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Research Article
Selection, Characterization and Application of Rhizobacteria and its Effect on Chili (*Capsicum annuum* L.) Plant Growth

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Abstract

Background and Objective: Phosphate solubilizing bacteria and nitrogen fixation bacteria as inoculant will increase the availability of phosphate and nitrogen for plants. The aim of this study was to isolate phosphate solubilizing rhizobacteria that could fix atmospheric nitrogen and to measure its effect on chili plant growth in a greenhouse experiment. **Materials and Methods:** Soil samples for bacterial isolation were collected from cultivation area of chili plants. All samples were selected and characterized each ability in solubilizing phosphate and fixing nitrogen. Selected isolates were identified by using gene 16S rRNA and potential isolate was used as inoculant on chili plants. **Results:** Three selected isolates of KD2.10, KD2.13 and KE2.15 showed the higher value of phosphate solubilizing index and produced soluble phosphate as much as 102.83, 102.5 and 101.5 mg L^{-1}, respectively. The nitrogenase activity was measured by acetylene reduction assay methods. Isolates KD2.10 and KE2.15 produced ethylene with the amount of 0.0614 and 0.0728 ppm h^{-1}, respectively, whereas isolate KD2.13 could not be measured. The three isolates were categorized as Gram-negative bacteria and isolate KD2.10, KD2.13 and KE2.15 closely related to *Burkholderia cepacia*, *Burkholderia diffusa* and *Enterobacter cloacae*, respectively. The use of *Burkholderia cepacia* KD2.10 on chili plant growth could increase the plant height, number of leaves, wet and dry weight and plant root length. **Conclusion:** Isolate *Burkholderia cepacia* KD2.10 as phosphate solubilizing and nitrogen fixing bacteria was suggested as an effective inoculant in improving the growth of chili plants in a greenhouse experiment.

Key words: Biofertilizer, nitrogen-fixation, phosphate solubilizing index, rhizobacteria

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biofertilizer contains living microorganism. When biofertilizer applied to seed, plant surfaces or soil, it colonizes the rhizosphere or the interior of the plant and promotes plant growth by increasing the availability of primary nutrients such as Phosphate (P) and Nitrogen (N). Biofertilizer from rhizobacteria group is one of the alternatives to reduce the use of chemical fertilizer[1]. The rhizobacteria known as Plant Growth Promoting Rhizobacteria (PGPR) is a group of soil bacteria that live around the roots of plants or rhizosphere to improve the quality of plant growth through (1) Producing the growth hormone, (2) Ability of N fixation to increase the supply of soil N, (3) Producing of certain compounds that to kill plant pathogens and (4) Ability of solubilizing insoluble P in the soil[2].

Phosphate generally will be bound to other elements such as iron (Fe), aluminum (Al), calcium (Ca) and magnesium (Mg), causing P cannot be used directly by plants[3]. While, gaseous N in the earth's atmosphere is about 79% of its volume but is not fully utilized by the plant[4]. Plants absorb N in the form of ammonium (NH_4^+) and nitrate (NO_3)[5].

Lynn *et al.*[6] studied that there were several strains of bacteria that had P-solubilizing activity[6]. The use of bacteria could increase the available P in soil and promote the growth of plants. *Burkholderia* and *Pseudomonas putida* which were isolated from the soil around limestone quarry area in Indonesia showed the highest phosphate solubilizing index on Pikovskaya medium containing $Ca_3(PO_4)$[7]. *Burkholderia* were also known as N-fixing bacteria and could form nodules in legume plants[8]. Sajan *et al.*[9] showed that application of biofertilizers such as N-fixing bacteria, P-solubilizing bacteria and mycorrhizae on chili plant in sandy loam soil could reduce the use of chemical (synthetic) fertilizer and had a beneficial effect compared to the application of chemical fertilizer or biofertilizer alone. However, this study used a single bacterial isolates which its specific capability had been known towards plants[9]. The aims of this study was to isolate phosphate solubilizing bacteria that could fix atmospheric N and its effect on chili plant growth at greenhouse experiments.

MATERIALS AND METHODS

Materials: Soil samples for bacterial isolation were collected from cultivation area of chili plants at Kuningan 6°57'42.29"S; 108°24'52.62"E, West Java, Indonesian. Ultisol soil for chili planting was obtained from farming land around Kabayan, Bogor Agricultural University. Chili seeds of Landung variety was obtained from the located IPB campus.

