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Screening, Isolation and Identification of a UV Resistant Phosphate-Solubilizing Bacteria from Soil

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Abstract: ***This research focuses on isolating phosphate-solubilizing bacteria from soil samples near Mumbai, with the aim of identifying strains capable of surviving high levels of ultraviolet irradiation. Soil and sewage samples were exposed to UV light, and subsequent screening led to the isolation of a Gram-positive, coryneform bacterium, closely resembling Corynebacterium and Arthrobacter species. Preliminary biochemical and staining tests confirmed its phosphate-solubilizing capacity and UV resistance. These findings highlight the potential application of such bacteria in sustainable agriculture and environments with elevated UV exposure, underlining their value in future biotechnological solutions.***

Keywords: Phosphate-solubilizing bacteria, Soil phosphate solubilization, UV resistance, Corynebacterium, sustainable agriculture

1. Introduction

Phosphorus, an essential macronutrient for all life on Earth, serves as a vital limiting nutrient element for plant growth and yield (Li et al., 2023). Phosphorus exists in the form of phosphates in the environment. Phosphates are present in soil as inorganic as well as organic forms. Inorganic forms include primary and secondary phosphate minerals, like calcium phosphates. Release of phosphate from these insoluble forms is regulated by soil pH, and ionic composition of soil particles (Timofeeva et al., 2022).

Chemical fertilizers are conventionally used to treat phosphate deficiency in soil, but most of the phosphorus added through chemical fertilizers becomes inaccessible to the growing plants due to rapid reactions with free cations in the soil. As a result, the efficiency of chemical phosphate fertilizers remains limited (Schnug & De Kok, 2016). Additionally, their widespread use can reduce soil fertility, deplete essential nutrients, and alter soil properties (Pahalvi et al., 2021). This opens the avenue for a more sustainable and efficient method to provide bioavailable phosphates to plants.

A majority of Phosphate Solubilizing Bacteria or PSBs produce organic acids that react with insoluble soil phosphates, forming soluble phosphate ions that can be readily taken up by plants (Sharma et al., 2013). Hence, these PSBs play a pivotal role in biogeochemical cycling of phosphorus and subsequently determine the extent of plant growth possible in that particular soil makeup.

Samples for the current study were collected from mangrove and organic farm soils. Mangroves experience periodic flooding which leads to a high salinity profile, along with absorption of insoluble phosphates brought by tides. (Behera et al., 2017). PSBs are present here in higher concentrations as compared to other soils so as to facilitate the mineralization of this insoluble phosphate for uptake by plants. Organic farms, with their absence of chemical fertilizer usage, also have a higher PSB population to facilitate phosphate breakdown into forms that crops can utilize (Boonlue, 2013).

Climate change and increased UV radiation, along with other stress factors, have caused substantial and possibly irreversible environmental changes. In these challenging settings, many PSBs may struggle to survive due to potential DNA damage and cellular harm. The current study focuses on isolating PSBs that demonstrate resilience to intense UV radiation, employing strategies such as pigment production (Shrivastava et al., 2010), biofilm formation (Ghorbal et al., 2022), or DNA repair mechanisms (Rastogi et al., 2010).

The ability of UV-resistant strains to thrive and function under the harsh conditions of UV radiation holds promise for improving phosphate availability in agricultural soils, reducing the dependency on chemical fertilizers, and mitigating environmental phosphorus pollution. Furthermore, with changing climate, UV radiation intensity is expected to increase drastically (McKenzie et al., 2011), and hence understanding the UV resistance mechanisms of PSBs becomes more imperative. The purpose of this study is to isolate and characterize UV-resistant phosphate-solubilizing bacteria from soil, with the goal of exploring their application in sustainable agriculture. This study is significant because it addresses the need for sustainable, eco-friendly alternatives to chemical fertilizers, particularly in environments exposed to high UV radiation. By identifying UV-resistant phosphate-solubilizing bacteria, the research contributes to advancing resilient agricultural practices and environmental stewardship.

2. Materials and Methodology

2.1 Sample Collection

Three samples were collected to isolate and identify UV-resistant PSBs. The choice of location was based on the ubiquitous availability of phosphate solubilizing bacteria in these sources. (1) Mangrove soil from Delhi Public School

Lake in Seawoods (Navi Mumbai, Maharashtra), (2) Farm soil from an agricultural plot in Lonavala (Maharashtra) and (3) Sewage water from an open drain in Air India Colony, Santacruz (Mumbai, Maharashtra). These samples were collected and brought to the laboratory for testing.

