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Shivangi H Zaveri
Biotechnology Department, Bhagwan Mahavir Center for Advanced Research (BMCAR), Surat, Gujarat, India

Dr. Sumita Dasgupta
Biotechnology Department, Bhagwan Mahavir Center for Advanced Research (BMCAR), Surat, Gujarat, India

Correspondence Author:
Shivangi H Zaveri
Biotechnology Department, Bhagwan Mahavir Center for Advanced Research (BMCAR), Surat, Gujarat, India

Isolation and plant growth promoting properties of bacterial endophytes from wild plant (*Wedelia urticifolia*) of south Gujarat region

Shivangi H Zaveri and Dr. Sumita Dasgupta

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Abstract
The diverse community of endophyte is a critical resource in enhancing plant growth. The endophytic bacteria belong to a larger group of microorganisms that have their life-cycle partly or entirely inside the plant and are located in intra and inter-cellular spaces or in the vascular tissue. Increasing nutritional content in staple crops either through agronomic biofortification or through conventional plant-breeding strategies continue to be a huge task for scientists around the globe. The microbial world of endophytes having the ability of nutrient mineralization has been proved a boon to mitigate hunger of the global population. This study aims to determine plant growth-promoting properties of total fourteen bacterial endophytes isolates from *Wedelia urticifolia*. WUR4 strain shows the best results out of all other isolates and shows the best plant growth-promoting activities. These includes potential to fix the nitrogen (+), solubilize phosphate (3.2 mm, 0.4636±0.0183 mg/ml), solubilize potassium (6.8 mm, 0.1438±0.0066 mg/ml) and siderophore production (zone +++, 69.9033±1.1509 SU). Our findings indicate that this isolate WUR4 have potential for future agricultural biotechnological applications through development for sustainable crop improvement and to formulate bio inoculant as well as can be used as biofertilizers.

Keywords: *Wedelia urticifolia*, bacterial endophytes, plant growth promotion, nutrient bio availability

1. Introduction
Due to its abundance of macro and micronutrients and its diversity of bacteria, healthy soil is crucial to the sustainability of planet Earth. Growing interest has been shown in the use of beneficial endophytic bacteria to improve plant health and crop output while preserving food quality and environmental viability as a result of the problems associated with the careless application of chemical fertilizers in farming. Global hunger has been lessened because to the microbial world of endophytes and their capacity to mineralize nutrients [1, 2].

The practice of maintaining mineral nutrients in soil and plant systems is known as nutrient management. This strategy derives from integrated nutrient management (INM), which concentrates on the nutrients nitrogen (N), phosphorus (P), and potassium (K) by combining agrochemicals with efficient microorganisms (EM) [3, 4]. Endophytes have been utilized to expand the range of soil fertility and mineralization or immobilization of various trace elements, such as N, P, K, Zn, Fe, Cu, Mg, and S, according to the principles of mineral nutrient management (MiNuM). Leaching and nutrient run-off, especially of N and P, brought on by synthetic fertilizers and pesticides resulted in environmental deterioration. In order to develop effective strategies for the control of the plant's mineral nutrients, endophytes must be addressed [5].

Wedelia, a flowering plant genus, classified in subtribe *Verbesinina* of *Asteraceae* (Composite), comprises about 60 species geographically spread in the tropical and warm temperate regions, including China, India, Burma, Malaysia, Ceylon, Japan, and Australia [6]. Among them, *W. Urticifolia*, and *W. Wallichii* are always considered in some studies. A number of plants in this genus are used as traditional herbal medicines throughout the world to treat a variety of diseases, such as headaches, fevers, infections, and pathologies of the respiratory tract. A study has demonstrated the potential antimicrobial effect of essential oils from various Wedelia species. *Wedelia urticifolia* is a perennial fragrant small herb, 60–80 cm tall, elliptic leaves, bright yellow flowers, terminal heads [7-9].

To the best of our knowledge, there is no report on the endophytic study of *Wedelia urticifolia*. The aim of this study was to isolate the endophytes and determine their biochemical as well as plant growth-promoting potentials of the isolates.

