number of bacterial pathogens. The capability of antagonistic bacteria to produce extracellular protease could be considered as an indicator of its antagonic capacity. The objective of this research was to study the optimum activity of protease produced by *Bacillus subtilis* B315 to control ~~chili~~ *Ralstonia solanacearum* causing wilt of chili. In this research optimum incubation time, pH and crude extract protease concentration of *B. subtilis* B315 was examined for maximum production of extracellular protease. The results showed that *B. subtilis* B315 was capable of producing protease at optimum incubation time of 30 min, pH 9 and crude extract protease concentration 0.50% with an activity of 1.3185 U/mL. Protease-producing capability of *B. subtilis* B315 was shown by the formation of a clear zone around the colony of *B. subtilis* B315 grown on skim-milk agar medium. *B. subtilis* B315 was capable of inhibiting the growth of *R. solanacearum* in vitro as indicated by 32 mm clear zone. Furthermore, *in planta* application of *B subtilis* showed that this bacteria was capable of controlling chili bacterial wilt caused by *R. solanacearum* with an effectiveness of 60,89%. The prospectically of *B. subtilis* B315 could formulated as biopesticide.

Key words: *Bacillus subtilis* B315, bacterial ~~wilt, biocontrol~~wilt, biocontrol, chilli, protease activity

INTRODUCTION

Bacterial wilt caused by *R*[AKG1]. *solanacearum* is an important disease in plants that may includes solanaceae[AKG2], bananas, potatoes, ginger and mulberries. In chili and tomato, bacterial wilt could reduce yields up to 80% (Vanitha et al. 2009). Curently, biological control using antagonistic bacteria has been widely studied, as it is more environmentally friendly and supports sustainable agriculture. Antagonistic bacteria could be explored from habitats of which the pathogen naturally lives.

B.[a3] *subtilis* B315 isolated from a healthy potato rhizosphere has been reported to be capable of controlling bacterial wilt in potatoes with an effectiveness of 64.9% (Prihatiningsih 2013), and 74.6% in tomato, chili and eggplant (Prihatiningsih and Djatmiko 2016). The potential of bacteria to be antagonist is shown by its ability to produce secondary metabolites (antibiotics and other compounds) and enzymes. Protease is a product of microbes which could functions to control plant pathogens, either fungi or bacteria. *Bacillus* sp. and other bacteria such as: *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Shumi and Anwar 2004) secretes extracellular enzymes including proteases. Saha et al. (2012) found two new strains of *B. subtilis* from eggplant rhizosphere that have the potential to be biocontrol agent which is shown by their capabilities of producing secondary metabolites, such as siderophore, and hydrolytic enzymes, such as chitinase, protease, lipase and amylase. *B subtilis* was also reported to be able to produce IAA (~~*indole*~~ *Indole* ~~*acetic*~~ *Acetic* ~~*acid*~~*Acid*) ranging from 57.56-79.33 ppm (Prihatiningsih et al. 2020). *B. subtilis* has ability to secrete amylase, protease, pullunase, chitinase, xylanase and lipase (Morikawa 2006; Lestari et al. 2017).

Protease is a hydrolytic enzyme that hydrolyses extracellular proteins ~~such that~~and increases the ability of cells to absorb useful hydrolysis products. *B. subtilis* has important bacteria because of its capability to secretes several hydrolytic enzymes, including protease, into the culture medium (Almas et al. 2009). ~~–~~Antagonistic *Bacillus* sp. significantly colonize~~d~~ rice plants and secrete~~d~~ various biocontrol determinants like protease of 1.1±5.5 U/mg of soil or U/mL of hydroponic solution. ~~-~~*Bacillus* spp. strains KFP-5 and KFP-17 produce~~d~~ variable quantities of siderophore, protease and glucanase in absence as well as in presence of rice blast pathogen *Pyricularia oryzae*. The protease production is ranging about 20.9-29.3 U/mL and 11.6-24 U/mL in KFP-5 and KFP-17, respectively (Rais et al. 2017).

B. subtilis B315 has important ~~to search as~~potential to produce protease ~~production~~ because this enzyme supported ~~to~~ the crops for enhanched resistance to disease. Nevertheles enzymatic treatment was secreted by *B. subtilis* B315 as

antagonist bacteria resulted in degradation of the EPS (*extracellular polymeric substances*) components and significant eradication of the biofilm of bacteria pathogens. —Biofilm bacteria pathogens were role on allow cell-to-cell communication, and responsible for adhesion to surface, its advantage to able survive in unfavorable environment conditions and it's much less susceptible to the antimicrobial agents (Mitrofanova et al. 2017). [a4]Most antibiotics cannot penetrate to biofilm but use the protease could degradation the biofilm of bacterial pathogens, so the optimum of protease production by *B. subtilis* B315 should be important to detection.

The objective of this research was to study the optimum activity of protease produced ~~by~~ extracellularly by *B. subtilis* B315 to control chilli *R. solanacearum*. In this research optimum incubation time, pH and concentration of crude extract protease *B. subtilis* B315 was examined for maximum production of extracellular protease.

MATERIALS AND METHODS

Preparation of *B. subtilis* B315 dan *R. solanacearum* isolates[AKG5]

Culture stock of *B. subtilis* B315 in 20% glycerol was used in this study. This isolate was previously reported to be capable of controlling against *R. solanacearum* of potato (Prihatiningsih et al. 2006; Prihatiningsih 2013). *R. solanacearum* was isolated from the diseased roots of chili suffered with bacterial wilt ~~chilli and~~ that was grown on CPG-TTC medium (Denny and Hayward 2001). *R. solanacearum* was characterized by creamy white fluidal colony with pink-red colour in the middle, irregular or regular shape, flat surface with uneven edges (Singh et al. 2010).

