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*Research Paper*

# Evaluation of Plant Growth Promoting Potential of Phosphate Solubilizing Bacteria Isolated from Digester Effluent of Vegetable Waste Based Bio-methanation Plant

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## A B S T R A C T

Green revolution enabled to increase yield from major grain crops with the help of chemical fertilizers in order to meet very serious food shortage in developing countries during the 1950s. Chemical fertilizers have been used continuously by farmers since the 1950s to improve crop productivity. The excessive and inappropriate use of these agro-chemicals has caused significant environmental harm, including to humans. Fertilizers of biological origin are considered environmentally friendly and their use is becoming increasingly important in the sustainable development of agriculture. The goal of the present study was to isolate and identify phosphate solubilizing bacteria from the digester effluent of the bio-methanation plant based on vegetable waste and to test their effect on crop plants for growth promotion. Seventeen bacteria were isolated from digester effluent, and these isolates were further screened on solid and also liquid media to determine their phosphate solubilizing ability. 16 S rRNA gene analyses identified the two most potent phosphate solubilizing bacteria and were selected further to determine their impact on crop plant growth by pot assay. The use of phosphate solubilizing bacteria as inoculants has been found to increase phosphorus absorption in crop plants in contrast to controls. Important effects were seen in the statistical analysis of the findings.

***Key words*:** Biofertilizers, Digester effluent, Phosphate solubilization, Plant growth promotion

Phosphorus is a second most vital nutrient for plants and microorganisms. In virtually all major cell metabolic processes, it plays an important role in [1]. Phosphorus is abundant in inorganic as well as organic types in soils. Inorganic phosphorus occurs mainly in soil that is not absorbable by plants in insoluble mineral complexes [2]. The significant reservoir of immobilized phosphorus that accounts for 20-80% of phosphorus in soils is organic matter [3]. There is just 0.1 percent of total phosphorus available for plant absorption in a soluble form [4]. For plants, it is a significant growth limiting factor. Chemical phosphorus fertilizers commonly used as supplement in agricultural soils are expensive and overuse has led to adverse environmental impacts in recent years: soil toxicity, loss of soil fertility, soil erosion, ecosystem destruction, contamination of air and underground water supplies, pollution of animal feed and fodder, increased human and livestock disease incidences [5-6]. Biofertilizers are microbial preparations that allow crop plants, when applied via seed or soil, to absorb nutrients through their interactions in the rhizosphere. Phosphate solubilizing biofertilizers play an important role in the environmentally beneficial and safe dissolution of insoluble and bound phosphorus in the soil [7]. The mechanisms of microbial phosphate solubilization include the release of complexing or dissolving mineral compounds such as organic acids, the release of extracellular enzymes and the release of P during substrate degradation [8-9]. Several species of bacteria and fungi have diverse capacity for solubilizing inorganic phosphates [10]. Phosphate solubilizing bacteria (PSB) have been isolated from various environmental locations, such as fertile soils, rhizospheric zones, agricultural waste compost and their effects on growth and crop yield has been reported by previous researchers [11-15].

Bio-methanation for waste treatment is a commonly used microbial technology. With the aid of the aerobic and anaerobic microorganism consortium, the process transforms organic portions of waste into biogas and digester effluent in anaerobic conditions [16]. By virtue of its nitrogen, phosphorus and potassium content and also the existence of beneficial microorganisms, the digester effluent is used as manure. The use of digester effluent has been found to increase the yield of crops [17]. There are very few reports on isolation of plant growth promoting bacteria from digester effluent and research on their stimulatory effect on crop growth. Thus, the study focused on isolating and identifying potent digester effluent phosphate solubilizing bacteria and evaluating their effect on crop plant growth.

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## MATERIALS AND METHODS

*Bio-methanation of vegetable waste*

The bio-methanation study was performed in locally produced digesters with a 5 litre size. At the organic loading rate of 0.320 g volatile solids/l.d, and pH was 7.0 under ambient temperature conditions with two cycles of 20 days hydraulic retention time, the digesters were worked with feeding of the vegetable waste slurry (consisting of equal mixture of potato, onion, tomato, brinjal, cauliflower and cabbage waste)

*Isolation of bacteria from digester effluent*

Using nutrient agar, bacteria were isolated by spread plate technique and representative distinct bacterial isolates obtained were preserved for further studies at cooling temperature.

*Screening of bacteria for phosphate solubilizing efficiency*

By spot inoculation of the loop of pure cultures on the medium of Pikovaskaya, the phosphate solubilizing ability of bacterial isolates was established [18]. These plates were incubated for 48 hours at room temperature and the clearing zones around the growth were detected. As per [19], the hydrolysis capacity (HC) was calculated.

