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Molecular Characterization of Phosphate Solubilizing Bacteria *Klebsiella variicola* PSEG-1 Associated with *Aporrectodea rosea* Gastrointestinal Tract

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Abstract: Phosphorus is a macronutrient crucially important for plant growth and development; its limited amount in soil and water poses bewildering concerns amongst agronomists. Externally applied phosphorus fertilizers can fulfil crops' phosphorus needs throughout essential growth stages; however, the overapplication of phosphorus fertilizers leads to diminished phosphorus acquisition efficiency (PAE), disrupts the delicate balance of nutrients in soil and water, leads to deficiencies in other essential elements, poses significant environmental risks, and accelerates the loss of phosphorus mineral supplies. Moreover, much of the applied phosphorus may become fixed as insoluble phosphates by combining with calcium, iron, aluminum, manganese, etc., present in soil, making it unavailable for the plants. Phosphate solubilizing bacteria (PSB) can render insoluble phosphate accessible to plants by solubilization and mineralization, hence enhancing crop yields while ensuring environmental sustainability. Earthworms are vital soil invertebrates that interact continuously with soil and soil microorganisms and play an essential role in maintaining soil fertility. The present study aims to screen and identify potential phosphate solubilizing bacteria from the intestinal tract of the earthworm *Aporrectodea rosea*. The experimental results indicate that the strain PSEG-1 was effective in phosphate solubilization, with a solubilization index of 1.6 in Pikovskaya (PVK)'s medium. The strain produced organic acid in the National Botanical Research Institute (NBRIP)'s medium. Phenotypic and genotypic studies of the isolate showed that the strain PSEG-1 belongs to *Klebsiella variicola*. Our results suggest that the vermi-bacterial strain *Klebsiella variicola* PSEG-1 possesses intrinsic abilities to solubilize phosphate, which could be exploited for formulating potential microbial biofertilizers to enhance crop production.

Keywords: phosphate solubilization; NBRIP medium; *Aporrectodea rosea*; *Klebsiella variicola*

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1. Introduction

Phosphorous is a vital nutrient that limits the growth of plants despite its abundance in soil in both organic and inorganic forms. No substantial atmospheric source exists for phosphorous like nitrogen that can be made available to plants from soil [1]. Nutrition is linked to many crucial metabolic activities of plants, such as photosynthesis, signal transduction, nitrogen fixation, respiration, disease resistance, crop quality, etc. [2]. Phosphorous is also essential for root proliferation and elongation, so its deficiency affects root architecture, plant maturity, and seed development [3]. Unfortunately, the concentration of soluble P in soil is often extremely low (400–1260 mg/kg) [4]), demanding chemical

fertilizers to ensure higher crop yields to meet the rising global demand. Because of this, the total annual need for P fertilizers has been rising by 2.5–3.0% [5]. The major problem with chemical fertilizers is that a significant portion of the inorganic phosphate is bound to soil and quickly immobilizes or is lost through leaching and erosion, forming eutrophicated water bodies and ultimately becoming unavailable to plants [6]. Further, augmented P availability in the soil reduces the assimilation of cadmium and modifies the adverse effects of Cd contamination on plant growth and human health.

Disproportionate fertilizer runoff from agricultural land due to rainfall, chemical discharge, and urban life are serious environmental issues evidenced in many water bodies of Chhattisgarh, India [7]. The rate at which human activity is destroying groundwater quality is increasing alarmingly in the Sarguja district of Chhattisgarh, India [8]. Wastewater discharged from coal mines contains high amounts of phosphorus (P) in the form of phosphate (PO_4) and nitrogen as ammonia (NH_3). When mixed with municipal wastewater, mine drainage impacts the stream ecosystems in mining regions [9]. Therefore, the isolation and application of phosphate solubilizing bacteria (PSB) as bioinoculants in the mining regions can be an effective and sustainable strategy to reduce the negative impacts of coal mines in terms of phosphate waste discharge. The ability of PSB to solubilize soil-insoluble phosphates can be resolved by a variety of processes, including the secretion of organic acids, the synthesis of enzymes, and the excretion of siderophores [10]. Further, PSB can stimulate the growth of plants by generating ethylene, hydrogen cyanide, and siderophores, and hormones such as auxins, cytokinins, and gibberellic acid [11], and also due to nitrogen fixation and resistance to soil pathogens [12]. Many bacterial species, including *Pseudomonas aeruginosa* (MK 764942.1) [13], *Paraburkholderia* sp. [14], *Burkholderia cepacia* Z-7, *Pantoea* sp. J-1, and *Acinetobacter baumannii* B-6 [15] have been identified and tested as potential phosphate solubilizers under field conditions and are now being used as phosphate solubilizing biofertilizers. The fungal counterparts of the soil ecosystem, on the other hand, mainly act as phosphate mobilizers and as bridges between plants and available soil phosphates. Bacterial species have demonstrated more efficacy in phosphate solubility than fungal strains [16]. The earthworm gut contains diverse microbial species that assist the physiological process of the host and improve the soil characteristics [17]. Considering the novel source of PSB from the earthworm gut, the present study aims to isolate and characterize potent PSB found in the gut of abundant indigenous earthworm species, such as *Aporrectodea rosea*. Studying the PSB from local earthworms' guts has an important justification for using locally sourced PSB that have previously adapted to Sarguja soil types to increase their application in this part of the country.

