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Research article

Some properties of protease extract derived from *Pseudomonas fluorescens* and its antifungal activity against *Fusarium* spp.

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Key words: *Pseudomonas fluorescens, Fusarium oxysporum, Fusarium solani,* protease extract, antifungal.

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Abstract

Soil-borne plant pathogenic fungi are of major concern problem in agriculture which affects yield and quality of agricultural products. As *Pseudomonas fluorescens* possess a variety of promising properties which make it a better biocontrol agent. In this current study, antagonistic effects of bacterial biocontrol agent, *Pseudomonas fluorescens* obtained from Microbiology Collection of Indonesian Institute of Sciences, was evaluated against plant pathogenic fungi *Fusarium solani* and *Fusarium oxysporum* causing wilt disease of plants. The ability of *Pseudomonas fluorescens* in antagonizing or inhibiting the growth of phytopathogenic fungi was tested by measuring the inhibition zone for the growth of the tested fungi using dual culture method. The protease extract derived from *P. fluorescens* showed to inhibit growth of *Fusarium solani* InaCC F76 and *Fusarium oxysporum* InaCC F78 as pathogens cause wilt disease on plants.

Introduction

Community awareness of environmental problem increasingly high and pressure from experts and environmentalists to make enzyme technology as one of alternative to substitute the different chemical process in industry sector [1]. The use of chemical fertilizers and pesticides has caused an incredible harm to the environment. These agents are both hazardous to animals and humans and may persist and accumulate in natural ecosystems [2]. An answer to this problem is replacing chemicals with biological approaches, which are considered more environment friendly in the long term. One of the emerging research area for the control of different phytopathogenic agents is the use of biocontrol plant growth promoting rhizobacteria (PGPR), which are capable of suppressing or preventing the phytopathogen damage [3]. Their applicability as biocontrol agents has drawn wide attention because of production of secondary metabolites such as siderophores, antibiotics, volatile compounds, HCN, enzymes and phytohormones [4]. *Pseudomonas fluorescens* is considered as biological biocontrol agent against root diseases colonized by *Fusarium* spp [5]. This fungus affects all parts of plants namely roots, stems, shoots, leaves and spikes. *Fusarium* spp. are a widespread cosmopolitan group of fungi and commonly colonize aerial and subterranean plant parts, either as primary or secondary invaders. *Fusarium* spp. can cause direct damages such as seedling foot and stalk rots, or indirect damages resulting from seedling blight or reduced seed germination; however, the most important diseases that may cause severe reduction in yield and quality [6]. They can be utilized in low-input sustainable agricultural applications, such as biocontrol, on account of their ability to synthesize secondary metabolites with antibiotic properties [7]. Antagonistic activity was also observed for Pseudomonas spp. in the rhizosphere has been recognizes as major factor in the suppression of many phytopathogens. Several antibiotic-like substance have been identified, including bacteriocins and phenazine antibiotics, but one of the most important mechanism responsible for the suppression of plant pathogens for Pseudomonas spp. is siderophore mediated competitions for iron. Pseudomonas comprise a large group of the active biocontrol strains as a result of their general ability to produce a diverse array of potent antifungal metabolites [8]. The aim of research is to know the ability of *P. fluorescens* bacteria, a collection of to produce protease that will be used to suppress a wilt disease of *Fusarium solani* and *Fusarium oxysporum* on plants.

Materials and methods

Isolates preparation

Isolate of *P. fluorescens* was a collection of Microbiology Division of Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Indonesia had been cultivated in media Nutrient Agar and incubated at 37°C in a shaker incubator for 3 days, while strains of *F.*

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solani InaCC F76 and *F. oxysporum* InaCC F78 were grown in Potato Dextrose Agar (PDA) [9].

Determination of protease activity on selective media

Selective media containing 0.5% skim milk, 0.5% pepton, 2% agar at pH 8.0 was used to determine the capability of bacteria on hydrolysis protein contained in the media during incubation at 37ºC for 24h. A positive result was qualitatively determined by indication of a clear zone surround bacterial colony [10].

Enzyme production

Bacterial cell production was carried out by growing bacteria on NA slant media, at room temperature for 3 days, and furthermore adds with a sterilized distilled water. An optical density (OD) of bacterial suspension was measured using spectrophotometer at 600 nm to obtain OD of 0.5. Protease production was carried out according to the method of Cappuccino and Sherman [11] by inoculating the production media for protease with bacterial suspension and incubated at room temperature for 2 days on a shaker at 120 rpm. As much as 2 mL of sample of bacterial culture was collected every day, and a filtrate was separated from a sludge, and then centrifuged with at 8000 rpm for 5 min. Supernatant was collected and used as crude enzyme solution and furthermore examined for its protease activity.