Isolation of P-solubilizing bacteria: Ten grams of soil samples were diluted into 90 mL physiological saline solution (NaCl 0.85%), then agitated on a shaker for 1 h at 120 rpm. Serial dilutions were prepared from 10^{-1} to 10^{-4}. One milliliter of 10^{-2}, 10^{-3} and 10^{-4} suspension respectively spread on Pikovskaya's agar. Each colony then purified using quadrant streak method and stored as a stock in Pikovskaya's agar.

Qualitative test ability to solubilize P and N fixing bacteria: The qualitative estimation of phosphate solubilizing bacteria was done by dot method and incubated for 3 days at room temperature. Phosphate Solubility Index (PSI) was measured on the value of halo zone diameter towards diameter of the bacterial colony, then the result was divided by diameter of the colony[7]. The ability of phosphate solubilizing bacteria as N fixer was tested by dot method on nitrogen free bromothymol blue (NfB) medium, incubated for 48 h at room temperature. The color change in the media was observed[10].

Hypersensitivity test on tobacco leaves: Hypersensitivity test was conducted by injecting the selected bacterial isolates (10^8 cells mL^{-1}) on the lower surface of tobacco leaves (*Nicotiana tabacum* L.) using 1 mL syringe (without needle). *Pseudomonas syringae* was used as positive control, while *Bacillus cereus* medium and water sterile were used as negative controls. Hypersensitive was scored as negative, if necrotic symptom did not develop in the infiltrated zone within 48 h after injection[10].

Quantitative test of P-solubilizing bacteria: Three selected isolates KD2.10, KD2.13 and KE2.15 were inoculated into 100 mL of Pikovskaya liquid medium and incubated in shaking incubator for 7 days at 37°C. Every 24 h, 1.5 mL of each bacteria culture was centrifuged at 10,600×g for 10 min. Approximately 1 mL supernatant was reacted with the color forming reagent (2.5 mL of 2.5% sodium molybdate and 1 mL of 0.3% hydrazine sulfate), then it was boiled for 10 min. After formation of blue color, the P-solubilizing activity was measured at 830 nm wavelength[6,7].

Nitrogenase activity assay: Nitrogenase activity of selected isolates was measured by Acetylene Reduction Assay (ARA) method by using gas chromatography instrument[10]. The selected isolates were grown in nitrogen-free bromothymol blue (NfB) semisolid media for 5 days at 30°C. After 5 days of incubation, the tube was covered by a rubber stopper and parafilm paper. The ARA measurement was conducted by removing out the air in the tube using 1 mL sterile syringe, then the acetylene gas (C_2H_2) was injected into the tube with

the same volume of the removed out air. After 2 h of incubation, ethylene gas in the tube was measured using gas chromatography instrument.

Identification of bacterial isolates: Each selected isolate was streaked on Nutrient Agar (NA) and incubated for 24 h at room temperature. Identification of bacteria was conducted by Gram staining to identify the type and shape of bacterial cells.

Molecular identification: A pure single bacteria was grown in 50 mL Nutrient Broth (NB) and incubated at 37°C for 24 h. Bacterial cells then centrifuged at 9,500×g for 1 min. Selected bacterial genomic DNA was extracted using PrestoTM gDNA Bacteria Mini Kit (Genaid)[7].

The 16S rRNA gene was amplified using Polymerase Chain Reaction (PCR) machine. Fifty microliters of PCR mix was created with composition: 25 µL GoTaq Green Master Mix 2 (Promega, Madison, WI, USA), 2.5 µL (100 pmol) for each primer: 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3')[11], 0.3 µL (64.4 ng μL^{-1}) of DNA templates and 19.7 µL nuclease free water. Polymerase Chain Reaction (PCR) was performed under the following conditions: Pre-denaturation at 94°C for 5 min, denaturation at 92°C for 30 sec, annealing at 58°C for 30 sec, elongation at 72°C for 1.5 min and final extension at 72°C for 5 min with 35 cycles. Finally the temperature was declined to 4°C for 10 min to stop PCR reaction. Polymerase Chain Reaction (PCR) products were purified and sequenced by sending it to sequencing service company (Genetika science). Sequences were analyzed using Bioedit program then aligned with the 16S rRNA gene data base using BLAST-N program. Phylogenetic analysis was performed using MEGA 6 program and Neighbour Joining (NJ) with the bootstrap method 1000x[12].