2. Materials and Methods

2.1. Sample Collection and Characterization of Earthworms

During April 2024, mature earthworms were collected from the Shantipara area in the Bhatko region of Batauli, located within the Surguja district of Chhattisgarh, India (Figure 1). The precise geographical coordinates of the collection site are 22°57′41.8″ north latitude and 83°25′03.4″ east longitude. This location was chosen due to its soil microbiome diversity, as indicated by its higher CFU count, which is ideal for studying earthworm-related microbiological activities.

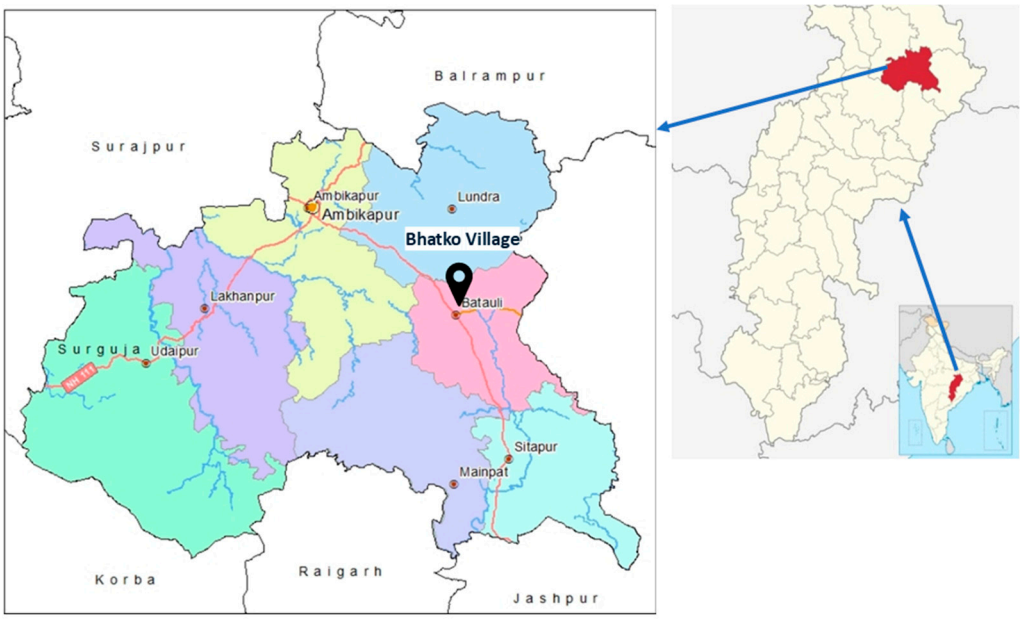

Figure 1. Location map indicating the study sites.

Sampling was carried out using a metal frame (40 × 40 cm) placed on the soil and earthworms were retrieved by digging soil with a hand sorting method. The collection was conducted manually to ensure minimal disturbance to the natural habitat of the earthworms. Approximately 100 mature individuals were sorted by hand. The development stage of each earthworm was examined. Earthworms were divided into three groups viz. sub-adult (with a full tubercula pubertatis but no clitellum), adult (with clitellate), and juvenile (without tubercula pubertatis or clitellum). The earthworms were placed in clean ventilated containers filled with moist soil to preserve their viability during transportation.

