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Screening of azotobacter, bacillus, and pseudomonas species for biofertilizer production

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Abstract
The increasing demand for sustainable agricultural practices has prompted the exploration of plant growth-promoting bacteria (PGPB) as eco-friendly alternatives to chemical fertilizers. This study aimed to isolate, characterize, and screen Azotobacter, Bacillus, and Pseudomonas species for their plant growth-promoting activities, including nitrogen fixation, phosphate solubilization, IAA production, and antifungal activity. Soil samples were collected from the rhizosphere of wheat, maize, and rice crops at SDMVMS College of Agricultural Biotechnology, Aurangabad. The isolates were characterized morphologically and biochemically, confirming their identity as Azotobacter, Bacillus, and Pseudomonas. Azotobacter demonstrated significant nitrogen fixation, as evidenced by ethylene production and ammonia detection. Bacillus and Pseudomonas exhibited phosphate solubilization, with Pseudomonas showing slightly higher solubilization indices. All isolates produced IAA, with Azotobacter showing the highest production. Antifungal activity was observed in all isolates, with Bacillus exhibiting the strongest inhibition against Fusarium oxysporum and Rhizoctonia solani. These findings highlight the potential of Azotobacter, Bacillus, and Pseudomonas species in enhancing crop growth and soil health, offering a sustainable approach to improving agricultural productivity.

Keywords: Sustainable agriculture, PGPB, azotobacter, bacillus, pseudomonas, IAA, antifungal

Introduction

The increasing demand for agricultural productivity to meet the food requirements of a growing global population necessitates the adoption of sustainable and eco-friendly practices. Excessive reliance on chemical fertilizers and pesticides has led to environmental degradation, reduced soil fertility, and negative impacts on human health (Tilman *et al.*, 2002). To mitigate these challenges, plant growth-promoting bacteria (PGPB) have emerged as a promising alternative, offering a sustainable approach to enhancing crop growth and yield while reducing chemical inputs (Vessey, 2003) [1]. Among the PGPB, *Azotobacter*, *Bacillus*, and *Pseudomonas* species have garnered significant attention for their beneficial roles in agricultural systems.

Azotobacter species are free-living nitrogen-fixing bacteria that enhance soil fertility by converting atmospheric nitrogen into forms accessible to plants (Wani *et al.*, 2016) [2]. They also produce growth-promoting substances such as gibberellins, cytokinins, and vitamins, which improve plant development and stress tolerance (Kannaiyan, 2002). *Bacillus* species are renowned for their ability to solubilize phosphorus, an essential nutrient often immobilized in soil (Rodríguez & Fraga, 1999) [3]. They also produce antimicrobial compounds, enzymes, and bioactive metabolites that suppress soil-borne pathogens, enhancing plant growth and resilience (Kloepper *et al.*, 2004) [4]. *Pseudomonas* species, particularly fluorescent pseudomonads, are vital in promoting plant health by producing siderophores that chelate iron and enhance its availability to plants (Sharma *et al.*, 2003) [5]. They also synthesize phytohormones like indole-3-acetic acid (IAA) and exhibit biocontrol properties against a wide range of pathogens through the production of secondary metabolites such as antibiotics and hydrogen cyanide (Compant *et al.*, 2005) [6].

This study aims to isolate, characterize, and screen *Azotobacter*, *Bacillus*, and *Pseudomonas* species for their plant growth-promoting activities, including nitrogen fixation, phosphate solubilization, siderophore production, and biocontrol capabilities. By identifying efficient strains, this research seeks to contribute to the development of eco-friendly biofertilizers and sustainable agricultural practices, aligning with global efforts to achieve environmental and food security.

Materials and Methods

Soil Sample Collection

Soil samples were collected from the rhizosphere of healthy crops, including wheat (*Triticum aestivum*), maize (*Zea mays*), and rice (*Oryza sativa*), in the agricultural field of SDMVMS College of Agricultural biotechnology, Chhatrapati Chhatrapati .sambhajinagar (Aurangabad) (MH). Samples were taken at a depth of 10-15 cm using sterile tools, placed in sterile containers, and stored at 4°C for further use (Mahato *et al.*, 2017) [15].

Isolation of Bacterial Strains Azotobacter: Isolated using Ashby's nitrogen-free agar medium supplemented with glucose. Plates were incubated at 28°C for 48-72 hours. Colonies with characteristic mucoid morphology were selected (Subba Rao, 1982) [16]. Bacillus: Soil samples were subjected to heat treatment at 80°C for 10 minutes to select spore-forming bacteria. Isolates were grown on a nutrient agar medium and incubated at 30°C for 24-48 hours (Hayat *et al.*, 2010) [17]. Pseudomonas: Isolated on King's B agar medium and incubated at 28°C for 24-48 hours. Fluorescent colonies were confirmed under UV light (King *et al.*, 1954) [9].

Morphological and Biochemical Characterization Morphological characteristics of the isolates were observed under a microscope after Gram staining. Biochemical tests, including catalase, oxidase, and starch hydrolysis, were performed to confirm preliminary identification (Cappuccino & Sherman, 2005).

Screening for Plant Growth-Promoting Traits

Nitrogen Fixation (Azotobacter)

Nitrogen fixation potential was evaluated by the acetylene reduction assay, which quantifies ethylene production as an indicator of nitrogenase activity (Hardy *et al.*, 1968) [7]. Ammonia production was further confirmed using Nessler's reagent (Bremner & Mulvaney, 1982) [8].

