Research Article

Isolation and Molecular Characterization of Potential Plant Growth Promoting *Bacillus cereus* GGBSTD1 and *Pseudomonas* spp. GGBSTD3 from Vermisources

Balayogan Sivasankari and Marimuthu Anandharaj

Department of Biology, Gandhigram Rural Institute-Deemed University, Gandhigram, Dindigul, Tamil Nadu 624 302, India

Correspondence should be addressed to Balayogan Sivasankari; balayogansivasankari@gmail.com

Received 1 May 2014; Accepted 28 July 2014; Published 1 September 2014

Academic Editor: Tibor Janda

Copyright © 2014 B. Sivasankari and M. Anandharaj. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Vermicompost was prepared from leaf materials of *Gliricidia sepium* + *Cassia auriculata* + *Leucaena leucocephala* with cow dung (1:1:2) using *Eudrilus eugeniae* (Kinberg) and *Eisenia fetida* for 60 days. Nineteen bacterial strains which have the capability to fix nitrogen, solubilize inorganic phosphate, and produce phytohormones were isolated from vermicompost, vermisources, and earthworm (fore, mid, and hind) guts and tested for plant growth studies. Among the bacterial strains only five strains had both activities; among the five *Bacillus* spp. showed more nitrogen fixing activity and *Pseudomonas* spp. showed more phosphate solubilizing activity. Hence these bacterial strains were selected for further molecular analysis and identified *Bacillus cereus* GGBSTD1 and *Pseudomonas* spp. GGBSTD3. Plant growth studies use these two organisms separately and as consortium (*Bacillus cereus* + *Pseudomonas* spp.) in (1:1) ratio at different concentrations using *Vigna unguiculata* (L.) Walp. at different day intervals. The germination percent, shoot length, root length, leaf area, chlorophyll a content of the leaves, chlorophyll b content of the leaves, total chlorophyll content of the leaves, fresh weight of the whole plant, and dry weight of the whole plant were significantly enhanced by the consortium (*Bacillus cereus* + *Pseudomonas* spp.) of two organisms at 5 mL concentrations on the 15th day compared to others.

1. Introduction

Nowadays, under the modern agricultural practices, chemical fertilizers are used to boost the crop production. But the application of chemical fertilizers affects the total productivity of the crops and in the long run the soil becomes sterile and unfit for cultivation practices. Hence, in order to enhance the fertility status of the soil, the natural way of feeding the soil with different types of organic inputs (composts, vermicomposts, biofertilizers, farmyard manure, etc.) has been developed so as to ensure sustained productivity [1]. However, a better understanding of nutrient cycling and the factors governing their decomposition in soil is imperative for implementing sustainable management practices. Nutrient cycling in soil involves chemical, biochemical, and physicochemical reactions, with the biochemical reactions being catalyzed by soil enzymes associated with viable cells

of microbial origin and plant roots. Therefore, any factor that affects soil microbial population will necessarily alter soil enzyme activity [2]. Sustainability of agricultural systems has become an important issue all over the world. Many of the issues of sustainability are related to soil quality and its change with time. It is well known that intensive cultivation has led to a rapid decline in organic matter and nutrient levels besides affecting soil physical properties. Conversely, management practices with organic materials influence agricultural sustainability by improving physical, chemical, and biological properties of soils [3].

Recently, there is increasing interest in the potential of vermicomposts, as plant growth media and as soil amendments. Vermicomposts are finely divided peat-like materials with high porosity, aeration, drainage, water-holding capacity, and microbial activity, which make them excellent soil amendments or conditioner. It is a sustainable source of

macro- and micronutrients, which enlivens the soil through partial substitution of the horticultural container media. Enhancement in plant growth after substitution of soils or greenhouse container media with composts is attributed to modifications in soil structure, change in water availability, increased availability of macro- and micronutrients, stimulation of microbial activity, and augmentation of the activities of critical substances by microorganisms through interactions with earthworms [4]. The vermicompost is rich in available nutrients, soil beneficial microbes, and plant growth promoting substances.

Research during the past few decades has led to the identification of certain biological organisms and their products that could potentially be used as fertilizer sources. This strategy of fertilizing the soil with biological sources has been widely accepted and recognized as a viable alternative to the application of chemical fertilizers. Nitrogen (N) fixing and phosphate (P) solubilizing bacteria may be important for plant nutrition by increasing N and P uptake by the plants and playing a significant role as plant growth promoting bacteria in the biofertilization of crops. Increasing and extending the role of biofertilizers could reduce adverse environmental effects caused by chemical fertilizers. Nitrogen is one of the basic requirements for the growth, productivity, and yield of plants. On a worldwide basis it is estimated that about 175 million tons of nitrogen per year is added to soil through biological nitrogen fixation. Meanwhile superphosphate fertilizer is expensive and is in short supply and hence biofertilizers can bridge the gap. Soluble fertilizer phosphorus (P) is the world's second largest bulk agricultural chemical under use and as such is absolutely essential for food production. However, most soil phosphorus, approximately 95–99 percent, is present in the form of insoluble phosphates and hence cannot be utilized by plants [5]. To increase the availability of phosphorus to plants, large amounts of fertilizers are used on a regular basis. But, after application, a large proportion of the fertilizer phosphorus is quickly transferred to the insoluble form [6]. Therefore, a very little percentage of the applied phosphorus only is used, making continuous application necessary. It has been reported that many soil fungi and bacteria can solubilize inorganic phosphates [7]. The *Bacillus* species offer several advantages over the other genera because of their capacity to produce spores in unfavorable environmental conditions. This characteristic facilitates the conversion of spore suspensions to powder formulations without killing bacteria [8]. Vermicompost is rich in NPK, plant growth promoters, and other nutrients and when applied, the soil will be enriched with a variety of nutrients that will become available for the indigenous microflora.

The PGPR have been known to directly enhance plant growth by a variety of mechanisms, namely, fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones [9]. Plant growth promoting bacteria (PGPB) stimulate plant growth by nitrogen fixation [10], solubilization of nutrients [11], and production of growth hormones and 1-aminocyclopropane-1-carboxylate (ACC).

The present paper describes the potential of PGPR isolates from vermisources and their plant growth stimulating activity in pot trials under ambient conditions. Further studies will clarify the potential use of these bacteria used as biofertilizers.

The main objective of this study was to isolate, identify, and characterize the potential plant growth promoting rhizobacteria from vermisources, as well as evaluating their plant growth potential under controlled environment and characterizing the isolated bacteria using various molecular techniques. These bacteria can be considered promising candidates for application in sustainable agricultural management.