Determination of protease activity

As much as 0.2 mL of enzyme solution was reacted with 0.2 mL 0.1% azocasein in buffer solution of glycine-NaOH 0.05 M at pH 8.0 and incubated at temperature 40°C for 20 min. A reaction was stopped by adding with 0.6 mL of 10% trichloroacetic acid (TCA), and then centrifuged at 10,160 *g* for 5 min and the filtrate was separated from the sludge. Tyrosine that was deliberated in the filtrate was determine by reading its OD at 280 nm. One unit of protease activity was defined as amount of enzyme that can produce 1 µg tyrosine in the reaction condition [12].

Effect of pH, temperature, and metal ion against protease activity

The effect of pH against protease activity was determined by reacting the enzyme solution with azocasein as substrate at the highest activity of the enzyme concentration in a different buffer of glycine-NaOH 0.05 M at pH 7.5; 8.0; 8.5; 9.0; 10.0 and 11.0. The effect of temperatue against protease activity was determined by reacting the enzyme solution with azocasein as substrate at the highest activity of enzyme concentration at the at temperature of 30-70°C, and to determine the stability of enzyme against pH and temperature then the respective enzyme solution were incubated at optimum pH and temperature for 10 min and furthermore were determined their residual activities. The effect of metal ions of Ca^{2+}, Mn^{2+}, K^{+}, Na^{+}, Cu^{2+}, Hg^{2+} in the form of salts $CaCl_2$, $MnCl_2$, KCl, NaCl, $CuCl_2$, $CoCl_2$, $ZnCl_2$, and $HgCl_2$ as an activator as well as an inhibitor against protease activity were determined by reacting the enzyme solution in the reaction mixture containing 1% of skim milk and 1 mM of metal ions and the reaction mixture without metal ion was used as control.

Results and discussions

P. fluorescens showed that it could degrade protein of the skim milk in the media (Figure 1). Skim milk contains casein was used as the only carbon and nitrogen source to enrich the medium with nutrient for microbial growth. Casein hydrolysate was used as an inducer for protease production of *Pseudomonas* 1A4R [13, 14]. Casein is a milk protein consists of phospho protein bound calcium to form insoluble calcium calsenic in water to form a whitish coloid in a solid media [15]. *P. fluorescens* could degrade protein as well lipid in the skim milk since it could produce lipase as well [16]. Protease activity on agar media containing skim milk had been shown with presence of the clear zone surrounding colony. According to Patil *et al.* [17] the skim milk agar media was suitable to use for selection of microbial producing protease qualitatively as well as microbial secretting extracellular proteolitic enzymes that could be recognized with the formation of the clear zone surrounding colony. *P. fluorescens* produced several extracellular enzymes such including protease, hydrolase, lechitinase and lipase [18-20]. Gradually decreasing of enzyme activity was observed along with increasing of incubation time, indicating role of enzyme as primer metabolite [21]. Protelytic activity of *P. fluorescens* that had been incubated for 6 days was range between 47.64 to 83.07 U/mL, and the optimum yield (83.07 U/mL) could be achieved after incubation for 2 days. However, Figure 2 showed that enzyme production dealing with proteolytic activity apparently fluctuated along with incubation on the day-3 up to day-6 due to extrinsic factors that could not be anticipated during cultivation.

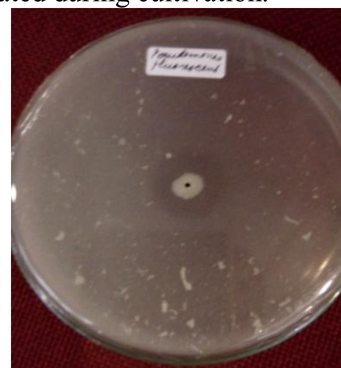

Figure 1. A clear zone around colony of *P. fluorescens* on a media containing skim milk.

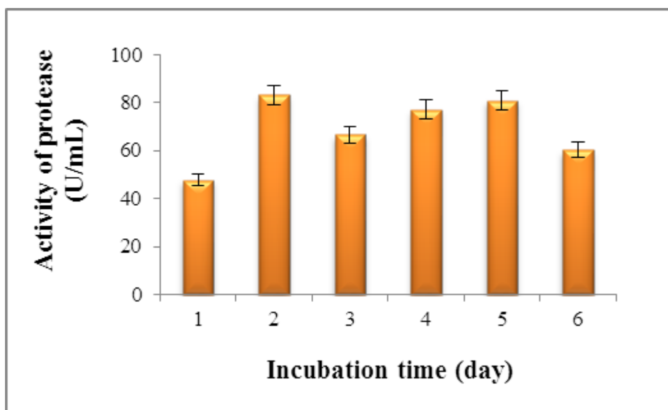

Figure 2. Profile of proteolytic activity of bacterial culture of *P. fluorescens*.