2.2. Identification of Earthworms

Earthworms were identified by species level according to standard protocols [18]. The collected earthworms were initially identified based on distinct physical and behavioral traits characteristic of the species. The earthworms were identified for their unique physical features: head coloration, body length, ventral pads, clitellum coloration, body shape, and behavioral response.

2.3. Isolation of Phosphate Solubilizing Bacteria from Earthworm Gut

The disinfected earthworms were placed in a sterile laminar flow hood, where their gut contents were surgically extracted with precision to prevent contamination. The gut contents were homogenized in sterile phosphate-buffered saline (PBS) and serially diluted up to 10^{-4}. Aliquots (0.1 mL) from each dilution were spread onto Pikovskaya's medium (pH 7.0). Qualitative analysis of the phosphate solubilization capability of the isolated strains was conducted by individually inoculating them onto Pikovskaya's agar plates. The plates were subsequently incubated at 37 °C for 3 days and monitored for the clearing zone surrounding the colonies, indicative of inorganic phosphate solubilization by bacteria.

2.4. Detection of Organic Acid Production

Colonies with a halo zone indicative of phosphate solubilization were selected and purified using the streak plate technique. Selected colonies were transferred to NBRIP-BPB agar plates containing bromophenol blue dye, which changes color from blue to yellow below pH 3.0. The plates were incubated at 30–35 °C for 72 h, with a color change indicating the release of organic acids by potential phosphate solubilizing bacteria (PSB).

2.5. Qualitative Assessment of Phosphate Solubilization

The ability of the phosphate solubilizing bacterium to break down tricalcium phosphate (TCP) was quantitatively evaluated on a specialized agar plate. A standardized formula was used to calculate the solubilization index (SI), representing the bacterium's efficiency in solubilizing TCP. The diameters of the bacterial colony and the surrounding clear area (halo zone) were measured to calculate the SI.

$$\text{Phosphate solubilizing index(SI)} = \frac{\text{colony diameter} + \text{Halo zone diameter}}{\text{colony diameter}} \quad (1)$$

2.6. Determination of Optimum pH for PSB Strain

The bacterial isolate PSEG-1 was inoculated into a Luria Bertani medium consisting of peptone 10 g, yeast extract 5 g, and sodium chloride 5 g to determine the optimum pH. Using 0.1 M HCl or NaOH, the medium's pH was changed to achieve various pHs: 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0. Three sets of experiments were run. The rise in optical density at 600 nm was used to determine optimal growth in the LB medium.

2.7. Quantitative Estimation of Phosphate and Ammonium Ion

The phosphate solubilizing potential of PSEG-1 was determined by measuring the quantity of available soluble phosphate in the Pikovskaya's broth supplemented with tri-calcium phosphate. The flask was inoculated with PSEG-1 and incubated at 35 °C, pH 7 for 5 days. The available phosphorous content of cell-free supernatant was determined, followed by the ascorbic acid method at 880 nm using a UV–VIS spectrophotometer [19]. PSEG-1 was inoculated in peptone water and incubated with constant shaking at 140 rpm for 7 days at 30 °C. An uninoculated medium served as the control. Every 24 h, aliquots of 3 mL were taken out of each culture flask. For ten minutes, the samples were centrifuged at 9500 rpm. The phenol hypochlorite method was used to assess the amount of ammonium ion colorimetrically generated. Triplicate sets were employed in the experiment.

2.8. Morphological and Biochemical Characterization of Potent Isolate

Morphological and biochemical tests of the potent phosphate solubilizing bacterial isolate were carried out for identification per the methods defined in *Bergey's Manual of Determinative Bacteriology*.

2.9. Molecular Characterization of Potent Isolate

Genomic DNA was extracted using the Xploregen gDNA Extraction Buffer™ (Xploregen Discoveries Pvt. Ltd., Bangalore, India), following the manufacturer's protocol, which includes vortexing, centrifugation, and purification steps. The acquired 16S rRNA gene sequence of the isolated strain was analyzed using NCBI–BLAST. The BLAST results identified the isolated strain as *Klebsiella variicola* based on its similarity percentage in NCBI. A phylogenetic tree was constructed using the neighbor-joining method in MEGA. The phylogenetic tree (Figure 2) verified the highest proportion of similarity with *Klebsiella variicola*.

