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Comparative Analysis of Phosphate-Solubilizing Bacteria from Rice Rhizosphere and Earthworm across Rice Field Management Systems as Biofilm-Based Biofertilizer Inoculants

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Abstract

Phosphate-solubilizing bacteria (PSB) are crucial microbial resources for improving phosphorus bioavailability and supporting low-input rice production. This study aimed to isolate and characterize high-performing PSB from rice rhizospheres and from the dominant earthworm species, *Pontoscolex corethrurus*. Samples were collected from 5 paddy field management systems in Central Java, Indonesia, including conventional, semi-organic, and 3 organic systems maintained for 7, 10, and 15 years, respectively. Sampling was conducted purposively at 5 points within each system, with 3 replicates per point. A total of 34 bacterial isolates were obtained using Nutrient agar: 20 from the rice rhizosphere and 14 from the gut of *P. corethrurus*. All isolates were screened for phosphate-solubilizing ability on Pikovskaya agar and in liquid assays. Morphological, physiological, and functional traits were assessed to evaluate diversity and potential. Rhizosphere isolates showed stronger phosphate-solubilizing activity than earthworm isolates in both assays. All isolates demonstrated biofilm-forming capacity. Four candidate isolates with the best PSB performance were identified: RZ5 and RZ16 (rhizosphere), and CAA and CAE (*P. corethrurus*). Their solubilization activity values were as follows: ROZ (qualitative = 5.10; quantitative = 6.90 µg ml^{-1}), RZ16 (2.94; 8.89 µg ml^{-1}), CAA (3.34; 5.23 µg ml^{-1}), and CAE (2.79; 6.32 µg ml^{-1}). These findings show ecological differentiation of PSB between rhizosphere and earthworm gut niches and across paddy field management systems. The superior strains identified here present strong potential as biofilm-based biofertilizer inoculants to enhance nutrient cycling and support sustainable rice agroecosystems, although further plant-level validation is still needed.

Keywords: biofilm-forming microorganisms; microbial inoculant development; organic rice systems; *Pontoscolex corethrurus*; soil microbial diversity

INTRODUCTION

Phosphorus (P) is one of the most essential macronutrients for plant growth, playing a central role in energy transfer, signal transduction, and metabolic regulation (Siedliska et al., 2021; Pan and Cai, 2023). It is particularly critical for root development and grain formation in cereal crops such as rice (*Oryza sativa* L.), a staple food for more than half of the global population (Yu et al., 2022; McDowell et al., 2023). Despite its abundance in soil, P is often bound to Ca, Fe, or Al compounds, making it largely unavailable to plants (Wang et al., 2017).

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This issue is especially pronounced in rice fields, where anaerobic conditions and high fixation rates further reduce P solubility (Zhang et al., 2025a).

To overcome this limitation, farmers commonly apply chemical phosphate fertilizers (Kile et al., 2025). However, the excessive and inefficient use of these inputs has led to environmental concerns, including eutrophication, soil degradation, and reduced microbial diversity (Preiner et al., 2020; Liu et al., 2022). Moreover, global reserves of rock phosphate are finite and geopolitically concentrated, raising concerns about long-term sustainability and food security (Dissanayaka et al., 2021). In response, there is growing interest in biological alternatives such as phosphate-solubilizing bacteria (PSB), which can mobilize insoluble P through the secretion of organic acids, chelating agents, and phosphatases (Rawat et al., 2021; Cheng et al., 2023).

PSB are naturally present in various soil niches, with the rice rhizosphere being one of the most studied environments because of its dynamic microbial interactions and nutrient fluxes. Root exudates in flooded rice systems stimulate microbial activity, creating favorable conditions for PSB proliferation (Pantigoso et al., 2023). Another promising yet underexplored niche is the earthworm gut, which acts as an ecosystem engineer in rice soils (Zhang et al., 2022). Earthworm activity enhances soil aeration, organic matter decomposition, and microbial turnover, and their gut may harbor unique PSB strains adapted to microaerophilic conditions (Bhakta et al., 2022; Akhila and Keshamma, 2023). Moreover, vermicompost derived from earthworms is known to contain beneficial microorganisms that contribute to soil fertility and microbial diversity (Arifah et al., 2021).

The diversity and functional traits of PSB are not only shaped by their ecological niche but also by the broader context of rice field management (Ughamba et al., 2025). Conventional systems often rely heavily on synthetic inputs, while organic and integrated systems promote biological processes and microbial resilience (Xing et al., 2024). Compared with conventional systems, organic fertilization fosters a richer, more diverse PSB community, which in turn enhances soil P availability and supports higher rice yields (Bi et al., 2020). Other studies have shown that sustained organic fertilization over extended periods enriches PSB populations (Chatterjee et al., 2021).

Previous research has shown that rhizobacterial isolates from rice plants possess phosphate-solubilizing ability, can tolerate drought conditions, and enhance rice growth (Rafedzi et al., 2024). Another study revealed that gut microbes in earthworms can enhance P mineralization in the soil and increase P availability (Biswas et al., 2018). Biofilms have also been recognized as a cohesive microbial consortium embedded within a thin yet resilient mucilaginous layer, generated through extracellular matrix production arising from microbial interactions. These biofilms help plants withstand various biotic and abiotic stresses (Dewi et al., 2023).

Unlike most previous studies that rely on rhizosphere isolates, this research employs isolates obtained directly from the earthworm gut, a source that remains underexplored for biofertilizer development. The earthworm gut represents a unique ecological niche where microbial communities are continuously exposed to organic matter transformation and nutrient mineralization. Exploring this source offers novel insights into PSB that may differ functionally from rhizosphere isolates, thereby providing distinct candidates for biofilm-based biofertilizer development. Furthermore, the influence of organic matter age across organic, conventional, and semi-organic systems deserves closer attention, particularly when comparing how earthworms and rhizosphere communities contribute to soil P availability.