Almost all biological reactions are accelerated or supported by a macromolecule referred as enzyme. Enzyme as catalysts that increase the rate of virtually all the chemical reactions within cells. Most biological reactions are catalyzed by proteins. In the absence of enzymatic catalysis, most biochemical reactions are so slow that they would not occur under the mild conditions of temperature and pressure that are compatible with life. Enzymes accelerate the rates of such reactions by well over a million-fold, so reactions that would take years in the absence of catalysis can occur in fractions of seconds if catalyzed by the appropriate enzyme. Cells contain thousands of different enzymes, and their activities determine which of the many possible chemical reactions actually take place within the cell [22]. Many studies of *P. fluorescens* have been concerned with the purification and properties of deleterious extracellular heat-stable proteases. The amount of protease and the degree of proteolysis which could cause gelation of milk protein have not been determined. Isolation was carried out with use medium contains casein, as a good substrate to isolate bacteria that produce protease enzyme and induce an alkali protease enzyme synthesis [23, 24]. Enzymes are biological catalysts that speed up biochemical reactions in living organisms, and which can be extracted from cells and then used to catalyze a wide range of commercially important processes. Various environmental factors are able to affect the rate of enzyme-catalyzed reactions through reversible or irreversible changes in the protein structure. The effects of pH and temperature are generally well understood [25]. The effect of temperature at 30-70ºC against enzyme activity was range between 18.05-79.58 U/mL and the enzyme stability was range between 11.73-50.54 U/mL. The enzyme showed optimum activity at temp 30ºC was 79.58 U/mL and showed stable was at 50,42 U/mL. The enzyme activity and stability were decreased at temp 35ºC to 70ºC (Figure 3). Wang and Jayarao [26] had shown that protease activity of *P. fluorescens* was higher at temp 22ºC rather than that of 7ºC and 32ºC. Decreasing enzyme yield at temp greater than 65°C could be linked to the enzyme conformation changes or degradation at higher temp. Temperature plays an important role in both the synthesis and excretion of the enzyme through the cell membrane for extracellular enzymes. The temperature requirement for growth may not necessarily be the same for optimum enzyme production as observed in this study where the growth temp was at 35°C while that of enzyme production was at 45°C. High enzyme yields achieved on a wide pH and temp range is an advantage for proper regulation of these parameters under large-scale production. [27]. The effect of pH against protease activity range between 33.39-72.32 U/mL. Optimum activity at pH 8.5 was 72.32 U/mL with stability enzyme was 47.00 U/mL. The enzyme activity and its stability initiated to decrease at pH 9 (Figure 4). The optimum enzyme activity against pH depends on experimental condition, such as buffer that was used, substrate particle, and its enzyme [28]. It should be noted that the optimum pH of an enzyme may not be identical to that of its normal intracellular surroundings. This indicates that the local pH can exert a controlling influence on enzyme activity [25]. Protease was classified have range of acid (pH 2.0-6.0), neutral (pH 6.0-8.0) and base (pH 8.0-13.0) [29; 30]. The genus *Pseudomonas* is well known for its production of both alkaline and acid proteases.

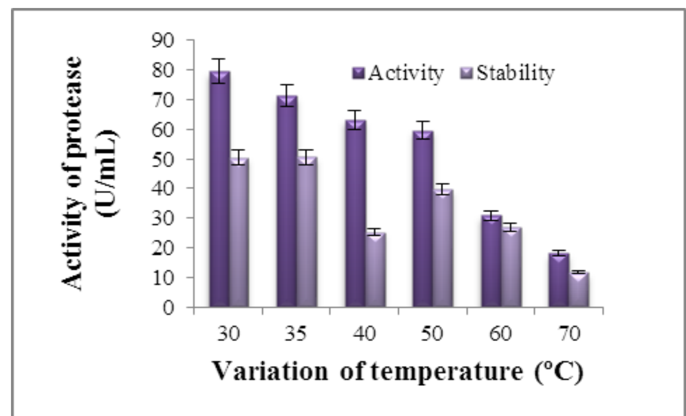

Figure 3. The effect of temperature against protease activity and enzyme stability.

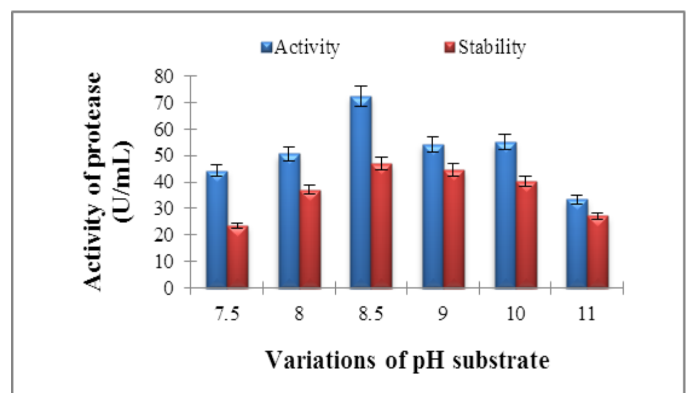

Figure 4. The effect of pH against protease activity and enzyme stability.

