
Potential enzymatic microbes for releasing plant nutrients and siderophore production

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Abstract *Burkholderia cepacia* strain S001-08, *Pseudomonas aeruginosa* strain S001-11 and *Pseudomonas fluorescens* strain S001-15 were isolated from soil planted to Durian and selected for the properties to solubilize phosphate for plant nutrient and siderophore production for bioremediation. These selected strains were morphological and molecular phylogenetic identification to confirm species level. *B. cepacia* strain S001-08, *P. aeruginosa* strain S001-11 and *P. fluorescens* strain S001-15 are found to be active responding as phosphorous solubilizers and producing siderophores. These active strains are being investigated to be synergistic effect and developing to be biological product as biostimulant and bioremediation. The developing biological product is promising to apply for crop production as an agricultural input for modern organic agriculture.

Keywords: Bioremediation, Biostimulant, Modern organic agriculture

Introduction

Soil microbes are generally maintained as symbiotic relationships with root plants by exudating antimicrobial agents, bioactive compounds, enzymes, growth promoters, siderophores to help in maintaining plant growth. In addition, microorganisms also recover the soil fertility by solubilizing insoluble nutrients in soil such as phosphorus, potassium, etc., and thereby stimulate the movement of nutrients to plants. It also acts against pathogens and pests and protects the plants crops biological damage (Janardhan *et al.*, 2014; Kaur *et al.*, 2016; Liu *et al.*, 2018; Khoshru *et al.*, 2020).

Rhizobacteria are a type of plant growth promoting bacteria that stimulate plant growth (Nouioui *et al.*, 2018). These microorganisms are widely distributed

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in soil but are most commonly found in soil around plant roots. Research finding for testing their phosphate solubilization efficiency found that fungi are more efficient at solubilizing phosphate than bacteria and actinomycetes, such as *Aspergillus niger*, *Aspergillus fumigatus*, and *Penicillium* sp., etc. (Kucey, 1983; Illmer and Schinner, 1992; Illmer and Schinner, 1995) These microorganisms have shown good efficacy for the growth of plants for enhancing the biological activities with releasing the biological metabolites into the rhizosphere soil, which are crucial for the yield (Wahyudi *et al.*, 2019). These microorganisms have an important role in fixing nitrogen, producing siderophore, solubilizing phosphorus, potassium, and producing indole-3-acetic acid etc. (Prakash and Cummings, 1988; Lee *et al.*, 2012; Anwar *et al.*, 2016; Myo *et al.*, 2019). These microorganisms may help to protect plants from pathogen infection. Soil enzymes become a major factor in biochemical activity of organic matter in rhizosphere soil, and their actions are closely associated to microbial reaction, organic matter in soil, and soil fertility properties (Jat *et al.*, 2021).

Aziz *et al.* (2016) stated that pesticide residues in soils in carrot, broccoli, corn, tomato, rice fields both in organic and conventional farms and compared available nitrogen, phosphorus, potassium. The residual chemical pesticides included organophosphates (diazinon, phentoin, methidathion, parasite, pronephros) and carbamates (carbofuran, MIPC, BPMC), and organochlorine (lindane 4, aldrin, heptachlor, dieldrin, DDT, endosulfan levels in soils. It found that soil organic carbon, nitrogen, available phosphorus, potassium, CEC and pH values showed greater in the organic farms than the conventional farms. Some chemical pesticide residues such as organophosphates and carbamates, organochlorines were detected in conventional farms, and in organic farms were not detected or detected in small quantities. Aziz *et al.* (2018) stated that soil microbe activities related to soil enzyme expressed dehydrogenase and cellulase in soli microbe activities in organic farming higher than in conventional farming. The quantity of soil microbe is related with dehydrogenase, urease, and cellulase in soil activities. Kobierski *et al.* (2020) stated that enzyme activities of the topsoil between organic farms planted to legume and using crop rotation, and animal dung compost application) while the conventional farms using simple crop together with crop rotation and applied chemical fertilizers. Result showed that soil organic matter was significantly increased in organic farm as compared with the conventional farm. Rieznik *et al.* (2021) reported that the various agricultural systems investigated the biological processes of soil enzymes e.g. catalase, cellulase, dehydrogenase, protease and urease showing the changes of soil enzyme activity in the soil layer thickness of 0-10 cm. Organic farm found to be increased the enzyme activity in uncultivated soils. The application of animal compost increased soil enzyme activities. Intensive farm found lower soil