2.2 Screening for UV Resistance and Phosphate Solubilizers

The samples were diluted with distilled water. For soil samples, 9 mL of distilled water was mixed with 1g of the soil and allowed to settle, while the sewage water sample was directly diluted 10X. 1 mL from each of the three diluted samples was transferred into two petri dishes. Further, one petri dish was subjected to 5 minutes of UV exposure while the other was exposed to 10 minutes of UV exposure.

The suspensions from these six plates were spread plated onto Pikovskaya's agar, a differential medium for PSB and incubated at room temperature for 3-4 days. A PSB colony is expected to develop a zone of clearance around it due to utilization of phosphate present as calcium phosphate in the medium (Pikovskaya, 1948).

Subsequent isolations were carried out on Pikovskaya's agar and nutrient agar plates. For staining and biochemical tests, the culture was inoculated in Pikovskaya's broth or peptone water. The pure culture was streaked on a nutrient agar slant for storage and preservation purposes. All of the media constituents and nutrient broths were laboratory grade, procured from Himedia India Private Limited.

2.3 Identification of possible bacterial genera

As a preliminary test, Gram staining was performed according to standard procedure (Tripathi, 2023). Gram nature was also confirmed by the KOH string test, performed as stated by Suslow et al., 1982. Gram nature and visible morphology were correlated with existing literature and dichotomous keys from Bergey's Manual of Determinative Bacteriology (Bergey, D. H., 1923), and suggested tests for further identification were shortlisted and performed based on the same. Results of performed tests were analyzed against the Online Advanced Bacterial Identification Software (ABIS) database (Sorescu & Stoica, 2021), and likely candidates of species were obtained from the same.

Suggested tests included endospore staining (Hussey et al., 2007), capsule staining (Hughes et al., 2007), catalase test (Reiner, 2010), which were performed according to standard procedure as referenced. Motility of the organism was tested by the soft agar assay method (American Society for Microbiology, 2011), and further confirmed by the hanging drop method following standard protocol (Palma et al., 2022).

The oxidase test for cytochrome oxidase production (American Society for Microbiology, 2010), and nitrate reduction test (Granger et al., 1996), were conducted according to standard protocol. The alkaline phosphatase assay using 4-nitrophenyl phosphate as a substrate was conducted according to the kit protocol provided by Erba Manheim (IFC Method Alkaline Phosphatase (ALP) Assay Kit) for cell free supernatant as well as cell suspension from the culture broth, and absorbance for both results was recorded colorimetric ally. Urease activity of the isolated bacterium was tested using urease agar streaked according to the standard procedure (Christensen W.B., 1946). Citrate utilization was tested by plating on Simmon's Citrate agar prepared according to standard protocol (Simmons J. S., 1926). Culture was streaked on Starch agar (Vedder E., 1915) to check for starch hydrolysis using Gram's Iodine for visualization.

Test for carbohydrate utilization was set up in sterile test tubes with inverted Durham tubes and Andrade's pH indicator according to standardized protocol (American Society for Microbiology, 2012). Utilization of glucose, lactose, arabinose, melibiose, fructose, raffinose, melezitose, sucrose, xylose, maltose, ribose, sorbitol, xylitol, trehalose, mannitol, inositol and cellobiose was tested in separate sets of control and inoculated test tubes.

All chemicals used for the biochemical tests were laboratory grade reagents procured from local suppliers SRL Pvt. Ltd. and Loba Chemie Pvt. Ltd. Solid media preparations and components were obtained from HiMedia Laboratories Pvt. Ltd.

3. Results

3.1 Isolation of Ultraviolet Radiation (U.V.) Resistant Phosphate Solubilizing bacteria

The media plates that were streaked with the samples showed some amount of growth within 24 hours but were incubated at room temperature for an additional three days to distinctly recognize probable phosphate solubilizing colonies. Incubation was carried out at room temperature, reflecting the original environments of the samples.

Distinct colonies displaying zones of clearance on Pikovskaya's agar were observed on the farm soil sample that was exposed to UV for 10 minutes. Zones of clearance on other plates were not distinct due to excessive growth, as well as fungal contamination. The farm soil sample that was plated on Pikovskaya's agar plate and underwent 10-minute UV exposure became the source of phosphate solubilizers for the rest of the project (Figure 1).Colonies showing zones of clearance were picked and streaked on nutrient agar plates. After a 24-hour incubation at room temperature, colonies were picked from nutrient agar plates and streaked onto Pikovskaya's agar plates (for colony characteristics refer Table 1). Pure isolates that showed zones of clearance were obtained after successive streaking.