*Quantitative measurement of phosphate solubilization in broth cultures*

The two bacterial isolates with the highest HC value were selected and grown separately for 10 days in 50 ml aliquots of Pikovskaya broth at 28 ± 2°C. By comparison to the standard graph of potassium dihydrogen orthophosphate, the amount of phosphorus released in culture broth was calculated by colorimetric technique.

*Effect of inoculation of potent PSB isolates on plant growth by pot assay*

Maize (*Z. mays* L.), Jowar (*S. bicolor*) and Wheat (*Triticum aestivum* L.) plant seeds were surface sterilized by immersion in 95% ethanol for 30 seconds and 0.2% mercury chloride for 3 minutes, accompanied by washing with sterile distilled water 5 times. Bacteria were grown at room temperature in nutrient broth on a rotary shaker (150 rpm) for 2 days. For seed coating and further drying, 0.5 ml of culture (OD at 600nm=0.9) was added to the seed surface. In each pot, five seeds were sown and triplicate experiments were performed for each isolate. The seeds that were uncoated were used as control. Sterile soil was added to each pot with 0.160 g of Tri-calcium phosphate (TCP) per kg of soil. The pots were irrigated every day with sterile plant nutritive solution and kept in sunlight. The pot labeled C-1 contained soil only, C-2 contained soil + TCP, B-1 contained soil + TCP + B-1 isolate, and soil + TCP + B-2 isolate contained B-2. Plants were uprooted after 2 weeks and seedlings were assessed for shoot length and root length. They were separated into root and shoot, and using an electrical digital balance, their fresh weight was recorded. The fresh plant materials were retained for 24 hours in a hot air oven at 80°C and then their dry weight was also calculated. In order to assess the significant effect of bacterial isolates on plant growth relative to the two controls C-1 and C-2, statistical data analysis was performed.

*Screening of potent PSB isolates for other PGPR traits*

In selected potent PSB isolates, namely B-1 and B-2, the production of siderophore, indole acetic acid, ammonia, catalase and hydrogen cyanide was detected by referring to standard methods [20-22].

*Identification of bacterial isolates*

Morphological, cultural and biochemical characterization, as per standard literature, was carried out to classify the potent PSB isolates up to species level [23-24]. In addition, molecular characterization using 16S rRNA gene analysis verified the recognition.

## RESULTS AND DISCUSSION

*Bio-methanation of vegetable waste*

The biogas yield at ambient temperature conditions for the mixture of six vegetable wastes was found to be 510-1340 (mL/d). The average yield was 0.633 L biogas / g VS.d and 59 percent of methane was detected.

*Isolation of bacteria from digester effluent*

The seventeen distinct bacterial isolates obtained were retained under refrigeration conditions for further studies.

*Determination of phosphate solubilization ability of bacterial isolates*

To determine their phosphate solubilizing ability, seventeen bacterial isolates obtained from digester effluent were tested. Bacterial isolates B-1 and B-2 were selected because, among other things, they exhibited the highest solubilizing capacity. B-2 isolate was found to be more potent in liquid media for solubilizing phosphate. Quantitative phosphate solubilization was shown in B-1 and B-2 at 7.02 mg/l and 7.59 mg/l respectively.

Table 1 Effect of inoculation of PSB isolates on the growth of maize (*Zea mays* L.)

| Isolate code | Shoot length (cm) | Root length (cm) | Shoot | | Root | |
|---|---|---|---|---|---|---|
| | | | Fresh weight (mg) | Dry weight (mg) | Fresh weight (mg) | Dry weight (mg) |
| C1 | 33.8±2.4587 | 28.98±2.0290 | 1226.2±138.572 | 100.2±10.8490 | 1336.2±176.8677 | 132.8±18.0748 |
| C2 | 33.28±2.8323 | 28.56±2.6025 | 1188.4±102.9699 | 96.2±15.9750 | 1269.4±244.6636 | 129.2±22.1179 |
| B-1 | 36.54±2.453 | 29.2±2.8089 | 1346±172.3833 | 103.4±10.7145 | 1425.8±140.3538 | 140.4±10.3344 |
| P value1 | 0.0085 | 0.7512 | 0.0050 | 0.1733 | 0.0156 | 0.1778 |
| P value2 | 0.0062 | 0.5084 | 0.0149 | 0.1200 | 0.0787 | 0.1363 |
| B-2 | 36.68±1.252 | 29.46±2.8997 | 1397±156.4049 | 104.8±7.2595 | 1417.8±244.1069 | 141±26.2202 |
| P value1 | 0.0273 | 0.4538 | 0.0314 | 0.2071 | 0.2264 | 0.2464 |
| P value2 | 0.0456 | 0.2921 | 0.0071 | 0.1773 | 0.1332 | 0.0670 |