2. Materials and Methods

2.1 Collection of sample plant

Plant sample was collected at flowering stage from Vesu, Surat, Gujarat (Lat 21.137939, Long 72.793596). The healthy plant used for the present study and was collected in bag and managed at Bhagwan Mahavir College of Science and Technology, Surat, Gujarat. The plant materials were brought to laboratory and were washed carefully under tap water to remove any adhering dirt and debris.

Fig 1: Sample plant (*Wedelia urticifolia)*

2.2 Isolation of endophytes

The leaves and flowers were surface sterilized with few modifications [10].

2.3 Morphological and Biochemical Characterization

Each purified colony was observed for their morphologically similar appearance of colour, elevation, size, shape, surface, margin, pigmentation and opacity. Gram staining of each isolate was determined by crystal violet and safranin, according to microbiological protocol. To determine metabolic activity of bacteria some of the routine biochemical tests were carried out using standard procedure and the name of biochemical tests mentioned as follows: Utilizations of carbohydrates and organic acids test was carried out using Methyl-Red (M-R) test, Voges-proskauer (V-P) test, Citrate utilization test, Urea hydrolysis test, Nitrate reduction test, Catalase test, Triple sugar iron agar test was carried out [11, 12].

2.4 Determination of PGP traits

The obtained endophytes were checked for their multiple plant growth-promoting activities such as the growth on nitrogen free media, Siderophore production and also phosphate (P) and Potassium (K) solubilization. It was further tested to quantify the plant growth-promoting activities.

2.4.1 Growth on nitrogen-free media

Semi-solid Rennie media [13-15] was used to screen the Nitrogen fixing capacity of the isolated entophytic strains. After autoclaving, filter-sterilized biotin and Para amino benzoic acid were added to final concentrations of 5 and 10 µg per liter. Plates were incubated at 37 °C for 48 hrs and observed for growth.

2.4.2 Phosphate solubilization

The qualitative phosphate solubilization test was carried out using spot inoculation technique on agar plate containing insoluble tricalcium phosphate (TCP) and incubated at 30 °C for 4 to 5 days and observed the zone of solubilization (15, 16). The phosphate solubilization index (PSI) was calculated using the following formula.

PSI = Colony diameter + Zone of clearance / Colony diameter

2.4.2.1 Quantitative phosphate solubilization

Quantitative estimation of P solubilize by the selected isolates was conducted by following [15, 17]. Isolates selected were cultivated in 100 mL Pikovskaya liquid medium and incubated in shaking incubator for 7 days at 37 °C. Every 24 h, 1.5 mL bacteria culture was centrifuged at 10,600 g for 10 min. One milliliter of supernatant was reacted with the colour-forming reagent (2.5 mL 2.5% sodium molybdate and 1 mL 0.3% hydrazine sulfate). After blue colour formed, phosphate solubilizing activity was then measured by visible spectrophotometer at 830 nm wavelength. The total amount of phosphate solubilize was calculated against K_2HPO_4 (1.0 to 10.0 µg/ml) standard calibration curve.

2.4.3 Potassium solubilization

Potassium solubilization studies were carried out in Aleksandrov liquid medium, as described by (15, 18). The composition of medium (g/L) was as follows: 5.0 glucose, 0.5 magnesium sulphate, 0.005 ferric chloride, 0.1 calcium carbonate, 2 calcium phosphate and 2 potassium alumino silicates minerals. The pH of the medium was adjusted to 7.2 by adding 1 N NaOH. The petri-plates were incubated at 30 °C for 4 to 5 days and observed the zone of solubilization. The potassium solubilization index (KSI) was calculated using following formula.

KSI = Colony diameter + Zone of clearance / Colony diameter

2.4.3.1 Quantitative potassium solubilization

A new spectrophotometric method was developed by [15, 19] for the quantification of potassium in the culture broth supernatant of K-solubilizing bacteria. The culture was prepared and inoculated into 100 ml of Aleksandrov broth medium and incubated in a shaker for 10 days at 28 °C. After 5 days, 10 ml of bacterial culture was centrifuged at 10,000 rpm, at 28 °C for 10 min. The supernatant was taken for K solubilization by using spectrophotometric method. The total amount of potassium solubilize was calculated against the KCL (1.0 to 10.0 µg/ml) standard calibration curve.