Table 1: Colony characteristics of the isolate on Nutrient Agar plates.

Colony Characteristics	Description
Size	2 mm
Shape	Circular and slightly elevated colonies
Colour	Cream or beige colored
Texture	Smooth
Optical Characteristics	Opaque

3.2 Identification of the bacterial species

Various tests were performed to determine the morphological features of the microbe of interest. The Gram staining procedure carried out multiple times, produced consistent conclusive results denoting the presence of Gram-positive bacteria. The Gram-positive nature was confirmed by the String KOH test. Further tests as mentioned in the table 2 were performed leading to results which suggested that the isolated microbe was a non-sporulating, non-capsulated, weakly motile, facultative anaerobe.

Several biochemical tests, shown in Table 2, were conducted to determine the microbe's biochemical activity. The positive Nitrate reduction test suggested that the isolate produce nitrate reductase which is characteristic of a facultative anaerobe. The bacterium was also observed to be positive for the Urease test and negative for the Oxidase and Citrate utilization tests. The Alkaline Phosphatase test produced a higher intensity of yellow coloration in the cell extract indicating intracellular enzymatic activity in our bacteria of interest. The absorbance measured at 400 nm was found to be 0.04 AU and 0.01 AU in the cell extract and cell free extract respectively.

A color change was observed in the carbohydrate utilization test solutions for glucose, fructose, sucrose, maltose, and trehalose, indicating that sugar utilization had taken place with formation of organic acids; however, no gas production was observed for any of the carbohydrates, indicating non-fermentative sugar utilization.

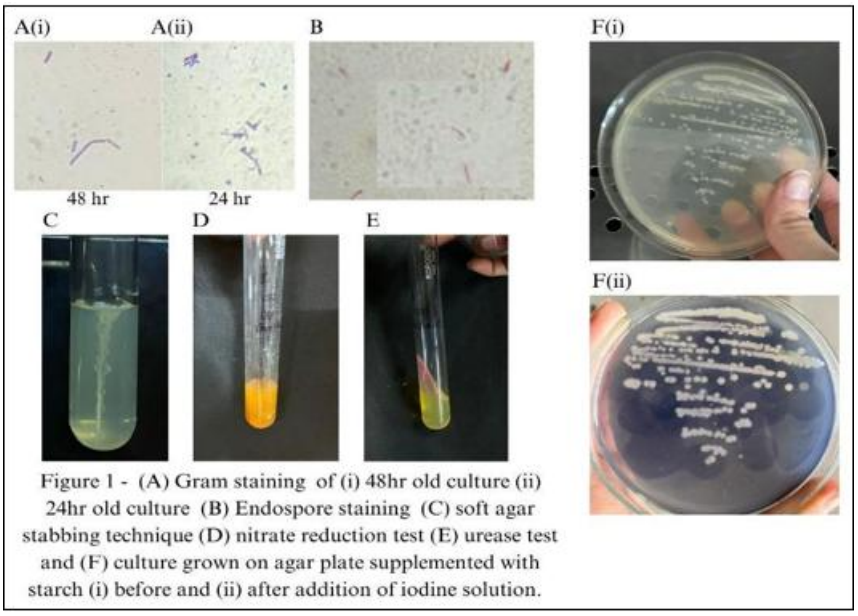

Figure 1 - (A) Gram staining of (i) 48hr old culture (ii) 24hr old culture (B) Endospore staining (C) soft agar stabbing technique (D) nitrate reduction test (E) urease test and (F) culture grown on agar plate supplemented with starch (i) before and (ii) after addition of iodine solution.