Table 2 Effect of inoculation of PSB isolates on the growth of Jowar (*Sorghum bicolor*)

| Isolate code | Shoot length (cm) | Root length (cm) | Shoot | | Root | |
|---|---|---|---|---|---|---|
| | | | Fresh weight (mg) | Dry weight (mg) | Fresh weight (mg) | Dry weight (mg) |
| C1 | 15.3±1.7847 | 11.66±1.9243 | 107±22.2036 | 9.8±3.2711 | 36.4±15.4532 | 7.2±4.9699 |
| C2 | 14.58±3.2935 | 15±2.3505 | 105.6±49.8678 | 9.4±4.0373 | 18.8±10.4976 | 5.4±3.5777 |
| B-1 | 16.8±4.6728 | 14.36±3.4275 | 149.8±56.7600 | 13.2±5.7619 | 30.6±13.6309 | 5.6± 4.0373 |
| P value1 | 0.3470 | 0.0287 | 0.0526 | 0.1328 | 0.1290 | 0.2272 |
| P value2 | 0.2245 | 0.3325 | 0.0442 | 0.0236 | 0.0175 | 0.8149 |
| B-2 | 18.36±1.4536 | 20.06±0.4827 | 164.4±26.4821 | 18±1.8708 | 51.4±8.9610 | 7±3.1623 |
| P value1 | 0.0003 | 0.0002 | 0.0004 | 0.0005 | 0.0125 | 0.8466 |
| P value2 | 0.0157 | 0.0041 | 0.0076 | 0.0018 | 0.0008 | 0.0161 |

Table 3 Effect of inoculation of PSB isolates on the growth of Wheat (*Triticum aestivum* L.)

| Isolate code | Shoot length (cm) | Root length (cm) | Shoot | | Root | |
|---|---|---|---|---|---|---|
| | | | Fresh weight (mg) | Dry weight (mg) | Fresh weight (mg) | Dry weight (mg) |
| C1 | 24.66±3.3193 | 13.76±2.7346 | 180.2±43.2400 | 20±3.7417 | 91±35.9235 | 22.8± 5.1672 |
| C2 | 24.14±1.0286 | 22.62±1.4412 | 178.6±24.1826 | 19.2±4.1473 | 120.4±41.3 95 | 20± 8.1548 |
| B-1 | 25.1±2.7668 | 20.1±2.3227 | 184.2±16.3768 | 20.4±4.2190 | 88.8±38.1798 | 14.2± 4.5497 |
| P value1 | 0.5593 | 0.0002 | 0.8166 | 0.7893 | 0.7916 | |
| P value2 | 0.3145 | 0.0081 | 0.4902 | 0.0705 | 0.0099 | 0.0416 |
| B-2 | 26.84±2.8667 | 20.78±0.7260 | 188.4±39.4246 | 20.6±3.9749 | 120.6±48.9673 | 17.8±5.0199 |
| P value1 | 0.1880 | 0.0021 | 0.2108 | 0.3739 | 0.0202 | 0.0185 |
| P value2 | 0.0725 | 0.0099 | 0.4361 | 0.2962 | 0.9902 | 0.4623 |

Table 4 Characterization and identification of potent PSB isolates

| Test | Result | |
|---|---|---|
| | Isolate code B-1 | Isolate code B-2 |
| Colony characters on Nutrient agar | Colony size 4mm, circular, irregular margin, flat, smooth consistency, white, opaque, Gram positive, rods, motile | Colony size 5mm, circular, regular margin, raised, smooth consistency, bluish-green, transparent, Gram negative, short rods, motile |
| Physiological and biochemical characteristics | | |
| Indole production | - | - |
| Methyl red | - | - |
| Voges Proskauer | + | - |
| Citrate utilization | + | - |
| $H_2S$ production | - | - |
| Catalase | + | + |
| Oxidase | + | + |
| Nitrate reduction | + | - |
| Casein hydrolysis | + | + |
| Starch hydrolysis | + | - |
| Gelatin hydrolysis | + | + |
| Urea hydrolysis | - | - |
| Egg yolk reaction | - | - |
| Arginine hydrolysis | + | + |
| Lysine decarboxylation | - | + |
| Acid from Galactose | + | - |
| Acid from Lactose | - | - |
| Acid from Fructose | - | - |
| Acid from Sucrose | + | - |
| Acid from Mannose | + | - |
| Acid from Maltose | + | - |
| Acid from D-Arabinose | + | - |
| Acid from D-Xylose | - | - |
| Acid from Trehlose | - | - |
| Identity of bacterial isolate | *Bacillus amyloliquefaciens* | *Brevundimonas diminuta* |

(+) = positive test; (-) = Negative test

*Effect of inoculation of PSB isolates on plant growth by pot assay*

Data depicted in (Table 1-3 and Fig 1-3) display the growth response shown by crop plants to the selected PSB isolates in the presence of insoluble phosphorus.