2. Materials and Methods

2.1. Vermicompost Preparation. For the present study epigeic earthworms, *Eudrilus eugeniae* (Kinberg) and *Eisenia fetida* (Savigny), were collected from the breeding stock of the Department of Biology, Gandhigram Rural Institute-Deemed University, Gandhigram, Tamil Nadu, India, and leaf materials of *Gliricidia sepium* Jacq, *Leucaena leucocephala* (Lam.) De Wit, and *Cassia auriculata* Linn. were collected from Gandhigram campus. The leaf materials were separately subjected to predigestion for 15 days by sprinkling water on the heap and covering it with gunny bag and turning it periodically in order to release out the initial heat produced during decomposition of organic material. The changes in temperature were observed every three days up to 15 days. The vermibeds were prepared in plastic containers of 45 × 35 × 15 cm size and the substrate was moistened to hold 60–80 percent moisture and kept for 24 hours stabilization. 20 numbers of healthy clitellate *E. eugeniae* and 30 numbers of *E. fetida* were separately introduced in the vermibeds. The vermicomposting trials were carried out in the rearing room with the relative humidity and the temperature of 75–85 percent and 26–28°C, respectively. The substrate was turned (mixed) once in a week and maintained up to 60 days. The experiment was carried out with three replicates for each substrate with proper control [12].

2.2. Microbial Study. The total microbial counts in terms of colony forming units (CFU) of bacteria in the vermicomposts, vermicasts, and earthworm (fore, mid, and hind) guts were determined every 15 days (0, 15, 30, 45, and 60 d) using standard plate count method. From the total colony forming units, only those bacterial colonies (19 bacterial colonies) which showed predominant growth were restreaked onto appropriate agar medium to obtain pure cultures and subjected to characterization and identification.

2.3. Screening for Biofertilization Activities

2.3.1. In Vitro Nitrogen Fixing Activity. The bacterial isolates were screened for nitrogen fixing ability using nitrogen-free liquid medium [13]. Nitrogen-free medium was prepared and sterilized. The strains were inoculated separately on selective nitrogen-free liquid medium. All the test organisms were incubated at 37°C ± 2°C for 7 days. The nitrogen fixing

activities were observed on the basis of the formation of turbidity in the flasks. Among all the nineteen bacterial isolates tested, only five bacterial isolates formed significantly higher rate of turbidity in the nitrogen-free liquid medium and selected for further molecular and plant growth studies.

2.3.2. In Vitro Phosphate Solubilization. All bacterial isolates were screened for inorganic phosphate solubilization using Pikovskaya's agar medium, the selective medium for phosphate solubilization test [14]. Pikovskaya's agar medium was prepared and sterilized and the bacterial strains were inoculated separately on the selective medium containing 0.5 percent of (w/v) tricalcium phosphate (Ca_3PO_4) as complex insoluble phosphate source. All the test plates of the bacteria were incubated at $37^\circ\text{C} \pm 2^\circ\text{C}$ for 10 days. After incubation for up to 7 days at 30°C , formation of yellow halos and/or clearing zones was evaluated. The results were expressed as solubilization index (SI) and they were measured using the following formula [15]:

$$\text{SI} = \frac{\text{Colony diameter} + \text{halozone diameter}}{\text{Colony diameter}}. \quad (1)$$

Five bacterial strains which showed phosphate solubilizing activity were identified and grouped as phosphate solubilizing bacteria (PSB). The bacterial strain which showed highest SI was selected for further molecular and plant growth studies.

2.4. Production of Phytohormones (IAA). The bacterial cultures were inoculated in nutrient broth with tryptophan ($5\text{ }\mu\text{g/mL}$) and incubated at $28 \pm 2^\circ\text{C}$ for 5 days. After incubation cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski's reagent (50 mL of 35% perchloric acid + 1 mL 0.5 FeCl_3) and incubated in the dark for 25 minutes. Development of pink colour indicates indole-3-acetic acid (IAA) production. The optical density was measured at 530 nm using Spectronic 200 (India). The quantity of IAA production was estimated using standard IAA graph and expressed as micrograms per milliliter [16, 17].

2.5. Siderophore Production. A qualitative assay of siderophore production was conducted in Chrome Azurol S (CAS) agar medium [18]. CAS agar plates were prepared and spot-inoculated with test organism and incubated at 30°C for 3–5 days. Change of blue color of the medium surrounding the bacterial growth to fluorescent yellow indicated the production of siderophore. *Bacillus subtilis* was chosen as a positive control [19, 20].

2.6. 16S rDNA Gene Amplification and Sequencing. Genomic DNA from each isolate was extracted using the modified method of Smoker and Barnum [21]. The extracted DNA was dissolved in $20\text{ }\mu\text{L}$ TE buffer and used as the template for the PCR reactions. PCR amplifications were performed in a total volume of $50\text{ }\mu\text{L}$ by mixing 20 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide

triphosphate and $1\text{ }\mu\text{M}$ of each universal primer of Bac8uf (5'-AGAGTTTGATCCTGGCTCAG-3') and Univ1492r (5'-CTACGGCTACCTTGTTCAG-3') described by Edwards et al. [22]. The thermocycling profile was carried out with an initial denaturation at 95°C (4 min) followed by 30 cycles of denaturation at 95°C (1 min), annealing at 56°C (30 s), extension at 72°C (1 min), and a final extension at 72°C (10 min) in an Eppendorf Gradient thermocycler. The PCR amplified rDNA were purified by using the Quick PCR purification kit (Bangalore Genie, India). 16S rRNA gene sequence of the isolate was compared with 16S rRNA gene sequences available by the BLAST search in the NCBI, GenBank database (<http://www.ncbi.nlm.nih.gov>). The analysis of alignment and homology of the partial nucleotide sequence of *Bacillus* sp. was carried out by the basic local alignment search tool (BLAST). The PCR products were stored at 4°C . Aliquots of the PCR products were separated by 2% agarose gel electrophoresis in TAE buffer (pH 8.0). A 100 bp DNA marker (Sigma, Bangalore) was used as a reference. Gels were stained with ethidium bromide (11 g/mL) and visualized under UV light.

2.7. Nucleotide Sequence Accession. The sequence obtained in this study was deposited in the GenBank (USA) nucleotide sequence database under the accession number GQ413962 (*B. cereus*) and HM753262 (*Pseudomonas* spp.).

2.8. Phylogenetic Analysis. The 16S rRNA gene sequences were obtained in the present study and the taxonomically related *Bacillus* spp. were retrieved from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>). All the sequences were aligned using multiple sequence alignment program CLUSTAL W developed by Higgins et al. [23]. The pairwise evolutionary distances were computed using the method of Kimura [24]. The multiple distance matrix obtained was then used to construct phylogenetic trees using neighbour joining method of Saitou and Nei [25]. Phylogenetic trees were constructed by using Mega 5 software (Molecular Evolutionary Genetic Analysis).

2.9. Prediction of RNA Secondary Structure. The secondary structure of 16S rDNA of *Bacillus* spp. was predicted using the bioinformatics Genebee tool.