2.4.4 Siderophore production

To produce 1 L of siderophore media, the glassware were defer rated by washing with 6-M HCl overnight to eliminate

the residual Fe contamination, then rinsed with double distilled water two to three times [15, 20]. To measure siderophores, the CAS (Chrome Azurol S) plate method was employed [21]. After solidifying, bacteria were spot inoculated on plate. Bacterial isolates inoculated on the CAS-blue agar were incubated at 30 °C for 4 to 5 days. Following incubation, these plates were observed for production of yellow-orange halo zone around the spot which indicates siderophore production.

2.4.4.1 Siderophore production by CAS liquid assay

To measure siderophores, the CAS (Chrome Azurol S) liquid assay method was employed [22]. The CAS assay solution was mixed with 0.5 ml of 72 hour old cell free supernatant and 10 µl shuttle solution (sulfosalicylic acid) was then added. After 15 min, the colour intensity of the solution was measured with UV-VIS spectrophotometer at 630 nm against reference. A decrease in blue colour as expressed in percent siderophore units (%SU) was seen as a result of siderophore synthesis [23].

Siderophore unit (SU) = Ar − As /Ar × 100%

Where, Ar - the absorbance of the reference at 630 nm.

As - the absorbance of the sample (bacterial cultures) at 630 nm.

3. Results

3.1 Isolation and primary screening of PGP traits of isolates

A total of fourteen endophytes were isolated from *Widelia urticifolia* plant samples, were collected and used for the isolation of different PGPR isolates. Out of fourteen isolates, six isolates were from roots which named as WUR1 to WUR6 and eight isolates were from leaves which named as WUL1 toWUL8.

3.2 Morphological and Biochemical characterization

In this study, we found that *Widelia urticifolia* plant harbored an abundance of culturable endophytic bacteria, and varied between leaves, and roots. A total of fourteen morphologically different endophytic bacteria was obtained and isolated from the sterilized surface of roots, and leaves of plant. Preliminary characterization of these isolates indicated that studied parts of *Widelia urticifolia* plant contained both gram negative and gram-positive bacteria.

Table 1: Distribution of bacterial endophytes obtain from plant

Plant Part	Isolate Code	Total Number
Leaf	WUL1, WU2, WUL3, WUL4, WUL5, WUL6, WUL7, WUL8	08
Root	WUR1, WUR2, WUR3, WUR4, WUR5, WUR6	06
Total		14

3.3 Growth on nitrogen free media

Nitrogen fixation is an ability exhibited by most of the endophytes. Five among the fourteen isolated bacterial strain WUL1 & WUL5 from leaf and WUR2, WUR3, WUR4 from root were found to grow in Jensen's medium, a selective medium for the detection and cultivation of nitrogen-fixing bacteria.

3.4 Phosphate solubilization

Phosphate solubilization in plants is essential. It converts the insoluble forms of phosphate like TriCalcium aluminum phosphate (Al_3PO_4), TriCalcium phosphate (Ca_3PO_4) in soluble forms which plants can easily take-up. In present study two isolated bacterial strains WUR4 and WUR5 among the fourteen were capable of solubilizing phosphate by showing zone of solubilization.

3.4.1 Quantitative estimation of souble phosphate

According to plate screening for clear zone formation, WUR4 and WUR5 strains gave clear zone formation around colony, except from WUR3 gave no clear zone formation around the well. Index for clear zone formation of these strains was shown in Table. As they gave clear zone, it can be assumed that these strains have phosphate solubilizing activity. In clear zone formation, WUR4 strain gave the largest zone formation. During seven days incubation, clear zone formation of all these strains was larger and larger.