*Screening of potent PSB isolates for other plant growth promoting traits*

The two PSB isolates selected showed production of siderophores, indole acetic acid, ammonia, HCN, as well as catalase.

*Identification of bacterial isolates*

The morphological, cultural and biochemical characterization of B-1 and B-2 bacterial isolates revealed the identity as Bacillus amyloliquifaciens and Brevundimonas diminuta (Table 4). The detection of *Bacillus amyloliquifaciens* B-1 and *Brevundimonas diminuta* B-2 was confirmed by 16 S rRNA analyses of selected bacterial isolates (Fig 4-5).


Fig 1 Effect of inoculation of PSB isolates on the growth of maize (from left-C1, C2, B-1 and B-2) (left image) and, shoot and root development in maize (from left-C1, C2, B-1 and B-2) (right image)


Fig 2 Effect of inoculation of PSB isolates on the growth of jowar (from left-C1, C2, B-1 and B-2) (left image) and, shoot and root development in jowar (from left-C1, C2, B-1 and B-2) (right image)


Fig 3 Effect of inoculation of PSB isolates on the growth of wheat (from left-C1, C2, B-1 and B-2) (left image) and, shoot and root development in wheat (from left-C1, C2, B-1 and B-2) (right image)

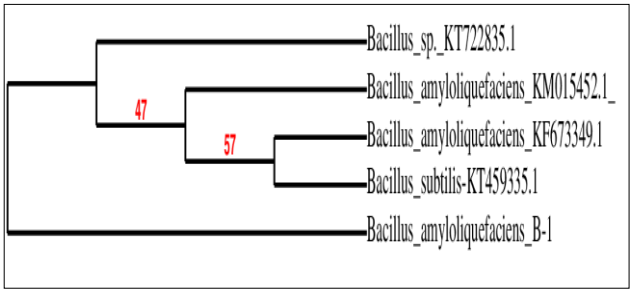


Fig 4 Dendrogram of B-1 isolate showing its phylogenetic position (identified as *Bacillus amyloliquefaciens* B-1)

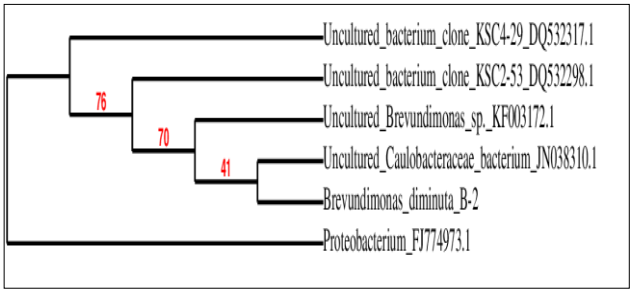


Fig 5 Dendrogram of B-2 isolate showing its phylogenetic position (identified as *Brevundimonas diminuta* B-2)

The seventeen bacteria were isolated using standard methods from the digester effluent of the vegetable waste-based bio-methanation plant. To evaluate insoluble phosphorus solubilization potential, they were further screened. As revealed in solid and liquid media, two bacterial isolates, namely B-1 (identified as *Bacillus amyloliquefaciens*) and B-2 (identified as *Brevundimonas diminuta*), were found to be potent phosphate solubilizers. In addition, the influence of these two isolates was checked by pot assay technique on the growth of maize, jowar and wheat plants.

In the case of maize plants, *Bacillus amyloliquefaciens* stimulated the shoot length, shoot fresh weight and shoot dry weight by 8.10, 9.77 and 3.19 percent respectively compared to Control-1 (C1), while 0.76, 6.70 and 5.72 percent respectively stimulated root length, root fresh weight and root dry weight. As compared to Control-2 (C2), *Bacillus*

*amyloliquefaciens* stimulated 9.80, 13.26 and 7.48 percent respectively of shoot length, shoot fresh weight and shoot dry weight, while 2.24, 12.32 and 8.67 percent respectively stimulated root length, root fresh weight and root dry weight. It was found that *Brevundimonas diminuta* was more stimulatory than B-1. As compared to C1, *Brevundimonas diminuta* stimulated 8.52, 13.93 and 4.59 percent respectively of shoot length, shoot fresh weight and shoot dry weight, while 1.66, 6.11 and 6.18 percent respectively stimulated root length, root fresh weight and root dry weight. As compared to C2, *Brevundimonas diminuta* stimulated 10.22, 17.55 and 8.94 percent of shoot length, shoot fresh weight and shoot dry weight respectively, while 3.15, 11.69 and 9.13 percent respectively stimulated root length, root fresh weight and root dry weight.