This study advances knowledge by isolating and characterizing PSB from rice rhizospheres and earthworm guts across conventional, semi-organic, and organic rice systems established for 7, 10, and 15 years in Central Java, Indonesia. By assessing their solubilization capacity and distribution under different management regimes, this research aims to identify high-performing PSB candidates and clarify how management practices shape microbial resource potential. Importantly, developing effective inoculants for biofilm-based biofertilizers can enhance plant resilience to a wide range of biotic and abiotic stresses.

MATERIALS AND METHOD

Research methods and study site

This study applied a descriptive-exploratory approach combining field surveys and laboratory analyses. Soil, rhizosphere, and earthworm samples were collected during the vegetative growth stage of rice in Ngampel, Gentungan Village, Mojogedang Sub-district, Karanganyar Regency, Central Java, Indonesia (coordinates 111°00’38” to 111°00’50” E; 7°32’49” to 7°32’51” S). The area is characterized by Inceptisols. Sampling was conducted across 5 rice management systems: conventional, semi-organic, and organic systems established for 7, 10, and 15 years.

Sampling procedures

Samples were collected using a purposive sampling design across 5 different management systems. PSB were isolated from 2 niches: the rice rhizosphere and the earthworm gut. Each rice management system comprised 5 biological replicates, with 3 subsamples collected per plot. Within each plot, the 3 subsamples were pooled to form a single representative sample per biological replicate, yielding 5 composite replicates for each management system and sample type. Rhizosphere samples were collected by carefully detaching soil adhering to rice roots before microbiological analysis. Earthworm populations were assessed using soil monoliths (25 cm × 25 cm × 30 cm) established adjacent to rice plants. *Pontoscolex corethrurus* was identified as the species and subsequently used for bacterial isolation (Ross et al., 2025).

Soil analysis

Soil samples collected from each rice field management system were first air-dried at room temperature, gently crushed, and passed through 2 mm and 0.5 mm sieves to remove coarse fragments and plant residues. The prepared soil was then homogenized and stored in clean containers to ensure consistency. Soil parameters were determined as follows: pH (pH meter), organic C (Walkley and Black method), available P (Olsen method), cation exchange capacity (CEC) and base saturation (ammonium acetate extraction), and soil texture (sedimentation method) (Sulaeman et al., 2005).

Bacterial isolation

Bacteria from the rhizosphere and the earthworm gut were isolated using serial dilution and the spread-plate method on nutrient agar (NA), and then incubated for 7 days at room temperature (Prashanthi et al., 2021). Colony morphology was recorded. Gram staining was performed for cellular characterization. For each isolate, frequency (F) and relative frequency (RF) were calculated to allow grouping by rice management systems (Mukhopadhyay, 2020).

Phosphate-solubilizing bacteria

Qualitative analysis used Pikovskaya agar, in which PSB colonies formed clear halo zones. The Phosphate Solubilization Index (PSI) was calculated as described by Fitriyanti et al. (2017). Quantitative analysis employed Pikovskaya broth, incubated for 7 days at room temperature, followed by filtration and centrifugation. The supernatant was analyzed for soluble P using the molybdenum blue method with spectrophotometric measurement at 693 nm (Alam et al., 2002).

Biochemical characterization

The PSB isolates were then subjected to a series of biochemical assays to characterize their physiological and functional traits. These assays included determination of oxygen requirement (aerobic, anaerobic, or facultative), the oxidation–fermentation (OF) test for glucose metabolism (Hanson, 2008), and the oxidase test for cytochrome oxidase activity (Shields and Cathcart, 2010). Salt tolerance was assessed at NaCl concentrations ranging from 2 to 10% (Sharma et al., 2021), while carbon source utilization was evaluated using glucose, fructose, lactose, and sucrose (Mengesha and Legesse, 2024). Temperature tolerance was tested at 10, 36, and 45 °C, and pH tolerance at 5, 7, and 9 (Mengesha and Legesse, 2024). Biofilm formation was examined using the crystal violet staining method (Adetunji and Isola, 2011), and pathogenicity was assessed by the hypersensitive response (HR) on tobacco leaves (Spiga et al., 2021). Finally, compatibility among isolates was determined using the streak method to detect inhibition zones (Fitriatin et al., 2020).

Statistical analysis

Data were analyzed in RStudio version 4.3.1 using one-way analysis of variance (ANOVA) at a 95% confidence level. Significant differences in soil chemical properties were further tested using the Duncan multiple range test (DMRT), whereas differences in P solubilization among PSB isolates were evaluated using the Scott–Knott test. The performance of the earthworm and rhizosphere groups was compared using Welch's independent two-sample t-test and a nested ANOVA. Pearson correlation analysis was applied to examine the relationships between P solubilization and soil properties.

RESULTS AND DISCUSSION

Soil characteristics under rice field management systems

Significant differences ($p < 0.05$) were observed in soil pH, soil organic carbon (SOC), available P, and C/N ratio (Table 1). The semi-organic system had the highest pH (6.92), whereas the 15-year organic system had the lowest (6.38). SOC peaked in the 7-year organic plots (2.64%), and available P was consistently higher in organic systems, with the peak observed in the 7-year plots (36.53 ppm). In contrast, available P declined after the 7-year peak, with lower values in the 10- and 15-year plots compared to the mid-term system. This decline was linked to crop uptake and microbial immobilization, although the 15-year plots showed a slight increase relative to the 10-year plots.

The 15-year organic plots also had the highest C/N ratio (7.03). Low C/N ratios (< 10) across all systems indicate rapid N turnover and leaching risks (Truong and Marschner, 2020). Prolonged organic inputs gradually acidify the soil through organic matter decomposition and organic acid production (Goulding, 2016). A previous study revealed that the steady addition of organic amendments enriches soil C and, due to their slow decomposition under flooded conditions, enhances long-term C storage (Wei et al., 2025).