2.10. Restriction Site Analysis in 16S rDNA. The restriction sites in DNA of *Bacillus* spp. were analyzed using the NEB Cutter program version 2.0 tools available online at <http://tools.neb.com/NEBcutter2/index.php>.

2.11. Fermentation Process. For the preparation of inoculum, 50 mL of nutrient broth medium (HiMedia, Mumbai, India) was inoculated with appropriate overnight bacterial culture and incubated at 37°C for 24 h at 120 rpm ($\text{OD}_{540} = 0.45; 10^7\text{--}10^8\text{ CFU mL}^{-1}$). The cell-free supernatant was obtained by centrifuging the fermented broth at

10,000 rpm for 10 min. The supernatant was analyzed for plant growth promotion at various concentrations.

2.12. Evaluation of Plant Growth Promoting Activity under Controlled Environment. The nitrogen fixing bacteria (*Bacillus* spp.) and the phosphate solubilizing bacteria (*Pseudomonas* spp.) were separately tested for plant growth promoting activity and also as consortium (1:1) at 5 different concentrations, that is, 1, 2, 3, 4, and 5 mL at three different day intervals (5, 10, and 15 days) with proper control through Exp1, Exp2, Exp3, and Exp0, respectively, using *Vigna unguiculata* (L.) Walp. The seeds (30 seeds in each pot) were sown in plastic pots containing sterilized vermiculite and arranged in a completely randomized factorial design. The seedlings were grown in a greenhouse at a temperature of 28–32°C and 85% relative humidity. The pots were watered to 50% water-holding capacity and were maintained at this moisture content by watering to weight every day and plant growth was observed for 15 days. Parameters such as germination percent, shoot length, root length, leaf area, chlorophyll content of the leaves, fresh weight of the whole plant, and dry weight of the whole plant were measured using standard procedure.

2.13. Statistical Analysis. The following statistical tools were used for the analyses and interpretation of the data. The experimental results are presented in the form of tables and graphs using Microsoft Excel 2007. Data obtained from the different treatments were statistically analyzed using the one-way ANOVA and the mean values were compared by the Duncan's multiple range test for multiple comparisons. Differences were considered significant at the 0.05 level using Origin software (Version 8.5.0) 2010, Origin Lab Corporation.

3. Results

3.1. Isolation and Characterization of Bacterial Isolates. A total of nineteen bacterial strains were isolated from vermicompost, vermicast, and earthworm (fore, mid, and hind) guts (Table 1). The morphological and biochemical characteristics of bacterial isolates were elucidated in Table 1. All the isolates were tested for plant growth promoting activity. Among the 19 bacterial isolates only five bacterial strains showed plant growth promoting activity. Based on colony morphology, microscopic observations, and cultural, biochemical, and physiological properties, the bacterium was given the name *Bacillus* spp., *Alcaligenes* spp., *Erwinia* spp., *Serratia* spp., or *Pseudomonas* spp. These five bacterial strains were able to grow in the nitrogen-free medium and they were also identified as phosphate solubilizing microorganisms on the basis of their solubilization index (Table 2). *Bacillus* spp. have the highest SI (38.55), followed by *Pseudomonas* spp. (35.22) and *Serratia* spp. (30.45). All the isolates produced a significant amount of IAA from the medium in the range of 11.3 to 24.2 µg/mL; *Bacillus* spp. showed higher IAA production (24.2 µg/mL) and *Serratia* spp. showed lower IAA production (11.3 µg/mL). All the strains (except *Alcaligenes*

spp.) were positive for siderophore production, showing a yellow zone on the CAS agar medium plate (Table 2).

3.2. Molecular Studies. The partial 16S rRNA sequences carried out in the present study for *Bacillus* spp. and *Pseudomonas* spp. covered a stretch of approximately 1500 nucleotides for each. About half of the sequences found in the clone library showed only slight relationship to other known sequences, while the other half were highly similar (approximately 95 percent sequence identity) to other database entries for *Bacillus* spp. and *Pseudomonas* spp. Less than 0.5 percent of all nucleotides was found to be unique within the conserved regions of the cloned sequence and could almost always be related to reading errors in ambiguous regions of the sequencing gel. On the basis of phylogenetic analysis of 16S rDNA partial sequences, *Bacillus* spp. GGBSTD1 is identified as *Bacillus cereus* and *Pseudomonas* spp. GGBSTD3 is identified as *Pseudomonas* spp. Phylogenetic analyses of the strains based on the neighbor joining method were represented in Figures 1 and 2. Among eubacteria, the mean guanine and cytosine (G + C) content of genomic DNA varies from approximately 25% to 75%. That the bacterial genomic G + C content is somehow related to phylogeny has been suggested [26, 27]. The phylogenetic tree of eubacterial 16S rRNA clearly indicates this relationship. Gram-positive bacteria *Bacillus subtilis* have genomic G + C, 42%. Gram-negative bacteria with intermediate G + C content, such as *Escherichia coli* (50%), *Serratia marcescens* (58%), *Salmonella typhimurium* (51%), and *Pseudomonas fluorescens* (60%), belong to the common Gram-negative bacteria [28]. *Bacillus cereus* have the G + C content 53% and A + T content 47%, similarly *Pseudomonas* spp. having G + C content 53% and A + T content 47%. Most functional RNA molecules have characteristic secondary structures that are highly conserved in evolution. In this study RNA secondary structures of *Bacillus cereus* and the *Pseudomonas* spp. were predicted, *Bacillus cereus* have free energy of −294.1 kkal/mol, and *Pseudomonas* spp. have free energy of −299.8 kkal/mol.

3.3. Evaluation of Biofertilization Abilities under Controlled Conditions. Germination percentage of *Vigna unguiculata* seeds sown in vermiculite supplemented with *Bacillus cereus* (Exp1), with *Pseudomonas* spp. (Exp2), and with consortium (Exp3) (1:1 ratio) at five different concentrations (1, 2, 3, 4, and 5 mL) and in the control (Exp0) is shown in Figure 3. The highest germination percentage was observed in Exp3 (consortium) at 5 mL concentration compared to others. The increase in germination percentage and in other growth parameters can be attributed to the combined effect of the nitrogen fixing and phosphate solubilizing microbes which are able to fix atmospheric nitrogen and solubilize P and also produce growth promoting substances. These organisms have been isolated from vermicompost and this observation indicates the reason for promotion and enhancement of crop growth and yield when vermicompost is applied. It is a proof that these symbiotic microbes present in vermicompost benefit the soil fertility. Observation on growth parameters such as shoot length, root length, and leaf area of *V. unguiculata*

TABLE 1: Predominant bacterial strains isolated from vermisources and their biochemical characteristics. T1—control—*G. sepium* + *L. leucocephala* + cow dung in (1:1:2) (worm free), T2—*G. sepium* + *L. leucocephala* + cow dung in (1:1:2) + *E. eugeniae*, T3—*G. sepium* + *L. leucocephala* + cow dung in (1:1:2) + *E. fetida*, T4—control (2)—*C. auriculata* + *L. leucocephala* + cow dung in (1:1:2) (worm free), T5—*C. auriculata* + *L. leucocephala* + cow dung in (1:1:2) + *E. eugeniae*, T6—*C. auriculata* + *L. leucocephala* + cow dung in (1:1:2) + *E. fetida*.