enzyme activity of enzymes in the layer thickness of 0-10 cm. Abbasi *et al.* (2022) found that five bacterial strains were strongest tolerance showing heavy metal-resistant strains which were *Kingella* sp., *Listeria* sp., *Bacillus* sp., *Pseudomonas putida*, *Cupriavidus necator*. With this, *Kingella* sp and *P. putida* expressed the strongest tolerance to copper (Cu), while *Listeria* sp., and *C. necator* expressed the strongest tolerance to chromium (Cr), and *Bacillus* sp. expressed the strongest tolerance to lead (Pb). The bioremediation of contaminated heavy metal in soil showed that *P. putida* removed Pb at 83.80%, *C. necator* removed Pb at 90.49%, *Kingella* sp. and *Listeria* sp. removed Cu at 81.87%. Akram *et al.* (2023) reported that *Aspergillus niger* was found to be more efficient with the greatest bioremediation activity for copper and lead removal efficiency and found to be bioleaching effect. Kondakindi *et al.* (2024) stated that bioremediation of heavy metals gets involve in microbial extracellular polymers (EPS). EPS forms the metal complexes as chelating agents and ion-binding sites to immobilized and precipitate heavy metals which decrease availability and toxicity of heavy metals. EPS act as a barrier to protect microbes from stress of heavy metals to facilitate survival and growth of microbes. The production of EPS increases heavy metal exposure to enhance bioremediation process efficiency. Phosphate solubilizing bacteria reported to change the insoluble phosphorus to soluble forms that uptake by plant roots which is a major factor for organic crop production (Aliyat *et al.*, 2022). *Burkholderia cepacia* was found to be salt tolerance and solubilizing several forms of phosphates. It revealed much phosphate solubilizing in phosphate containing pesticides tested in pot experiments. *Pseudomonas aeruginosa* can also be reported as phosphate-solubilizing microbe to transit insoluble phosphorus to soluble forms. *P. aeruginosa* is recorded to be growth-promoting rhizobacteria and increase the nutrient uptake. *P. fluorescens* is also reported to be solubilized phosphate which promoted the growth of plant (Ramani and Patel, 2011). *P. fluorescens* is found to solubilize the insoluble P by proton excretion with ammonium assimilation and organic acid production. (Park *et al.*, 2009). *P. aeruginosa* is successfully applied to promote the growing groundnut which in combination of *P. aeruginosa* and phosphorous fertilizer resulted to higher pod yield from 7.36 to 13.18% (Mohapatra *et al.*, 2024). The research findings were aimed to isolate, identify soil microbes and screened for phosphorous solubilizing and producing siderophores targeted to decrease heavy metal contamination in water and soil.

Materials and methods

Isolation and identification

The samples of soil planted to Durian were collected, brought to laboratory, then air dried and sieved to be small soil particles before isolation. SCNA medium which consists of casein 0.3g, starch 10 g, KNO₃ 2 g, NaCl₂ 2 g, K₂HPO₄ 2 g, MgSO₄.7 H₂O 0.01g, agar 20g, and distilled water 1000 ml and adding nalidixic acid to avoid fungal contamination, then sterilized in autoclave at 15 lbs/inch² for 30 minute. The fine particles of soil sample were serially diluted from 10⁻⁴ to 10⁻⁷, then 1 ml of each dilution was poured and spreaded with glass rod over starch-casein agar plates. The single colony was transferred into starch-casein nitrate agar plates with single streak until get pure culture (Jayanthi *et al.*, 2023).