The findings of this research suggest that mid-term organic management, like a 7-year organic system, optimizes P release through enhanced microbial activity, organic acid production, and rapid mineralization of fresh organic inputs. In contrast, the decline in available P in the 15–10-year systems likely reflects increased crop uptake, microbial immobilization, and a shift toward moderately labile P pools. These observations align with long-term fertilization studies showing that sustained organic–inorganic inputs gradually redistribute P among labile, moderately labile, and stable fractions, thereby regulating its availability to plants (Mitran et al., 2016; Meena et al., 2022).

CEC showed no significant differences because the soil texture in nearly all systems was either clay loam or clay, resulting in relatively stable CEC values. In this study, the long-term organic plots also showed higher clay content, pointing to gradual improvements in soil structure, porosity, and moisture retention driven by sustained organic inputs (Assefa and Tadesse, 2019). Overall, the results of this research align with previous findings that organic systems generally deliver superior soil fertility compared with semi-organic and conventional practices (Romadhon et al., 2024).

Bacterial isolation from the rhizosphere and earthworm

A total of 20 rhizosphere PSB isolates were identified (Table 2), with the highest number found in the 15-year organic system (15 isolates) and the lowest in the 7-year organic system (6 isolates). Interestingly, the 7-year organic system exhibited the lowest number of PSB isolates compared to other management systems. This condition reflects the mid-term transition phase, where high P availability coincides with unstable microbial communities, leading to reduced detectable diversity of PSB (Sun et al.,

Table 1. The chemical properties and soil texture under rice field management systems

Rice field management system	pH H_2O	SOC (%)	Available P (ppm)	CEC (cmol kg^{-1})	C/N	Texture
Conventional	6.75±0.06^{b}	1.85±0.03^{c}	7.77±0.70^{d}	21.34±0.89^{a}	3.88±0.83^{b}	Clay loam
Semi-organic	6.92±0.04^{a}	2.20±0.25^{b}	9.74±0.37^{cd}	21.61±0.32^{a}	5.11±0.28^{b}	Clay loam
15-year organic	6.38±0.05^{c}	2.61±0.12^{a}	14.37±0.66^{b}	23.00±1.84^{a}	7.03±1.42^{a}	Clay
10-year organic	6.82±0.12^{ab}	2.43±0.12^{ab}	11.17±3.46^{c}	22.76±0.21^{a}	5.01±0.64^{b}	Clay loam
7-year organic	6.73±0.03^{b}	2.64±0.05^{a}	36.53±0.54^{a}	21.34±0.88^{a}	5.01±0.88^{b}	Clay
ANOVA (p-value)	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p \geq 0.05$	$p < 0.05$	

Note: Means followed by the same letter in the same column are not significantly different

Table 2. Distribution of rhizosphere PSB isolates across different rice field management systems

Rice field management system	Isolate	Total isolate
Conventional	RZ1, RZ2, RZ3, RZ5, RZ6, RZ11, RZ12, RZ19, RZ20	
Semi-organic	RZ3, RZ4, RZ5, RZ9, RZ17, RZ18, RZ19, RZ20	8
15-year organic	RZ1, RZ2, RZ3, RZ4, RZ5, RZ6, RZ8, RZ9, RZ11, RZ13, RZ14, RZ15, RZ16, RZ17, RZ19	15
10-year organic	RZ1, RZ3, RZ5, RZ6, RZ7, RZ8, RZ9, RZ10, RZ11, RZ12	10
7-year organic	RZ1, RZ2, RZ3, RZ4, RZ5, RZ18	6

Table 3. Distribution of earthworm PSB isolates across different rice field management systems

Rice field management system	Isolate	Total isolate
Conventional	CAA, CAB, CAC, CAD, CAE, CAF, CAG, CAH, CAI, CAJ, CAK, CAL, CAM, CAN	14
Semi-organic	CAB, CAD, CAE, CAF, CAG, CAH, CAK, CAL, CAM, CAN	10
15-year organic	CAB, CAD, CAE, CAG, CAH, CAI, CAJ, CAK, CAL, CAM	10
10-year organic	CAB, CAE, CAF, CAG, CAH, CAI, CAK, CAL, CAM	9
7-year organic	CAB, CAC, CAD, CAE, CAH, CAI, CAJ, CAL, CAM	9

2026). Abundance in long-term organic plots reflects continuous organic inputs that enhance microbial diversity, whereas conventional systems showed higher earthworm PSB diversity (Table 3). Previous research suggests that earthworms consuming lower-quality organic matter, which is typical of conventional systems, harbor more diverse bacterial communities than those consuming higher-quality organic matter in organic systems (Chao et al., 2025).

RF analysis revealed distinct distribution patterns of PSB isolates between rhizosphere and earthworm gut habitats. In the rhizosphere (Table 4), only RZ3 and RZ5 were consistently present (100%), while most isolates showed moderate (40 to 60%) or low (20%) frequencies. This suggests that while certain PSB strains are well adapted to the rice rhizosphere environment, many exist as opportunistic or niche members of the microbial community. Such variability is consistent with findings that microbial abundance and persistence in the rhizosphere are strongly influenced by soil nutrient status, root exudates, and management practices (Mayadunna et al., 2023). In contrast, 4 earthworm isolates (CAB, CAE, CAL, and CAM) reached 100%, confirming the stability of earthworm gut microhabitats (Table 5). Earthworm guts and casts are known to provide stable microhabitats enriched with organic matter and microbial interactions, which may favor the persistence of PSB consortia (Bhakta et al., 2022).