Oh *et al.* [31] reported protease production in *P. aeruginosa* that was active in the pH 7.0-9.0 range, with optimum activity at pH 8.0. Koka and Weimer [32] reported metalloprotease production in *P. fluorescens*, with an optimum activity at pH 5.0 and incubation temperature of 35ºC. Protease optimum activity of *P. fluorescens* RO98 was at pH 5.0 and 35ºC, while the enzyme stability was at 15ºC-55ºC and pH 4.5-9.0. The majority species of *Pseudomonas* have the optimum pH of 6.5-8.0 [33-35]. *P. fluorescent* could be classified as alkali bacteria. Reaction rate of enzyme increased in alkali and then decreased after a maximum pH had been reached. Zambare *et al.* [36] reported that had fermented *P. aeruginosa* MCM-327, where the maximum production of protease was obtained at pH 8.0, which corresponds to over 80% of the activity observed in the present study. The optimum enzyme yield was reached at a wide ranges of pH and temperature give advantage to regulation this parameter with accurate for a big scale and can also influence characteristic, morphology and physiological of organisms [27]. Metal ions give a significantly effect on enzyme activity as a vital nutrition for all life organisms and usually protein-bonded that plays an important role in structure and function of protein, probably can increase activity or inhibit a reaction of enzyme-substrate [37, 38]. Table 1, showed that protease activity of *P. fluorescent* activated by divalent cations of $CaCl_2$, $MnCl_2$, while divalent cations of $CuCl_2$, $CoCl_2$, $ZnCl_2$, $HgCl_2$ and monovalent cations of KCl and NaCl were as enzyme inhibitor. General type of protease that produced by *P. fluorescens* is a metal protease [39].

Tabel 1. The effect of metal ion supplementation against enzyme activity.

Metal supplementation	Protease Activity (U/mL)	Activator/ Inhibitor
Control	60.03	
Ca^{2+}	62.12	Activator
Mn^{2+}	64.23	Activator
K^{+}	48.45	Inhibitor
Na^{+}	53.23	Inhibitor
Cu^{2+}	43.87	Inhibitor
Co^{2+}	-	Inhibitor
Zn^{2+}	-	Inhibitor
Hg^{2+}	-	Inhibitor

Metal ion very important for all of life form, it is needed in form of monovalent and divalent cations as activators to increase the specific enzymes activity. However, ion-the ions can also act as inhibitors on the certain concentration [40]. Inhibition by ions of K^+, Na^+, Cu^{2+}, Co^{2+}, Zn^{2+}, and Hg^{2+}, protease activity may be caused by interaction of protein sulphur group that change enzyme conformation so that decrease protease catalytic capacity [41]. The results according to Widhyastuti *et al.* [42] showed that ions of Ag^+ and Ca^{2+} of 1 mM were enzyme activators, while ions of Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, K^+, Na^+, Sr^{2+}, and Sn^{2+} were as protease inhibitors against some bacterial isolates and the results had been reported by Baehaki *et al.* [43] showed that metal ions of Mn^{2+} (5 mM) and Ba^{2+} (5 mM) were the protease strong activator on *Staphylococcus epidermidis* that could increase enzyme activity, while ions of Na^+ (1 mM), K^+ (1 mM), Fe^{3+} (1 mM and 5 mM), Zn^{2+} (5 mM), and Ca^{2+} (1 M) were as the enzyme inhibitors. It was reported by Siddalingeshwara *et al.* (2010), protease of *Bacillus* sp. showed the optimum activity at pH 10 and temp 65°C and the metal ions of Fe^{3+}, Cu^{2+}, Mn^{2+} and Ca^{2+} were inhibitors. Zhang [24] reported that protease of *P. fluorescens* showed optimum activity at pH 7.0-8.5 and temp 45-50°C and its activity could be increased by ions Ca^{2+} and Zn^{2+}, however inhibited by ion Co^{2+}. The biotechnological applications of proteases are rapidly growing as the proteases with novel properties and wide substrate specificities were documented in recent years. Proteases capable of functioning at extreme pH (3-11), temperature (40-80°C) and in organic solvents are considered as robust proteases and suitable for industrial applications. Currently, thermo-, organic solvent stable, alkaline proteases were preferred for various industrial and therapeutic applications [44]. *P. fluorescens* bacteria can give advantage effect against development and growth of plants as PGPR which are capable of suppressing or preventing the phytopathogen damage [3]. Phytopathogenic fungi, as the most common plant pathogens, are capable of infecting different types of plant tissues. Among the main aims in agriculture is finding adequate strategies for their suppression. One of these strategies is biological control of plant diseases that relies on the use of natural antagonists of phytopathogenic fungi [45]. A special place among the natural antagonists of phytopathogenic fungi belongs to rhizobacteria that show beneficial effects on PGPR [46]. *Pseudomonas* species are capable of inhibiting the phytopathogenic fungi that belong to genus *Fusarium* [47]. Majority species of *Fusarium* are pathogenic and cause the damage in plants, thus can cause crop failure including *F. solani* that frequently cause a disease on leaves of paddy, tomatoes, sugarcane, soybean and banana. The food damage due to the diseases caused by *F. solani* on potaoes usually called as dry rot. The dry rot attacks a bark of crop and the damage frequently occurred upon harvesting. Due to the soil-borne nature of the diseases caused by *Fusarium* species the use of chemical methods for the control of disease is rarely successful. Inconsistencies in biocontrol under varying environmental conditions have been a common limitation of soil-borne pathogens. The present research was conducted to evaluate the efficacy of indigenous *Pseudomonas* isolates against these pathogens. Figure 5 showed *in vitro* antifungal activity of selected *P.*