Morphological characterization

The isolated bacteria were morphological observed their characterization such as elevation, color, shape and colony characters grown on a Nutrient Agar (NA) medium and incubated under aerobic conditions at 30°C for 24-48 hour to obtain isolated colonies. Colony morphology was observed based on macroscopic character included colony shape, pigment, elevation, and optical appearance, according to standard bacterial identification procedures. The microscopic character was done by using the Gram staining. A pure culture of bacterial colony was sub-cultured to a cleaned glass slide, then suspended in sterile distilled water, air-dried, and heat-fixed. The bacterial smear was stained with crystal violet for 1 min, followed by Gram's iodine for 1 min, decolorized with 95% ethanol for 10-15 seconds, and counterstained with safranin for 1 min. The stained smear was gently rinsed with distilled water between each step and air-dried before investigation. Cell morphology and gram reaction were observed under binocular compound microscope at 100X with oil immersion objective lens (total magnification ×1000) (Beveridge, 2001).

Molecular phylogenic identification

The strains were molecular phylogenic identification by 16S rRNA gene sequence analysis. The amplification of 16S rRNA gene was done by polymerase chain reaction (PCR) using U2Bio® with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (De Lillo *et al.* 2006). Comparison of nucleotide sequences were done by the database from the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The program showed bacterial strains with similarities of sequence to the selected strains to determine the phylogenetic relationships. The sequences showing the highest identity and maximum coverage were chosen and selected sequences which are aligned using Clustal X2 software. The phylogenetic trees were done using MEGA 6 software. The neighborhood-joining method was used in the poisson model with Nearest Neighbor Interchange and a Bootstrap test of phylogeny. The bootstrap was used 1000 replicates to confirm the reliability of the phylogeny.

Screening of P solubilizing

The strains of bacteria were done by cross-streak onto Pikovskaya's agar medium, which consists of 0.5g yeast extract, 10g dextrose, and 5g $\text{Ca}_3(\text{PO}_4)_2$ in 1 liter of sterilized water and added, 2 0.5g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g of KCl, 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001 g of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.0001 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ to identify the solubilizes phosphate microbes. Phosphorus solubilizing actinobacteria (PSA) strains were noticed to produce clear zone surrounding the bacterial colony until 7 days at 30 °C. Phosphorus solubilization Index (PSI) was evaluated the efficiency of microbes in converting insoluble inorganic phosphorus into available forms. It is calculated by dividing the total diameter (colony + clear zone) by the colony diameter on a solid agar medium. Aliyat *et al.* (2022) stated the activity level of PSI as $\text{PSI} < 1.5 =$ low efficiency, $1.5 \leq \text{PSI} < 2.0$, medium efficiency and $\text{PSI} \geq 2.0 =$ high efficiency. With this, PSI of 3.5 expressed significant potential for promoting plant growth by enhancing nutrient availability in phosphorus deficient.

Screening of siderophore production

The production of siderophore was observed on Chrome azurol S (CAS) agar plates for qualitative assay. 50 μl of bacterial suspension was spotted on CAS agar plates and incubated for 4-5 days at 30°C. A positive result is expressed by the medium changing in color from yellow to dark brown around colony.

Statistical analysis

All experiments were conducted using Completely Randomized Design (CRD) with 4 replications. Data were computed analysis of variance (ANOVA) and treatment means were separated by Duncan's Multiple Range Test (DMRT) at $p = 0.05$ and 0.01 .

Results

Isolation and identification

Burkholderia cepacia strain S001-08, *Pseudomonas aeruginosa* strain S001-11 and *Pseudomonas fluorescens* strain S001-15 were isolated from soil which confirmed morphological (Figure 1) and molecular phylogenetic identification.