High-frequency isolates are considered adaptive and ecologically stable, making them strong candidates for biofilm-based biofertilizers, whereas low-frequency isolates may still contribute unique functional traits. The frequent occurrence of dominant strains across samples suggests that they are not only abundant but also

Table 4. Relative frequency of rhizosphere PSB isolates

Isolate	F	RF (%)
RZ1	4	80
RZ2	3	60
RZ3	5	100
RZ4	3	60
RZ5	5	100
RZ6	3	60
RZ7	1	20
RZ8	2	40
RZ9	3	60
RZ10	1	20
RZ11	3	60
RZ12	2	40
RZ13	1	20
RZ14	1	20
RZ15	1	20
RZ16	1	20
RZ17	2	40
RZ18	2	40
RZ19	3	60
RZ20	2	40

Note: F = Frequency; RF = Relative frequency

Table 5. Relative frequency of earthworm PSB isolates

Isolate	F	RF (%)
CAA	1	20
CAB	5	100
CAC	2	40
CAD	4	80
CAE	5	100
CAF	3	60
CAG	3	60
CAH	4	80
CAI	4	80
CAJ	3	60
CAK	3	60
CAL	5	100
CAM	5	100
CAN	3	60

Note: F = Frequency; RF = Relative frequency

ecologically stable. Biofilms formed by such dominant PSB can enhance P availability and improve plant tolerance to biotic and abiotic stresses (Dewi et al., 2023). Overall, RF data emphasize the importance of combining dominant and functionally diverse isolates to strengthen biofilm resilience and improve phosphate solubilization efficiency in tropical rice agroecosystems.

Bacterial morphology of rhizosphere and earthworm isolates

Bacteria isolated on NA medium showed diverse morphological traits. Rhizosphere isolates (Table 6) were dominated by white colonies (n = 11), followed by yellow (n = 6), with occasional orange, cream, and beige. Shapes were mainly round, although irregular, rhizoid, and filamentous forms occurred. Margins and elevations varied widely, and textures were mostly smooth, with a few rough isolates. All rhizosphere isolates were Gram-negative, comprising coccus (n = 8) and bacillus (n = 12). Earthworm isolates (Table 7) also showed dominant white colonies (n = 7), with additional colors such as yellow-brown and orange. Shapes were round or irregular, with rhizoid (CAK) and filamentous (CAL) forms present. Margins included lobate and serrate types, and elevations were convex, raised, or flat. Most textures were smooth, although 4 isolates were rough. All earthworm isolates were Gram-negative, comprising coccus (n = 9) and bacillus (n = 5).

Many common Gram-negative soil bacteria are well-known candidates for PSB, due to their ability to release P from insoluble compounds and enhance plant nutrient uptake, such as *Pseudomonas*, *Burkholderia*, and *Enterobacter*

Table 6. Morphology, Gram reaction, and cell shape of rhizosphere isolates

Isolate	Color	Form	Margin	Elevation	Texture	Gram staining	Cell shape
RZ1	White	Rhizoid	Entire	Raised	Smooth	-	Coccus
RZ2	White	Irregular	Lobate	Raised	Smooth	-	Bacillus
RZ3	White	Round	Round	Convex	Smooth	-	Bacillus
RZ4	White	Round	Convex	Raised	Smooth	-	Coccus
RZ5	Yellow	Round	Round	Convex	Smooth	-	Coccus
RZ6	Yellow	Filamentous	Wavy	Crateriform	Rough	-	Bacillus
RZ7	White	Round	Scalloped	Draugtsman Colony	Rough	-	Bacillus
RZ8	Orange	Round	Undulate	Umbonate	Smooth	-	Bacillus
RZ9	White	Rhizoid	Filiform	Umbonate	Rough	-	Bacillus
RZ10	Yellow	Round	Undulate	Crateriform	Smooth	-	Bacillus
RZ11	Cream	Round	Round	Convex	Smooth	-	Coccus
RZ12	Yellow	Round	Round	Convex	Smooth	-	Coccus
RZ13	White	Round	Undulate	Raised	Smooth	-	Coccus
RZ14	White	Irregular	Filamentous	Raised	Rough	-	Bacillus
RZ15	Yellow	Round	Round	Convex	Smooth	-	Bacillus
RZ16	White	Round	Entire	Flat	Smooth	-	Coccus
RZ17	Yellow	Filamentous	Wavy	Crateriform	Rough	-	Bacillus
RZ18	White	Round	Undulate	Flat	Smooth	-	Bacillus
RZ19	Beige	Round	Round	Convex	Smooth	-	Bacillus
RZ20	White	Round	Round	Raised	Smooth	-	Coccus

Table 7. Morphology, Gram reaction, and cell shape of earthworm isolates

Isolate	Color	Form	Margin	Elevation	Texture	Gram staining	Cell shape
CAA	White-brown	Round	Lobate	Convex	Rough	-	Bacillus
CAB	White	Irregular	Entire	Raised	Rough	-	Coccus
CAC	Yellow	Round	Round	Convex	Smooth	-	Coccus
CAD	White-yellow	Irregular	Lobate	Raised	Smooth	-	Coccus
CAE	Orange	Round	Round	Convex	Smooth	-	Coccus
CAF	Cream	Irregular	Lobate	Raised	Smooth	-	Coccus
CAG	White	Irregular	Lobate	Raised	Smooth	-	Bacillus
CAH	White-orange	Irregular	Lobate	Raised	Smooth	-	Bacillus
CAI	White	Round	Round	Raised	Smooth	-	Coccus
CAJ	White	Irregular	Lobate	Flat	Rough	-	Coccus
CAK	White	Rhizoid	Serrate	Flat	Rough	-	Coccus
CAL	White	Filamentous	Filiform	Flat	Smooth	-	Bacillus
CAM	White	Round	Round	Raised	Smooth	-	Bacillus
CAN	Glossy white	Round	Round	Convex	Smooth	-	Coccus

(Janati et al., 2023). The predominance of Gram-negative isolates observed in this study may partly reflect methodological factors, such as incubation time and the composition of the culture medium (Pham et al., 2026). This trend is consistent with earlier reports showing that rhizosphere PSB communities are often dominated by Gram-negative bacteria, which possess metabolic advantages for P solubilization and greater adaptability to environmental fluctuations (Awais et al., 2017).