fluorescens toward strains *F. solani*. The hydrolytic enzymes get great attention because they play a significant role in controlling diseases by excreting cell wall hydrolases [48]. Several rhizobacteria, including genera of *Pseudomonas*, are known to produce a battery of hydrolases such as chitinase which help in the maceration of cell walls of those plant pathogens [49-52]. The selected isolates were evaluated for their antagonistic effect against *Fusarium*. It showed that protease extract of *P. fluorescens* could inhibit the growth of *F. solani* InaCC F76 (Figure 5A) and *F. oxysporum* InaCC F78 (Figure 5B), thus the fungi could not grow well and thrive by covering the entire surface of the media, although both fungi had been grown for more than a week. The potential antagonistic activity of the bacteria *P. fluorescens* could be correlated with the highest protease activity of the bacteria. The involvement of antifungal activity compounds produced by *P. fluorescens* in the inhibition of fungal growth was confirmed by the ability of cell-free culture filtrate of this strain to inhibit the hyphal growth of *Fusarium sp.* Conducted tests revealed the direct influence of *P. fluorescens* on the growth rate of the tested fungus under study. Studies shows that prolonging the bacterial culturing time and at the same time increasing the amount of secondary metabolites affect the inhibition the growth of the fungus. Selective activity of *P. fluorescens* against of phytopathogens has been described in many research papers. The researchers report that the antifungal properties of Pseudomonas largely depend on the capability of secretion of secondary metabolites, notably lytic enzymes and antibiotics [53]. Koche *et al* [54] found that *P. fluorescens* was most efficient in inhibiting the mycelial growth. Whereas, Toua *et al* [55] demonstrated restricting the growth of the two species of *F. oxysporum* by five strains of *P. fluorescens.* Moreover conidial germinartion and germ tube elongation were inhibited and reduced. The results of the experiments by [53] indicated the importance synthesized by *P. fluorescens* of salicylic acid in reducing of fungal phytopathogens. The inhibition of mycelial growth amounted to successively for *F. greaminarum*, *F. avenaceum* and *F. solani* in the range 80-100%, depending on the tested *Pseudomonas* strain [53]. The inhibitory properties of *P. fluorescens* obtained could be probably due to the production by own strain of secondary metabolites and/or lytic enzymes that can degrade cell wall. Although [53] reported that fungal lysis need not necessarily be caused by lytic enzymes capable of decomposition of glycosidic bonds-chitinase and β 1,3 glucanase but also by other substances which are manufactured by bacteria from the *Pseudomonas* which include intensively secreted siderophores, hydrogen cyanide as well as exogenous proteases.