Application of selected bacteria on chili plants (*Capsicum annum* L.): Chili seeds were soaked in the water for 24 h and seeded for 4 weeks before planting. Four weeks old of chili seedling plants are planted in 5 kg autoclaved soil in polybag. The most effective bacterial isolate (10^8 cell mL^{-1}) was applied towards red chili plant by completely randomized design. The first treatment was planting media on soil added with NPK fertilizer based on the recommendation and without fertilizer used. The second treatment were inoculum addition and without inoculum addition. Each treatment was repeated 5 times with 20 experiments. The observation parameters were plant height, leaves number, wet weight, dry weight and root length.

Statistical analysis: Data were analyzed by using analysis of variance (ANOVA). Significantly different Tukey test was used for comparison among treatments at confident level 95%.

RESULTS

Characterization of P-solubilizing and N-fixing isolates: There were 137 bacterial isolates that had the ability to solubilize P on Pikovskaya media based on the clear zone formation around the colony. Twenty five of P-solubilizing isolates were successfully selected based on the highest Phosphate Solubility Index (PSI) (Fig. 1a, Table 1). All of 25 isolates were tested their hypersensitivity towards tobacco leaves. After 48 h, only 16 isolates showed negative reactions, indicated by unforming necrosis without leaf discoloration to yellowish or brownish in the injection area (Table 1). Negative reactions were also shown by injection of sterile water, medium and *B. cereus* which were used as a negative control.

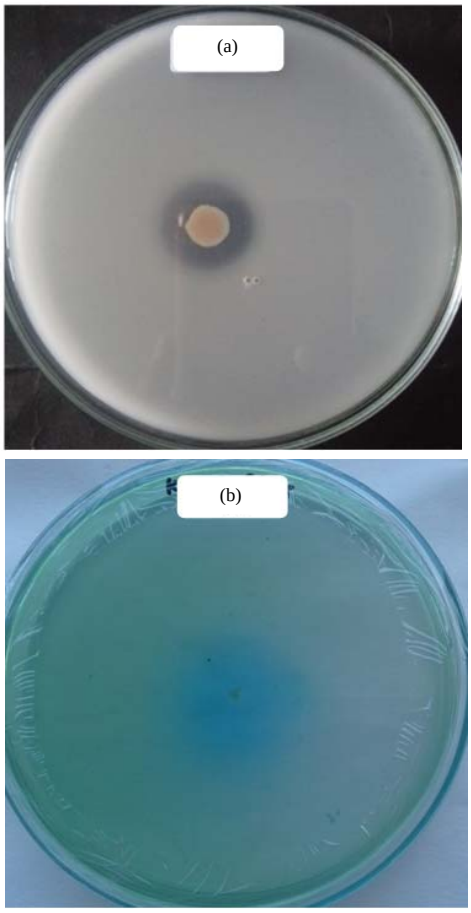

Fig. 1(a-b): Soil rhizobacteria isolate that able to solubilize P and N fixation with (a) Pikovskaya and (b) Nitrogen free bromothymol blue (NfB)