Protease Assay of *B. subtilis* B315 on skim milk agar medium

For ~~The~~ qualitative test of protease producing by *B. subtilis* B315, ~~was prepared it's~~the bacterial culture was grown on skim milk agar medium (casein 5g, yeast extract 2,5g, glucose 1g, agar 15g, destilled water 1000 mL, skim milk 7% was added as inducer (Majumdar and Chakraborty 2017). Protease assay of *B. subtilis* B315 was carried out through protease screening using a skim milk agar medium with scratched inoculated method of one loopfull (Vijayaraghavan and Vincent 2013; Majumdar and Chakraborty 2017). Clear zone appeared around scratch~~ed~~ was protease secreted by *B. subtilis* B315.

Protease producing assay of *B. subtilis* B315

Protease activity was measured using a modified Kunitz method (Majumdar and Chakraborty 2017). For the assay, a total of 0.5 ml Casein substrate (0.6 w/v) was added in 0.1 M Tris-HCl buffer at pH 8.0. Enzymatic reaction was initiated by adding 0.1 ml of protease solution to the Tris-HCl buffer at 45ºC and incubation time of 30 minutes. The protease was prepared using ethyl acetate extraction method of Alnahdi (2012; Abu-Mejdad et al. 2013). -The reaction was stopped by adding 0.5 ml cold TCA (*Trichloroacetic Acid*) followed by centrifugation at 4000 rpm at 4ºC for 15 minutes. –The dissolved peptide in the supernatant was further measured using spectrophotometer at ƛ 275 nm. The control solution is made with the same treatment but the substrate and the cold TCA were first dissolved and then protease solution was added. The standard solution used to measure proteolytic activity was tyrosine 1-120 µg /mL. One unit of protease activity (U) is defined as the amount of enzyme needed to produce 1 µg of tyrosine/minute ml of the enzyme solution from the Casein substrate at certain pH and temperature conditions, calculated by the formula

$$\text{Activity} = \frac{(a) - (b)}{30 \text{ min.} \times \text{mL enzyme}} \times \text{dilution factor}$$

Note: (a) = µg tyrosine/mL sample, (b) = µg tyrosine/mL control, dilution factor = total volume /(enzyme volume + filtrate sample volume).

In this research 4 factors, namely: incubation periods, incubation pH, and crude extract protease concentration were studied to know the optimum condition for protease activity of *B subtilis* B315. The incubation periods used were 15, 30, 45 and 60 min. The incubation pH: 7 of Phosphate buffer, pH 8 and 9 of Tris-HCl buffer, pH 10 of NaHCO, and pH 11 of NaOH buffer. While the crude extract protease concentrations were 0,1, 0,3, 0,5 and 0,7%. For determination of optimum temperature for enzyme activity, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time and 1 ml protease extract of *B. subtilis* B315). For determination of optimum pH, incubation condition was set following standard protocol (30 minutes incubation time, 45ºC and 1 ml protease extract of *B. subtilis*) except the pH was varied at pH 7, 8, 9, 10 and 11. For determination of optimum enzyme concentration, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time at 45ºC) except the protease extract concentration was varied at 0.1, 0.3, 0.5, and 0.7%.

Biocontrol potency test against *R. solanacearum in vitro*

Potency of *B. subtilis* B315 as a biocontrol of *R. solanacearum in vitro* was done by the two-layer medium method as stated by Ghosh et al. (2007). *B. subtilis* B315 was inoculated ~~a point~~ on the YPGA medium (*yeast peptone glucosa agar*), after 24 hours the Petri dish was turned over and droped with 500 µl chloroform, incubated for 3 hours until the chloroform evaporated. Pathogenic bacteria of *R. solanacearum* chilies isolates that have been grown on YPGA medium ~~are~~ were harvested ~~with~~ in 10 ml of sterile water, then 200 µl are put into 4 mL of 0.6% water agar solution which slowly poured into the Petri dish. After 48 hours of incubation an inhibition zone was formed.

Plant Resistance of bacterial wilt on chilli by *B. subtilis* B315

For testing ~~C~~control bacterial wilt of chilli with *B. subtilis* B315, ~~is carried out by~~ soaking of chilli seeds in *B. subtilis* B315 suspension with a population density of 10^8 cfu/ml before seedling ~~in *B. subtilis* B315 suspension with a population density of 10^8 cfu/ml~~ was carried out, ~~the next treatment is~~then watering with *B. subtilis* B315 suspension at the same concentration when moving planting age 10, 20 and 30 HST (days after planting) at a dose of 100 ml/plant. The observation of the effectiveness of wilt disease control ~~is~~ was done by assessing the intensity of the disease using the formula DI = a / b x 100%, with a: number of wilted plant; b: total number of plants observed (Aslam et al. 2015). Then, calculation of control effectiveness is to compare the intensity of control disease with the intensity of treatment disease according to Pawaskar et al. (2014) E = (control DI - treatment DI)/control DI x 100%.

RESULTS AND DISCUSSION

The qualitative test of protease

The qualitative protease was produced by *B. subtilis* B315 showed ~~that~~ clear zone around scratche~~d was~~ which indicated the production of protease secreted by *B. subtilis* B315 (Fig. 1). The role of this protease in biocontrol plant pathogens and enhanced plant resistant to pathogens infection[AKG6] (Jha and Bhattacharyya 2012; Mitrofanova et al. 2017).

Figure 1. Clear zone around the scratches of *B. subtilis* B315 on skim milk medium to show its potency to produce protease

The clear zone in the protease-producing test of *B. subtilis* B315 means that ~~*Bacillus*~~ this microorganism is capable to produce proteases. It was indicated by a zone width ranging from 12-26 mm. In qualitative plate assay, isolate S1 exhibited the largest clear zone (30±1.13 mm) in skim milk agar and isolate S5 exhibited the lowest (18±1.41 mm), which S1 and S5 isolates are Gram positive bacteria (Bhowmik et al. 2015; Joshi and Ghike 2019).

