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Research Article

Identification and Characterization of Fluorescent Pseudomonas Producing Active Compounds Controlling Plant Pathogens

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Abstract: Fluorescent pseudomonad is one of the biocontrol agents against plant pathogens. Various compounds reportedly can be produced by fluorescent pseudomonad, including chitinase, β-1,3-glucanase, HCN, siderophore, antibiotics, Indole Acetic Acid (IAA), phosphate solvent compounds, and 2,4-diacetylphloroglucinol (DAPG). In this study, it was identified and characterized six isolates of fluorescent pseudomonad (PfPj1, PfPj2, PfKd7, PfCas, PfCas3, and LAHp2). All isolates were isolated from the rhizosphere of various types of plants. The results showed that six isolates were identical to *Pseudomonas aeruginosa* (93-94%). All bacterial isolates tested were able to produce siderophore, HCN, and solubilize phosphates. The highest siderophore was produced by isolate PfPj2. Whereas isolate PfKd7 had the highest at HCN production and the ability to dissolve phosphates.

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1. Introduction

Bacteria are the most commonly found organisms and their existence is not evenly distributed. The number of bacteria in the rhizosphere is much higher than in the soil. This could be related to the plant's roots' secreting exudate as a food source for bacteria. Certain bacteria have the ability to control plant pathogens, and can also affect plant growth (Olanrewaju et al., 2017; Sakci et al., 2021; Kaba and Bektas, 2022; Yerli and Sahin, 2022). Bacteria that affect plant growth directly or indirectly are referred to as Plant Growth Promoting Rhizobacteria (PGPR) (Alizadeh, 2011). Several genera of bacteria such as *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Bradyrhizobium,* and *Rhizobium* show PGPR activity in different plants (Ahemad and Kibret, 2014).

Chemical compounds are commonly used to control various plant pathogens. It is difficult to do since pathogens are able to live in diverse environments and very broad host ranges. Another alternative that can be used as a control for plant pathogens is biocontrol agents. Nguyen and Ranamukhaarachchi (2010) reported *Bacillus megaterium*, *Enterobacter cloacae*, *Pichia guillermondii*, and *Candida ethanolica* which were isolated from the soil and suppressed effectively growth of the *Ralstonia solanacearum*.

Various factors can affect the ability of biocontrol agents to reduce the intensity of plant diseases. The biocontrol agents are capable of producing several compounds involved in antagonistic mechanisms. According to Viveros et al. (2010), the ability of biocontrol agents to suppress plant diseases is caused by their colonizing plant roots, and the ability to produce antibiotics, siderophore, HCN, hydrolytic enzymes (chitinase, protease, lipase, etc.) or through activities plant defense mechanism. Yanti et al. (2017) reported that the biocontrol agent *Bacillus subtilis* CIFT-MFB-4158A can produce a siderophore, IAA, and it is able to dissolve phosphate.

Other biocontrol agents that can be used to control plant diseases are a group of fluorescent pseudomonad bacteria. The beneficial effect of the use of fluorescent pseudomonad is that it can produce chitinase, β-1,3-glucanase, HCN, siderophore, antibiotics, phytohormones, phosphate solvent compounds, induction of systemic resistance to various pathogens (Podile and Kishore, 2006), producing 2,4 -diacetylphloroglucinol (DAPG), phenazines, and pyrrolnitrin (Haas and Défago, 2005), Indole Acetic Acid (IAA), and show the activity of boosting plant growth (Deshwal and Kumar, 2013).