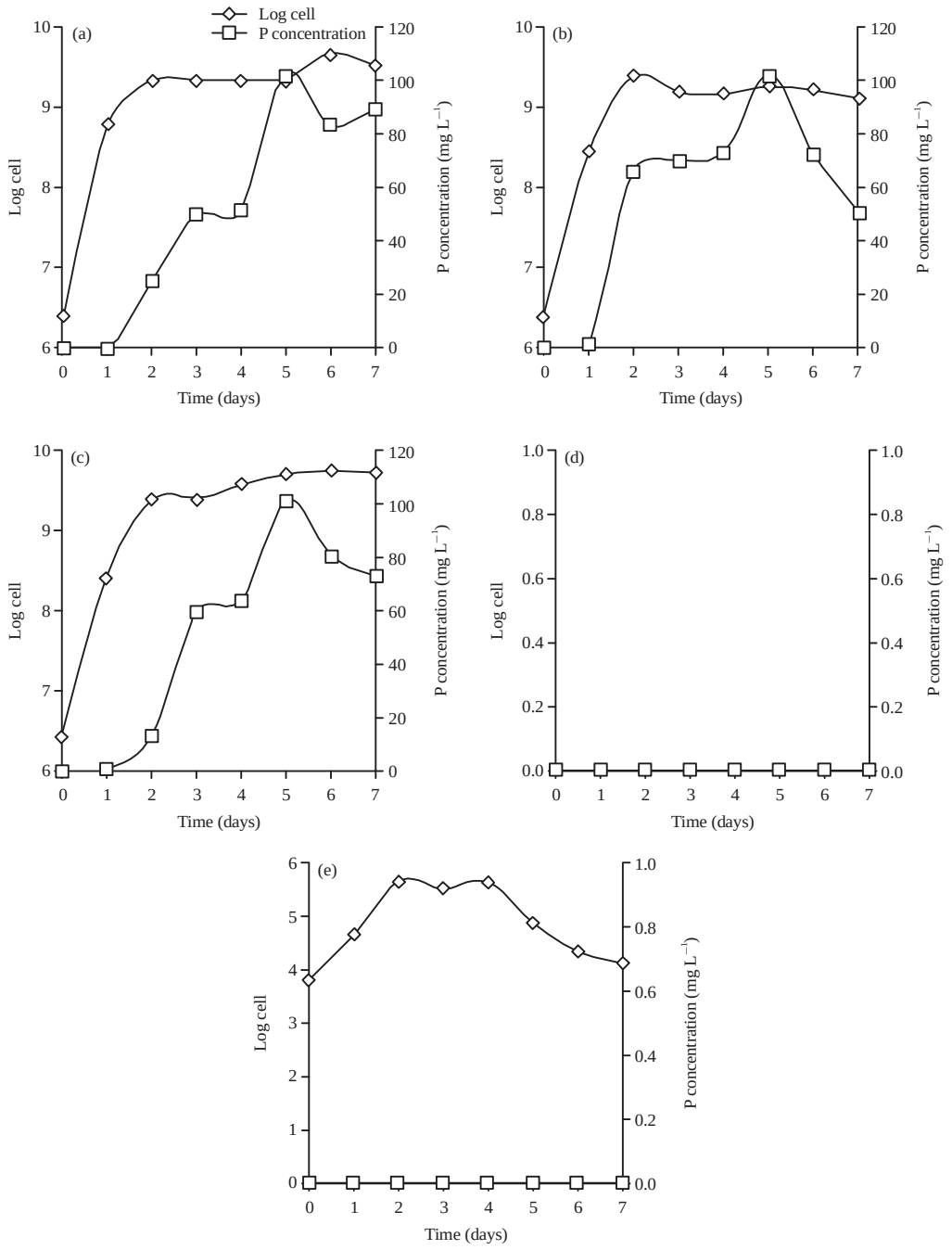

Fig. 2(a-e): Cell number and soluble P concentration of three isolates (a) KD2.10, (b) KD2.13, (c) KE2.15, (d) Without the culture and (e) Without the phosphate in the media but added with culture

In contrast, injection area of *P. syringae* showed a positive reaction in the presence of yellow spot which indicated symptoms of necrosis. Furthermore, the selected bacteria were used to qualitative test of nitrogen fixing ability in the nitrogen-free bromothymol blue (NfB) agar. There were 12 isolates were able to fix the nitrogen which indicated by the changing of medium color from green to blue (Fig. 1b, Table 1).

Quantitative test of phosphate solubilizing and nitrogenase activity: Three selected isolates were used for further test based on the highest Phosphate Solubilizing Index (PSI) and negative hypersensitivity. The results of the quantitative test showed that isolates of KD2.10, KD2.13 and KE2.15 produced maximal solubilized P on the 5 days incubation with ranged between 101.5-102.83 m gL^{-1} (Fig. 2). There were two types of control treatments i.e., without the

Table 1: Screening and characterization of rhizobacteria from chili plants area

Isolates	P-solubility index	Nitrogen fixation	Plant hypersensitivity	Isolates	P-solubility index	Nitrogen fixation	Plant hypersensitivity
KA3.1	1.33	-	-	KD1.11	1.50	-	+
KA3.2	1.77	-	-	KD2.2	1.33	+	-
KA3.3	1.66	-	-	KD2.3	1.09	-	-
KA3.4	2.00	-	-	KD2.10	1.40	+	-
KA3.5	1.75	-	+	KD2.13	1.44	+	-
KB2.10	1.75	-	+	KD3.1	1.22	+	-
KC1.1	1.88	-	-	KD3.2	1.00	+	+
KC1.2	1.88	-	-	KE2.5	1.00	+	-
KC2.5	1.00	+	-	KE2.7	1.00	+	-
KD1.3	1.50	-	+	KE2.13	1.09	+	+
KD1.4	1.50	-	+	KE2.14	1.27	+	+
KD1.10	1.60	-	+	KE2.15	1.77	+	-
				KE3.5	1.20	+	-