Phosphate-solubilizing ability of rhizosphere and earthworm isolates

Functional assays confirmed that all rhizosphere isolates solubilized phosphate, with significant differences ($p < 0.01$) in both PSI and dissolved P (Table 8). Qualitatively, 15 of 20 isolates formed halo zones, with PSI ranging from 2.12 to 5.10; RZ5 (Figure 1a) was the highest (5.10), while 5 isolates were inactive. Quantitatively, all isolates solubilized phosphate (6.01 to 8.89 µg ml^{-1}), with RZ16 showing the highest activity (8.89 µg ml^{-1}). All isolates from earthworm guts were capable of solubilizing phosphate, with highly significant differences ($p < 0.01$) observed in both qualitative and quantitative measures (Table 9). Among the 14 earthworm isolates, 12 formed halo zones in the qualitative test, and all were positive in the quantitative test. The highest value in the quantitative method was found in isolate CAA (Figure 1b), with a value of 3.34.

Based on PSI values, bacterial isolates were categorized as low (PSI < 2), moderate (2 ≤ PSI < 4), and high (PSI ≥ 4) (Teles et al., 2023). Among

Table 8. Results of the functional analysis of PSB in the rhizosphere

Isolate	Qualitative (PSI)	Quantitative (µg ml^{-1})
RZ1	2.74±0.19^{b}	7.05±0.12^{b}
RZ2	2.24±0.17^{c}	7.38±0.22^{b}
RZ3	2.52±0.07^{c}	7.49±1.10^{b}
RZ4	NHZ	7.84±0.94^{a}
RZ5	5.10±0.42^{a}	6.90±0.38^{b}
RZ6	3.04±0.21^{b}	6.10±0.24^{c}
RZ7	3.35±0.83^{b}	6.79±0.17^{b}
RZ8	2.12±0.00^{c}	7.36±0.64^{b}
RZ9	2.97±0.35^{b}	8.31±0.43^{a}
RZ10	2.40±0.08^{c}	7.62±0.06^{b}
RZ11	2.69±0.10^{b}	6.04±0.05^{c}
RZ12	2.53±0.14^{c}	6.12±0.10^{c}
RZ13	3.13±0.21^{b}	7.28±0.50^{b}
RZ14	NHZ	7.42±0.69^{b}
RZ15	NHZ	8.28±0.22^{a}
RZ16	2.94±0.04^{b}	8.89±0.24^{a}
RZ17	NHZ	8.65±0.50^{a}
RZ18	2.24±0.02^{c}	8.75±0.32^{a}
RZ19	NHZ	7.94±0.14^{a}
RZ20	2.13±0.07^{c}	8.18±0.58^{a}
ANOVA (p-value)	$p < 0.01$	$p < 0.01$

Note: NHZ = No halo zone observed. Means followed by the same letter in the same column are not significantly different

Table 9. Results of the functional analysis of PSB in the earthworm

Isolate	Qualitative (PSI)	Quantitative (µg ml^{-1})
CAA	3.34 ± 0.16^{a}	5.23 ± 0.02^{c}
CAB	NHZ	5.13 ± 0.39^{c}
CAC	2.53 ± 0.25^{c}	6.35 ± 0.45^{b}
CAD	2.50 ± 0.13^{c}	7.22 ± 0.23^{a}
CAE	2.79 ± 0.07^{b}	6.32 ± 0.15^{b}
CAF	2.50 ± 0.18^{c}	5.23 ± 0.94^{c}
CAG	2.64 ± 0.06^{b}	5.08 ± 0.19^{c}
CAH	2.65 ± 0.12^{b}	7.73 ± 1.33^{a}
CAI	NHZ	5.76 ± 0.47^{c}
CAJ	2.36 ± 0.17^{c}	6.66 ± 0.28^{b}
CAK	2.31 ± 0.08^{c}	3.69 ± 0.32^{d}
CAL	2.45 ± 0.11^{c}	4.14 ± 0.42^{d}
CAM	2.43 ± 0.05^{c}	4.87 ± 0.06^{c}
CAN	2.49 ± 0.11^{c}	5.39 ± 0.72^{c}
ANOVA (*p*-value)	$p < 0.01$	$p < 0.01$

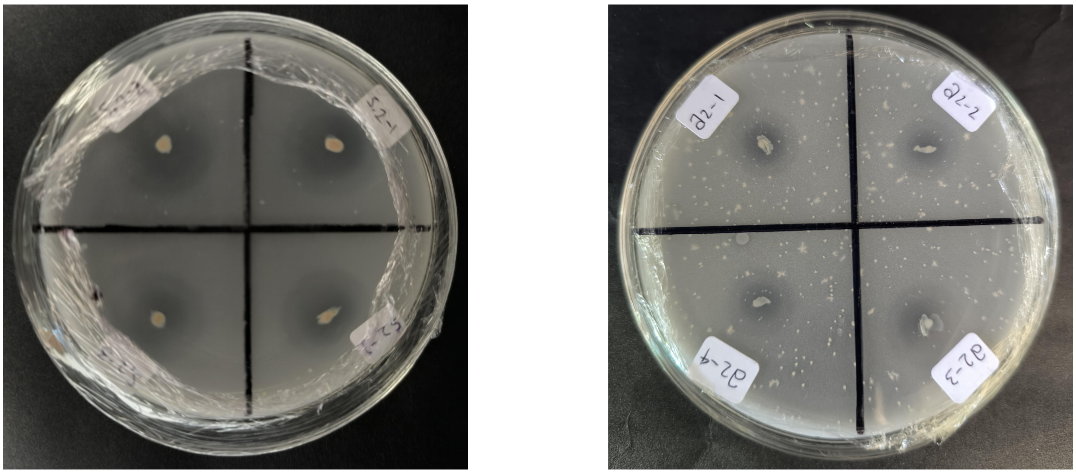
Note: NHZ = No halo zone observed. Means followed by the same letter in the same column are not significantly different

