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(RESEARCH ARTICLE)

Isolation and biochemical screening of Phosphate Solubilizing Bacteria from Kans grass rhizosphere of Fly Ash Dump Sites near NTPC, Angul

Karubakee Dhal, B S Sipra, Bandita Pati and Sanhita Padhi *

Biochemistry and Acoustics Laboratory, PG Department of Botany, Ravenshaw University, Cuttack, Odisha-753003, India.

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Abstract

Soil bacteria, capable of converting insoluble phosphorus into soluble, plant-accessible forms, collectively known as phosphate-solubilizing bacteria (PSB), have emerged as an eco-friendly and cost-effective solution for supplying phosphorus to plants. This study presents preliminary insights into the rhizospheric bacterial population associated with the vigorously growing kans grass (*Saccharum spontaneum* L.) at different fly ash disposal sites of NTPC, Kaniha in Angul district, Odisha. In the quest for efficient plant growth promoting rhizobacteria (PGPR) strains with diverse beneficial activities, a total of sixteen bacterial isolates were collected from the rhizosphere of chosen plant species and identified, with only ten isolates underwent biochemical characterization. Among these, four isolates exhibiting a higher phosphate-solubilizing index were further characterized, serving as a foundation for future exploration of the bio-prospective potential of rhizosphere bacteria in this unique ecological niche. Further assessment of these isolates, showcasing several plant growth-promoting (PGP) traits, is essential to determine their efficacy as potent PGPR agents.

Keywords: Phosphate solubilizing bacteria; Kans grass; Fly ash; Rhizosphere; PGPR

1. Introduction

The rhizosphere, a thoroughly studied ecological microenvironment, refers to the soil volume surrounding plant roots characterized by its abundant bacterial populations influenced by root exudates [8]. It is a common phenomenon to observe bacterial populations within the rhizosphere that are 100 to 1,000 times more abundant compared to the surrounding bulk soil. This disparity in population can be attributed to the metabolic adaptability of these bacteria, enabling them to efficiently utilize the diverse root exudates. The role of microorganisms of rhizosphere in exerting both direct and indirect impacts on soil characteristics through their advantageous or detrimental actions.

Phosphorus (P) stands as one of the pivotal plant nutrients, significantly shaping overall plant growth by influencing on a range of critical metabolic processes, including cell division and development, macromolecular synthesis, photosynthesis, energy transfer, signal transmission, and plant respiration [24]. In contrast to other nutrients, the concentration of P in the soil solution is notably lower, typically falling within the range of 0.001 to 1 mg/l [2]. Phosphorus (P) serves as a primary nutrient that frequently restricts plant growth, and there is no significant atmospheric source that can be readily converted into a biologically accessible form [3]. Some species of bacteria have the potential to mineralize and solubilize organic and inorganic phosphorus in soil [9]. The predominant group of reputable PGPR mainly comprises genera such as *Acinetobacter, Agrobacterium, Arthobacter, Azotobacter, Xanthomonas, Rhodococcus, Azospirillum, Burkholderia, Bradyrhizobium, Rhizobium, Frankia, Serratia, Chryseobacterium, Gordonia, Phyllobacterium, Thiobacillus, Pseudomonads, and Bacillus* [15]. These genera are recognized for their substantial phosphorus (P) solubilization capabilities in addition to a range of activities that promote plant growth [4][5][23]. Many studies have focused on identifying highly efficient phosphate-solubilizing bacteria (PSB). The

* Corresponding author: Sanhita Padhi

majority of PSB fall into the category of Gram-negative bacteria, primarily *Pseudomonas, Acinetobacter and Pantoea* [10][11][18]. Additionally, some PSB are Gram-positive bacteria, such as Bacillus [7][25].

The finest particulate byproduct resulting from the coal combustion in various thermal power plants and industrial facilities is well known as fly ash. During combustion process, the alternation of mineral components within coal particles leads to the formation of fly ash. Consequently, the substantial production of fly ash, presents a multitude of challenges associated with disposal. It is worth noting that fly ash is still predominantly regarded as a waste byproduct. In India, accounting for 85% of the total fly ash generated, which is expected to surge to approximately 180 million metric tons per annum following the completion of proposed power plants, necessitating the allocation of 120,000 hectares of area for dumping [1]. The state of Odisha stands out with its substantial coal reserves, accounting for over 24 percent of the nation's total coal reserves. Notably, the largest individual coalfield in India, known as the Talcher coalfield, boasts approximately 39.64 billion metric tons of coal reserves [21].