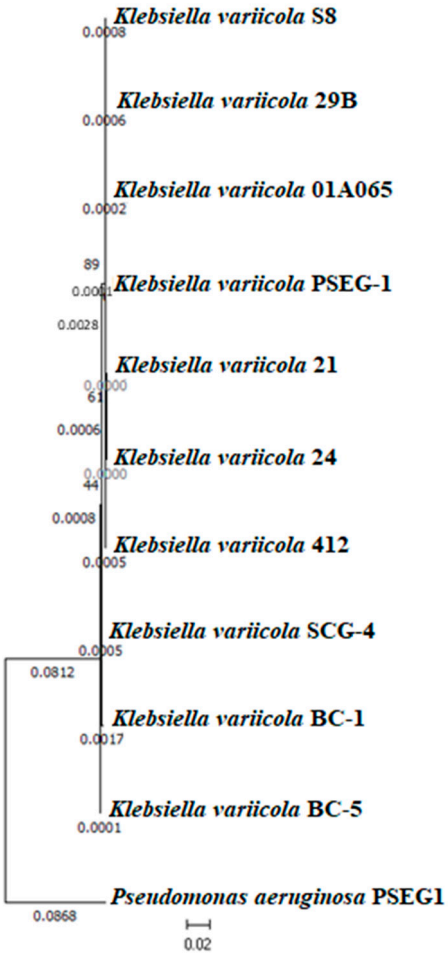

Figure 2. Phylogenetic tree showing the evolutionary relationship of *Klebsiella variicola* PSEG-1. The tree was constructed using the neighbor-joining method, with 1000 bootstrap replicates.

3. Results

3.1. Morphological Characteristics of the Earthworm

The anterior segment of the earthworm exhibited a vibrant rosy-pink color, which is a key diagnostic feature of this species. The earthworms measured 10–13 cm, typical for mature individuals of *Aporrectodea rosea*. The ventral surface of the earthworms showed whitish-red colored pads associated with the setae, further supporting the species identification. The clitellum, a saddle-like structure near the anterior end of the earthworm, displayed a light orange tint, another distinctive characteristic of the species. The earthworm's body was slightly wider towards the posterior end, a common morphological feature of *Aporrectodea rosea*. Upon handling, the earthworms exhibited a typical defensive behavior by curling into a tight protective posture, a typical response to stress disturbance (Figure S1).

3.2. Isolation of Phosphate Solubilizing Bacteria

The collected gut samples were plated on Pikovskaya (PKV)'s agar plates for PSB. Out of eight colonies, PSB isolate PSEG-1 was a potent phosphate solubilizer, showing a clear halo zone around its colony. The isolate PSEG-1 showed a 2.00 mm halo zone around its colony (Table 1). The solubilization index (SI) of all isolates was calculated at the end of the incubation period, and the PSEG-1 isolate showed the highest phosphate solubilization index (SI) of 1.6. The halo zone indicates the production of organic acids or the presence of phosphatase enzymes that solubilize phosphates [20]. The creation of the halo zone

may result from chemicals generated by neighboring bacteria during growth, which can solubilize phosphate. The effectiveness of phosphate solubilization by specific bacteria may fluctuate depending on the types of secondary metabolites and the rate at which they are released and disseminated in the medium. Zhu et al. (2011) asserted that the development of a distinct zone surrounding the colony signifies the qualitative testing [21].

Table 1. Qualitative estimation for phosphate solubilization efficiency of isolates obtained from earthworm gut.

S. No.	Mean Colony Diameter (cm)	Mean Halo Zone Diameter (cm)	Phosphate Solubilization Index
1	0.5	0.2	1.4
2	0.3	0.2	1.6
3	0.6	0.3	1.5
4	0.4	0.2	1.5
5	0.5	0.1	1.2
6	0.8	0.3	1.3
7	0.5	0.2	1.4
8	0.5	0.1	1.2

3.3. Effect of pH on Isolated PSB Strain

The optimum pH for attaining the maximum growth was 7 (Figure S2). However, the organisms might also flourish in a pH range of 6 to 9. PSEG-1 could withstand slightly greater pH levels of alkalinity (8–9).

3.4. Quantitative Estimation of Phosphate Solubilization

The PSEG-1 strain was found to possess high intrinsic phosphate solubilizing efficiency. The quantitative analysis was recorded at optimized conditions up to the fifth day of incubation. The findings showed that PSEG1 can solubilize phosphates up to 200.46 µg/mL. The solubilized phosphate concentration increased gradually from 24 h to 120 h. The phosphate was solubilized at its maximum at 96 h of incubation (Figure S3).