Table 2: Phosphate solubilization zone (in mm)

Isolate	Day 1	Day 2	Day 3	Day 4	Day 5
WUR4	0.7	1.5	2.1	2.7	3.2
WUR5	0.5	1.1	1.6	1.9	2.2

Phosphate solubilizing activity of all selected strains was quantitatively determined by UV-VIS spectrophotometric method at 830 nm using KH2PO4 as standard. Amount of solubilize phosphate of all selected strains were shown in Figure 2. Although WUR4 strain gave the largest clear zone in plate screening method after 5 day incubation Table 2, it showed the highest amount of solubilize P in quantitative analysis. In comparison to broth assay, the solubilize phosphate concentration is 0.4636±0.0183 mg/ml.

According to quantitative analysis, strains WUR3, WUR4 and WUR5 showed phosphate solubilizing activity in broth assay. Although strain WUR3 strain gave no clear zone formation on plate screening.

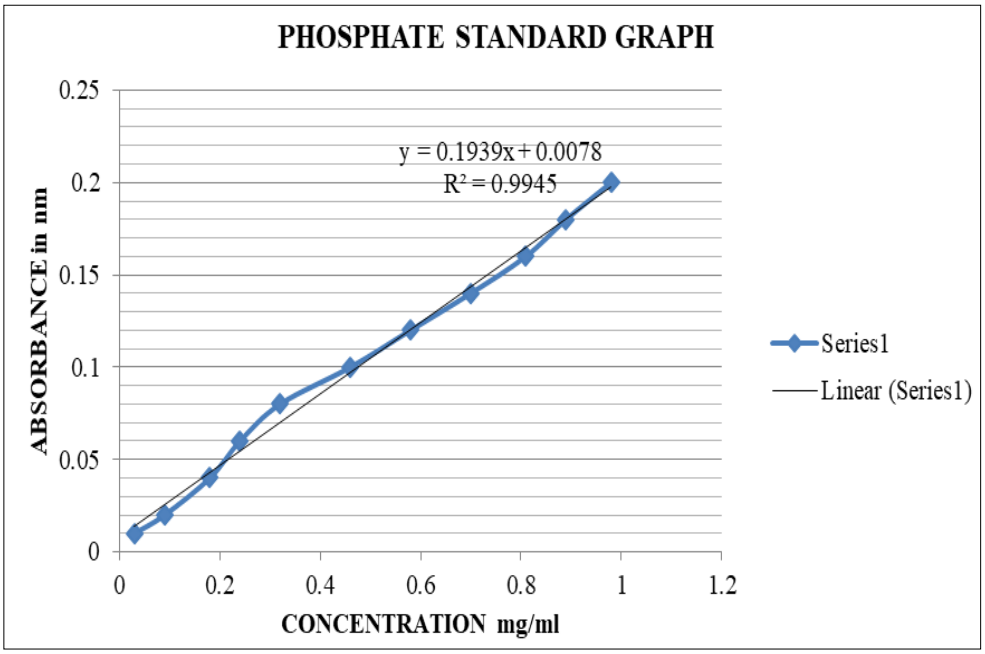

Fig 2: Standard graph of phosphate

3.5 Potassium solubilization

KSB can dissolve silicate minerals and release K through the production of organic and inorganic acids. Three isolated bacterial strains WUR1, WUR4 and WUR5 among fourteen isolates showed positive results.

3.5.1 Quantitative estimation of souble potassium

According to plate screening for clear zone formation WUR1, WUR4 and WUR5 strains gave clear zone formation around colony, except from WUR6 gave no clear zone formation around the well. Index for clear zone formation of these strains was shown in Table 3. As they gave clear zone, it can be assumed that these strains have phosphate solubilizing activity. In clear zone formation, WUR4 strain gave the largest zone formation. During five days incubation, clear zone formation of all these strains was larger and larger.

Potassium solubilizing activity of all selected strains was quantitatively determined by UV-VIS spectrophotometric method at 830 nm using KCL as standard. Amount of solubilize potassium of all selected strains were shown in Figure 3. Although WUR4 strain gave the largest clear zone in plate screening method after 5 day incubation (Table 3). In comparison to broth assay, solubilize potassium concentration is 0.1438±0.0066 mg/ml.