The different characteristics possessed by biocontrol agents make them good candidates for suppressing plant pathogens. Fluorescent pseudomonads are non-pathogenic rhizobacterial groups, and some of them are *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa*, and *P. aureofaciens* which have a wide ability to suppress soil-borne pathogens with different mechanisms of action (Karthikeyan et al., 2006). The isolates PfPj1, PfPj2, PfKd7, PfCas, PfCas3, and LAHp2 of fluorescent pseudomonad produced the HCN in the medium added by ZnSO4.7H2O (Advinda et al., 2018). IAA can be produced by *P. fluorescens* and *B. subtilis* (Reetha et al., 2014). Furthermore, Anhar et al. (2011) reported that fluorescent pseudomonad can increase plant height, number of tillers, and wet weight of rice plants. Whereas Qessaoui et al. (2019) reported that *Pseudomonas* sp. strain Q6B can increase seed germination, while *Pseudomonas* spp. (Q6B, Q14B, Q7B, Q1B, and Q13B) stimulated the increase in tomato seedling height. The Fluorescent pseudomonad can control root rot disease in black pepper plants caused by *Phytophthora capsici* (Paul and Sarma, 2006). *In vitro*, the growth of *Xanthomonas axonopodis* pv. *malvacearum* can be inhibited by various strains of *P. fluorescens* and *B. subtilis* (Salaheddin et al., 2010).

In the previous study, we reported that the effectiveness of fluorescent pseudomonad isolates of PfPj1, PfPj2, PfPj3, PfPb1, PfPb2, PfPb3, and PfPm1 to control the Blood Disease Bacteria (BDB) in banana seeds (Advinda, 2009). However, the detailed information about these isolates is still inadequate. Then in the present study, we report the identification and characterization of isolates PfPj1, PfPj2, PfKd7, PfCas, PfCas3, and LAHp2 of fluorescent pseudomonad.

2. Material and Methods

2.1. Condition of fluorescent pseudomonad isolates

The isolates PfPj1, PfPj2, PfKd7, PfCas, PfCas3, and LAHp2 of fluorescent pseudomonad were isolated from the rhizosphere of various types of plants and deposited in the L. Advinda collections of the Biology Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Padang, West Sumatra, Indonesia. Isolates were stored at room temperature in Eppendorf tubes containing sterile aquadest. Rejuvenation of all isolates using a solid King's B medium, and multiplication of inoculums in a liquid King's B medium.

2.2. 16s rRNA gene PCR and Sequencing

Isolation of bacterial genomes was done by using the boiling method (Dashti et al., 2009). As much as 2-3 Loopful of bacterial colonies were put into a 1.5 mL Eppendorf tube containing 200 µL 1/10 TE buffer pH 8. The suspension was vortexed until it was homogeneous. The boiling process is carried out in a water bath at a temperature of 95-100ºC, for 15 minutes. The suspension is then centrifuged at 12,000 rpm for 10 minutes to separate the supernatant and pellet. The supernatant is transferred into a new Eppendorf tube and stored at - 20ºC until it is about to be used.

The amplification process is carried out by the PCR method using thermal cycle tools with universal primers 27F (forward) and 1492R (reverse). The PCR reaction was carried out in 25 µL reactions, consisting of 6.5 µL ddH2O, 12.5 µL Dream Taq PCR (Thermo Scientific) Kit, 2 µL for each forward and reverse primer, 2 µL DNA template. The temperature used was an initial denaturation of

95ºC for 3 minutes, followed by 35 cycles consisting of denaturation of 95ºC for 45 seconds, primary attachment (annealing) at 55ºC for 30 seconds, and elongation at 72ºC for 2 minutes. The final extension step of the reaction was carried out at 72ºC for 7 minutes (Frank et al, 2008). PCR product analysis was performed by electrophoresis using 1% agarose gel. The electrophoresis results were then checked with Gel Documentation System. The amplification results are sent to Macrogen Singapore Sequencing for sequencing using an automated DNA sequencer (ABI Prism 3100 Analyzer, Applied Biosystem, USA).

2.3. Phylogenetic analysis

The sequences of 16S rRNA gene of the tested bacterial isolates were compared with the gene sequences listed in the NCBI database using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Singh et al., 2014) and sequence alignment DNA between strains was carried out using the ClustalW program. The phylogenetic tree was constructed using the Maximum Likelihood statistical method and the Tamura-Nei model, 1000 pseudoreplicate bootstrap integrated in MEGA X software (Kumar et al., 2018). Reference to the 16S rRNA gene sequence was obtained from data in GenBank (Table 1).