S. number	Sample source	Gram's reaction ^a	Cell shape	Motility ^b	Indole test	Methyl Red test	Voges Proskauer test	Biochemical characteristics ^c							Name of the strain
								Citrate Utilization test	Catalase test	Urease test	Gelatin hydrolysis test	Nitrate reductase test	Starch hydrolysis test	Casein hydrolysis test	
1.	Vermicompost (T2)	+	Cocci	—	—	+	—	+	—	+	+	—	—	—	<i>Staphylococcus</i> spp.
2.	Vermicompost (T3)	+	Rod	+	—	—	—	—	+	—	+	—	—	+	<i>Corynebacterium</i> spp.
3.	Vermicompost (T5)	+	Rod	—	+	+	+	—	—	+	+	+	+	+	<i>Bacillus</i> spp.
4.	Vermicompost (T6)	+	Cocci	—	+	+	—	—	+	—	—	—	—	+	<i>Micrococcus</i> spp.
5.	Vermicast (T3)	+	Cocci	—	+	+	—	—	+	+	+	—	+	—	<i>Klebsiella</i> spp.
6.	Vermicast (T5)	+	Rod	—	+	—	—	—	+	—	—	—	—	+	<i>Streptococcus</i> spp.
7.	Vermicast (T6)	+	Cocci	—	+	—	+	+	—	—	+	+	—	+	<i>Enterobacter</i> spp.
8.	Earthworm hind gut (T2)	+	Cocci	—	—	+	+	—	—	—	—	+	+	+	<i>Tricoccus</i> spp.
9.	Earthworm hind gut (T3)	+	Cocci	—	—	+	—	+	+	—	+	—	—	—	<i>Yersinia</i> spp.
10.	Earthworm hind gut (T5)	+	Cocci	—	—	—	—	—	+	+	+	—	—	—	<i>Micrococcus</i> spp.
11.	Vermicompost (T1)	—	Rod	—	+	+	—	—	—	+	+	—	—	+	<i>Escherichia</i> spp.
12.	Vermicompost (T2)	—	Rod	—	—	+	—	—	+	+	+	—	—	+	<i>Proteus</i> spp.
13.	Vermicompost (T4)	—	Rod	—	—	—	+	—	—	—	+	+	+	+	<i>Paracoccus</i> spp.
14.	Vermicompost (T5)	—	Rod	+	+	—	—	—	+	+	+	—	—	—	<i>Pseudomonas</i> spp.
15.	Vermicast (T2)	—	Rod	+	—	+	—	—	+	—	+	—	—	—	<i>Citrobacter</i> spp.
16.	Vermicast (T3)	—	Cocci	—	+	+	—	—	+	—	—	—	—	—	<i>Erwinia</i> spp.
17.	Vermicast (T5)	—	Rod	+	+	+	+	+	+	+	+	—	—	—	<i>Alcaligenes</i> spp.
18.	Vermicast (T6)	—	Cocci	—	—	+	+	+	+	+	+	—	—	—	<i>Serratia</i> spp.
19.	Earthworm mid gut (T2)	—	Rod	+	—	—	—	—	—	—	—	—	—	—	<i>Pediococcus</i> spp.

^aGram's staining: (+) positive, (–) negative.

^bMotility: (+) motile, (–) nonmotile.

^cBiochemical characteristics: (+) positive, (–) negative.

FIGURE 1: Neighbor joining phylogenetic tree of full 16S rRNA gene sequences showing the relationship among *Bacillus cereus* GGBSTD1 and representatives of some related taxa. The sequence data for several closely related *Bacillus* cultures were recovered from GenBank and included in the tree.

TABLE 2: The phosphate solubilizing activity, phytohormone (IAA) production, and siderophores production of the bacterial isolates.

Name of the organism	Phosphate solubilization index (SI)	IAA production ($\mu\text{g/mL}$) ^a	Siderophores production ^b
<i>Bacillus</i> spp.	38.55	24.2	+++
<i>Alcaligenes</i> spp.	29.30	14.7	–
<i>Erwinia</i> spp.	28.90	11.3	+
<i>Serratia</i> spp.	30.45	16.8	++
<i>Pseudomonas</i> spp.	35.22	19.6	+++

^aIndole acetic acid production in culture media supplemented with tryptophan (5 $\mu\text{g/mL}$) after 5 days.

^b*In vitro* siderophores production: – represents the absence of siderophores production and +++ represents >10 mm wide yellow-orange zone.

is given in Tables 3 and 4, respectively. The shoot length (11.47 ± 0.06 cm), root length (10.27 ± 0.06 cm), and leaf area (9.05 ± 0.26 cm²) of the plant get significantly ($P < 0.001$) increased in Exp3 (consortium of *Bacillus cereus* + *Pseudomonas* spp. [1:1]) at 5 mL concentration on the 15th day compared to other experiments. The chlorophyll a, b and total chlorophyll content of the leaves on d 5, d 10, and d 15 are given in Table 5. The chlorophyll a (0.10 ± 0.09 mg/g of fresh leaf), chlorophyll b (0.85 ± 0.16 mg/g of fresh leaf), and total chlorophyll (10.95 ± 0.07 mg/g of fresh leaf) content

of leaves were significantly ($P < 0.001$) higher in Exp3 (consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1)) at 5 mL concentration on the 15th day compared to other experiments. Observations on the fresh weight of the whole plant are given in Table 6. The fresh weight of the whole plant (0.92 ± 0.00 g) was significantly ($P < 0.001$) higher in Exp3 (consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1)) at 5 mL concentration on the 15th day compared to other experiments. Observations on the dry weight of the whole plant are given in Table 6. The dry weight of the whole

FIGURE 2: Neighbor joining phylogenetic tree of full 16S rRNA gene sequences showing the relationship among *Pseudomonas* spp. GGBSTD3 and representatives of some related taxa. The sequence data for several closely related *Pseudomonas* cultures were recovered from GenBank and included in the tree.

plant (0.09 ± 0.00 g) was significantly ($P < 0.001$) higher in Exp3 (consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1)) at 5 mL concentration on the 15th day compared to other experiments.