The protease activity of *B. subtilis* B315

The protease activity of *B. subtilis* B315 was calculated based on the regression equation obtained from the absorbance value of the tyrosine standard at ƛ 275 nm, as follows: Y = 0.0045x + 0.0016 (Fig. 2), the protease activity of *B. subtilis* B315 at various incubation period, pH and crude extract protease concentration are shown in Fig. 3, 4 and 5. The activity of protease *B. subtilis* B315 showed at 30 minutes incubation period was the highest by 1.259 U/ml (Fig 3) and at pH incubation 9 was maximum protease activity by 1.422 U/ml (Fig 4).

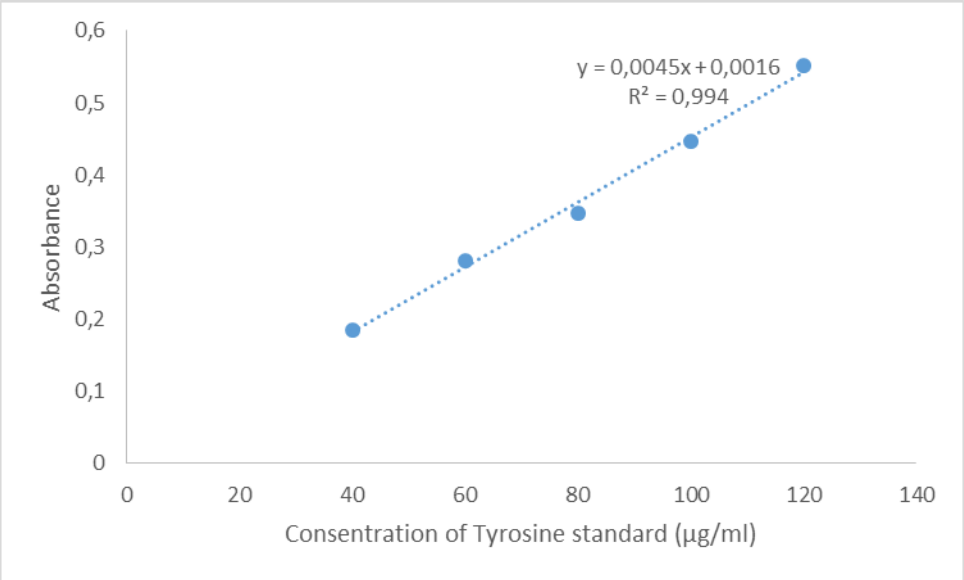

Figure 2. Absorbance of tyrosine standard at ƛ 275 nm

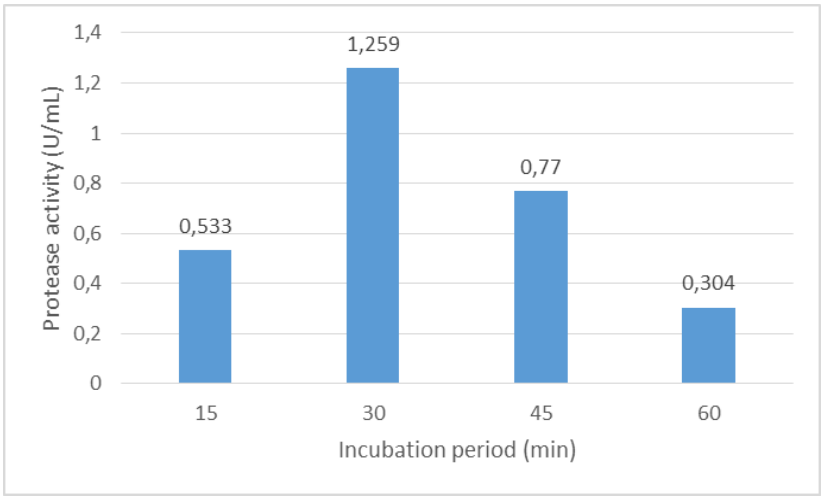

Figure 3. Protease activity of *B. subtilis* B315 at variation incubation periods