According to quantitative analysis, strains WUR1, WUR4, WUR5, and WUR6 showed potassium solubilizing activity in broth assay. Although strain WUR6 strain gave no clear zone formation on plate screening.

Table 3: Potassium solubilization zone (in mm)

Isolate	Day 1	Day 2	Day 3	Day 4	Day 5
WUR1	1.1	2.3	3.5	4.6	5.2
WUR4	1.6	2.9	4.2	5.3	6.8
WUR5	0.8	1.9	2.7	3.6	4.8

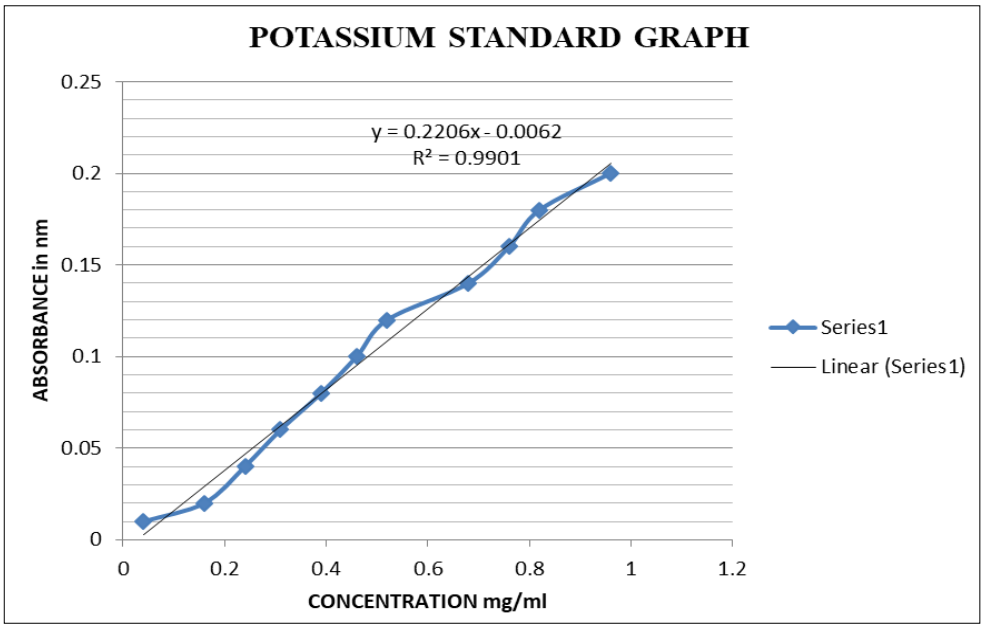

Fig 3: Standard graph of potassium

3.6 Screening of siderophore production by plate assay

Out of total, all root isolates were found to be positive for siderophore and selected for quantitative estimation of siderophore production.

3.6.1 Siderophore production by CAS liquid assay

Among the all fourteen isolated bacterial strain WUR4 strain produced significantly higher quantities of siderophores above 60% siderophore units (SU). Percentage wise siderophore production by endophytic strains from plant is shown in Table 4. Strains were producing siderophore in range of 50% to 70% (Table 4). Among them WUR4 produced significantly higher quantities of siderophore of 69.9033±1.1509.

Table 4: Percentage siderophore production of bacterial endophytes

Isolate	Siderophore Production (Cas Plate Assay)	% Siderophore production (siderophore unit su), (Cas Liquid Assay)
WUR4	+++	69.9033±1.1509

Table 5: Biochemical characterizaion of selected isolate

Biochemical tests	Isolate WUR4
M-R test	+
V-P test	-
Citrate utilization	+
Urea hydrolysis	-
TSI	+
Catalase	+
H_2S production	+
Nitrate reduction	+
Ammonia production	+
Indole production	+

Table 6: Plant growth promoting activity of wur4 strain

Plant growth promoting activity	Isolate WUR4
Nitrogen fixation	+
Phosphate solubilization	Zone - 3.2 mm, Concentration - 0.4636±0.0183 mg/ml
Potassium solubilization	Zone - 6.8 mm, Concentration - 0.1438±0.0066 mg/ml
Siderophore production	Zone - +++, Liquid assay - 69.9033±11509 SU