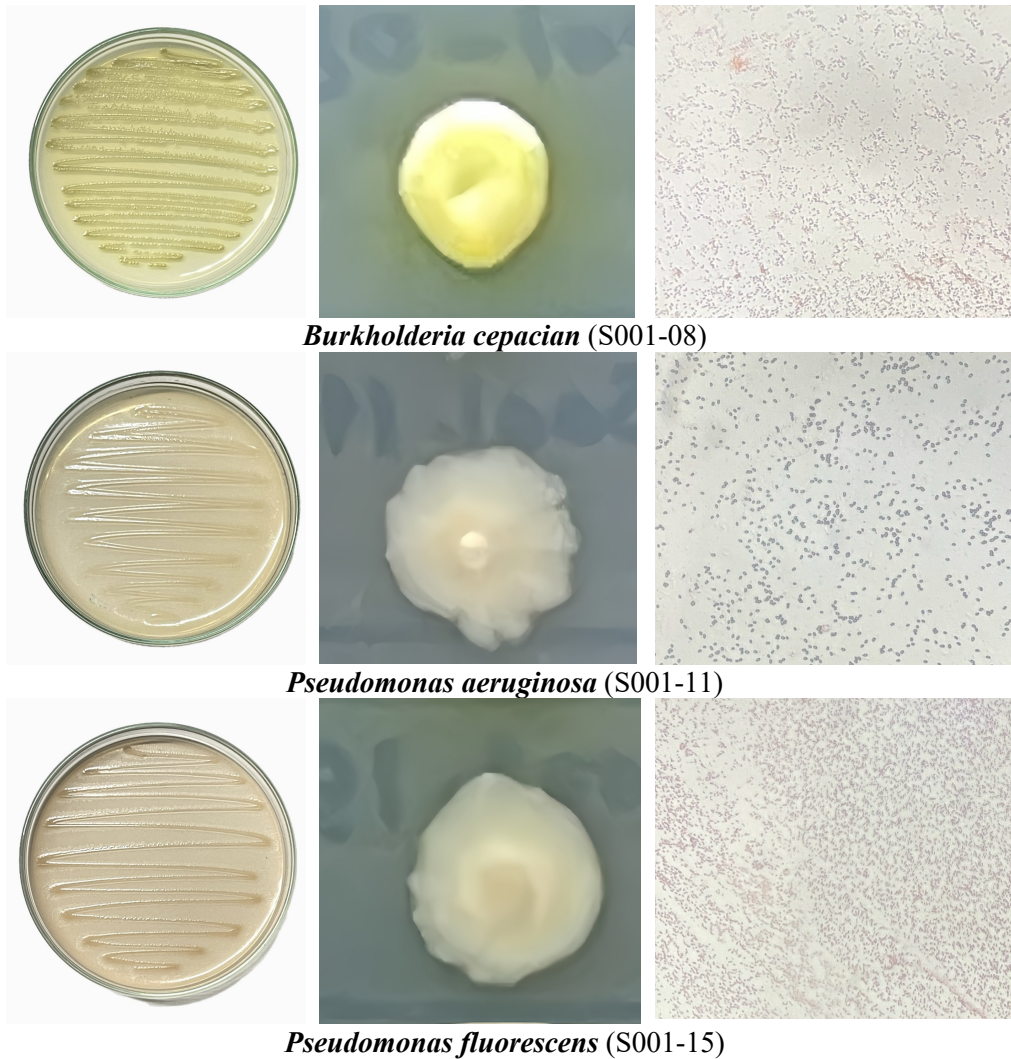


Figure 1. Morphological characters of *Burkholderia cepacia* strain S001-08, *Pseudomonas aeruginosa* strain S001-11 and *Pseudomonas fluorescens* strain S001-15

Morphological characterization

Morphological characterization resulted that all isolates formed circular and opaque colonies but differed in colony color and elevation (Figure 1; Table 1). Isolate S001-08 (*Burkholderia cepacia*) exhibited light yellow colonies with raised elevation, whereas isolates S001-11 (*Pseudomonas aeruginosa*) and S001-15 (*Pseudomonas fluorescens*) produced cream-colored colonies with flat elevation. Microscopic observations showed that all isolates were gram-negative, non-spore-forming, and rod-shaped.

Molecular phylogenic identification

Burkholderia cepacia strain S001-08 that is recorded as an accession number PO781596.1 identified as *Burkholderia cepacia* at percentage of similarity of 99% which *Enterobacter cloacae* as an outgroup (Figure 2). *Pseudomonas aeruginosa* strain S001-11 is recorded as an accession number QR708584.1 confirmed identification as *Pseudomonas aeruginosa* at percentage of similarity of 93% which *Rhizobium* sp. as an (Figure 3) *Pseudomonas fluorescens* strain S001-15 is recorded as an accession number MN256396.1 confirmed identification as *Pseudomonas aeruginosa* at percentage of similarity of 100 % which *Rhizobium* sp. as an outgroup (Figure 4) as seen in Table1.