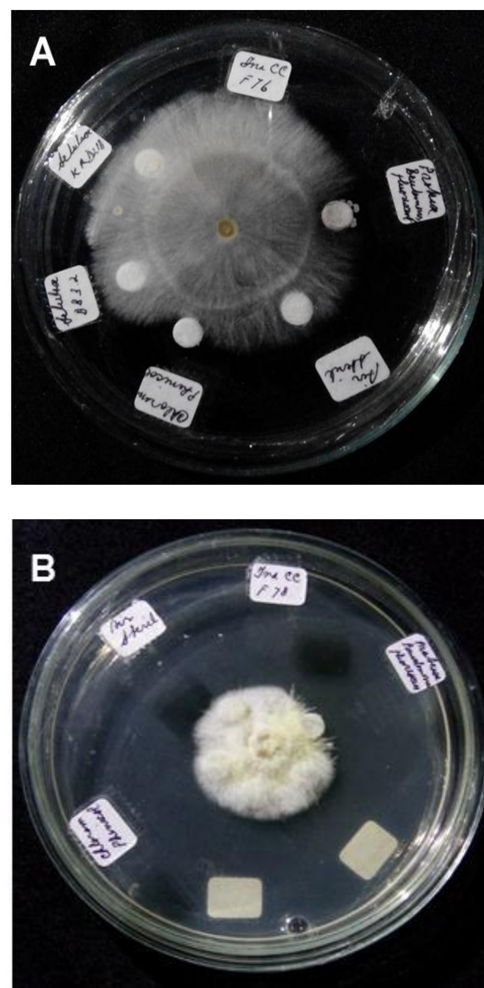

Figure 5. Inhibitory effect of protease extract of *P. fluorescens* on growth of *F. solani* InaCC F76 (A) and *F. oxysporum* InaCC F78 (B).

Conclusion

The results obtained in this study pointed out the possible use of strains of *Pseudomonas* as biocontrol agents against *Fusarium* sp.. However, further research is needed under pot culture as well as field conditions to elucidate the mechanism of action of the potential antagonistic bacteria in detail. Our investigation confirmed more or less pronounced antifungal activity of all tested *Pseudomonas fluorescens*. Regarding the *Fusarium* species, the highest sensitivity to antibiotic-producing *Pseudomanas* isolates was observed for *F. oxysporum* and *F. solani*. Our findings impose that the studied *Pseudomonas* isolates have potential in controlling plant diseases caused by *Fusarium* spp., whereby the bacterial isolates with the highest inhibitory potential will be selected for further experiments. Conducted research confirmed fungistatic properties of *P. fluorescens* against *F. oxysporum* and *F. solani* strains and prove that growth inhibition of the fungi depends not only on the biological properties and age of the bacterial culture and also susceptibility of the fungus to bacterial metabolites. Therefore, promising method to crop protection against

Fusarium sp. may be the application of *P. fluorescens* as the biocontrol agents. However, antagonist Pseudomonas with the antagonistic activity in vitro may not act *in vivo* due to environmental conditions and competition with other microorganisms. Therefore, it is important that biocontrol potential under field conditions should be further evaluated.