the rhizosphere isolates, most exhibited moderate PSI values, with only one isolate (RZ5) reaching the high category (5.10). In contrast, all PSB isolated from the earthworm fell within the moderate classification. Some bacterial isolates exhibited high solubilization activity in the qualitative method but low activity in the quantitative method, and vice versa. PSB isolates that were inactive in qualitative assays were quantitatively verified to ensure accuracy and avoid overlooking potential candidates. Differences between methods may result from nutrient availability, growth requirements, and organic acid diffusion (Walpola and Hettiarachchi, 2020). Overall, rhizosphere isolates demonstrated stronger solubilization capacity, reinforcing the role of PSB in enhancing P availability in root zones. This is driven by several rhizosphere-induced changes, including rhizosphere acidification, the release of organic acids and phosphorus-hydrolyzing enzymes, soil respiration, and the modulation of microbial activity around the rhizosphere (Elhaissoufi et al., 2020).

Table 10 shows that earthworm gut isolates exhibited lower quantitative phosphate-solubilizing activity than rhizosphere PSB. This difference reflects the ecology of each environment. The rhizosphere is continually enriched by root exudates, including organic acids, sugars, and amino acids, which fuel microbial metabolism and stimulate P release (Bilal et al., 2021; Sun et al., 2026). In contrast, the earthworm gut is a short-term, nutritionally limited habitat in which microbes pass through quickly and have fewer resources to sustain high solubilization activity (Kerketta et al., 2025).

Biochemical and biological characteristics of bacterial isolates

Rhizosphere PSB isolates (Table 11) were predominantly aerobic and fermentative. Most tolerated up to 8% NaCl, used lactose as their main carbon source, grew optimally at 36 °C



a. b.

Figure 1. Appearance of PSB in qualitative phosphate solubilization analysis. (a) RZ5 isolate from the rhizosphere, (b) CAA isolate from the earthworm

Note: These figures have been previously published in the proceedings (Mukmina et al., 2025)

Table 10. Comparison of qualitative index (PSI) and quantitative phosphate-solubilizing activity (µg ml^{-1}) between earthworm gut and rhizosphere isolates

Parameter	Earthworm (mean±SD)	Rhizosphere (mean±SD)	t-test (*p*-value)
Qualitative index (PSI)	2.58±0.31	2.82±0.45	0.060 (ns)
Quantitative activity (µg ml^{-1})	5.63±0.52	7.52±0.61	< 0.001***

Note: An independent-sample t-test was used to compare groups. ns = not significant ($p > 0.05$); *** = highly significant ($p < 0.001$)

as mesophiles, preferred neutral pH (pH 7), and 5 isolates exhibited strong biofilm formation (+++). All isolates were non-pathogenic and mutually compatible. In comparison, earthworm PSB isolates (Table 12) were also aerobic and fermentative but tolerated only 2% NaCl, relied on lactose, grew best at 45 °C as thermophiles, and preferred pH at 5 and 7. Two isolates (CAE and CAG) formed strong biofilms (+++), while one isolate (CAJ) showed pathogenic activity. All isolates of earthworm guts were mutually compatible. These isolates demonstrate strong potential to colonize plant roots and establish stable microbial communities, as biofilms provide protection against fluctuations in nutrient availability, pH, and temperature, as well as competition with other microbes (Bhat and Roach, 2025). Their adaptive diversity highlights functional differences that support the development of multi-isolate biofertilizers, enabling synergistic phosphate solubilization and improved soil resilience (Zhang et al., 2025b).

Several isolates were found exclusively in specific rice field management systems (RF 20%, Table 4). RZ7 and RZ10 occurred only in the 10-year organic system, while RZ13, RZ14, RZ15, and RZ16 were restricted to the 15-year system (Table 2). These 6 isolates displayed distinct functional and physiological traits (Table 11). RZ7 is an aerobic isolate that utilizes glucose, grows best at 36 °C and pH 7, and shows relatively high phosphate-solubilizing activity (PSI = 3.35) despite weak biofilm formation. RZ10 is a facultative anaerobe capable of forming moderate biofilms (++), indicating adaptability to fluctuating oxygen conditions, and exhibits strong quantitative phosphate solubilization (7.62 µg ml^{-1}). RZ13 forms strong biofilms (+++) but demonstrates considerable phosphate-solubilizing activity (PSI = 3.13; 7.28 quantitative µg ml^{-1}) and relies on sucrose as its C source. RZ15 shows no qualitative activity but has high quantitative solubilization (8.28 µg ml^{-1}), making it a unique anaerobic isolate with hidden functional potential. RZ16 is the strongest performer, with the highest quantitative value (8.89 µg ml^{-1}), aerobic metabolism, fructose utilization, and strong biofilm formation (+++), highlighting its promise as a bioinoculant. RZ19, a facultative anaerobe, also shows high quantitative activity (7.94 µg ml^{-1}), moderate biofilm formation (++), and metabolic flexibility under diverse conditions. Together, these traits highlight that each isolate carries unique strengths, which can be strategically harnessed in developing biofilm-based biofertilizers for sustainable rice production.

Two rhizosphere isolates, RZ3 and RZ5, were present across all rice field management systems (RF 100%) (Table 2; Table 4). RZ3 is aerobic, shows strong phosphate-solubilizing activity (PSI = 2.52; 7.49 µg ml^{-1}), utilizes lactose, tolerates up to 8% NaCl, grows optimally at 36 °C, and forms strong enough biofilms (++). This combination of high salt tolerance, robust biofilm formation, and phosphate solubilization makes RZ3 highly resilient under variable field conditions. RZ5, on the other hand, had the highest qualitative phosphate-solubilizing value (PSI = 5.10) among all isolates, with moderate quantitative activity (6.90 µg ml^{-1}). It is anaerobic, utilizes sucrose, grows optimally at 36 °C and pH 7, and forms moderate biofilms (++). Its ability to function under anaerobic conditions, combined with strong qualitative solubilization, makes RZ5 particularly suited to flooded rice soils with limited oxygen availability. Thus, RZ3 excels in environmental tolerance and biofilm formation, whereas RZ5 stands out for solubilization capacity and adaptation to anaerobic niches.