The present research was conducted on the fly ash landfill that has been spontaneously revegetated with *Saccharum spontaneum* L. grass, which are great soil binders, minimize erosion, and improve slope stabilisation of fly ash dumps. *S. spontaneum* L. is a potential grass known to be an effective coloniser of wastelands and marginal regions, due to its high biomass yield and robust root structure [16][17]. The choice of resilient plant species, alongside microorganisms that serve a key role in initiating early ecosystem development, is influenced by their functional capabilities, which encompass nitrogen fixation and organic matter recycling, and their potential to facilitate the establishment of plants. With a focus on addressing the environmental considerations mentioned above, this research was conducted to investigate the ability of rhizobacteria in the rhizosphere of prolifically growing *S. spontaneum* within a heavily metal-contaminated fly ash dump sites to solubilize phosphate and exhibit diverse plant growth-promoting traits.

2. Materials and methods

2.1. Sample collection

Plant samples, collectively with their attached rhizosphere fly ash samples (aggregates adhering to the roots), collected in from different fly ash dump sites of Thermal Power Station of Kaniha (NTPC) is in Angul district of Odisha, India. Geographical co-ordinates of NTPC, Kaniha is 21.0930^0 north, 85.0818^0 east. After collection of Soil samples, the further procedure follows immediate storage at 4 °C in plastic bags. These bags were loosely knotted to enable proper aeration and moisture retention until the bacterial colony was isolated.

2.2. Isolation of phosphate solubilizing bacterial strain

1 g dry weight of samples of the rhizosphere zone was suspended in 9.0 ml of sterile distilled water used to provide a dilution series from 10^{-1} up to 10^{-7}. The bacterium was isolated, then preliminary screening continued with serial dilution using a standard spread plate technique on Pikovskaya's agar (PKA) medium consisting of constituents: glucose 10 g; tri-calcium phosphate (TCP) 5 g; yeast extract 0.5 g; ammonium sulphate 0.5 g; potassium chloride 0.2 g; sodium chloride 0.2 g; magnesium sulphate 0.1 g; ferrous sulphate trace; manganese sulphate trace; agar agar 15 g; distilled water 1 L [19]. pH 7.0±0.2 was maintained. Incubation period of 48 hours with maintained temperature at 28± 2 °C, leads to the formation of halo zone around the culture. The distinct colony with halo zones was collected. The bacterial colonies showing clear zones form around them, were considered as phosphate solubilizing bacteria (PSB). Pure cultures of the isolates were made by repeated subculturing for 2–3 times on fresh PKA plates and were maintained on PKA slants at 4 °C. PSB were expressed as colony forming unit (CFU) per gram of dry soil weight. These isolated colonies were maintained on Pikovskaya's agar medium with 30% glycerol on cryopreservation tubes at -20 °C for further study.

2.3. Qualitative and quantitative estimation of phosphate solubilization

The Phosphate solubilizing index (PSI) was used to provide a qualitative estimate of phosphate solubility. Each isolate's 10 µl inoculum was spotted on Pikovskaya's agar solid medium with 5.0 g/L TCP as a sole source of P and cultured at 30 °C for 7 days. The sterile media served as a control.

PSI was calculated according to formula by Purnomo et al., (2021)

$$PSI = (C+H)/C, \quad (C = \text{Colony diameter}; H = \text{Halo zone diameter}).$$

The phospho-molybdate blue colorimetric technique, developed by Murphy and Riley in 1962, was used to quantitatively test tricalcium phosphate (TCP) solubilization. Pikovskaya's broth (100 ml) was adjusted to pH 7 and made with sucrose and TCP (0.3 g/100 ml) and distributed into 250-ml flasks. Following sterilisation, the flasks were inoculated with exponentially growing bacterial cells and put on a rotary shaker for 120 hours. At certain time (24hrs) interval, 5 ml of culture broth was taken and centrifuged (10,000 rpm/15 minutes). The resultant supernatant was collected and analysed. The available phosphorus (P) concentration in the supernatant was quantified using a spectrophotometer (882 nm), and the results were calibrated using standard KH_2PO_4 curves. The pH of the supernatant was recorded as well at each time point [13].

2.4. Preliminary screening of bacterial strains

Morphological and biochemical tests were performed on the chosen phosphate solubilizing bacterial isolate to identify it using the procedures described in Bergey's Manual of Determinative Bacteriology.