Table 2: Tests used for Identification of the isolates

Staining/ Test	Observation
Gram staining	Purple colored rod shaped bacterial cells either forming short chains or clusters or singly present
String KOH test	No viscosity observed in the bacterial suspension
Endospore staining	No retention of malachite green by bacterial cells
Capsule staining	No colorless capsule observed between the bacterial cells stained red and the blue background
Soft agar method motility test	Substantial bacterial cell growth on the upper surface of the agar as well as along the stab line in having a zig-zag appearance
Hanging drop method motility test	Movement seen (although not darting motility
Nitrate Reduction	No color change observed in the nitrate broth tube; nitrate positive
Oxidase Test	Filter paper soaked in Kovac's reagent did not turn purple; oxidase negative
Urease Test	Media containing urea (yellow) turned pinkish red in color; urease positive
Citrate Utilization	No color change to blue in the Simmon's citrate medium; citrate negative
Starch Hydrolysis	Iodine added to the culture medium plate supplemented with starch produced zones of clearances around the colonies; positive starch hydrolysis
Alkaline Phosphatase (ALP) Test	Yellow coloration exhibited by both, the cell extract and cell free extract; ALP positive
Carbohydrate utilization	Presence of pink coloration in the Peptone water Andrade medium containing glucose, fructose, sucrose, xylose, maltose, trehalose separately, which indicates breakdown of these to organic acids, but no gas production was seen in any of the tubes. The tubes containing lactose, arabinose, melibiose, raffinose, melezitose, ribose, sorbitol, xylitol, mannitol, inositol, cellobiose showed no colour change, indicating inability of the isolate to utilize these sugars.

4. Discussion

The results were compared with published literature to support the identification process. (Funke et al., 1997; Bernard K., 2012) as well as Bergey's Manual of Determinative Bacteriology (Bergey, D. H., 1923). The isolated bacterium showed characteristics commonly observed in Coryneform bacteria, being a Gram positive, catalase producing, non-spore forming, and non-capsulated rod (Bergey, D. H., 1923).

The Online Advanced Bacterial Identification Software (ABIS) database (Sorescu & Stoica, 2021) was used to analyze test results for prospective Corynebacterium and related species. The database returned results with maximum similarity to Corynebacterium spp., followed by Arthrobacter species.

Both *Corynebacterium* as well as Arthrobacter species can be found in soil (Mandell et al., 2015), and these genera are also known to produce phosphatase enzymes and hence act as Phosphate Solubilizing Bacteria in various natural ecosystems (Seshadri et al., 2002; Timofeeva et al., 2022), with Timofeeva et al. (2022) also stating the presence of PSB Arthrobacter species in the rhizosphere of plants. As a result of these findings, further studies confirming species level identification of the organism isolated are likely to return one of these two genera.

The ABIS online database (Sorescu & Stoica, 2021) returned various confirmatory wet-lab tests for both of these genera, which can be conducted in the future to further narrow down the organism. Albert's staining for metachromatic granules can be conducted to confirm *Corynebacterium* spp., as outlined by Chaudhary and Pandey (2023). Rod-coccus cycling is a characteristic feature of Arthrobacter species (Bergey, D. H., 1923), and while it was not explicitly observed during routine staining in the course of the study, it can be tested for by the method outlined by Luscombe and Gray (1971).

As the motility observed for the singular cells was not a common darting motility, flagellar characterization with the help of flagellar staining (American Society of Microbiology, 2016) could also be done to justify this abnormal motility observed.

5. Conclusion

The current study was successful in narrowing down the prospective genus of the isolated organism to either *Corynebacterium* spp. or *Arthrobacter* spp. using primarily wet-lab tests. Sequencing the 16S rRNA from the isolated bacterium (Weisburg, 1991) and subsequent phylogenetic analysis against rRNA databases can concretize the genus-level identity of the organism, and even species level in some cases (Petti, 2007).

Although the bacterium survived 10 minutes of UV exposure, future studies should quantify its UV tolerance using a survival curve to clarify its potential applications. A technique similar to ones used by Karki and Ham (2016), taking into account the time of exposure, as well as the intensity of UV irradiation and temperature would yield significant results. Subsequent studies investigating the mechanism of UV tolerance by this bacterium are of importance, since the bacterium does not produce any pigments, nor require to form biofilms in order to survive the irradiation.

A soil bacterium that can conduct its phosphate solubilizing activity in the presence of high doses of UV radiation could be used as a biofertilizer in farmlands exposed to high degrees of sunlight, and could help in conversion of previously arid lands into arable, mineral rich soils. Terraforming in the distant future would require mineral solubilization under high levels of solar UV irradiation, and microbial agents in combination with plants will be essential for soil formation (Conde-Pueyo, 2020). PSBs can also be engineered to be eco-friendly, efficient, and sustainable tools for bioremediation efforts, contributing to a cleaner and healthier environment. The isolated bacterium shows characteristics that would be beneficial for such applications and while the current study laid out an extremely preliminary foundation, further research on this bacterium holds immense scope for breakthroughs in these fields.

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