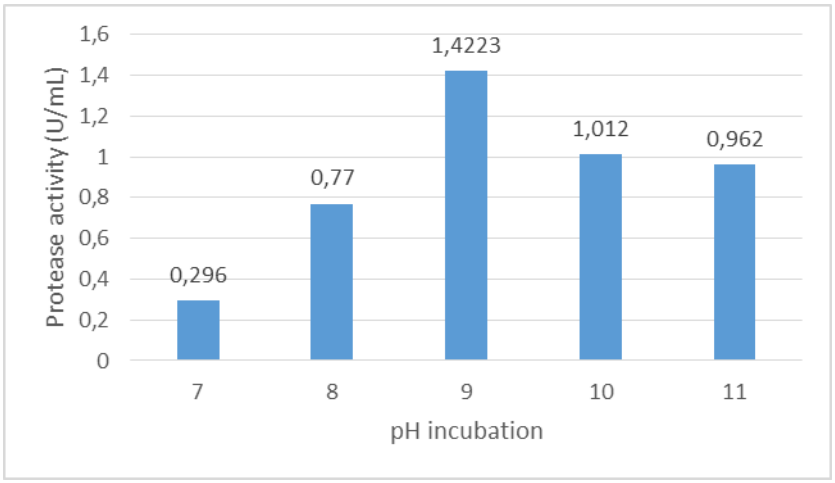

Figure 4. Protease activity of *B. subtilis* B315 at variation pH

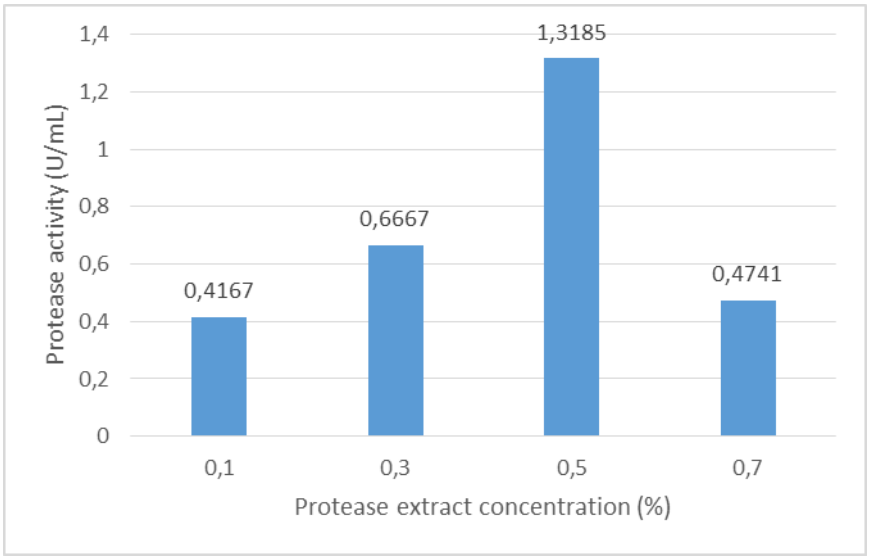

Figure 5. Protease activity of *B. subtilis* B315 at variation crude extract protease concentration

The incubation time variations showed that 30 minutes duration was the best treatment with the protease activity ~~was~~ 1.259 U/ml. The treatment of variations in incubation pH showed the highest protease activity was at pH 9 of 1.422 U/ml. Protease activity at various concentrations at pH 9 with 30 min. incubation time of 1.318 U/ml was at a concentration of 0.5% solution. The effect of extract enzyme concentration on protease activity is shown in Figure 5 based on the best pH and incubation time (9 and 30 min.). *B. subtilis* ~~is the bacteria~~is resistance to growth at a high temperature. The activity crude protease at 40ºC, pH 8 by 255 U/ml (Jadhav et al. 2014). ~~-~~The protease activity was 236.37 U/ml and 175,083 U/ml with carbon source and nitrogen source at pH 7.4 and incubation temperature 50ºC respectively (Pant et al. 2015). The activity of protease depends on the *Bacillus* strain~~–~~, medium, physical and chemical factor (Queiroga et al. 2013; Tebyanian et al. 2018). *Bacillus* spp. strains KFP-5, KFP-7 and KFP-17 produced variable quantities of siderophore, protease and glucanase in absence as well as presence of rice blast pathogen *Pyricularia oryzae*, protease (20.9±29.3U/mL) presence *P oryzae*, and protease (11.6±24 U/mL) if absent *P oryzae* (Rais et al. 2017).

Biocontrol potency test against *R. solanacearum in vitro*

The potency of *B. subtilis* B315 as a biocontrol of *R. solanacearum in vitro*, ~~is shown that the~~showed largest inhibition zone (32 mm) for *R. solanacearum in vitro* of chilli ~~by 32 mm~~ (Fig. 6.). *In vitro* inhibition test results of *R. solanacearum* isolat chilli was smaler than its suppress for potato isolates of *R. solanacearum* because *B. subtilis* B315 ~~is~~ was isolated from healthy potato plants as there habitat, the bacterial have adaptif to the condition. *B. subtilis* B315 can suppressed the *R. solanacearum* isolated from potato was 36 mm. However, *B. subtilis* B315 was found capable to control against *R. solanacearum* ~~family of solanaceae~~.

Figure 6. The inhibition zone of *B. subtilis* B315 against chilli *R. solanacearum*

Protease secreted by *B. subtilis* B315 has effect for the virulence of bacterial pathogens with combating biofilms formation, and induce stress tolerance in plants. *Bacillus* sp. which the genomics, proteomics and metabolics were

required to elucidate the mechanism of *Bacillus*-plant interaction for biotic and abiotic stress management in crops Radhakrishnan et al. (2017). *Bacillus* is also known to produce an array of extracelullar proteolytic enzymes. *Bacillus* proteases have high stability and low pathogenicity and can be easily purified and obtained in industrial quantities. These features make them promising matrix-degrading agents for combating bacterial biofilms (Mitrofanova et al. 2017)**.** The effect of *B. subtilis* B315 treatment to inhibition of *R. solanacerum in vitro* and *in planta* showed at Table 1 with plant resistance factor to bacterial wilt showed by the effectiveness.

Table 1. Inhibition effect of *B. subtilis* B315 *in vitro*, *in planta*[AKG7], and the effectiveness

Treatment *in vitro*	Inhibition (mm)	Disease intensity (%)	Effectiveness (%)
Control	0	7.34	-
B. subtilis B315	36	2.87	60,89
Bactericide	24	3.06	58.31

Note: Control (without *B. subtilis* B315, without bactericide)

Protease from *Bacillus* sp. can promote of plant resistance to pathogen, showed the decreased of disease intensity (Table 1). The plant resistance indicated by phenol compound as an indicator of increase of plant resistance to pathogens and decrease of disease intensity with the effectiveness more than 50%. The induced plant resistance by *B. subtilis* strain FZB24 could improve plant strength and furthermore avoid to pathogens infection (Jha and Bhattacharyya 2012). ~~–~~*B. subtilis* B298 in microencapsulated formula that applied to chilli can reduce~~d~~ the bacterial wilt caused by *R. solanacearum* and induced systemic resistance by increased phenol compound up to 6.27% (Prihatiningsih et al. 2019).

In conclusion, the bacteria of potato rhizosphere *B. subtilis* B315 was capable to produce protease with an activity of 1.3185 U/mL. *B. subtilis* B315 was an effective control of bacterial wilt by 60,89% in chilli. Protease from *B. subtilis* B315 could indicates of potency for control bacterial wilt because this enzyme serves to increase *B. subtilis* B315 activity as biological control agents and inductical resistance of plant varieties. ~~–~~The future *B. subtilis* B315 formulated as biopesticide to supported sustainable agriculture, and protease from *B. subtilis* B315 can produce as well as inductrically to control bacterial wilt of solanaceae.