3. Results and discussion

Following screening for p-solubilizing activity, the top ten PSB were chosen from a total of sixteen PSB for biochemical assays and future research. The isolates were tested for phosphate solubilization activity using tricalcium phosphate as the only source of phosphorus. The abundance of numerous plant growth-promoting bacteria (PSB) in the soil serves as a crucial indicator of their ability to effectively enhance crop growth and support sustainable agricultural development [26]. Clear halo zones formed surrounding the colony, indicating its ability to solubilize tricalcium phosphate. This might be caused by the formation of organic acids or polysaccharides, or the action of phosphatase enzymes [5]. The average solubilization index of the most effective isolate of each species is presented in Table1. The findings were established based on the zone of clearing, computing the solubilization index in mm. The SI of the isolates ranges from 6mm to 18mm. Isolate PSB5 showed highest PSI and PSB10 showed lowest PSI (table 1). From the results it is concluded that PSB5 was efficient phosphate solubilizer and PSB10 was less efficient phosphate solubilizer as compared to rest other nine PSB isolates of the region. The colonies showing maximum diameter of halo zone were further selected, sub-cultured, purified and maintained in agar plates.

Rhizodeposition promotes the growth of gram-negative bacteria, making them more mobile, whereas it inhibits the activity of gram-positive bacteria. The rhizosphere of various crop species demonstrates a stronger association with gram-negative rhizobacteria compared to gram-positive rhizobacteria [14]. Morphological and biochemical characterization of PSRB strains revealed that the eight isolates, out of the below mentioned 10 bacteria strains, were gram-negative rods, while PSB4 and PSB6 was identified as gram-positive rods (table1). These findings are comparable with those reported by Mujahid et al. (2014), who found that gram-negative PSRB strains outnumber gram-positive ones.

Table 1 Phosphate solubilizing index (PSI) at 5th day of incubation

Isolates	Gram staining	colony diameter (mm)	Halozone diameter (mm)	Phosphate Solubulizing Index (PSI)
PSB1	-ve	5	11.5	3.3
PSB2	-ve	5	13	3.6
PSB3	-ve	4	9	3.25
PSB4	+ve	5	14	3.8
PSB5	-ve	4	15	4.75
PSB6	+ve	6	10	2.66
PSB7	-ve	7	9	2.28
PSB9	-ve	5	10	3
PSB10	-ve	6	9	2.5
PSB11	-ve	4	7	2.75

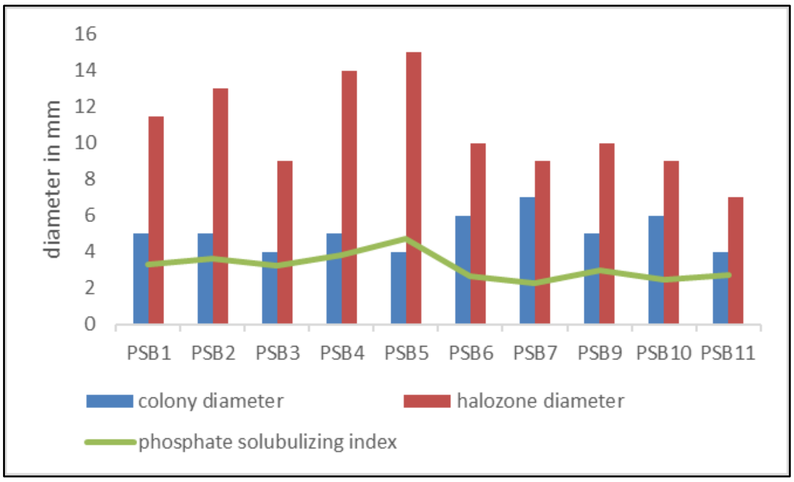

Figure 1 Phosphate solubilizing activity of different isolates

Figure 2 Pure culture of selected bacteria strains (PSB2, PSB7, PSB3, PSB1 respectively) showing growth with halo zone on selective media

Most colonies had a clear opacity, resulting in milky, white, or yellow tint on the agar plates. The bacterial colonies had round to irregular forms with elevated elevations. Of the ten PSB isolates, eight were gram-negative and two were gram positive. The results of different biochemical tests for four PSB isolates with comparatively higher phosphate solubilizing index were summarized in figure 1.

The biochemical test conducted for all the ten isolates and the result of only four isolates with higher phosphate solubilizing index are represented in table 2. Except PSB4, rest three isolates (PSB1, PSB2, PSB3) are gram negative. gram-negative bacteria are attracted to root exudates, which increases their population around the roots and results in the release of substances beneficial for plant growth. In contrast, gram-positive bacteria, being aerobic, experience a decrease in their population around the roots due to the limited oxygen availability [6]. Plants constantly face environmental stress due to their stationary nature. To combat these challenges and counteract the impact of hydrogen peroxide, PGPR strains generate an enzyme known as catalase [6]. Among the selected four isolates, three isolates (PSB1, PSB2, PSB5) were found to be catalase positive and PSB4 was found to be catalase negative.