4. Discussion

Inoculation of these newly identified nitrogen fixing and phosphate solubilizing microbes in the plant growth medium enhanced various physiological activities culminating in higher biomass production in *V. unguiculata*. Since this is a nonhazardous way of fertilization of crop plants, it is very relevant to a developing country like India. This technique can save the farmers of India from the problem posed by high cost of fertilizers by offering a comparatively inexpensive alternative (biofertilizers), while additionally preventing the degradation of the soil and thereby ensuring sustainable agriculture. Bess (1999) reported that the content in composting needs to be determined through the concentration of six functional groups of microorganisms such as aerobic bacteria, anaerobic bacteria, fungi, actinomycetes, *Pseudomonas*, and nitrogen fixing bacteria [29]. Now there are ways to evaluate the concentrations of these organisms in the finished compost and that can serve as an interpretation guide to

determine the quality of the compost as an inoculant of soil microorganisms. Graff (1981) and Atlavinyte have reported a higher P content in the cast than in the surrounding soil [30]. Satchell (1983) has linked the phosphatase activity in the gut of worms to the availability of bound P in soils. The bacterial strains which have the capability to fix nitrogen only can grow in the nitrogen-free medium [31]. Hence the nitrogen fixing ability was tested in the present study based on the growth of bacterial strain in nitrogen-free medium. Five bacteria strains showed more nitrogen fixation; among all the five bacterial strains tested only one strain (i.e., *Bacillus* spp.) showed significantly more turbidity compared to the other strains. The ultimate source of the nitrogen used by plants is N_2 gas, which constitutes 78% of the Earth's atmosphere. Unfortunately higher plants cannot metabolize N_2 directly into protein. N_2 gas must be converted to a plant available form [32] isolated many species of nitrogen fixing *Bacillus* (*B. megaterium*, *B. cereus*, *B. subtilis*, *B. licheniformis*, and *B. azotoformans*) from the rhizospheric zone of rice fields in Central China. The widely studied *Bacillus* genus represents one of the most diverse genera in the bacilli group. Numerous *Bacillus* and *Paenibacillus* strains express plant growth promoting (PGP) activities and a number of these strains have already been commercially developed as biological fungicides, insecticides, and nematicides or generic plant

TABLE 3: Shoot length and Root length of *Vigna unguiculata* grown on vermiculite supplemented with *Bacillus cereus*, with *Pseudomonas* spp., and with their consortium (1:1) at five different concentrations (1, 2, 3, 4, and 5 mL) on the 5th, the 10th, and the 15th day and in the control.

Experiments ^a	Quantity of bacterial cultures added	Shoot length (cm) ^b			Root length (cm) ^b		
		5th day	10th day	15th day	5th day	10th day	15th day
Exp0	1 mL	2.97 (ab)	3.97 (a)	4.97 (ab)	1.97 (de)	2.83 (ab)	3.97 (bcd)
	2 mL	3.03 (bcd)	4.03 (ab)	5.00 (ab)	2.00 (abc)	2.87 (de)	4.00 (a)
	3 mL	3.10 (abc)	4.07 (a)	5.03 (ab)	2.03 (de)	2.90 (abc)	4.03 (ab)
	4 mL	3.10 (bcd)	4.10 (bcd)	5.07 (bcd)	2.07 (a)	2.97 (de)	4.10 (abc)
	5 mL	3.13 (a)	4.13 (ab)	5.10 (ab)	2.10 (bcd)	3.00 (ab)	4.13 (de)
Exp1	1 mL	3.40 (ab)	4.40 (abc)	5.40 (ab)	2.33 (abc)	3.27 (bcd)	4.33 (f)
	2 mL	4.50 (ab)	5.40 (abc)	6.47 (ab)	3.33 (ab)	4.30 (a)	5.40 (abc)
	3 mL	5.50 (de)	6.30 (ab)	7.50 (a)	4.40 (de)	5.50 (abc)	6.20 (a)
	4 mL	6.53 (ab)	7.30 (a)	8.33 (ab)	5.50 (bcd)	6.53 (de)	7.50 (abc)
	5 mL	7.40 (ab)	8.40 (abc)	9.23 (f)	6.40 (abc)	7.53 (ab)	8.27 (ab)
Exp2	1 mL	2.77 (bcd)	3.57 (f)	4.40 (de)	1.60 (de)	2.17 (abc)	3.50 (f)
	2 mL	3.43 (abc)	4.47 (ab)	5.53 (ab)	2.53 (abc)	3.40 (bcd)	4.27 (bcd)
	3 mL	4.37 (de)	5.43 (abc)	6.33 (abc)	3.40 (ab)	4.43 (f)	5.33 (ab)
	4 mL	5.47 (bcd)	6.47 (de)	7.33 (a)	4.20 (bcd)	5.50 (ab)	6.33 (ab)
	5 mL	6.40 (f)	7.33 (bcd)	8.33 (bcd)	5.33 (ab)	6.30 (ab)	7.30 (bcd)
Exp3	1 mL	5.30 (bcd)	6.47 (ab)	7.30 (ab)	4.33 (de)	5.50 (abc)	6.20 (ab)
	2 mL	6.43 (ab)	7.40 (bcd)	8.27 (f)	5.47 (a)	6.30 (de)	7.33 (ab)
	3 mL	7.37 (bcd)	8.40 (bcd)	9.27 (ab)	6.40 (ab)	7.40 (bcd)	8.40 (bcd)
	4 mL	8.47 (abc)	9.30 (a)	10.43 (abc)	7.40 (f)	8.33 (a)	9.37 (abc)
	5 mL	9.57 (ab)	10.30 (f)	11.47 (bcd)	8.33 (abc)	9.40 (ab)	10.27 (bcd)

^aExp0: nutrient solution (control), Exp1: *Bacillus cereus*, Exp2: *Pseudomonas* spp., Exp3: consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1).

^bMean of two repeated experiments (30 plants). Different letters within parenthesis indicate significant difference between treatments for each growth parameter using Duncan's multiple range test ($P = 0.05$).

growth promoters. The use of these strains in agriculture has recently been reviewed [33]. Those strains besides having several PGP properties can also fix nitrogen and also do phosphate solubilization. The symbiotic fixation of nitrogen through inoculation of legume crops with effective rhizobia is well known [34]. Asymbiotic nitrogen fixing bacteria, which live in the rhizosphere and/or endophytically, often increase yields of crops. Many bacterial species have nitrogen fixing properties, including *Bacillus* spp., *Azotobacter* spp., *Azospirillum* spp., *Beijerinckia* spp., and *Pseudomonas* spp. [35]. Nitrogen fixing bacteria have been used in foliar applications in mulberry. The solubilization index (SI) was formed due to solubilization of insoluble phosphates by organic acid secretion [36]. El-Komy (2005) indicated that *Pseudomonas fluorescence* and *Bacillus megaterium* strains were the most powerful phosphate solubilizers on PVK plates as well as on Pikovskaya's broth [37]. The P concentration in Pikovskaya's broth increased gradually, achieving a peak on the sixth day and declined slowly during the late days. In this study, pH values decreased gradually in PVK broth during the early incubation days and no revival was observed during later days for all the tested bacterial strains. Several bacteria, particularly those belonging to the genus *Bacillus* spp., convert insoluble phosphate into soluble forms by secreting organic acids such as formic acid, acetic acid, propionic acid, citric acid, fumaric acid, gluconic acid, glyoxylic acid, ketobutyric

acid, malonic acid, succinic acid, and tartaric acid. These acids lower the pH and bring about the dissolution of bound forms of phosphate. Some of the hydroxyl acids may chelate with calcium and iron resulting in effective solubilization and utilization of phosphates [38].