3.5. Quantitative Estimation of Ammonium Ion

The production of ammonium ions rose with the increase in the incubation period. PSEG-1 produced 124 $mg \cdot L^{-1}$ ammonium ions after 5 days of incubation. The production reached its saturation point after 5 days of incubation (Figure S3).

3.6. Morphological, Biochemical, and Molecular Characterization of the Bacterial Isolates

The morphological and biochemical characteristics of isolate PSEG-1 are outlined in Table 2. 16S rRNA sequencing was performed to identify the PSEG-1 isolate. The sequenced 16S rRNA gene fragment was analyzed using BLAST from the NCBI GenBank database, revealing a high similarity (99.68%) with *Klebsiella variicola* strain 01A065 isolate SB1 (Sequence ID: HG933294). BLAST analysis revealed the alignment of the 16S rRNA sequence with several species of the genus *Klebsiella*; we used the 10 closest homologs to construct a phylogenetic tree using the neighbor-joining method (Figure 2). It was evident from the phylogenetic tree that the isolate PSEG-1 lay in the gamma-proteobacteria order of Klebsiella and shared a close relation with its nearest neighbors. Therefore, it was concluded that the isolated organism was *Klebsiella variicola* strain PSEG-1. Biswas et al. [22] previously isolated three phosphate solubilizing bacterial strains, designated as PSB1, PSB2, and PSB3, from the gut of the earthworm *Metaphire posthuma*. The three stains were identified as *Bacillus megaterium* (MF589715), *Staphylococcus hemolyticus* (MF589716), and *Bacillus licheniformis* (MF589720) through 16S rRNA gene sequencing and biochemical characterization.

Table 2. Biochemical characteristics of isolate PSEG1.

S. No	Biochemical Tests	Results
1	Cell morphology	Rod-shaped
2	Gram staining reaction	−ve
3	Oxidase activity	+ve
4	Catalase activity	+ve
5	Indole production test	−ve
6	Methyl red test	−ve
7	Voges–Proskauer test	−ve
8	Citrate utilization test	+ve
9	H_2S production test	−ve
10	Urease activity	−ve

4. Discussion

Phosphorus is a major limiting nutrient in soil and water, and phosphorus fertilization in agricultural land is required for fulfilling its deficiency. However, soil phosphorus becomes reduced in due course due to leaching, conversion of phosphorus to insoluble form, immobilization of phosphorus in biomass, and run-off in rainwater. Increases in soil pH, improper drainage and aeration, low temperature, reduced organic content of soil, etc., can further complicate phosphorus deficiency. Integrated soil nutrient management employing phosphate solubilizing bacteria could help in ameliorating soil phosphate deficiency in an eco-friendly manner. Phosphate solubilizing bacteria has garnered interest in the past few decades and its use as a biofertilizer has been found to serve a multifaceted role in improving soil phosphorus utilization efficiency, fostering crop growth and development, and facilitating stress tolerance [23]. Soil inoculated with these strains significantly improves the phosphate utilization for plant fertilization, which could bypass the use of hazardous chemical fertilizers and lower the economic burden on agriculture. However, most phosphate solubilizing bacteria lose their solubilization capability during subculturing, so identifying a potent phosphate solubilizing bacteria is difficult [24]. Therefore, identifying potential PSB is vital for sustainable agricultural practice. Previous studies by [16] reported several bacterial strains, such as *Bacillus megaterium*, *Staphylococcus haemolyticus*, and *Bacillus licheniformis*, from the gut of the endogeic earthworm *Metaphire posthuma*, which can possess strong phosphate solubilization abilities. In the present study, the isolated earthworm gut bacteria *Klebsiella variicola* PSEG-1 demonstrated considerable phosphate solubilization activity even after repeated subcultures. Similarly, Saranya et al., 2022 [25], isolated a potent PSB *Curtobacterium luteum* from the marine environment that retains solubilization potential even after multiple subcultures, similar to the present study.