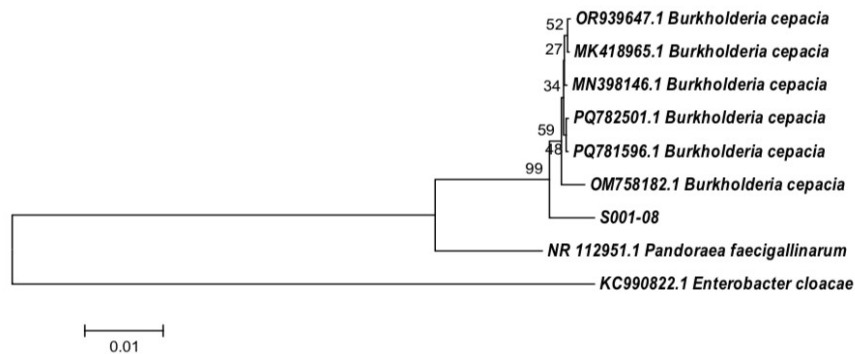


Figure 2. *Burkholderia cepacia* strain S001-08 that is recorded as an accession number PO781596.1 identified as *Burkholderia cepacia* at percentage of similarity of 99%

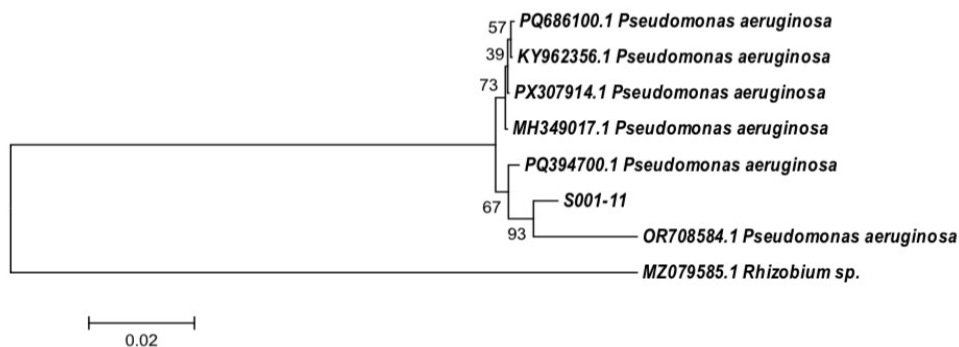


Figure 3. *Pseudomonas aeruginosa* strain S001-11 is recorded as an accession number QR708584.1 confirmed identification as *Pseudomonas aeruginosa* at percentage of similarity of 93%

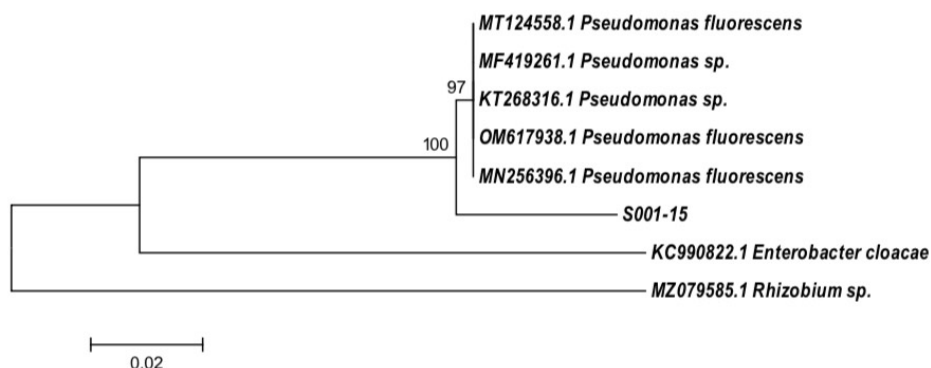


Figure 4. *Pseudomonas fluorescens* strain S001-15 is recorded as an accession number MN256396.1 confirmed identification as *Pseudomonas aeruginosa* at percentage of similarity of 100 %

Table 1. Accession number and percentage of similarity of *Burkholderia cepacia* strain S001-08, *Pseudomonas aeruginosa* strain S001-11 and *Pseudomonas fluorescens* strain S001-15

Isolates	Accession number	Name of Identified bacteria	Percentage of similarity
S001-08	PQ781596.1	<i>Burkholderia cepacia</i>	99%
S001-11	OR708584.1	<i>Pseudomonas aeruginosa</i>	93%
S001-15	MN256396.1	<i>Pseudomonas fluorescens</i>	100%

Screening of *P solubilizing*

Burkholderia cepacia strain S001-08, *Pseudomonas aeruginosa* strain S001-11 were significantly higher clear zone and colony diameters which were 15.63, 10.13 and 5.15 mm, respectively than *Pseudomonas fluorescens* strain S001-15 which clear zone and colony diameters of 11.11 and 3.43 mm, respectively in 3 days (Figure 5 and Table 2). Phosphorous solubilization index (PSI) were 35, 5.7 and 4.1 mm., respectively.