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Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chili

Abstract. Antagonistic bacteria isolated from a healthy potato are potential biocontrol agents against a number of bacterial pathogens. The capability of antagonistic bacteria to produce extracellular protease could be considered as an indicator of their antagonistic capacity. The objective of this research was to study the optimum activity of protease produced by *Bacillus subtilis* B315 to control *Ralstonia solanacearum* causing wilt of chili. In this research were optimum incubation time, pH and crude extract protease concentration of *B. subtilis* B315 examined for maximum production of extracellular protease. The results showed that *B. subtilis* B315 was capable of producing protease at optimum incubation time of 30 min, pH 9 and crude extract protease concentration 0.50% with an activity of 1.3185 U/mL. Protease-producing capability of *B. subtilis* B315 was shown by the formation of a clear zone around the colony of *B. subtilis* B315 grown on skim-milk agar medium. *B. subtilis* B315 was capable of inhibiting the growth of *R. solanacearum* in vitro as indicated by 32 mm clear zone. Furthermore, in chili application of *B subtilis* showed that this bacteria was capable of controlling chili bacterial wilt caused by *R. solanacearum* with an effectiveness of 60.89%. The prospective of *B. subtilis* B315 could be formulated as biopesticide.

Key words: *Bacillus subtilis* B315, bacterial wilt, biocontrol, chili, protease activity

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is an important disease in plants that may include tomato, chili, eggplant, bananas, potatoes, ginger and mulberries. In chili and tomato, bacterial wilt could reduce yields up to 80% (Vanitha et al. 2009). Currently, biological control using antagonistic bacteria has been widely studied, as it is more environmentally friendly and supports sustainable agriculture. Antagonistic bacteria could be explored from habitats of which the pathogen naturally lives.

Bacillus subtilis B315 isolated from a healthy potato rhizosphere has been reported to be capable of controlling bacterial wilt in potatoes with an effectiveness of 64.9% (Prihatiningsih 2013), and 74.6% in tomato, chili and eggplant (Prihatiningsih and Djatmiko 2016). The potential of bacteria to be antagonist is shown by its ability to produce secondary metabolites (antibiotics and other compounds) and enzymes. Protease is a product of microbes which could functions to control plant pathogens, either fungi or bacteria. *Bacillus* sp. and other bacteria such as: *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Shumi and Anwar 2004) secrete extracellular enzymes including proteases. Saha et al. (2012) found two new strains of *B. subtilis* from eggplant rhizosphere that have the potential to be biocontrol agent which is shown by their capabilities of producing secondary metabolites, such as siderophore, and hydrolytic enzymes, such as chitinase, protease, lipase and amylase. *B subtilis* was also reported to be able to produce IAA (*Indole Acetic Acid*) ranging from 57.56-79.33 ppm (Prihatiningsih et al. 2020). *B. subtilis* has the ability to secrete amylase, protease, pullunase, chitinase, xylanase and lipase (Morikawa 2006; Lestari et al. 2017).

Protease is a hydrolytic enzyme that hydrolyses extracellular proteins and increases the ability of cells to absorb useful hydrolysis products. *B. subtilis* has important bacteria because of its capability to secrete several hydrolytic enzymes, including protease, into the culture medium (Almas et al. 2009). Antagonistic *Bacillus* sp. significantly colonize rice plants and secretes various biocontrol determinants like protease of 1.1±5.5 U/mg of soil or U/mL of hydroponic solution. *Bacillus* spp. strains KFP-5 and KFP-17 produce various quantities of siderophore, protease and glucanase in absence as well as in presence of rice blast pathogen *Pyricularia oryzae*. The protease production is ranging about 20.9-29.3 U/mL and 11.6-24 U/mL in KFP-5 and KFP-17, respectively (Rais et al. 2017).

Bacillus subtilis B315 has important potential to produce protease because this enzyme support the crops for enhanched resistance to disease. Nevertheles enzymatic treatment was secreted by *B. subtilis* B315 as antagonist bacteria resulted in degradation of the EPS (*extracellular polymeric substances*) components and significant eradication of the biofilm of bacteria pathogens. One of the hydrolytic enzymes can penetrate to biofilm, the use of protease can degrade

pathogenic bacteria biofilms. Therefore, the optimum protease production by *B. subtilis* B315 should be important to detection. Biofilm bacteria pathogens have a role to allow cell-to-cell communication, and responsible for adhesion to surface, its advantage to be able to survive in unfavorable environment conditions and it's much less susceptible to the antimicrobial agents (Mitrofanova et al. 2017).

The objective of this research was to study the optimum activity of protease produced extracellularly by *B. subtilis* B315 to control *R. solanacearum* of chili. In this research optimum incubation time, pH and concentration of crude extract protease *B. subtilis* B315 examined for maximum production of extracellular protease.

MATERIALS AND METHODS

Culture of *B. subtilis* B315 and *R. solanacearum* of chili for protease effect test

Culture stock of *B. subtilis* B315 in 20% glycerol was used in this study. This isolate was previously reported to be capable of controlling *R. solanacearum* of potato (Prihatiningsih et al. 2006; Prihatiningsih 2013). *R. solanacearum* was isolated from the diseased roots of chili suffered with bacterial wilt that was grown on CPG-TTC medium (Denny and Hayward 2001). *R. solanacearum* was characterized by creamy white fluidal colony with pink-red colour in the middle, irregular or regular shape, flat surface with uneven edges (Singh et al. 2010).

Protease Assay of *B. subtilis* B315 on skim milk agar medium

For qualitative test of protease producing by *B. subtilis* B315, the bacterial culture was grown on skim milk agar medium (casein 5g, yeast extract 2.5g, glucose 1g, agar 15g, destilled water 1000 mL, skim milk 7% was added as inducer (Majumdar and Chakraborty 2017). Protease assay of *B. subtilis* B315 was carried out through protease screening using a skim milk agar medium with scratched inoculated method of one loopfull (Vijayaraghavan and Vincent 2013; Majumdar and Chakraborty 2017). Clear zone appeared around scratch was protease secreted by *B. subtilis* B315.