Regarding the incubation time needed for P solubilization, the results in the preliminary investigation were consistent with previous publications, which found that the maximum phosphate solubilization efficiency of isolated *Bacillus* sp. was after 96 h of incubation [26]. This could result from the strains' exponential phase of cellular growth. Additionally, it was noted that the change in pH has been directly linked to phosphate solubilization, similar to the findings obtained in *Pseudomonas aeruginosa* and fungus *Trichoderma* sp. [27]. The current study determined that *Klebsiella variicola* PSEG-1 grows in the pH range of 6–9, corroborated by several prior investigations. Zhu et al. (2011) observed the optimal development of a PSB *Kushneria* sp. YCWA18 at pH 8 [21]. The qualitative study indicated that the solubilization index (SI) was 1.6 (for strain PSEG-1). The creation of the halo zone may result from chemicals generated by neighboring bacteria during growth, which can solubilize phosphate. The effectiveness of phosphate solubilization by specific bacteria may fluctuate depending on the types of secondary metabolites and their rate of release and dispersion in the medium.

Previous studies demonstrated that various low molecular weight organic acids such as 2-keto gluconic and gluconic acid, lactic acid, oxalic acid, isovaleric acid, isobutyric acid, acetic acid, and citric acid, etc., produced by microorganisms are the primary cause of phosphate solubilization [28]. Phosphates in soluble form and ammonia directly support plant growth as macronutrients [29]. *Klebsiella variicola* PSEG-1 was able to produce ammonia with a longer incubation period (3–5 days).

Despite the ecological roles of phosphate solubilizing bacteria, their efficacy in agricultural settings diverges from laboratory results, presenting hurdles for practical implementation [30]. The unique ability of *Klebsiella varicola* PSEG-1 to retain its phosphate solubilizing capacity even after multiple subcultures is of great significance for agricultural applications. This characteristic indicates that the isolated strain can be used in agricultural applications without losing its effectiveness over time and, therefore, serves as a potential candidate for the preparation of biofertilizers. This led researchers to investigate avenues for further research on the genome and metabolic characterization of PSEG-1 to identify the mechanism behind the unique characteristics. An in-depth understanding of the mechanisms of action, colony dynamics, and functions of PSB is needed to facilitate their use as biofertilizers, expand their contribution to sustainable agriculture, and address farmers' concerns.

5. Conclusions

In the present study, we isolated potent PSB from the gut of the earthworm *Aporrectodea rosea*. The isolated strain *Klebsiella variicola* PSEG-1 showed promising results in plant growth, promoting potentials like organic acid production. This bacterium provides a sustainable substitute for chemical fertilizers by transforming insoluble phosphorus into a form that is accessible to plants, enhancing soil health, and lowering pollution levels in the environment. Although the utilization of PSB to address soil phosphorus needs yields gratifying results in laboratory or greenhouse settings, farmers may encounter certain difficulties in field trials. Some concerns that scientists and farmers must address for the development and effective deployment of microbial biofertilizers are as follows: (i) the intricate interactions and competition between inoculated PSB strains and other microbial inhabitants of the soil must be considered to prevent inadequate colonization or exclusion; (ii) soil constituents influence the solubilization and mineralization of phosphorus by microorganisms. Comprehending the type of phosphorus source in the soil and the appropriate phosphorus solubilization mechanism related to that source will facilitate the effective selection of phosphorus solubilizing isolates. More research is necessary to fully understand the benefits of PSB, including how best to apply them in various agricultural situations and how they interact with other soil microbes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bacteria4010005/s1, Figure S1: Morphological characteristics of *Aporrectodea rosea*. Figure S2: Effect of pH on the growth of PSEG1. Figure S3: Phosphate solubilization and ammonia production potential of PSEG1.

Author Contributions: Conceptualization, A.K.P., S.D.M. and S.S.B.; methodology, V.K. and A.K.P.; software, A.K. and A.K.P.; writing—original draft preparation, A.K.P., S.D.M. and S.S.B.; writing—review and editing, A.K.P., S.D.M. and S.S.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for the study entitled "Molecular Characterization of Phosphate-Solubilizing Bacteria *Klebsiella variicola* PSEG-1 associated with *Aporrectodea rosea* gastrointestinal tract" which includes earthworms. As per the Guidelines on the Regulation of Scientific Experiments on Animals, Ministry of Environment & Forests

(Animal Welfare Division) Government of India, June 2007 the approval of the Ethics Committee is not required for species of invertebrates (which include earthworms), anything higher than invertebrates in terms of the level of sentience requires regulation.

Informed Consent Statement: Not Applicable.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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