Table 2: Sequence analysis gene of three selected isolates sequences compared to NCBI database

Isolate description	Query cover	Identity (%)	E-value	Accession
KD2.10				
Burkholderia cepacia strain BIP 184	100	99	0.0	KU161306.1
Burkholderia anthina strain S25-8	100	99	0.0	KU049648.1
KD2.13				
Burkholderia diffusa strain IHB B 7016	100	99	0.0	KJ721213.1
Burkholderia ambifaria strain DZBT 06	100	99	0.0	KM191298.1
KE2.15				
Enterobacter cloacae strain 41	100	99	0.0	KX156583.1
Enterobacter aerogenes strain HC050612-1	100	99	0.0	EU047701.1

Table 3: Percentage of plant height, leaves number, wet weight, dry weight and plant root length of chili plant on 12 weeks after planting

	Effect on chili plant growth at greenhouse experiments				
Treatments	Plant height	No. of leaves	Root length	Wet weight	Dry weight
TNPKB	38.2^{b}	29.2^{c}	7.92^{ab}	5.38^{a}	1.03^{ab}
TNPK	30.6^{b}	23.6^{b}	9.0^{b}	4.40^{a}	0.79^{ab}
TB	39.2^{b}	29.6^{c}	9.6^{b}	11.92^{b}	1.84^{b}
T	7.6^{a}	5.4^{a}	4.3^{a}	0.70^{a}	0.07^{a}

Values followed by the same letter are not significantly different by Tukey test at 95% confident level. TNPKB: Treatments with KD2.10 isolate and NPK, TNPK: Treatment with NPK alone, TB: Treatment with KD2.10 alone and T: Without NPK and KD2.10 isolate treatments

culture and phosphate in the media but added with culture. Both controls were yielded a value of 0 m gL^{-1} in P solubilization test.

Nitrogenase activity of isolates KD2.10 and KE2.15 were measured by Acetylene Reduction Assay (ARA). The amount of ethylene production by KD2.10 and KE2.15 were approximately 0.0614 and 0.0728 ppm h^{-1}, while isolate KD2.13 could not detected.

Identification of selected bacteria: All of three isolates KD2.10, KD2.13 and KE2.15 were belonged to Gram negative and cell-shapes were rod (Fig. 3). The amplification results of 16S rRNA gene of the three isolates using 63f and 1387r primers[11], showed the amplicon size is about 1300 kb (Fig. 4). The phylogenetic tree analysis of three isolates showed that isolates KD2.10, KD2.13 and KE2.15 had the closest relationship with *Burkholderia cepacia*, *Burkholderia diffusa* and *Enterobacter cloacae*, respectively (Fig. 5, Table 2).

Application of isolates KD2.10 on the growth of chili plants: Isolate *Burkholderia cepacia* KD2.10 could solubilize the highest P in the Pikovskaya broth. It was selected to be used as inoculant on the growth of chili plants at greenhouse experiments. Since the first week after planting until 12 weeks after planting, 5 mL of KD2.10 isolate (10^{8} cell mL^{-1}) was poured around rhizosphere of the chili plants. Planting media was given by two treatments that were soil with NPK fertilizer (TNPK) and without NPK (T). The second treatment was the inoculum addition (TNPKB) and without inoculum addition (TB). After 12 weeks after planting, the chili plant which added with *Burkholderia cepacia* KD2.10 showed good respond on the plant height, leaves number, wet and dry weight and length of plant root (Table 3).