Protease producing assay of *B. subtilis* B315

Protease activity was measured using a modified Kunitz method (Majumdar and Chakraborty 2017). For the assay, a total of 0.5 mL Casein substrate (0.6 w/v) was added in 0.1 M Tris-HCl buffer at pH 8.0. Enzymatic reaction was initiated by adding 0.1 mL of protease solution to the Tris-HCl buffer at 45^{o}C and incubation time of 30 minutes. The protease was prepared using ethyl acetate extraction method of Alnahdi (2012; Abu-Mejdad et al. 2013). The reaction was stopped by adding 0.5 mL cold TCA (*Trichloroacetic Acid*) followed by centrifugation at 4000 rpm at 4^{o}C for 15 minutes. The dissolved peptide in the supernatant was further measured using spectrophotometer at ƛ 275 nm. The control solution is made with the same treatment but the substrate and the cold TCA were first dissolved and then protease solution was added. The standard solution used to measure proteolytic activity was tyrosine 1-120 µg /mL. One unit of protease activity (U) is defined as the amount of enzyme needed to produce 1 µg of tyrosine/minute mL of the enzyme solution from the Casein substrate at certain pH and temperature conditions, calculated by the formula

$$\text{Activity} = \frac{(a) - (b)}{30 \text{ min.} \times \text{mL enzyme}} \times \text{dilution factor}$$

Note: (a) = µg tyrosine/mL sample, (b) = µg tyrosine/mL control, dilution factor = total volume /(enzyme volume + filtrate sample volume).

In this research 3 factors, namely: incubation periods, incubation pH, and crude extract protease concentration were studied to know the optimum condition for protease activity of *B subtilis* B315. The incubation periods used were 15, 30, 45 and 60 min. The incubation pH: 7 of Phosphate buffer, pH 8 and 9 of Tris-HCl buffer, pH 10 of NaHCO, and pH 11 of NaOH buffer. While the crude extract protease concentrations were 0.1, 0.3, 0.5 and 0.7%. For determination of optimum temperature for enzyme activity, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time and 1 mL protease extract of *B. subtilis* B315). For determination of optimum pH, incubation condition was set following standard protocol (30 minutes incubation time, 45^{o}C and 1 mL protease extract of *B. subtilis*) except the pH was varied at pH 7, 8, 9, 10 and 11. For determination of optimum enzyme concentration, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time at 45^{o}C) except the protease extract concentration was varied at 0.1, 0.3, 0.5, and 0.7%.

Biocontrol potency test against *R. solanacearum in vitro*

Potency test of *B. subtilis* B315 as a biocontrol of *R. solanacearum in vitro* was done by the two-layer medium method as stated by Ghosh et al. (2007). *B. subtilis* B315 was inoculated on the YPGA medium (*yeast peptone glucosa agar*), after 24 hours the Petri dish was turned over and droped with 500 µl chloroform, incubated for 3 hours until the chloroform

evaporated. Pathogenic bacteria of *R. solanacearum* chilies isolates that had been grown on YPGA medium were harvested in 10 mL of sterile water, then 200 µl are put into 4 mL of 0.6% water agar solution which slowly poured into the Petri dish. After 48 hours of incubation an inhibition zone was formed.

Plant Resistance of bacterial wilt on chili by *B. subtilis* B315

For testing control bacterial wilt of chili with *B. subtilis* B315, chili seeds were soaking in *B. subtilis* B315 suspension with a population density of 10^8 cfu/mL before seedling was carried out, then watering with *B. subtilis* B315 suspension at the same concentration when reaching planting age 10, 20 and 30 DAP (days after planting) at a dose of 100 mL/plant. The observation of the effectiveness of wilt disease control was done by assessing the intensity of the disease using the formula DI = a / b x 100%, with a: number of wilted plant; b: total number of plants observed (Aslam et al. 2015). Then, control effectiveness calculation was done to compare the intensity of control disease with the intensity of treatment disease according to Pawaskar et al. (2014), E = (control DI - treatment DI)/control DI x 100%.

RESULTS AND DISCUSSION

The qualitative test of protease

The qualitative protease produced by *B. subtilis* B315 showed clear zone around scratche which indicated the production of protease secreted by *B. subtilis* B315 (Fig. 1). The role of this protease from antagonistic bacteria were controlling of plant pathogens, promoting of plant growth, and enhanching of plant resistant by inhibiting biofilm plant pathogens (Jha and Bhattacharyya 2012; Mitrofanova et al. 2017)**.**

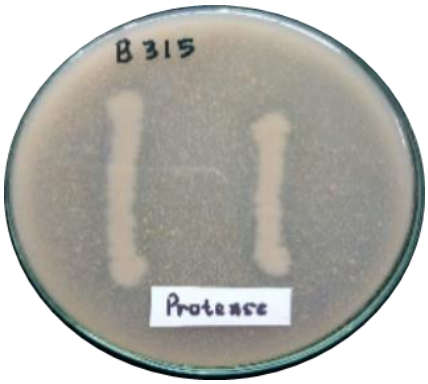

Figure 1. Clear zone around the scratches of *B. subtilis* B315 on skim milk medium to show its potential to produce protease

The clear zone in the protease-producing test of *B. subtilis* B315 means that this microorganism is capable of producing proteases. It was indicated by a zone width ranging from 12-26 mm. In qualitative plate assay, isolate S1 exhibited the largest clear zone (30±1.13 mm) in skim milk agar and isolate S5 exhibited the lowest (18±1.41 mm), which S1 and S5 isolates are Gram positive bacteria (Bhowmik et al. 2015; Joshi and Ghike 2019).