Table 2 A-shape, B- nitrate reduction, C- citrate utilization, D- IAA production, E- hydrogen sulphide test, F- starch hydrolysis test, G- catalase activity, H- Voges Proskauer test

Strain	A	B	C	D	E	F	G	H
PSB1	Rod	+ve	+ve	-ve	-ve	+ve	+ve	+ve
PSB2	Rod	-ve	+ve	-ve	+ve	+ve	+ve	-ve
PSB4	Rod	+ve	+ve	-ve	-ve	+ve	-ve	-ve
PSB5	Rod	-ve	+ve	-ve	+ve	+ve	+ve	-ve

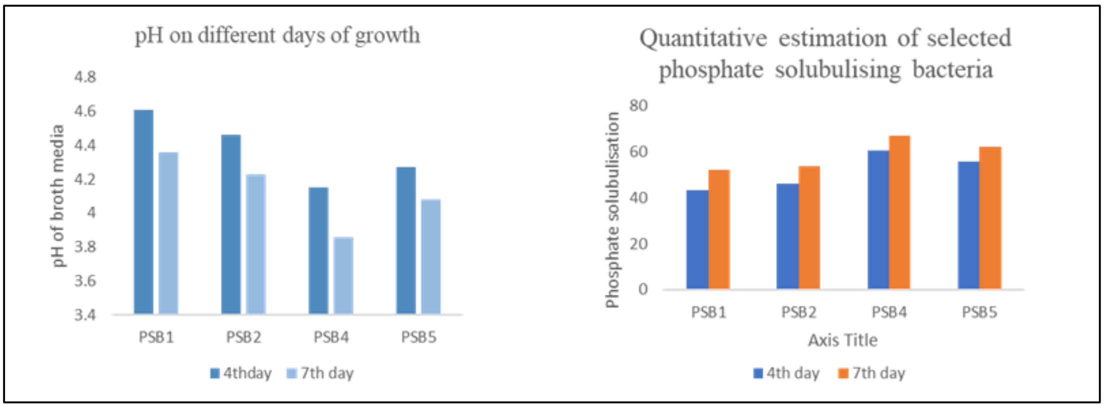

Figure 3 The phosphate solubilization efficiency (shown as concentration of free phosphate), and the concomitant decrease of pH in the PKV medium

The selected strains exhibited TCP solubilization in the liquid medium, leading to a decrease in pH from initial neutral value of 7.0 to different acidic levels ranging between 4.15 and 4.85 after 4 days of incubation. In the control treatment, no soluble phosphorus (P) was detected, and there was no observable change in pH. Conversely, the test cultures showed a notable decrease in pH. On the fourth day of incubation, strain PSB4 exhibited the highest phosphorus (P) solubilization, recording 64 mg/100ml with a corresponding pH decrease to 4.15. This was followed by strain PSB5, which recorded 56 mg/100ml of solubilized P and a pH drop to 4.27. In contrast, strain PSB1 showed the lowest concentration of soluble P at 43.5 mg/100ml, accompanied by a minimal pH drop to 4.61 on the same day of incubation. The observed decrease in the pH of the culture medium could serve as indirect evidence suggesting the presence of organic acids. Phosphorus solubilization is an important trait implicated in plant growth-promoting rhizobacteria that boost the nutrient availability to the host plant [22]. P is a significant constraint on agricultural productivity. The addition of a large quantity of phosphorus (P) fertilizer to soil not only raises the cost of agriculture but also leads to environmental issues [7][27], which is damaging to agriculture's sustainable growth. PSB possesses a remarkable capacity to convert insoluble phosphorus in the soil into a soluble form, making it readily available. This feature makes PSB very promising for implementation in eco-agriculture.

4. Conclusion

As we have isolated and characterized different bacteria capable of plant growth promoting traits, considering more efficient phosphate solubilizing activity, we go by the fact that all the four strains PSB1, PSB2, PSB4 and PSB5 are potential candidate for further investigation. These bacterial strains can give useful insights on their modes of action, effectiveness in varied soil conditions, and possible uses in sustainable agriculture. It would be beneficial to conduct experiments to understand the interactions between these bacterial strains and *S. spontaneum* in more detail. This could involve research on their colonization patterns, their effect on plant growth under different environmental conditions, and their ability to remediate specific contaminants in soil. Overall, these findings open exciting possibilities for using *S. spontaneum* of fly ash dump sites and its associated phosphate-solubilizing bacteria in eco-friendly agricultural practices and soil remediation strategies.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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