In the earthworm microhabitat, only one isolate, CAA, was unique to a specific management system in the conventional system (RF 20%) (Table 3; Table 5). CAA showed good phosphate-solubilizing ability (PSI = 3.34; 5.23 µg ml^{-1}), is aerobic with an O+/F+ pattern, tolerates 2% NaCl, utilizes lactose, grows

Table 11. Results of P functional, biochemical, and biological assays on rhizosphere PSB

Isolate	Biochemical assay							Biological assay		
	Oxygen requirement	OF	Oxidase	NaCl (%)	Carbon source	Temperature (°C)	pH	Biofilm	HR	Compatibility
RZ1	Aerobic	O+/F+	-	2	Lactose	45	7	+++	-	+
RZ2	Aerobic	O+/F+	-	2	Lactose	45	7	++	-	+
RZ3	Aerobic	O+/F+	-	8	Lactose	36	7	++	-	+
RZ4	Aerobic	O+/F+	-	2	Glucose	36	5	+	-	+
RZ5	Anaerobic	O-/F-	-	2	Sucrose	36	7	++	-	+
RZ6	Anaerobic	O+/F+	-	2	Fructose	36	7	++	-	+
RZ7	Aerobic	O+/F+	-	2	Glucose	36	7	+	-	+
RZ8	Anaerobic	O+/F+	-	2	Glucose	36	9	++	-	+
RZ9	Aerobic	O+/F+	-	2	Lactose	45	5	++	-	+
RZ10	Anaerobic Facultative	O+/F+	-	2	Glucose	36	9	++	-	+
RZ11	Aerobic	O+/F-	-	2	Fructose	36	9	+++	-	+
RZ12	Anaerobic	O+/F+	-	8	Fructose	36	7	+++	-	+
RZ13	Aerobic	O+/F-	-	2	Sucrose	36	9	+++	-	+
RZ14	Anaerobic Facultative	O+/F+	-	8	Lactose	36	7	++	-	+
RZ15	Anaerobic	O+/F+	-	2	Glucose	10	7	+	-	+
RZ16	Aerobic	O+/F-	-	2	Fructose	36	7	+++	-	+
RZ17	Aerobic	O+/F+	-	2	Lactose	45	7	++	-	+
RZ18	Anaerobic	O+/F+	-	2	Lactose	45	5	++	-	+
RZ19	Anaerobic Facultative	O+/F+	-	2	Glucose	45	7	++	-	+
RZ20	Aerobic	O+/F+	-	2	Fructose	45	7	+	-	+

Note: O+/F+ = Fermentative; O-/F- = Non-oxidizer/Non-fermenter; O+/F- = Oxidative

Table 12. Results of P functional, biochemical, and biological assays on earthworm gut PSB

Isolate	Biochemical assay							Biological assay		
	Oxygen requirement	OF	Oxidase	NaCl (%)	Carbon source	Temperature (°C)	pH	Biofilm	HR	Compatibility
CAA	Aerobic	O+/F+	-	2	Lactose	45	7	+	-	+
CAB	Aerobic	O+/F+	-	2	Fructose	45	7	++	-	+
CAC	Aerobic	O+/F+	-	2	Fructose	45	7	++	-	+
CAD	Anaerobic Facultative	O+/F+	-	2	Fructose	45	7	++	-	+
CAE	Anaerobic Facultative	O+/F-	-	2	Glucose	45	5	+++	-	+
CAF	Aerobic	O+/F+	-	2	Lactose	45	7	++	-	+
CAG	Anerobic	O-/F-	-	2	Fructose	45	5	+++	-	+
CAH	Aerobic	O+/F-	-	2	Lactose	45	5	++	-	+
CAI	Aerobic	O+/F+	-	2	Lactose	45	7	++	-	+
CAJ	Anaerobic Facultative	O+/F+	-	2	Fructose	45	7	++	+	+
CAK	Anaerobic Facultative	O+/F+	-	2	Glucose	45	7	++	-	+
CAL	Aerobic	O+/F+	-	2	Lactose	45	7	++	-	+
CAM	Aerobic	O+/F+	-	2	Lactose	45	7	++	-	+
CAN	Aerobic	O+/F+	-	2	Lactose	45	7	+	-	+

Note: O+/F+ = Fermentative; O-/F- = Non-oxidizer/Non-fermenter; O+/F- = Oxidative optimally at 45 °C, and forms weak biofilms (+), indicating adaptation to warm, neutral environments (Table 12). Four earthworm isolates were present across all systems (RF 100%): CAB, CAE, CAL, and CAM (Table 3; Table 5). These isolates exhibited diverse functional traits (Table 12). CAB is aerobic (O+/F+), tolerates 2% NaCl, utilizes fructose, grows at 45 °C, forms moderate biofilms (++), and shows quantitative phosphate solubilization (5.13 µg ml^{-1}). CAE is a facultative anaerobe (O+/F–), utilizes glucose, grows at 45 °C and pH 5, forms strong biofilms (+++), and shows moderate solubilization (PSI = 2.79; 6.32 µg ml^{-1}). CAI, CAL, and CAM are aerobic (O+/F+), utilize lactose, tolerate 2% NaCl, grow at 45 °C and pH 7, and form moderate biofilms (++), with varying phosphate-solubilizing capacities. Overall, the physiological diversity observed among these isolates, across oxygen requirements, C utilization, temperature, pH tolerance, and biofilm formation, reflects their adaptation to distinct microhabitats and underscores their potential in biofilm-based biofertilizer formulations.