The protease activity of *B. subtilis* B315

The protease activity of *B. subtilis* B315 was calculated based on the regression equation obtained from the absorbance value of the tyrosine standard at ƛ 275 nm, as follows: Y = 0.0045x + 0.0016 (Fig. 2), the protease activity of *B. subtilis* B315 at various incubation period, pH and crude extract protease concentration are shown in Fig. 3, 4 and 5. The activity of protease *B. subtilis* B315 showed at 30 minutes incubation period was the highest by 1.259 U/ml (Fig 3) and at pH incubation 9 was maximum protease activity by 1.422 U/ml (Fig 4).

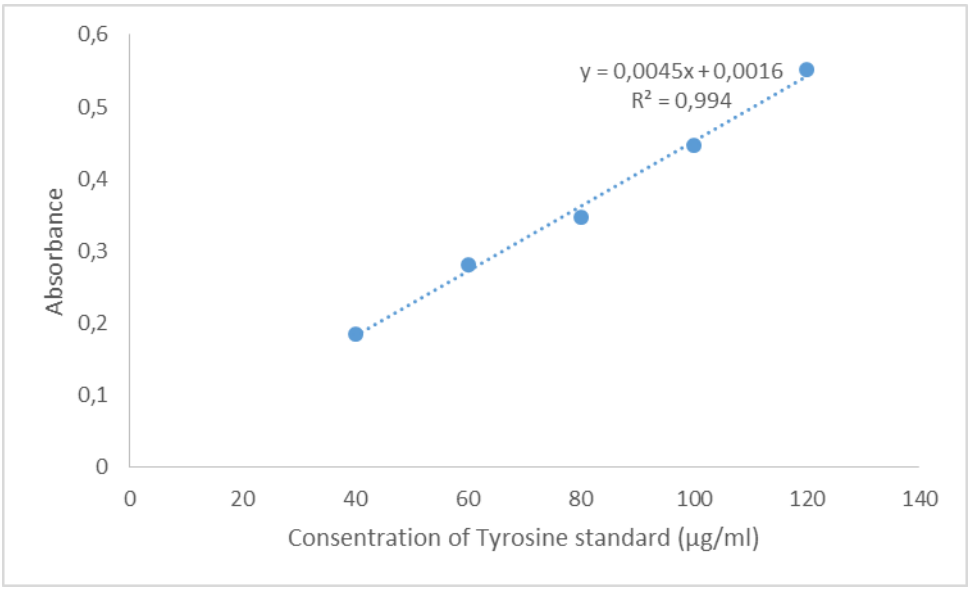

Figure 2. Absorbance of tyrosine standard at ƛ 275 nm

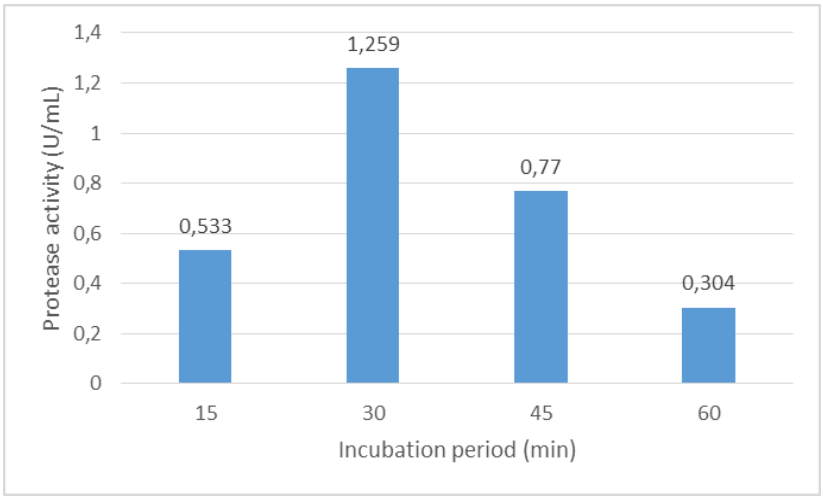

Figure 3. Protease activity of *B. subtilis* B315 at variation incubation periods