Table 1. The 16S rRNA gene reference sequence that was obtained from the NCBI website

Reference sequence of *Trichoderma*		
1	*Pseudomonas aeruginosa*	NR117678
Reference sequence *outgroup*		
1	*Pseudomonas fluorescens*	NR043420
2	*Pseudomonas putida*	NR043424
3	*Rhizobacter gummiphilus*	NR132677
4	*Rugamonas rubra*	NR104915

2.4. Siderophore production

The level of siderophore of isolates is determined quantitatively. Siderophore production is known by growing fluorescent pseudomonad on low-iron synthetic medium containing 20 g sucrose, 2 g L-asparagine, 1 g K_2HPO_4, and 0.5 g $MgSO_4$ dissolved into distilled water up to 1 liter. Then the medium was sterilized in an autoclave at 121 ^oC and a pressure of 1 atm for 15 minutes (Elad and Baker, 1985).

To find out the siderophore production of each isolate was carried out by taking 1 mL of fluorescent pseudomonad suspension, then inoculating it in 25 mL of low-iron synthetic medium and incubating it for 24 hours on a shaker. The resulting suspension is centrifuged at 11,000 rpm for 30 minutes, then the supernatant is filtered with filter paper. Detection of siderophore is done by adding 1 mL of FeCl 0.01 M to 3 mL of the supernatant. Siderophore detection is measured using a spectrophotometer at a wavelength (λ) of 405 nm (Cody and Gross, 1987).

2.5. HCN production

The procedure of Vanitha and Ramjegathesh (2014) was modified and used to determine HCN production of fluorescent pseudomonad. Fluorescent pseudomonad was grown on TSA + glycine medium (10 g TSA + 4.2 g glycine), which was dissolved with distilled water up to 1 L volume. Indicators of HCN production were picric acid solution consisting of 2 g picric acid and 8 g sodium carbonate dissolved into distilled water up to a volume of 200 mL.

To determine the production of cyanide acid, it was done by taking 0.1 mL of fluorescent pseudomonad suspension (population $3x10^8$ cfu mL^{-1}, 1 Mc Farland's scale), then inoculating the petri dish which contained glycine medium. On the lid of the petri dish is attached a piece of filter paper that has been dropped with 1 mL of picric acid solution. Bacterial cultures were incubated at room temperature for 2x24 hours. The color of filter paper which remained yellow showed that the tested isolates did not produce cyanide acid, whereas the light brown, dark brown, and brick red colors indicated increasing cyanide acid production.

2.6. Phosphate solubilization

The ability of fluorescent pseudomonad to dissolve phosphate can be determined by inoculating it on Pikovskayas agar medium. Sterile disc paper is placed in a petri dish and dropped with 0.1 mL of a pseudomonad fluorescent suspension (population $3x10^8$ cfu mL^{-1}, scale 1 Mc Farland's). Then the disc was placed in the middle of the medium so that Pikovskayas was in a petri dish, and incubated for 48 hours at room temperature. The ability to solubilize phosphate is indicated by the formation of a halo zone.

3. Results and Discussion

3.1. Molecular identification

A total of six isolates were isolated from the rhizosphere of various types of plants and were successfully identified by molecular methods. BLASTN results of 16s rRNA gene sequences with reference sequences in the NCBI Database can be seen in Table 2.

Table 2. The results of the identification of 16S rRNA gene of bacterial isolates using BLAST

Isolate	BLAST Results				Access no
	Description	Max Score	E Value	Ident (%)	
LAHf2 (901 bp)	*Pseudomonas aeruginosa* strain DSM 50071, 1527 bp (NR117678)	992	0.0	93.97	SAMN29359229
PfCa5 (910 bp)	*Pseudomonas aeruginosa* strain DSM 50071, 1527 bp (NR117678)	1007	0.0	94.05	SAMN29359230
PfCa53 (904 bp)	*Pseudomonas aeruginosa* strain DSM 50071, 1527 bp (NR117678)	1013	0.0	94.19	SAMN29359231
PfPj1 (900 bp)	*Pseudomonas aeruginosa* strain DSM 50071, 1527 bp (NR117678)	1033	0.0	94.17	SAMN29359232
PfPj2 (893 bp)	*Pseudomonas aeruginosa* strain DSM 50071, 1527 bp (NR117678)	1000	0.0	94.13	SAMN29359233
PpKd7 (904 bp)	*Pseudomonas aeruginosa* strain DSM 50071, 1527 bp (NR117678)	1031	0.0	93.82	SAMN29359234