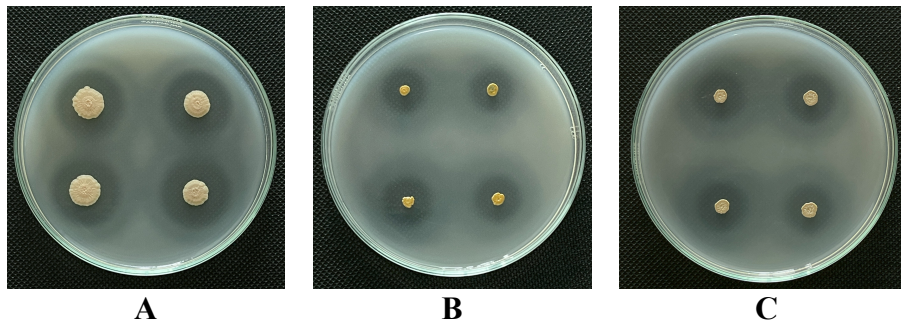


Figure 5. Phosphorous solubility tests of *Burkholderia cepacia* strain S001-08 (A), *Pseudomonas aeruginosa* strain S001-11 (B) and *Pseudomonas fluorescens* strain S001-15(C) in 3 days

Table 2. Phosphorous solubility index of *Burkholderia cepacia* strain S001-08, *Pseudomonas aeruginosa* strain S001-11 and *Pseudomonas fluorescens* strain S001-15 in 3 days

Species Name (Isolates Code)	Clear zone Diameter (mm) ¹	Colony Diameter (mm) ¹	Phosphorus Solubilization Index (PSI)
<i>Burkholderia cepacia</i> (S001-08)	15.63 ^a	10.13 ^a	3.5
<i>Pseudomonas aeruginosa</i> (S001-11)	12.65 ^b	5.15 ^b	5.7
<i>Pseudomonas fluorescens</i> (S001-15)	11.13 ^b	3.43 ^b	4.1
F	8.02	8.02	
Grand mean	13.13	6.23	
CV%	7.14	14.37	

¹/ Average from 4 replicates, and Mean values with different letters in the same column are significantly different at $p < 0.01$ based on the Duncan's multiple range test.

Screening of siderophore production

B. cepacia strain S001-08 is found to be the strongest significantly differed in siderophore production with showing clear zone of 21.48 mm within

3 days as strongest reaction and followed by *P. aeruginosa* strain S001-11 which showing clear zone 14.84 mm as medium reaction (Figure 6, Table 3).

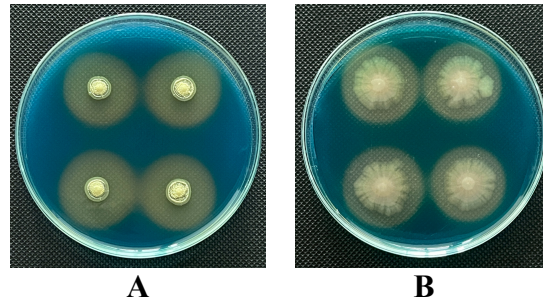


Figure 6. Siderophore production of *Burkholderia cepacia* S001-08(A) and *Pseudomonas aeruginosa* S001-11(B) in 3 days

Table 3. Reaction of siderophore production of *Burkholderia cepacia* S001-08(A) and *Pseudomonas aeruginosa* S001-11(B) in 3 days

Species Name (Isolates Code)	Clear zone Diameter (mm) ¹	Siderophore Production ²
<i>Burkholderia cepacia</i> (S001-08)	21.48 ^a	Strongest
<i>Pseudomonas aeruginosa</i> (S001-11)	14.84 ^b	Moderate
F	13.75	
Grand mean	18.15	
CV%	6.23	