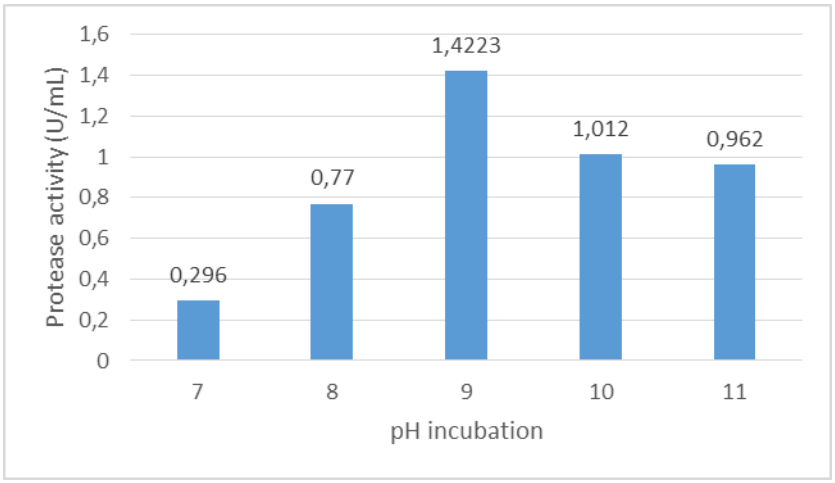

Figure 4. Protease activity of *B. subtilis* B315 at variation pH

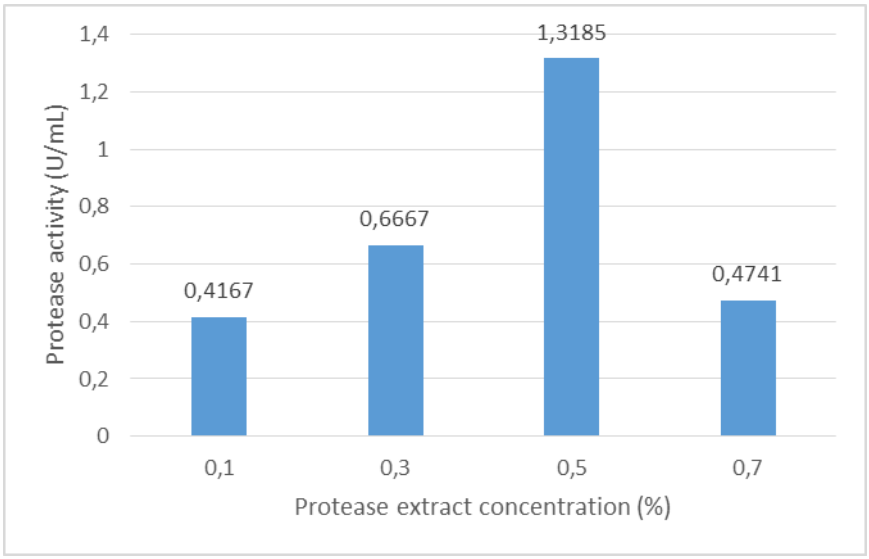

Figure 5. Protease activity of *B. subtilis* B315 at variation crude extract protease concentration

The incubation time variations showed that 30 minutes duration was the best treatment with the protease activity 1.259 U/ml. Variations in pH incubation treatment showed that the highest protease activity was at pH 9 of 1.422 U/ml. Protease activity at various concentrations at pH 9 with 30 minute incubation time of 1.3185 U/ml was at a concentration of 0.5% solution. The effect of extract enzyme concentration on protease activity is shown in Figure 5 based on the best pH and incubation time (9 and 30 min.). *B. subtilis* is resistance to growth at a high temperature. The activity crude protease was at 40ºC, pH 8 by 255 U/ml (Jadhav et al. 2014). The protease activity was 236.37 U/ml and 175,083 U/ml with carbon source and nitrogen source at pH 7.4 and incubation temperature 50ºC respectively (Pant et al. 2015). The activity of protease depends on the *Bacillus* strain, medium, physical and chemical factors (Queiroga et al. 2013; Tebyanian et al. 2018). *Bacillus* spp. strains KFP-5, KFP-7 and KFP-17 produced variable quantities of siderophore, protease and glucanase in absence as well as presence of rice blast pathogen *Pyricularia oryzae*, protease (20.9±29.3U/mL) in presence *P. oryzae*, and protease (11.6±24 U/mL) in absence of *P. oryzae* (Rais et al. 2017).

Biocontrol potency test against *R. solanacearum in vitro*

The potency of *B. subtilis* B315 as a biocontrol of *R. solanacearum in vitro*, showed largest inhibition zone (32 mm) for *R. solanacearum in vitro* of chilli (Fig. 6.). *In vitro* inhibition test results of *R. solanacearum* isolat chilli was smaler than its suppress for potato isolates of *R. solanacearum* because *B. subtilis* B315 was isolated from healthy potato plants as their habitat, the bacterial have adaptive to the condition. *B. subtilis* B315 can suppress the *R. solanacearum* isolated from potato of 36 mm. However, *B. subtilis* B315 was found capable of controlling *R. solanacearum*.

Figure 6. The inhibition zone of *B. subtilis* B315 against chili *R. solanacearum*

Protease secreted by *B. subtilis* B315 has effect on the virulence of bacterial pathogens with combating biofilms formation, and induce stress tolerance in plants. *Bacillus* sp. which the genomics, proteomics and metabolics were required to elucidate the mechanism of *Bacillus*-plant interaction for biotic and abiotic stress management in crops

Radhakrishnan et al. (2017). *Bacillus* is also known to produce an array of extracelullar proteolytic enzymes. *Bacillus* proteases have high stability and low pathogenicity and can be easily purified and obtained in industrial quantities. These features make them promising matrix-degrading agents for combating bacterial biofilms (Mitrofanova et al. 2017)**.** The effect of *B. subtilis* B315 treatment to inhibition of *R. solanacerum in vitro* and in chili showed at Table 1 with plant resistance factor to bacterial wilt showed by the effectiveness.

Table 1. Inhibition effect of *B. subtilis* B315 *in vitro*, in chili, and the effectiveness

Treatment	Inhibition (mm) *in vitro*	Disease intensity (%)in chili	Effectiveness (%)
Control	0	7.34	-
B. subtilis B315	36	2.87	60,89
Bactericide	24	3.06	58.31

Note: Control (without *B. subtilis* B315, without bactericide)

Protease from *Bacillus* sp. can promote of plant resistance to pathogen seen by the decreased of disease intensity (Table 1). The plant resistance is indicated by phenol compound as an indicator of the increase of plant resistance to pathogens and the decrease of disease intensity with the effectiveness more than 50%. The induced plant resistance by *B. subtilis* strain FZB24 could improve plant strength and furthermore avoid pathogens infection (Jha and Bhattacharyya 2012). *B. subtilis* B298 in microencapsulated formula that is applied to chilli can reduce the bacterial wilt caused by *R. solanacearum* and induced systemic resistance by increased phenol compound up to 6.27% (Prihatiningsih et al. 2019).

In conclusion, the bacteria of potato rhizosphere *B. subtilis* B315 was capable of producing protease with an activity of 1.3185 U/mL. *B. subtilis* B315 was an effective control of bacterial wilt by 60.89% in chili. Protease from *B. subtilis* B315 could indicate the potency to control bacterial wilt because this enzyme serves to increase *B. subtilis* B315 activity as biological control agents and induces resistance of plant varieties. The future *B. subtilis* B315 formulated as biopesticide to support sustainable agriculture, and protease from *B. subtilis* B315 can be produced as well as induced to control bacterial wilt of solanaceae.

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The authors declare no Conflic of interest.

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