4. Discussion

There are some endophytes which facilitate in nutrient uptake and minerals which contribute to the growth of plants such as nitrogen and phosphorus fixation which are essential macronutrients required for the biological growth and development of plants [23]. In the present work total fourteen bacterial endophytes were isolated from *Widelia urticifolia* plant. The endophytes contribute to plant growth by solubilizing inorganic phosphorus which is insoluble and making it available to plants. This trait is common amongst many microbial communities' associate with various crop plants including wheat, rice, maize and legumes [2, 3, 24]. Several endophytes have been reported to have plant growth promoting properties such as members of the *Burkholderia*, *Enterobacter*, *Halolamina*, *Pantoea*, *Pseudomonas*, *Citrobacter* and *Azotobacter* [2, 5, 25].

Nitrogen is major component of chlorophyll and amino acids. The primary reasons of interaction between biological nitrogen fixation and plant are the nutritional exchange because plant provide carbon source for microbial growth and in return microorganism provide the fixed N to plants and promote their growth [14, 15, 26]. In the present study, five among the fourteen isolates were able to grow on nitrogen free media. In previous study, 343 endophytes were isolated from stem, root, and leaves of soybean cultivar. About 60% of endophytes grow on NFb medium and 21% of endophytes revealed containing nifH gene by PCR analysis. The endophytic isolates identified as *A. Calcoaceticus*, *Burkholderia* sp., *Pseudomonas* sp., and *Ralstonia* sp. [26].

Phosphorus is one of the essential nutrients for plant growth, but it may be relatively unavailable to plants because of its chemistry. In soil, the majority of phosphorus is present in the form of a phosphate, usually as metal complexes making it bound to minerals or organic matter. Therefore, inorganic phosphate solubilization is an important process of plant growth promotion by plant associated bacteria and fungi [27]. In the present study, two among fourteen isolates were able to solubilize phosphate by showing zone of solubilization. In previous studies of phosphate solubilization experiments confirmed that endophyte strains WP5 and WP42 had the ability to solubilize Ca3 (PO_4) 2. Endophytic bacteria *A. calcoaceticus* and *Burkholderia sp*. isolated from soybean cultivars showed significant growth promotion of plant by solubilization of mineral phosphate [28-30].

According to qualitative analysis, WUR3, WUR4 and WUR5 showed positive results in broth assay though strain WUR3 showed no clear zone formation on plate assay. So, it was known that clear zone diameter formation was not directly proportional to the amount of solubilize phosphate concentration in broth assay. All these findings revealed that one should not rely only on qualitative method i.e. zone formation on plate assay, while isolating and screening the P solubilizing microorganisms. It is wise to supplement qualitative method with quantitative measurement of P solubilizing for getting more reliable inferences. Similar results have been reported in [31]. It has also been reported that many isolates which did not show any clear zone in qualitative method i.e. NBRIP medium- agar plate assay solubilize insoluble inorganic phosphates in quantitative method. Thus, the plate screening method fails where the clear zone is inconspicuous or absent. This may be because of the varying diffusion rates of different organic acids secreted by an organism [29]. Phosphate solubilizing activity is determined by microbial biochemical ability to produce

and release organic acids, which their carboxylic groups chelate the cations (Mainly Ca) bound to phosphate converting them into soluble forms [17].