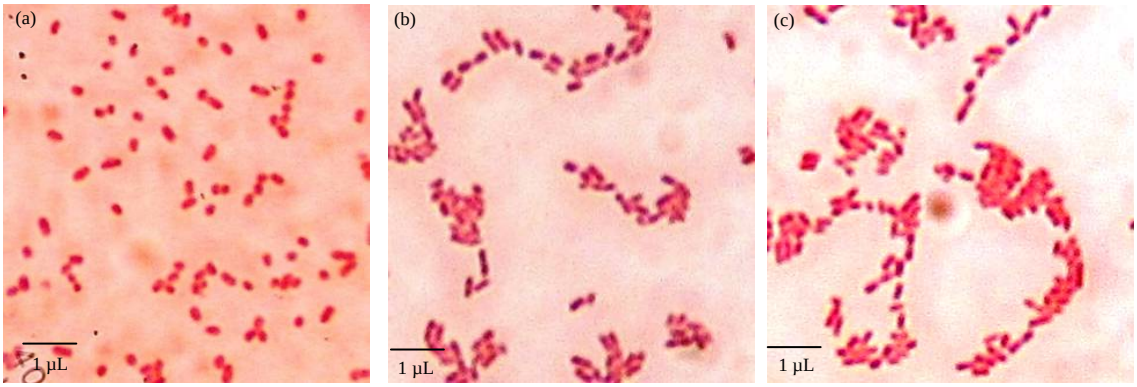

Fig. 3(a-c): Gram staining of three selected isolates (a) KD2.10, (b) KD2.13 and (c) KE2.15 at 1000x magnification

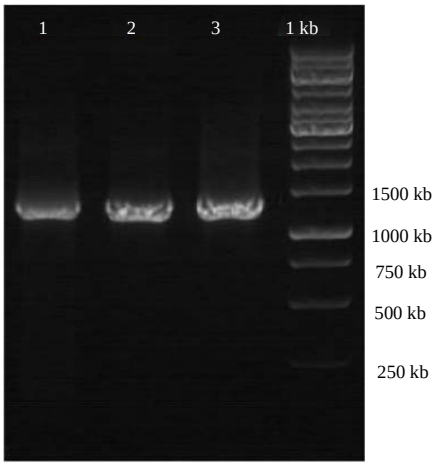

Fig. 4: Amplification of three selected isolates through electrophoresis based on 16S rRNA gene
M: Marker 1 kb, 1: Isolate KD2.10, 2: KD2.13 and 3: Isolate KE2.15

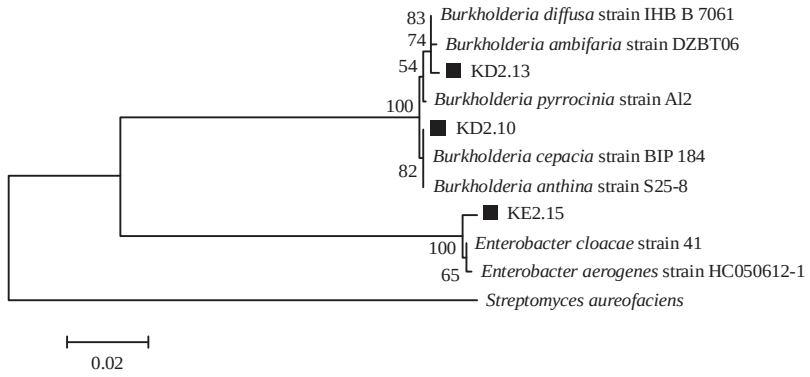

Fig. 5: Phylogenetic tree construction of three selected isolates by 16S rRNA gene

DISCUSSION

Microbe had beneficial effects as phosphate solubilizer and also as nitrogen fixer simultaneously (Fig. 1). The mechanism of P-solubilization was determined by the ability of microbes producing the organic acids and protons, through binding of the hydroxyl group with a phosphate cation thus converted into a soluble form[13]. Bashan *et al.*[14] stated that the process of P solubilization occurs because of the release of proton H^+ that reacts with calcium phosphate $Ca_3(PO_4)_2$. In

addition, the variation of the value of the P-solubilization test, based on clear zone index was occurred because of the ability of bacteria to produce organic acids such as citric, malic, oxalate and acetate that serves as catalysts, chelating and P absorbent agent[15]. The characteristic of nitrogen fixing is discoloration of the medium around the colonies into blue on nitrogen free bromothymol blue (NfB) medium. This characteristic showed that bacteria had the ability to fix N_2 and to change the pH of medium become alkaline[10].