Based on the measurement of sequential kinship through the average nucleotide identity (ANI) value in the BLAST program, all isolates of the tested bacteria were suspected to be not identical to *Pseudomonas aeruginosa* because they were below the threshold of similarity between species for the bacterial and archae categories. According to Reller et al. (2007) if the percentage of homology is close to 100% or > 97%, it can be confirmed as the same species but conversely if homology is less than 97% this isolate might be a new species or unconfirmed species. Kim et al. (2014) through their research revealed that there were significant differences in the overall ANI distribution between intra- and interspecies relationships in ANI 95-96%. Then they determined which level of similarity in 16S rRNA gene sequences was within the ANI threshold that is currently accepted for species demarcation using more than one million comparisons. Two-fold cross-validation statistical tests reveal that the similarity in the 16S rRNA gene sequence of 98.65% can be used as a threshold to distinguish between two species. From the results of BLAST it can also be observed that there are differences in base length between isolates and reference sequences. According to Sukweenadhi et al. (2019) this could affect the accuracy of the prediction of test bacterial isolates.

3.2. Phylogenetic analysis

Figure 1 is a molecular phylogenetic tree arranged from the sequence of nucleotide genes encoding 16S rRNA regions from the tested bacterial isolates and *Pseudomonas aeruginosa* which have

the highest similarity values based on BLAST analysis (Figure 1). The chosen outgroups come from different species and genera, these outgroups help provide character polarization (apomorphy and plesiomorphy characters). Apomorphy characters are characters that change and are inherited in test bacterial isolates (ingroup), while plesiomorphy characters are primitive characters found in outgroups. Bootstrap values which are represented by numbers on tree branches show high tree reliability. Hillis and Bull (1993) in Lemoine et al. (2018) stated that phylogenetic trees with high bootstrap values above 70% are good phylogenetic trees.

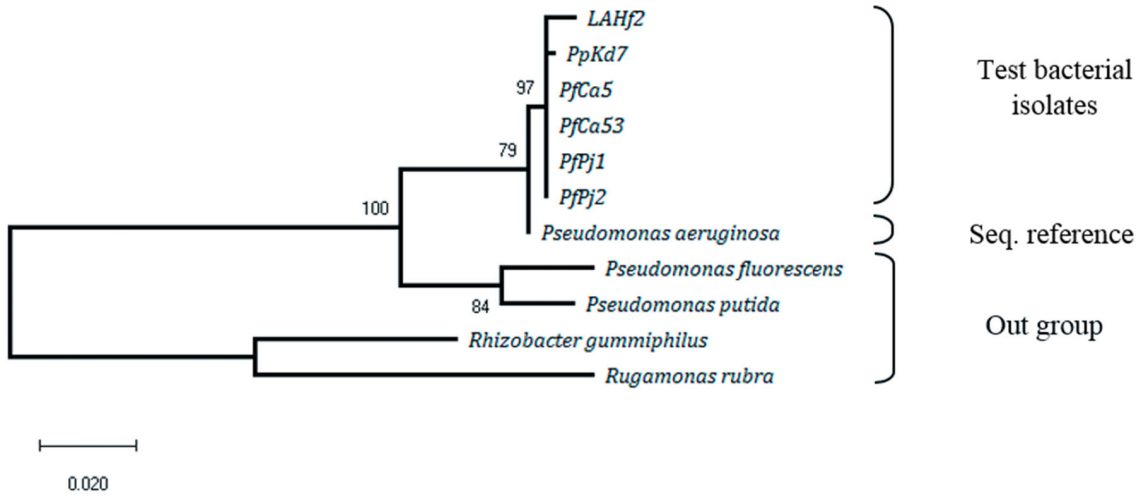

Figure 1. The phylogenetic tree (Neighbor-joining tree) of the 16S rRNA gene sequences of the *Pseudomonas* bacteria which was constructed using the Maximum Likelihood statistical method and bootstrap analysis (1,000 replicates) in MEGA X 10.0.5 software (Kumar et al., 2018).