Beneduzi et al. (2008) had isolated the plant growth promoting strain SVPR30 and identified by 16S rRNA gene sequence as *Bacillus* spp. and they had tested it for plant growth through *in vivo* experiments [39]. This strain was characterized as a high IAA producer, able to solubilize phosphate and also fix a considerably high amount of nitrogen. The inoculation of rice with *Bacillus* spp. SVPR30 strain showed a significant increase in the root and shoot parts when compared with the controls within 15 and 30 days after sprouting. Numerous *Bacillus* and *Paenibacillus* strains expressed plant growth promoting (PGP) activities and a number of these strains have been commercially developed as generic plant growth promoters. The use of these strains in agriculture has recently been reviewed [33].

Various reports indicate that coinoculation of beneficial organisms generally increased plant growth relative to single inoculation with a sole beneficial organism [40]. Most of the effects of the individual microorganisms coinoculation are additive, although a synergistic effect has been reported in some cases. The average root to shoot ratio was higher in *B. subtilis* treated yam minisetts in comparison to those

TABLE 4: Leaf area of *Vigna unguiculata* grown on vermiculite supplemented with *Bacillus cereus*, with *Pseudomonas* spp., and with their consortium (1:1) at five different concentrations (1, 2, 3, 4, and 5 mL) on the 5th, the 10th, and the 15th days and in the control.

Experiments ^a	Quantity of bacterial cultures added	Leaf area (cm ²) ^b		
		5th day	10th day	15th day
Exp0	1 mL	0.27 (ab)	0.94 (abc)	4.07 (b)
	2 mL	0.35 (ab)	1.02 (ab)	4.19 (abc)
	3 mL	0.40 (abc)	1.25 (ab)	4.71 (bcd)
	4 mL	0.48 (ab)	1.38 (abc)	5.10 (ab)
	5 mL	0.54 (abc)	1.61 (b)	5.38 (b)
Exp1	1 mL	0.18 (bcd)	1.23 (b)	6.01 (abc)
	2 mL	0.25 (ab)	1.38 (abc)	6.32 (ab)
	3 mL	0.33 (abc)	1.50 (ab)	6.84 (b)
	4 mL	0.38 (ab)	1.68 (ab)	6.86 (abc)
	5 mL	0.44 (abc)	1.91 (bcd)	7.33 (ab)
Exp2	1 mL	0.11 (b)	1.07 (ab)	5.34 (b)
	2 mL	0.14 (ab)	1.19 (ab)	5.59 (ab)
	3 mL	0.17 (abc)	1.27 (ab)	5.79 (ab)
	4 mL	0.21 (ab)	1.53 (abc)	6.31 (abc)
	5 mL	0.26 (ab)	1.66 (b)	6.67 (b)
Exp3	1 mL	0.26 (bcd)	2.97 (ab)	7.53 (bcd)
	2 mL	0.33 (b)	3.25 (ab)	7.87 (ab)
	3 mL	0.40 (ab)	3.50 (bcd)	8.11 (bcd)
	4 mL	0.44 (b)	3.84 (b)	8.61 (b)
	5 mL	0.62 (ab)	4.00 (bcd)	9.05 (ab)

^aExp0: nutrient solution (control), Exp1: *Bacillus cereus*, Exp2: *Pseudomonas* spp., Exp3: consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1).

^bMean of two repeated experiments (30 plants). Different letters within parenthesis indicate significant difference between treatments for each growth parameter using Duncan's multiple range test ($P = 0.05$).

FIGURE 3: Germination percentage of *Vigna unguiculata* seeds sown in vermiculite supplemented with *Bacillus cereus*, with *Pseudomonas* spp., and with their consortium at five different concentrations (1, 2, 3, 4, and 5 mL) and in the control. Exp0: nutrient solution (control), Exp1: *Bacillus cereus*, Exp2: *Pseudomonas* spp., Exp3: consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1).

not treated with the bacterial culture [41]. In earlier reports, root elongation was found to occur in *Sesbania aculeata* by inoculation with *Azotobacter* spp. and *Pseudomonas* spp. in

Vigna radiata by *Pseudomonas putida* [16] and in *Pennisetum americanum* by *Azospirillum brasilense* [42]. Esitken et al. (2006) had found out that *Bacillus* OSU-142 and *Pseudomonas* BA-8 alone or in combination had a great potential to increase the growth, yield, and nutrition of sweet cherry plant [43]. *Bacillus* M3, *Bacillus* OSU-142, and *Micobacterium* FS01 separately or in combination were found to have great potential for use as plant growth promoting rhizobacteria to increase production in apples and in many other crops [44]. These phosphate solubilizing microbes consist predominantly of fungal, bacterial, and actinomycetes species, collectively called phosphate solubilizing microorganisms—PSM [45]. There are several microorganisms which can also solubilize the cheaper sources of phosphorous such as rock phosphate. Bacteria such as *Bacillus* are widely used in plant production system and are important phosphorus solubilizing microorganisms, resulting in improved growth, yield of crops, and metabolic activities, especially in synthesis of protein [34, 46].

5. Conclusions

This study illustrates the isolation of plant growth promoting bacteria from vermisources, screening under *in vitro* conditions for multiple PGPR traits and their evaluation under controlled conditions in a pot experiment. The findings of this study indicated that the consortium of *B. cereus* and

TABLE 5: Chlorophyll content of leaves of *Vigna unguiculata* grown on vermiculite supplemented with *Bacillus cereus*, with *Pseudomonas* spp., and with their consortium (1 : 1) at five different concentrations (1, 2, 3, 4, and 5 mL) on the 5th day, the 10th day, and the 15th day and in the control.