Acknowledgments

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References

1. Erickson B, Nelson JE and Winters P. Perspective on opportunities in industrial biotechnology in renewable chemicals. Biotechnology Journal 2012; 7(2): 176–185.
2. Ajmal M, Ali HI, Saeed R, Akhtar A, Tahir M, Mehboob MZ and Ayub A. Biofertilizer as an Alternative for Chemical Fertilizers. Research & Reviews: Journal of Agriculture and Allied Sciences 2018; 7(1): 1-7.
3. Nihorembere V, Ongena M, Smargiass M, Beneficial effect of the rhizosphere microbial community for plant growth and health. Biotechnol.Agron. Soc. Environ. 2011; 15 (2): 327-337.
4. Gupta CP, Dubey RC, Kang SC, Maheh Wari DK. Antibiosis-mediated necrotrophic effect of Pseudomonas GRC2 against two fungal plant pathogens. Current Science 2001; 81: 91-94.
5. Ursula SK, Arnaud S, Monika M, Caroline B, Brion D, Cecile G.B, Cornelia R, Regina N, Genevie VDF, Dieter H and Christoph KL. Autoinduction of 2, 4 Diacetylphloroglucinol biosynthesis in biocontrol agent *Pseudomonas fluorescens* CHAO and repression by the bacterial metabolites salicylate and pyoluteorin. Journal of Bacteriology 2000; 182(5): 1215-1225.
6. Munkvold GP. Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. European Journal of Plant Pathology. 2003; 109: 705–713.
7. Franks A, Ryan RP, Abbas A, Mark GL, and O'Gara F Molecular tools for studying plant growth promoting rhizobacteria. In: Cooper JE, Rao JR (eds) Molecular approaches to soil, rhizosphere and plant microorganisms analysis. Biddes Ltd, Kings Lynn, UK 2006; 116–131.
8. Shalini RS. Antifungal Activity of *Pseudomonas fluorescens* against different plant pathogenic fungi. The Internet Journal of Microbiology 2008; 7(2): 1-7.
9. Enya J, Togawa M, Takeuchi T, Yoshida S, Tsushima S, Arie T and Sakai T. Biological and Phylogenetic Characterization of *Fusarium oxysporum* Complex, Which Causes Yellows on *Brassica* spp., and Proposal of F. *oxysporum* f. sp. *rapae*, a Novel Forma Specialis Pathogenic on *B. rapa* in Japan. Phytopathology 2008; 98(4): 475-483.
10. Naiola E, and Widhyastuti N. Semi purification and characterization of protease from *Bacillus* sp. Journal of Periodic Biological Research 2007; 13(1): 51-56 (*in Indonesian*).
11. Cappuccino JG and Sherman N. Microbiology: A Laboratory Manual. Addison- Wesley Publishing Company. California USA. 1983.
12. Yang SS and Huang CI. Proteases production by amylolytic fungi in solid state fermentation. Journal of Chinese Agricultural Chemical Society 1994; 32(6): 589-601.
13. Susanti EVH. Isolation and Characterization of Proteases from *Bacillus subtilis* 1012M15. Jurnal Biodiversitas 2003; 4(1): 12-17. (in Indonesian).
14. Correa APF, Daroit DJ, Velho RV and Brandelli A. Hydrolytic potential of a psychrotrophic *Pseudomonas* isolated from refrigerated raw milk. *Brazilian* Journal of Microbiology 2011; 42: 1479-1484.
15. Pakpahan R. Isolation of bacteria and testing of thermophilic protease activity from hot water sources Sipoholon North Tapanuli, Northern Sumatra. Postgraduate School, University of North Sumatra, Medan. [Thesis]. 2009. (in Indonesian).
16. Soeka YS. Optimazation of lipase from *Pseudomonas fluorescens*. First Edition Proceeding of National Seminar on Biodiversity, Airlangga University, Surabaya. 2016. (in Indonesian).
17. Patil P, Sabale S and Devale A. Isolation and Characterization of Protease Producing Bacteria from Rhizosphere Soil and Optimization of Protease Production Parameters. International Journal of Current Microbiology and Applied Sciences 2015; 2: 58-64.
18. Wiedmann M, Weilmeier D, Dineen SS, Ralyea R and Boor KJ. Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. Applied and Environmental Microbiology 2000; 66(5): 2085-2095.
19. Dogan B and Boor KJ. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Applied and Environmental Microbiology 2003; 69(1): 130-138.
20. Dufour D, Nicodème M, Perrin C, Driou A, Brusseaux E, Humbert G, Gaillard J-L and Dary A. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology 2008; 125(2): 188-196.
21. Rauf A, Irfan M., Naleem M., Ahmed I. and Iqbal HMN. Optimization of growth conditions for acidic protease productions from *Rhizopus oligosporus* through solid state fermentation of sunflower meal. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 2010; 4(12): 898-901.
22. Bisswanger H. Enzyme assays. Perspectives in Science 2014; 1(1–6): 41-55.
23. Rodarte MP, Dias DR, Vilela DM and Schwan RF. Proteolytic activities of bacteria, yeasts and filamentous fungi isolated from coffee fruit (*Coffea arabica* L.). Acta Scientiarum Agronomy Maringá 2011; 33(3): 457-464.
24. Zhang WW, Hu YH, Wang HL and Sun L. Identification and characterization of a virulence-associated protease from a pathogenic *Pseudomonas fluorescens* strain. Veterinary Microbiology 2009; 139 (1-2): 183–188.
25. Robinson PK. Enzymes: principles and biotechnological applications. Essays Biochemistry 2015; 59: 1–41.
26. Wang L and Jayarao BM. Phenotypic and genotypic characterization of *Pseudomonas fluorescens* isolated from bulk tank milk. Journal of Dairy Science 2001; 84(6): 1221-1229.
27. George-Okafor UO and Mike-Anosike EE. Screening and optimal protease production by *Bacillus* sp. Sw-2 using low cost substrate medium. Research Journal of Microbiology 2012; 7(7): 327-336.
28. Bacha E, Sant'Anna V, Daroit DJ, Correa APF, Segalin J, Brandelli A. Production, one-step purification, and characterization of a keratinolytic protease from *Serratia marcescens* P3. Process Biochemistry 2012; 47: 2455–2462.
29. Gupta R, Beg QK and Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Applied Microbiology and Biotechnology 2002; 59(1): 15-32.
30. Sabotic J and Kos J. Microbial and fungal protease inhibitors-current and potential applications. Applied Microbiology and Biotechnology 2012; 93(4): 1351-1375.
31. Oh YS, Shih IL, Tzeng, YM, Wang, SL. Protease produced by *Pseudomonas aeruginosa* K-187 and its application in the deproteinization of shrimp and crab shell wastes. Enzyme and Microbial Technology. 2000; 27(1): 3-10.
32. Zambare V, Nilegaonkar S and Kanekar P. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-3 27: enzyme production and its partial characterization. New Biotechnology 2011; 28(2): 173–181.
33. Koka R and Weimer BC. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. Journal of Applied Microbiology 2000; 89: 280-288.
34. Chen L, Daniel RM, Coolbear, T. Detection and impact of protease and lipase activities in milk and milk powders. International Dairy Journal 2003; 13(4): 255-275.
35. Nielsen SS. Plasmin system and microbial proteases in milk: characteristics, roles, and relationship. Journal of Agriculture and Food Chemistry 2002; 50(22): 6628-6634.
36. Stepaniak L. Dairy enzymology. International Journal of Dairy Technology 2004; 57(1-2): 153-171.
37. Sattar H, Aman A and Ul Qader SA. Effect of Metal Ions, Solvents and Surfactants on the Activity of Protease from *Aspergillus niger* KIBGE-IB36 Journal of Basic & Applied Sciences 2017; 13:491-495.
38. Priebatsch KM, Kvansakul M, Poon IKH and Hulett MD. Functional regulation of the plasma protein histidine-rich glycoprotein by Zn^{2+} in settings of tissue injury. Biomolecules 2017; 7(1): 1-25.
39. Martins ML, Pinto UM, Riedel K and Vanetti MCD. Milk-deteriorating exoenzymes from *Pseudomonas fluorescens* 041 isolated from refrigerated raw milk. Brazilian Journal of Microbiology 2015; 46(1): 207-217.
40. Blaby-Haas CE and Merchant SS. Lysosome-related Organelles as Mediators of Metal Homeostasis. Journal of Biological Chemistry 2014; 289(41): 28129–28136.