*Bacillus amyloliquefaciens* stimulated the shoot length, shoot fresh weight and shoot dry weight by 9.80, 40 and 34.70 percent respectively in the case of Jowar plants compared to C1, while only root length was stimulated by 23.15 percent. Compared to C2, the shoot length, shoot fresh weight and shoot dry weight were stimulated by 15.23, 41.86 and 40.43 percent respectively by *Bacillus amyloliquefaciens*, while 62.77 and 3.70 percent respectively stimulated root fresh weight and root dry weight. The notable impact on the length of the root was not noted. It has been found that *Brevundimonas diminuta* is more stimulatory than *Bacillus amyloliquefaciens*. As compared to C1, *Brevundimonas diminuta* stimulated 20, 53.65 and 83.67 percent of shoot length, shoot fresh weight and shoot dry weight respectively, while 72 and 41.21 percent respectively stimulated root length and root fresh weight. Compared to C2, *Brevundimonas diminuta* stimulated 25.93, 55.68 and 91.49 percent respectively of the shoot length, shoot fresh weight and shoot dry weight, while 33.73, 173.40 and 29.63 percent respectively of the stimulated root length, root fresh weight and root dry weight.

In case of wheat plants, compared to C1, *Bacillus amyloliquefaciens* stimulated 1.78, 2.22 and 2 percent respectively of the shoot length, shoot fresh weight and shoot dry weight, while 46.08 percent stimulated only root length. *Bacillus amyloliquefaciens* stimulated the shoot length, shoot fresh weight and shoot dry weight by 3.98, 3.14 and 6.25% respectively compared to C2, although the stimulatory effect on root length, root fresh weight and root dry weight was not observed.

It has been found that *Brevundimonas diminuta* is more stimulatory than *Bacillus amyloliquefaciens*. As compared to C1, *Brevundimonas diminuta* stimulated 8.84, 4.55 and 3 percent respectively of the shoot length, shoot fresh weight and shoot dry weight, while 51.02 and 32.53 percent respectively stimulated root length and root fresh weight. Compared to C2, Brevundimonas diminuta stimulated 11.19, 5.49 and 7.29 percent respectively of shoot length, shoot fresh weight and shoot dry weight, although no stimulatory effect was observed on root length, root fresh weight and root dry weight.

The statistical analysis of the P test results showed that these isolates significantly stimulate crop plant growth in relation to controls. Other plant growth promoting characteristics such as siderophores, ammonia, catalase and hydrogen cyanide were also produced by these two isolates. It is understood that the digester effluent improves soil fertility and crop yield [25]. Bacteria that solubilize phosphate have been documented to play an important role in promoting plant growth [26-29]. *B. amyloliquefaciens* strains have gain attention as plant growth promoting bacteria because of their ability to generate multiple plant growths promoting compounds [30]. *Brevundimonas diminuta* was previously known as *Pseudomonas diminuta* and has the potential to promote plant growth. *Brevundimonas diminuta* was previously known as *Pseudomonas diminuta* and has the potential to promote plant growth. It is clear that these two bacteria directly promote plant growth through phosphate solubilization and can also indirectly promote growth through the removal of pathogen through siderophore and cyanide production [31-32].

## CONCLUSIONS

The indiscriminate and excess abuse of chemical fertilizers in recent years has caused serious damage to agriculture and environment. In the present study, *Bacillus amyloliquifaciens* and *Brevundimonas diminuta* isolated and identified from digester effluent of vegetable waste-based bio-methanation plant were found to be most potent phosphate solubilizing among the seventeen bacterial isolates. These two isolates exerted their noticeable effect on the shoot and root development of crop plants as revealed in pot assay. Further, these isolates showed production of siderophores, IAA, ammonia, catalase and hydrogen cyanide. The present study suggests that these isolates can be used for development of efficient biofertilizers in ecofriendly and sustainable development of agriculture that will help to reduce environmental pollution by avoiding excessive applications or substitute to chemical inorganic phosphate-based fertilizers.

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