Experiments ^a	Quantity of bacterial Cultures added	Chlorophyll content (mg/g of fresh leaf) ^b								
		5th day			10th day			15th day		
		Chlorophyll a	Chlorophyll b	Total chlorophyll	Chlorophyll a	Chlorophyll b	Total chlorophyll	Chlorophyll a	Chlorophyll b	Total chlorophyll
Exp0	1 mL	0.21 (ab)	2.85 (abc)	3.07 (a)	0.20 (abc)	4.81 (a)	5.01 (ab)	0.15 (bcd)	6.12 (abc)	6.27 (bcd)
	2 mL	0.22 (abc)	2.93 (ab)	3.15 (abc)	0.21 (ab)	4.89 (ab)	5.10 (bcd)	0.12 (abc)	6.18 (ab)	6.30 (ab)
	3 mL	0.23 (abc)	3.01 (ab)	3.15 (abc)	0.22 (bcd)	4.97 (bcd)	5.18 (a)	0.14 (a)	6.33 (cde)	6.47 (bcd)
	4 mL	0.24 (ab)	3.08 (f)	3.32 (a)	0.22 (a)	5.04 (b)	5.27 (abc)	0.15 (bcd)	6.41 (cde)	6.56 (a)
	5 mL	0.25 (f)	3.16 (abc)	3.41 (bcd)	0.19 (ab)	5.11 (ab)	5.30 (abc)	0.11 (cde)	6.45 (ab)	6.50 (abc)
Exp1	1 mL	0.35 (b)	4.00 (cde)	4.35 (abc)	0.11 (b)	5.81 (cde)	5.93 (cde)	0.10 (abc)	7.04 (abc)	7.15 (f)
	2 mL	0.26 (abc)	4.62 (bcd)	4.88 (f)	0.11 (abc)	6.47 (b)	6.58 (abc)	0.06 (ab)	7.79 (bcd)	7.85 (a)
	3 mL	0.21 (bcd)	5.26 (abc)	5.47 (ab)	0.08 (bcd)	7.18 (ab)	7.26 (bcd)	0.06 (f)	8.47 (f)	8.53 (ab)
	4 mL	0.08 (f)	5.87 (cde)	5.95 (b)	0.11 (bcd)	7.78 (abc)	7.89 (abc)	0.04 (bcd)	9.33 (b)	9.37 (bcd)
	5 mL	0.26 (abc)	6.41 (bcd)	6.67 (abc)	0.25 (cde)	8.34 (cde)	8.59 (f)	0.07 (abc)	9.76 (cde)	9.83 (abc)
Exp2	1 mL	0.30 (abc)	3.54 (ab)	3.84 (b)	0.12 (a)	5.45 (abc)	5.56 (ab)	0.07 (bcd)	6.81 (abc)	6.88 (bcd)
	2 mL	0.29 (bcd)	4.20 (f)	4.49 (abc)	0.11 (bcd)	6.10 (ab)	6.21 (abc)	0.07 (abc)	7.44 (f)	7.52 (bcd)
	3 mL	0.22 (ab)	5.04 (abc)	5.27 (bcd)	0.15 (a)	6.77 (cde)	6.92 (bcd)	0.06 (bcd)	8.12 (ab)	8.17 (bcd)
	4 mL	0.10 (f)	5.66 (a)	5.76 (cde)	0.18 (abc)	7.44 (f)	7.62 (ab)	0.05 (ab)	8.85 (a)	8.90 (abc)
	5 mL	0.06 (bcd)	6.22 (abc)	6.28 (abc)	0.18 (b)	8.10 (abc)	8.28 (a)	0.04 (cde)	9.48 (a)	9.52 (f)
Exp3	1 mL	0.27 (ab)	4.70 (bcd)	4.96 (cde)	0.09 (ab)	6.68 (b)	6.77 (cde)	0.10 (a)	8.14 (ab)	8.25 (a)
	2 mL	0.20 (ab)	5.48 (a)	5.68 (f)	0.03 (bcd)	7.46 (bcd)	7.49 (ab)	0.09 (ab)	8.87 (f)	8.97 (ab)
	3 mL	0.05 (bcd)	5.84 (abc)	5.89 (ab)	0.08 (bcd)	8.04 (abc)	8.12 (cde)	0.10 (ab)	9.53 (abc)	9.63 (bcd)
	4 mL	0.23 (f)	6.63 (ab)	6.86 (cde)	0.07 (ab)	8.70 (b)	8.77 (f)	0.10 (abc)	10.19 (bcd)	10.29 (f)
	5 mL	0.22 (ab)	7.44 (cde)	7.66 (a)	0.09 (a)	9.32 (abc)	9.40 (ab)	0.10 (a)	10.85 (ab)	10.95 (abc)

^aExp0: nutrient solution (control), Exp1: *Bacillus cereus*, Exp2: *Pseudomonas* spp., Exp3: consortium of *Bacillus cereus* + *Pseudomonas* spp. (1 : 1).

^bMean of two repeated experiments (30 plants). Different letters within parenthesis indicate significant difference between treatments for each growth parameter using Duncan's multiple range test ($P = 0.05$).

TABLE 6: Fresh weight and dry weight of whole plant of *Vigna unguiculata* grown on vermiculite supplemented with *Bacillus cereus*, with *Pseudomonas* spp., and with their consortium (1:1) at five different concentrations (1, 2, 3, 4, and 5 mL) on the 5th, the 10th, and the 15 days and in the control.

Experiments ^a	Quantity of bacterial cultures added	Fresh weight of the whole plant (g) ^b			Dry weight of the whole plant (g) ^b		
		5th day	10th day	15th day	5th day	10th day	15th day
Exp0	1 mL	0.58 (bcd)	0.61 (abc)	0.64 (ab)	0.06 (a)	0.06 (abc)	0.06 (ab)
	2 mL	0.58 (ab)	0.61 (a)	0.64 (abc)	0.06 (bcd)	0.06 (ab)	0.06 (ab)
	3 mL	0.58 (abc)	0.61 (ab)	0.64 (bcd)	0.06 (abc)	0.06 (bcd)	0.06 (b)
	4 mL	0.41 (abc)	0.61 (ab)	0.64 (ab)	0.06 (abc)	0.06 (ab)	0.06 (abc)
	5 mL	0.58 (abc)	0.61 (abc)	0.64 (bcd)	0.06 (a)	0.06 (bcd)	0.06 (def)
Exp1	1 mL	0.62 (abc)	0.65 (abc)	0.68 (ab)	0.06 (bcd)	0.06 (ab)	0.07 (bcd)
	2 mL	0.65 (bcd)	0.68 (bcd)	0.71 (def)	0.06 (abc)	0.07 (a)	0.07 (ab)
	3 mL	0.68 (abc)	0.71 (def)	0.74 (bcd)	0.07 (abc)	0.07 (ab)	0.07 (abc)
	4 mL	0.71 (ab)	0.74 (abc)	0.77 (abc)	0.07 (bcd)	0.07 (b)	0.08 (bcd)
	5 mL	0.74 (abc)	0.77 (def)	0.80 (ab)	0.07 (def)	0.08 (a)	0.08 (b)
Exp2	1 mL	0.56 (ab)	0.59 (bcd)	0.62 (ab)	0.06 (ab)	0.06 (ab)	0.06 (abc)
	2 mL	0.59 (abc)	0.62 (abc)	0.65 (def)	0.06 (ab)	0.06 (abc)	0.06 (bcd)
	3 mL	0.62 (bcd)	0.65 (a)	0.68 (bcd)	0.06 (abc)	0.06 (bcd)	0.07 (abc)
	4 mL	0.65 (abc)	0.68 (ab)	0.71 (b)	0.06 (ab)	0.07 (abc)	0.07 (b)
	5 mL	0.68 (abc)	0.71 (b)	0.74 (a)	0.07 (def)	0.07 (abc)	0.07 (bcd)
Exp3	1 mL	0.74 (ab)	0.77 (ab)	0.80 (abc)	0.07 (a)	0.08 (bcd)	0.08 (ab)
	2 mL	0.77 (bcd)	0.80 (a)	0.83 (bcd)	0.08 (a)	0.08 (bcd)	0.08 (abc)
	3 mL	0.80 (bcd)	0.83 (bcd)	0.86 (abc)	0.08 (ab)	0.08 (b)	0.09 (b)
	4 mL	0.83 (bcd)	0.86 (abc)	0.89 (ab)	0.08 (abc)	0.09 (ab)	0.09 (bcd)
	5 mL	0.86 (ab)	0.89 (b)	0.92 (def)	0.09 (bcd)	0.09 (bcd)	0.09 (b)