^{1/} Average from 4 replicates, and Mean values with different letters in the same column are significantly different at $p < 0.01$ based on the Duncan's multiple range test. ^{2/} Siderophore production was assayed in agar plates; results are expressed in function of the diameter of clear zone formed around bacterial colonies on CAS medium. Symbols: - = No clear zone, (no reaction), + +1-10 mm (mild reaction), ++ = 11 to 20 mm (moderate reaction), and +++ = over 21 mm diameter of clear zone (strongest reaction)

Discussion

Burkholderia cepacia S001-08, *Pseudomonas aeruginosa* S001-11 and *Pseudomonas fluorescens* were isolated from soil planted to Durian. *B. cepacia*, *P. aeruginosa* and *Pseudomonas fluorescens* are confirmed species by phylogenetic trees at similarity of 99, 93 and 100 %, respectively. *B. cepacia* strain S001-08, *Pseudomonas aeruginosa* strain S001-11 were significantly wider clear zone and colony diameters than *P. fluorescens* 3 days. Song *et al.* (2008) found that *Burkholderia cepacia* DA23 was affected by phosphate regulation for Phosphate-solubilizing activities of insoluble phosphate. Aliyat *et al.* (2022) stated phosphate solubilizing bacteria which changing insoluble phosphorus to available forms to enhance plant growth acts as a key factor of organic

agriculture. Ramani and Patel (2011) stated that *Bacillus sphaericus* and *Burkholderia cepacia* were salt tolerance and showing phosphate solubilizing activity with various forms of phosphates. They expressed a significant result in pot experiments in green house which revealed more phosphate solubilizing activity in phosphate containing pesticides, while *B. cepacia* gave more phosphate solubilizing activity with different pesticides which may candidate as microbial inoculants. However, *P. aeruginosa* is reported as good effective, multifunctional phosphate-solubilizing bacterium to convert insoluble phosphorus to available forms through organic acid production. *P. aeruginosa* reported to act as plant growth-promoting rhizobacteria to enhance nutrient uptake and crop yield and *P. fluorescens* is recorded as a highly effective, gram-negative, phosphate-solubilizing bacterium which used as a biofertilizer to promote plant growth by changing insoluble phosphate to available phosphorus and improve rhizosphere soil. Moreover, Park *et al.* (2009) found that *P. fluorescens* strain RAF15 can be solubilized insoluble P through proton excretion with ammonium assimilation and production of organic acid. P solubilization is also promoted with glucose. *P. fluorescens* RAF1 expressed the mechanism to promote plant growth as producing pectinase, protease, lipase, siderophore, hydrogen cyanide, and indoleacetic acid. However, Mohapatra *et al.* (2024) stated that plant-growth-promoting rhizobacteria, *Pseudomonas aeruginosa* strain MK 764942.1 can be applied to promote the growth of groundnut for agricultural safety and sustainability, instead of synthetic fertilizers. The combination of *P. aeruginosa* with Phosphorous fertilizer gave significantly higher pod yield from 7.36 to 13.18%.

B. cepacia strain S001-08 expressed to be the strongest significantly differed in siderophore production in this investigation with expressing the clear zone of 21.48 mm within 3 days as the strongest activity and followed by *P. aeruginosa* strain S001-11 which a medium activity. Sasirekha and Srividya (2016) found that *P. aeruginosa* strain FP6 isolated from rhizosphere soil expressed confirmed siderophore production through a chrome-azurol S agar plate with blue to orange red. The maximum siderophore production was obtained in succinate medium (125 μ M) followed by King's B medium (105 μ M). The presence of sucrose and mannitol increased siderophore production is increased by presenting sucrose and mannitol. Our research investigation, these active strains of *Burkholderia cepacia* S001-08, *Pseudomonas aeruginosa* S001-11 and *Pseudomonas fluorescens* are being conducted for synergistic activity and developing to be bioinoculant product as bio stimulant and bioremediation. The developing biological product is promising to apply for crop production as an agricultural input for modern organic agriculture.

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Conflicts of interest

The authors declare no conflict of interest.

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