Phosphate Solubilization (Bacillus and Pseudomonas)

The ability to solubilize inorganic phosphate was tested on Pikovskaya's agar medium. Clear zones around bacterial colonies indicated positive phosphate solubilization, and the solubilization index was calculated (Pikovskaya, 1948) [48].

Indole-3-Acetic Acid (IAA) Production (All Isolates)

IAA production was quantified using Salkowski's reagent. Cultures were supplemented with L-tryptophan (5 mM) and incubated for 48 hours. The pink color intensity was measured at 530 nm (Gordon & Weber, 1951) [11].

Antifungal Activity (All Isolates)

Antifungal activity was tested against *Fusarium oxysporum* and *Rhizoctonia solani* using the dual-culture method. Zones of inhibition were measured to assess the biocontrol potential (Dennis & Webster, 1971) [10].

Results and discussion

Isolation and Morphological Characterization

Azotobacter: The colonies isolated from Ashby's nitrogen-free agar medium exhibited typical mucoid morphology, a characteristic of Azotobacter species. The isolates were Gram-negative and showed positive reactions for catalase and oxidase enzymes, confirming their identity as Azotobacter. These findings align with previous reports of Azotobacter's nitrogen-fixing potential and its role in enhancing soil fertility through atmospheric nitrogen fixation (Wani *et al.*, 2016) [2].

Bacillus

Bacillus isolates were obtained after heat treatment, consistent with the method used to enrich spore-forming bacteria. The colonies grew on nutrient agar as large, opaque, circular colonies. These isolates were Gram-positive rods, which is typical for Bacillus species. These findings align with previous reports by Rodríguez & Fraga, 1999 [3].

Pseudomonas

Pseudomonas isolates were obtained from King's B agar medium. The colonies exhibited fluorescence under UV light, confirming their identity as Pseudomonas species. These Gram-negative bacteria are well-known for their production of siderophores, which enhance iron availability to plants.

Biochemical Characterization

Azotobacter

The Azotobacter isolates showed catalase and oxidase positivity, which are standard biochemical tests. Additionally, ammonia production was detected using Nessler's reagent, indicating that these isolates possess nitrogen-fixing capabilities, consistent with findings by Hardy *et al.* (1968) [7] and Wani *et al.* (2016) [2].

Bacillus

Biochemical tests for Bacillus isolates showed catalase activity and positive results for starch hydrolysis, indicating their ability to produce enzymes that may aid in plant growth by breaking down complex organic matter in the soil.

Pseudomonas

The Pseudomonas isolates were catalase and oxidase-positive, confirming their identity.

Screening for Plant Growth-Promoting Traits

Nitrogen Fixation (Azotobacter)

The nitrogen fixation potential of Azotobacter was evaluated using the acetylene reduction assay, which showed significant ethylene production, indicating active nitrogenase activity. This result supports earlier studies showing that Azotobacter plays a crucial role in nitrogen fixation in agricultural soils. Furthermore, ammonia production was confirmed using Nessler's reagent, which is a widely used method for detecting nitrogen fixation in soil bacteria. These results are consistent with Azotobacter's known ability to enhance soil fertility by fixing atmospheric nitrogen (Wani *et al.*, 2016) [2].

Phosphate Solubilization (Bacillus and Pseudomonas) Both Bacillus and Pseudomonas isolates exhibited significant phosphate solubilization on Pikovskaya's agar medium. The formation of clear zones around bacterial colonies indicated positive phosphate solubilization, with Bacillus isolates showing solubilization indices ranging from 1.5 to 2.2, while Pseudomonas isolates showed indices between 1.7 and 2.5. These results are in line with those of Rodríguez and Fraga (1999) [3] who demonstrated Bacillus' ability to solubilize phosphate, thereby improving phosphorus availability to plants.

Indole-3-Acetic Acid (IAA) Production (All Isolates)

All bacterial isolates produced IAA, a plant growth-promoting hormone. The highest IAA production was observed in Azotobacter (55 µg/mL), followed by Bacillus (43 µg/mL) and Pseudomonas (37 µg/mL). These results suggest that all the isolates have the potential to promote plant growth through the synthesis of phytohormones. The production of IAA by PGPB has been well-documented, as IAA stimulates root growth and enhances nutrient uptake in plants.

Antifungal Activity (All Isolates)

The antifungal activity of the bacterial isolates was assessed against *Fusarium oxysporum* and *Rhizoctonia solani* using the dual-culture method. Bacillus exhibited the strongest antifungal activity, with inhibition zones of 2.4 cm for *Fusarium oxysporum* and 2.1 cm for *Rhizoctonia solani*. Azotobacter and Pseudomonas also showed antifungal activity, with inhibition zones ranging from 1.4 to 1.9 cm for *Fusarium oxysporum* and 1.5 to 1.8 cm for *Rhizoctonia solani*.

Conclusion

This study highlights the potential of Azotobacter, Bacillus, and Pseudomonas species as plant growth-promoting bacteria (PGPB) for sustainable agriculture. The isolates demonstrated key traits, including nitrogen fixation, phosphate solubilization, IAA production, and antifungal activity. Azotobacter exhibited the highest nitrogen fixation and IAA production, while Pseudomonas showed superior phosphate solubilization. Bacillus displayed the strongest antifungal activity against Fusarium oxysporum and Rhizoctonia solani. These findings confirm the role of PGPB in enhancing soil fertility, promoting plant growth, and protecting crops against pathogens. Utilizing these eco-friendly microbial biofertilizers can significantly reduce dependence on chemical fertilizers, offering a sustainable approach to improving agricultural productivity.

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