Hypersensitivity test of selected isolates toward tobacco leaves after 48 h showed negative reactions related to negative control treatments that did not show necrosis or color changes to yellow or brown in the injection area. *Bacillus cereus* is one of the bacteria that had a specific host, not harmful to natural enemies of pests and other non-target organisms, easily biodegradable by the environment and the pathogenicity can be increased by genetic engineering techniques[16]. *Pseudomonas syringae* as positive control showed a positive reaction in the presence of yellow spots as the symptoms of necrosis around the injection area. Hypersensitivity reaction is a process of rapid cell death and is localized. This reaction appears on infected plants during the introduction of pathogens which is an attempt to inhibit the growth of pathogens. Induction of both hypersensitive and pathogenicity is influenced by *hrp* gene commonly found in plant pathogenic Gram-negative bacteria, including *P. syringae* group[17].

Phosphate solubilization ability of bacteria isolate was observed by clear zone forming around its colony. Karti *et al*.[18] stated that the large clear zone formed by bacteria showed larger ability of P-solubilization. Although the clear zone wide could determine the phosphate solubilization of bacteria, the estimation amount (concentration) of soluble phosphate could not be determined[6]. Quantitative assay of P solubilization from three isolates showed that solubilizing activity was observed when the amount of the cell raised and reached stationary phase (Fig. 2). Isolate KD2.10 had the highest P-solubilization ability among two other isolates. It might because of the diffusion level variation of different organic acid that is produced by different microbes[7]. Phosphate solubilizing bacteria that could mineralize P in solid medium was not always able to solubilize P in liquid medium[6].

Based on the Acetylene Reduction Assay (ARA) with reduced acetylene showed the nitrogenase activity. Nitrogen fixation involves an enzyme called nitrogenase, which consists of two enzyme, nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe protein). The nitrogenase (Mo-Fe protein) composed by two pairs of different subunits encoded by the *nifD* and *nifK* genes, while nitrogenase reductase (Fe protein) encoded by the *nifH* gene[19].

The genera *Burkholderia* and *Enterobacter* are plant growth-promoting rhizobacteria (PGPR) that could solubilize phosphate, fix nitrogen, produce both phytohormones and siderophores[20,21]. Strains of *Burkholderia* are categorized as rhizobia that encodes *nifH* gene and *nodA* of nitrogenase[22] and *Burkholderia diffusa* have the ability to soluble phosphate[23]. *Enterobacter cloacae* is able to fix nitrogen[24], had a role as a biocontrol strain and able to synthesize antibiotics[25]. Based on some of the descriptions to provide information that rhizobacteria presence in the soil have a beneficial effect on plant growth and increase agricultural production. One rhizobacteria used in experiments in the greenhouse is the *B. cepacia* has been known that had the ability to soluble phosphate[26] and the ability in nitrogen fixation[27].

The use *Burkholderia cepacia* of KD2.10 culture on chili plants showed good response on the plant height, number of leaves, wet and dry weight and plant root length up to 12 weeks after planting. Collavino *et al*.[20] reported that some bacteria belonging to *Burkholderia* could increase photosynthesis and the P and N content of leaves on beans *Phaseolus vulgaris*. Microorganisms belong to PGPR had been used as bio-fertilizers and recognized for its potential use in agriculture and horticulture[28]. The use of rhizobacteria at chili seed treatment could increase the quality of seed, growth and fruit total amount compared with control[29]. This study showed that the use of bacteria have a significant effect in improving the growth of chili plants therefore it can be used as a biofertilizer. This study revealed that the single use of *B. cepacia* inoculum as phosphate solubilizer and nitrogen fixer could give the same effect towards plant, almost similar to multiple use of bacteria inoculum.

CONCLUSION

Bacterial isolates recovered from soil samples chili cultivation in Kuningan Indonesia. *Burkholderia cepacia* KD2.10 selected and show the characteristics: Capable solubilized P, capable of fixing nitrogen and no hypersensitivity in plants. *Burkholderia cepacia* KD2.10 isolates can be used as a biological fertilizer, thereby could reduce the excessive use of chemical fertilizers. The formulations and the viability of the bacteria to be effective should have been known to improve plant growth.

SIGNIFICANT STATEMENTS

One of the rhizobacteria is *Burkholderia* most commonly found in soil, able to solubilize minerals and produce organic acids and increase the availability of nutrients in the soil. The

indigenous *Burkholderia cepacia* could solubilize phosphate and fix nitrogen and also increase the plant growth of chili plants. This study could support the previous study that rhizobacteria could reduce the use of chemical fertilizer. The use of bacteria as biofertilizer is more beneficial than chemical biofertilizer, because it could increase the growth, plant height, number of leaves, wet and dry weight and plant root length compared to chemical fertilizer.

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