Table 3. Percentage of homology of *P. aeruginosa* isolate nucleotide sequence against data at Genbank

Seq->	P.A	LAHf2	PfCa5	PfCa53	PfPj1	PfPj2	PpKd7
P.A	ID	0.93	0.934	0.935	0.935	0.933	0.934
LAHf2		ID	0.992	0.994	0.994	0.991	0.992
PfCa5			ID	0.998	0.998	0.998	0.997
PfCa53				ID	1	0.997	0.998
PfPj1					ID	0.997	0.998
PfPj2						ID	0.995
PpKd7							ID

Info: P.A = *Pseudomonas aeruginosa* DSM 50071.

From the results of phylogenetic tree construction, it is known that the tested bacterial isolates and *Pseudomonas aeruginosa* are from common ancestors and have a very close kinship, but then a separation of gene evolution lines occurs so that the tested bacterial isolates form new groups which are shown by short branches on the meeting point (node) between the test bacterial isolate and *P. aeruginosa*. Among the 6 bacterial isolates tested, LAHf2 and PpKd7 isolates had longer evolution than other isolates. It is assumed that the tested bacterial isolate is a derivative of *P. aeruginosa* that has undergone a change or evolution. Hillis and Bull (1993) stated that branch length shows the number of changed sequences that occurred before the level of separation. The greater the length of the branch, the more sequential changes occur.

Various developments regarding the observations of various PGPR traits have been widely reported. The production of diverse microbial metabolites such as antibiotics, siderophore, ammonia, HCN, pyrrolnitrin, phenazine, 2,4-diacetyl phloroglucinol, and lytic enzymes by *P.fluorescens* against several pathogenic bacteria has been reported (Maleki et al., 2010; Subramanian and Satyan, 2014). This

study reports the identification and characterization of the isolates PfPj1, PfPj2, PfKd7, PfCas, PfCas3, and LAHp2 of fluorescent pseudomonad. Based on the results of phylogenetic tree construction, these fluorescent pseudomonad isolates were closely related to *P. aeruginosa*, but with some genetic variations. Further specific studies and more analysis were needed to confirm these isolates.

From the observations of siderophore production, all isolates produced siderophores (Figure 2). The siderophore levels were determined using a spectrophotometer at a wavelength (λ) of 405 nm, and figures indicated Optical Density (OD) absorbance values related to results. The difference in OD produced showed that each isolate was able to produce a siderophore at different levels. Isolate PfPj2 produced the highest siderophore, 3.240 (OD_{405}), and the lowest was produced by isolate PfPj1, 2.185 (OD_{405}).

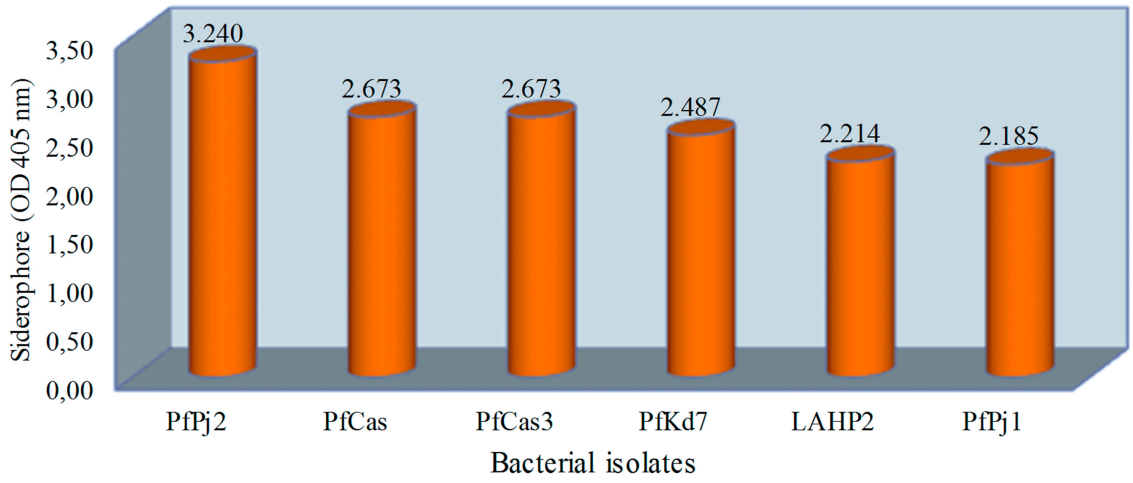

Figure 2. The ability of bacterial isolates to produce siderophores.

