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by Biodiversitas Journal Of Biological Diversity Vol. 22 (3)

Submission date: 08-Mar-2022 12:17PM (UTC+0700)
Submission ID: 1779201374
File name: Extracel._protease_Bs_B315_Biodiv_22_3_2021.pdf (333.34K)
Word count: 3880
Character count: 20991

BIODIVERSITAS
Volume 22, Number 3, March 2021
Pages: 1291-1295

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220327

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

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Manuscript received: 22 January 2020. Revision accepted: 14 February 2021.

Abstract. *Prihatiningsih N, Asnani A, Djatmiko HA. 2021. Extracellular protease from* Bacillus subtilis *B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chili. Biodiversitas 22: 1291-1295.* 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 effectiveness of 60.89%. The prospective of *B. subtilis* B315 could be formulated as biopesticide.

Keywords: *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 that could function 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 agents 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, pullulanase, 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 supports the crops for enhanced resistance to disease. Nevertheless, 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 in allowing cell-to-cell communication, and responsible for adhesion to surface, its advantage to survive in unfavorable environmental 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 from 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 color 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 produced by *B. subtilis* B315, the bacterial culture was grown on skim milk agar medium (casein 5g, yeast extract 2.5g, glucose 1g, agar 15g, distilled 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 loop full (Vijayaraghavan and Vincent 2013; Majumdar and Chakraborty 2017). Clear zone that 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 °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 °C for 15 minutes. The dissolved peptide in the supernatant was further measured using spectrophotometer at λ 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°C, and 1 mL protease extract of *B. subtilis*) except the pH was varied at pH 7, 8, 9, 10 and 11. For optimum enzyme concentration determination, incubation condition was set following standard protocol (pH 8, 30 minutes incubation time at 45 °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 glucose agar*), after 24 hours the Petri dish was turned over and dropped 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 plants; 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 scratches which indicated the production of protease secreted by *B. subtilis* B315 (Figure 1). The role of this protease from antagonistic bacteria was controlling plant pathogens, promoting plant growth, and enhancing plant resistance by inhibiting biofilm plant pathogens (Jha and Bhattacharyya 2012; Mitrofanova et al. 2017).

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 λ 275 nm, as follows: Y = 0.0045x + 0.0016 (Figure 2), the protease activity of *B. subtilis* B315 at various incubation period, pH and crude extract protease concentration are shown in Figures 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 (Figure 3) and at pH incubation 9 was maximum protease activity by 1.422 U/ml (Figure 4).

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 resistant to growth at a high temperature. The activity crude protease was at 40°C, 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 and nitrogen sources at pH 7.4 and incubation temperature 50°C respectively (Pant et al. 2015). The protease activity 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 (Figure 6.). In vitro inhibition test results of *R. solanacearum* isolate chili was smaller 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*.

Protease secreted by *B. subtilis* B315 affects the virulence of bacterial pathogens with combating biofilms formation, and induces stress tolerance in plants. *Bacillus* sp. with the genomics, proteomics, and metabolomics 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 extracellular 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 inhibit *R. solanacearum* in vitro and in chili showed at Table 1 with plant resistance factor to bacterial wilt shown by the effectiveness.

Table 1. Inhibition effect of *Bacillus 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)

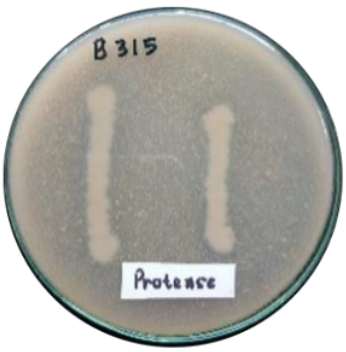

Figure 1. Clear zone around the scratches of *Bacillus subtilis* B315 on skim milk medium to show its potential to produce protease

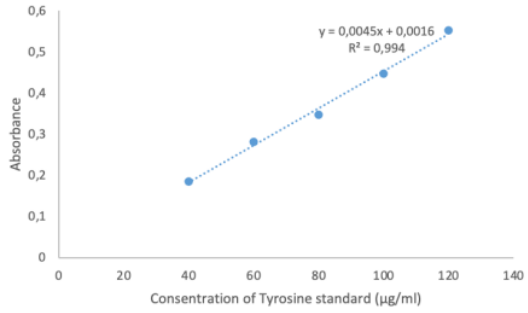

Figure 2. Absorbance of tyrosine standard at λ 275 nm

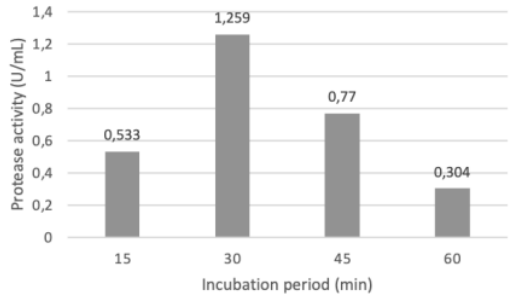

Figure 3. Protease activity of *Bacillus subtilis* B315 at variation incubation periods

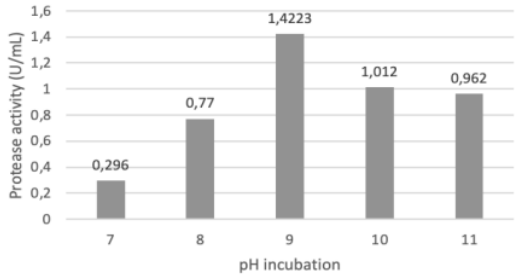

Figure 4. Protease activity of *Bacillus subtilis* B315 at variation pH

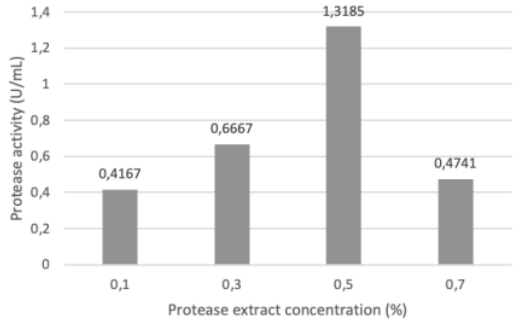

Figure 5. Protease activity of *Bacillus subtilis* B315 at variation crude extract protease concentration

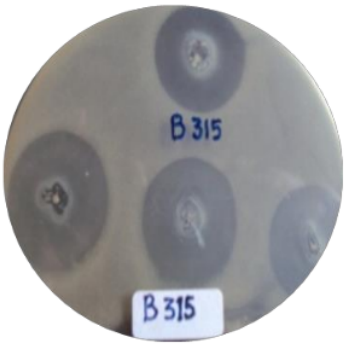

Figure 6. The inhibition zone of *Bacillus subtilis* B315 against chili *Ralstonia solanacearum*

Protease from *Bacillus* sp. can promote plant resistance to pathogen seen by the decrease 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 of 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 chili 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 effectively controlled bacterial wilt by 60.89% in chili. Protease from *B. subtilis* B315 could indicate the potency to control bacterial wilt. This enzyme increases *B. subtilis* B315 activity as biological control agents and induces plant varieties resistance. 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.

ACKNOWLEDGEMENTS

The authors were grateful to the Indonesian Ministry of Research, Technology and Higher Education (DRPM Kemenristekdikti) for supporting this research in fundamental scheme.

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