Isolates detected across all rice field management systems, whether from the rhizosphere or earthworms, are particularly valuable for biofilm biofertilizer (BFBF) development, as their stable occurrence indicates strong ecological adaptability. These generalist isolates contribute to the reliability of BFBF functions through their phosphate-solubilizing ability, tolerance to NaCl, utilization of diverse C sources, and capacity to form biofilms that support root colonization and community resilience under stress. In contrast, isolates found only in specific management systems act as ecological specialists, offering unique functional advantages such as highly efficient phosphate solubilization or adaptation to particular soil conditions. Their presence enriches the functional diversity of the biofilm community, enabling BFBF to remain stable and flexible across different rice field management practices.

Based on this study, the superior isolates selected as potential phosphate-solubilizing BFBF inoculants are RZ5 and RZ16 from the rhizosphere, and CAA and CAE from earthworms. All 4 isolates demonstrated strong phosphate-solubilizing capacity in both qualitative and quantitative assays, with distinct metabolic and environmental tolerance profiles. RZ5 is an anaerobic, non-oxidizer/non-fermenter (O–/F–) capable of growing in 2% NaCl, utilizing sucrose, and thriving at 36 °C and pH 7. RZ16 is aerobic with an oxidative (O+/F-) profile and uses fructose as its C source. Among earthworm isolates, CAA is aerobic and fermentative (O+/F+), utilizing lactose at 45 °C and neutral pH, while CAE is a facultative anaerobe with an oxidative (O+/F-) profile that metabolizes glucose under acidic conditions. Biofilm formation also varied among isolates. RZ5 produced moderate biofilms (++), RZ16 produced strong biofilms (+++), whereas CAE formed strong biofilms (+++) and CAA formed weak biofilms (+) but exhibited high phosphate-solubilizing activity. These physiological differences reflect each isolate's adaptation to its native habitat, rhizosphere or earthworm gut, and likely influence their functional performance, particularly in phosphate solubilization and biofilm-based inoculum development.

Correlation between PSB in the rhizosphere and earthworm and soil chemistry

Pearson correlation analysis revealed associations between soil properties and PSB activity in rhizosphere and earthworm habitats (Table 13). The negative correlation between rhizosphere PSI and SOC (r = –0.70) suggests that higher organic C may reduce the apparent phosphate-solubilizing activity, possibly due to organic matter binding P. However, organic matter appears capable of breaking down slowly decomposing organic P compounds for PSB, including phytic acid, polyphosphates, and phosphonates (Rodríguez and Fraga, 1999). Rhizosphere isolates showed significant positive correlations: PSI-R with available P (r = 0.56), and Qt-PSBR with SOC (r = 0.62), CEC (r = 0.59), and C/N ratio (r = 0.54), indicating that higher SOC, a balanced C/N ratio, and greater CEC support PSB performance. In contrast, earthworm isolates (Qt-PSBE) exhibited a strong negative correlation with soil pH H_2O (r = –0.81*), consistent with their adaptation to acidic gut environments that enhance phosphate solubilization (Bhakta et al., 2022).

Overall, these findings demonstrate that organic systems enhance PSB presence and function, thereby improving soil fertility and nutrient availability for rice cultivation. PSB

Table 13. Coefficient correlation (r) between PSB in the rhizosphere and earthworm and soil chemistry

PSB	pH H_2O	SOC	Available P	CEC	C/N
PSI-R	0.16	-0.70*	0.56*	-0.06	-0.29
PSI-E	-0.45	-0.19	0.04	-0.20	0.48
Qt-PSBR	0.32	0.62*	0.13	0.59*	0.54*
Qt-PSBE	-0.81*	0.61	-0.06	0.46	0.42

Note: *Indicates a statistically significant correlation. PSI-R = the phosphate-solubilizing index of rhizosphere isolates; PSI-E = the phosphate-solubilizing index of earthworm isolates; Qt-PSBR = the quantitative analysis of phosphate-solubilizing bacteria in the rhizosphere; Qt-PSBE = the quantitative analysis of phosphate-solubilizing bacteria associated with earthworms

presence and function differ significantly among rice management systems because organic matter inputs and microbial diversity are key drivers. Organic and integrated management systems were found to enhance PSB activity, thereby improving P availability and ultimately boosting rice yields (You et al., 2026). In contrast, conventional systems tend to suppress microbial contributions (Rawat et al., 2022). These findings emphasize the importance of sustainable management practices that nurture beneficial microbial communities, ensuring long-term soil fertility and productivity (Rasul et al., 2024).

CONCLUSIONS

This study shows that PSB from the rhizosphere and earthworms vary across rice field management systems and are strongly shaped by soil fertility, particularly SOC and soil pH. These findings indicate that rice management systems strongly shape PSB presence and function, with organic practices fostering microbial traits that enhance P availability and soil fertility. This underscores the broader implication that sustainable rice production benefits from management strategies that enhance microbial diversity and soil health. Rhizosphere isolates exhibited higher phosphate-solubilizing activity, whereas earthworm isolates showed greater thermotolerance and tolerance of acidic environments. Four isolates, RZ5 and RZ16 from the rhizosphere and CAA and CAE from earthworms, demonstrated superior functional traits and strong potential as biofilm-based biofertilizer candidates. However, further validation under plant and field conditions is required to confirm the agronomic effectiveness of these PSB candidates and to explore their long-term role in nutrient cycling and crop productivity.

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DECLARATION OF GENERATIVE AI USE

The authors declare that generative AI tools (Microsoft Copilot and ChatGPT) were used solely for language refinement, grammar correction, sentence restructuring, and improving the manuscript's readability. No content, data interpretation, or scientific conclusions were generated by AI. The authors take full responsibility for the accuracy and integrity of the manuscript.

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