41. Rajmohan S, Dodd CE, and Waites WM. Enzymes from isolates of Pseudomonas fluorescens involved in food spoilage. Journal of Applied Microbiology 2002; 93(2), 205-213.
42. Widhyastuti N, Hotimah B and Achmadi SS. Properties of extracellular protease of bacterial P.1 strain isolated from palm wine. Indonesian Jurnal for Biology 2001; 3(1): 80-89. (in Indonesian).
43. Baehaki A, Nurhayati, T and Suhartono MT. Characteristics of protease from pathogenic bacteria *Staphylococcus epidermidis*. Bulletin of Fisheries Product Technology 2005; 8(2); 25-34. (in Indonesian).
44. Li Q, Yi L, Marek P, and Iverson BL. Commercial proteases: Present and future. FEBS Letters. 2013; 587(8): 1155–1163.
45. Heydari A and Pessarakli M. A review on biological control of fungal plant pathogens using microbial antagonists. Journal of Biological Sciences. 2010; 10(4): 273-290.
46. Zehnder GW, Murphy JF, Sikora EJ, Kloepper JW. Application of rhizobacteria for induced resistance. European Journal of Plant Pathology. 2001; 107(1): 39-50.
47. Showkat S, Murtaza I, Laila O and Ali A. Biological control of *Fusarium oxysporum* and *Aspergillus* sp. by *Pseudomonas fluorescens* isolated from wheat rhizosphere soil of Kashmir. IOSR Journal of Pharmacy and Biological Sciences 2012; 1(4): 24-32.
48. Chernin L and Chet I. Microbial enzymes in biocontrol of plant pathogens and pests. R.G. Burns, R.P. Dick, Eds., Enzymes in the environment: activity, ecology, and applications. Marcel Dekker, inc., New York. 2002: 171–225.
49. Huang CJ, Wang TK, Chung SC, and Chen CY. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28–9. Journal of Biochemistry *and* Molecular Biology 2004; 38(1): 82–88.
50. Hoster F, Schmitz JE, Daniel R. Enrichment of chitinolytic microorganisms isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel Streptomyces strain. Applied Microbiology and Biotechnology 2005; 66: 434–442.
51. Aktuganov GE, Galimzyanova NF, Melent' Ev AI, and Kuz' Mina LY. Extracellular hydrolases of strain *Bacillus* sp.739 and their involvement in the lysis of micromycetecell walls. Mikrobiologiia 2007; 76(4): 471–479.
52. Bogas AC, Watanabe MAE, Barbosa A, Vilas-Boas LA, Bonatto AC, Dekker R, Souza EM, and Fungaro MHP. Structural characterization of the *bglH* gene encoding a beta glucosidase-like enzyme in an endophytic *Bacillus pumilus* strain. Genetics and Molecular Biology 2007; 30(1): 100–104.
53. Jankiewicz U, Gołąb D and Frąk M. Effect of salicylic acid, synthesized by the bacteria Pseudomonas fluorescens and P. chlororaphis on phytopathogenic fungi Fusarium. Polish Journal of Agronomy 2013; 15: 65–68.
54. Koche MD, Gade RM and Deshmukh AG. Antifugal activity of secondary metabolites produced by *Pseudomonas fluorescens*. The Bioscan. Supplement on Medicinal Plants 2013; 8 (2): 723-726.
55. Toua D, Benchabane M, Bnsaid F and Bakour R. Evaluation of *Pseudomonas fluorescens* for the biocontrol of fusarium wilt in tomato and flax. Glob J Microbiol Res. 2013; 1(1): 075-084.