The ability of bacteria to produce siderophore is an important trait in Plant Growth Promoting Rhizobacteria (PGPR) because siderophore is able to bind iron ($Fe^{3+)}$ into siderophore-iron bonds that become available to plants. According to Ahmed and Holmström (2014), the siderophores produced by microorganisms can inhibit the growth of pathogens. This happens because $Fe^{3+}$is already bound to siderophore, so pathogens have Fe^{3+} deficiency.

The fluorescent pseudomonad isolates JUPF31 and JUPF37 of Anitha and Kumudini (2014) were able to produce a siderophore. Isolate JUPF37 were the highest in its ability to produce siderophores followed by isolate JUPF32 (Anitha and Kumudini, 2014). Advinda et al. (2019) stated that siderophore production can be influenced by the growth medium of fluorescent pseudomonad. Fluorescent pseudomonad PfCas3 isolates grown on glucose medium produce higher siderophore than fructose medium. According to Yeole and Dube (2001), the differences in the levels of siderophore produced by fluorescent pseudomonad can be caused by various things, such as the type of isolate, changes in time, space, nutrition, and the growth environment of these microorganisms.

In the present study, all bacterial isolates showed different abilities to produce HCN. The ability to produce HCN from isolates PfPj1, PfPj2, PfKd7, PfCas, PfCas3, and LAHp2 is characterized by the resulting color changes in filter paper pieces that have been dropped with 1 mL of cyanide acid detection solution. The color of the filter paper which remained yellow showed that the tested isolates did not produce cyanide acid, whereas the light brown, dark brown, and brick red colors indicated increasing cyanide acid production (Figure 3).

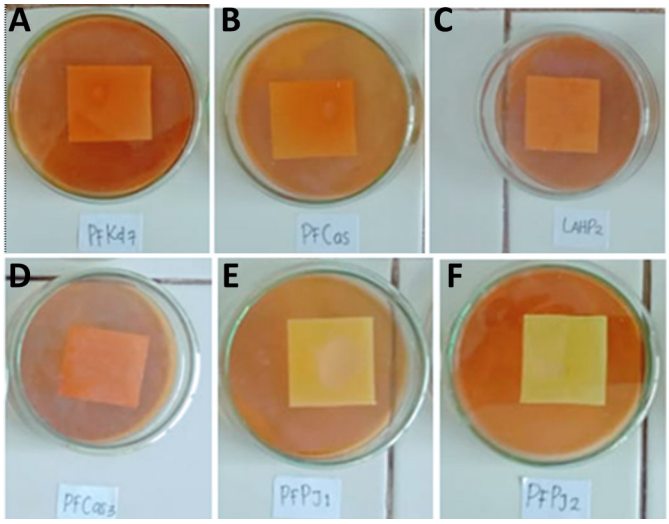

Figure 3. The ability of bacterial isolates to produce HCN: A, isolate PfKd7; B, isolate PfCas; C, isolate LAHp2; D, isolate PfCas3; E, isolate PfPj1; F, isolate PfPj2.

The PfKd7 isolate produces the highest HCN, characterized by filter paper which is brick red. While the one that did not produce HCN was isolated PfPj2 (Figure 3). This is supported by research results reported by Prasad et al. (2017) that there are differences in the ability to produce HCN by *P. fluorescens-10* and *B. subtilis-2* which are isolated from the tomato plant rhizosphere. Thus, these two bacteria are very useful as plant pathogen biocontrol agents.