After nitrogen (N) and phosphorus (P), potassium (K) is most important plant nutrient that has key role in growth, metabolism and development of plant. The K in the soil exists in the form of feldspar (90-98%) and mica [17]. The phenomenon of solubilization of potassium from soil by endophytic microbes mostly dependent on the pH and type of soil [18]. Endophytic bacteria belonging to genera Achromobacter, Acinetobacter, *Bacillus*, *Burkholderia* [19, 20, 22, 32] have been reported for potassium-solubilization. In the present study, three among fourteen isolates were able to solubilize potassium by showing zone of solubilization. In previous studies, *Bacillus* licheniformis and Pseudomonas azotoformans which were isolated from the soil of rice plants, using two potassium solubilizing bacteria, could increase the availability of potassium in the soil [20]. Another study concluded that among all investigated *Actinobacteria strains*; *Streptomyces alboviridis*, *Streptomyces griseorubens* and *Nocardiopsis Alba* were able to solubilize potassium [22]. Comparable results of K solubilization were reported in [33], a total of 10 endophytic bacterial isolates have been isolated from samples of roots, stems, and leaves of maize (*Zea mays* L.) on aleksandrow agar *Bacillus* sp. KF668 isolate could show clear zones at an incubation time of 48- 96 hour.

According to qualitative analysis, WUR1, WUR4, WUR5 and WUR6 showed positive results in broth assay though strain WUR6 showed no clear zone formation on plate assay. Similar results were found in [17], in their research B1 and Y strains cannot give clear zone formation when screened on media, but they can solubilize potassium mica by giving positive results in quantitative assay. In potassium decomposing activity, Y strain gave the highest amount of soluble potassium concentration and B1 strain was the second highest. The ability of bacteria to release K largely depends on the nature of the mineral compounds [34]. The variability among the bacteria indicates the importance of exploration of different mineral potassium solubilizing bacteria and their solubilizing mechanisms [31].

Microbial siderophore are subject of more interest because of chelating ferric iron, produced under low iron stress. Siderophores are produced by several microorganisms such as *Pseudomonas* spp., *Enterobacter* spp. and *Escherichia coli* [35]. In the present study, all root bacterial endophytes were screened for siderophore production. They showed orange colour halo zone on CAS agar plate. Production of siderophores is an important attribute of plant growth promoting endophytes and facilitates growth of plants under iron limiting conditions through iron sequestration [36]. This media is very sensitive to variations in pH or FeCl3 concentrations. Schwyn and Neiland developed a universal siderophore assay using chrome azurol S (CAS) and hexadecyl trimethyl ammonium bromide (HDTMA) as indicators. The CAS/ HDTMA complexes tightly with ferric iron to produce a blue color. When a strong iron chelators such as a siderophore removes iron from the dye complex, the color changes from blue to orange. There are many reports in literature on siderophore producing capacity of endophytes affirmed using CAS assay [21, 37, 38]. Comparable results of strains of B. Pumilus and B. Safensis were positive for siderophore production on CAS agar medium [39].

The production of siderophore was roughly estimated on the basis of size of halo formation on CAS agar. CAS agar method can only give rough idea and is not a perfect method for quantification of siderophore production. Hence, quantitative estimation of siderophore is done using liquid culture media and CAS reagent [40]. In the present study WUR4 isolates were selected for quantitative estimation. Amount of siderophore produced by all the root strains was checked. For control or absorbance reference (Ar) uninoculated broth and CAS reagent were kept. Concentration of siderophore production by bacterial strain WUR4 was above 60% siderophore units (SU). The maximum concentration of siderophore production was found in bacterial strain WUR4 with 69.9033±1.1509 SU. Similar results were found with (39), with concentration of siderophore production n range of 68.102±0.056 to 60.241±0.102 SU. Quantitatively also P. aeruginosa (KA19) produced maximum amount of siderophore 60.241±0.102 SU by spectrophotometer (traditional method) [41].

5. Conclusion

In current scenario, due to the abundant use of synthetic chemicals on crops, the sustainability of agriculture systems has distorted; the cost of cultivation has increased at a high rate. The use of endophytes as an integral component of agricultural practices is quickly gaining momentum worldwide. Improvements are required regarding the input of biological agents i.e. plant growth promoting bacteria (PGPB). The evidence obtain from present study is the stain WUR4 can be subjected to molecular sequencing and application to plants are necessary because it is best for solubilizing phosphate and potassium as well as siderophore production and fix the nitrogen, which could be used as bio fertilizer. Further research in fields may lead to development of novel agricultural practices such as microbial inoculant for crop productivity and sustainability.

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