HCN production by *P. fluorescens* CPF1 to CPF10 isolated from *Coleus rhizosphere* has been reported by Vanitha and Ramjegathesh (2014). Fluorescent pseudomonad growth medium can affect its ability to produce HCN. The growth medium of pseudomonad fluorescent isolates PfCas and PfCas3 added by $ZnSO_4.7H_2O$ produced the best HCN (Advinda et al., 2018). While Kumar et al., (2012) analyzed HCN produced by bacterial isolates FBJ6 and FBS4 can induce the resilience of *Pisum sativum* and *Zea mays* plants.

From observations of the ability to dissolve phosphates in Pikovskaya's medium, it was found that all bacterial isolates showed different abilities (Figure 4). Isolate PfPj1 has the highest ability to dissolve phosphates, indicated by the diameter of the halo zone formed is 1.38 cm (Figure 4). While the lowest at the ability of dissolving phosphates is isolate PfKd7. Similar observations have been made on *B. subtilis* and *B. cereus* derived from the groundnut rhizosphere (Maheswar and Sathiyavani, 2012). *P. fluorescens* isolates PSM1, PSM2, PSM3, PSM4, PSM5, and *B. megaterium* isolate MTCC 8755 isolated by Yadav et al. (2016) from wheat rhizosphere are able to dissolve phosphates in Pikovskaya's medium.

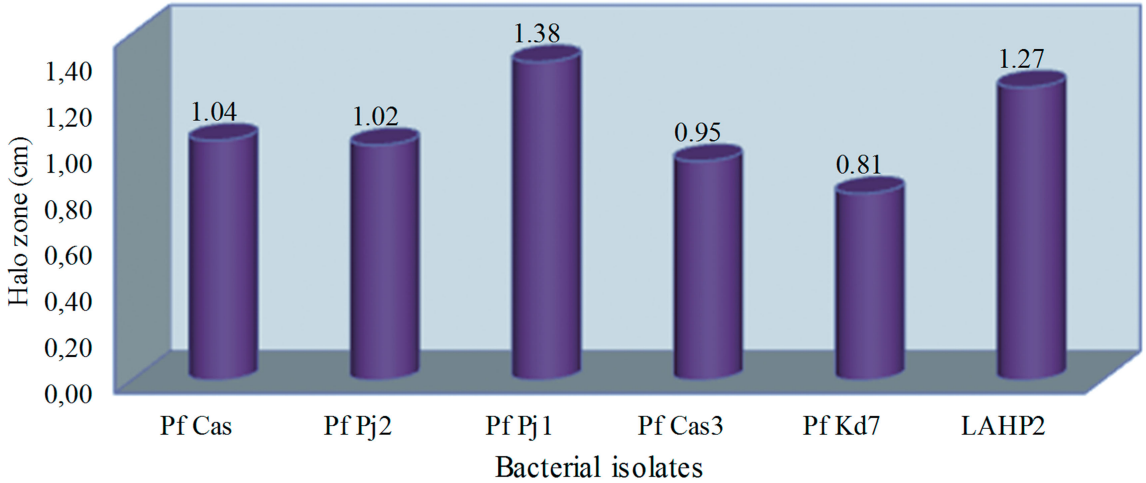

Figure 4. The ability of bacterial isolates to dissolve phosphate.

Phosphate solubilizing microorganisms are able to secrete organic acids such as gluconate acid, malic acid, succinic acid, lactic acid, formic acid, and citric acid which form a chelate with cations such as Al and Fe. These acids affect the dissolution of phosphates so that phosphorus becomes available and can be absorbed by plant roots (Vyas and Gulati, 2009). According to Widnyana and Javandira (2016), phosphate solubilizing microorganisms can also produce several enzymes including phosphatase. Phosphatase is an enzyme that is produced when the phosphate availability is low. In the process of mineralization of organic matter, organic phosphate compounds are converted into inorganic phosphates with the help of enzymes that are available to plants.

Conclusion

This research successfully identified *P. aeruginosa* which was isolated from the rhizosphere of various types of plants. All bacterial isolates tested were able to produce siderophore, HCN, and dissolve phosphates. The highest siderophore was produced by isolate PfPj2. Whereas isolate PfKd7 was the highest at producing HCN and at the ability to dissolve phosphates.

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