^aExp0: nutrient solution (control), Exp1: *Bacillus cereus*, Exp2: *Pseudomonas* spp., Exp3: consortium of *Bacillus cereus* + *Pseudomonas* spp. (1:1).

^bMean of two repeated experiments (30 plants). Different letters within parenthesis indicate significant difference between treatments for each growth parameter using Duncan's multiple range test ($P = 0.05$).

Pseudomonas spp. acts as a potential biofertilizer, due to its various plant growth promotion abilities such as solubilization of phosphate and production of IAA and siderophore; it can be developed as potential microbial inoculant for various crops.

Conflict of Interests

The authors declared that there is no conflict of interests regarding this paper.

Acknowledgments

The authors are thankful to the Department of Biology, Gandhigram Rural Institute—Deemed University, Gandhigram. This research was funded by University Grants Commission under UGC Research Fellowship in Science for Meritorious Students (BSR) Grant no. F.4-1/2008.

References

- [1] U. S. Bhawalkar, *Vermiculture Ecotechnology*, Earthworm Research Institute, Pune, India, 2nd edition, 1996.
- [2] S. Kiss, D. M. Bularda, and D. Radulescu, "Biological Significance of Enzymes Accumulated in Soil," *Advances in Agronomy*, vol. 27, pp. 25–87, 1975.
- [3] D. L. Karlen, M. J. Mausbach, J. W. Doran, R. G. Cline, R. F. Harris, and G. E. Schuman, "Soil quality: a concept, definition, and framework for evaluation," *Soil Science Society of America Journal*, vol. 61, no. 1, pp. 4–10, 1997.
- [4] R. M. Atiyeh, C. A. Edwards, S. Subler, and J. Metzger, "Earthworm processed organic wastes as components of horticultural potting media for growing manifold and vegetable seedlings," *Compost Science and Utilization*, vol. 8, no. 3, pp. 215–223, 2000.
- [5] N. Vassilev and M. Vassileva, "Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes," *Applied Microbiology and Biotechnology*, vol. 61, no. 5-6, pp. 435–440, 2003.
- [6] S. A. Omar, "The role of rock-phosphate-solubilizing fungi and vesicular-arbuscular-mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate," *World Journal of Microbiology and Biotechnology*, vol. 14, no. 2, pp. 211–218, 1998.
- [7] J. A. J. Paul and T. Daniel, "Standardization of sampling method for physical characterization of municipal solid waste," *Asian Journal of Water, Environment and Pollution*, vol. 5, no. 1, pp. 95–98, 2007.
- [8] R. Laloo, D. Maharajh, J. Görgens, and N. Gardiner, "A downstream process for production of a viable and stable *Bacillus cereus* aquaculture biological agent," *Applied Microbiology and Biotechnology*, vol. 86, no. 2, pp. 499–508, 2010.
- [9] B. Sivasankari, N. Kavikumar, and M. Anandharaj, "Indole-3-acetic acid production and enhanced Plant growth promotion by Indigenous bacterial species," *Journal of Current Research in Science*, vol. 1, no. 5, pp. 331–335, 2013.

- [10] J. Han, L. Sun, X. Dong et al., "Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens," *Systematic and Applied Microbiology*, vol. 28, no. 1, pp. 66–76, 2005.
- [11] H. Rodríguez and R. Fraga, "Phosphate solubilizing bacteria and their role in plant growth promotion," *Biotechnology Advances*, vol. 17, no. 4-5, pp. 319–339, 1999.
- [12] T. Daniel and N. Karmegam, "Bio-conversion of selected leaf litters using an African epigeic earthworm, *Eudrilus eugeniae*," *Ecology, Environment and Conservation*, vol. 5, no. 3, pp. 271–275, 1999.
- [13] J. Dobereiner, I. E. Marriel, and M. Nery, "Ecological distribution of *Spirillum lipoferum*, Beijerinck," *Canadian Journal of Microbiology*, vol. 22, no. 10, pp. 1464–1473, 1976.
- [14] R. I. Pikovskaya, "Mobilization of phosphorus in soil in connection with vital activity of some microbial species," *Microbiology*, vol. 17, pp. 362–370, 1948.
- [15] M. Edi Premono, A. M. Moawad, and P. L. G. Vlek, "Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere," *Indonesian Journal of Crop Science*, vol. 11, pp. 13–23, 1996.
- [16] C. L. Patten and B. R. Glick, "Bacterial biosynthesis of indole-3-acetic acid," *Canadian Journal of Microbiology*, vol. 42, no. 3, pp. 207–220, 1996.
- [17] N. Amaresan, V. Jayakumar, K. Kumar, and N. Thajuddin, "Isolation and characterization of plant growth promoting endophytic bacteria and their effect on tomato (*Lycopersicon esculentum*) and chilli (*Capsicum annum*) seedling growth," *Annals of Microbiology*, vol. 62, no. 2, pp. 805–810, 2012.
- [18] B. Schwyn and J. B. Neilands, "Universal chemical assay for the detection and determination of siderophores," *Analytical Biochemistry*, vol. 160, no. 1, pp. 47–56, 1987.
- [19] P. Rahi, P. Vyas, S. Sharma, and A. Gulati, "Plant growth promoting potential of the fungus *Discosia* sp. FIHB 571 from tea rhizosphere tested on chickpea, maize and pea," *Indian Journal of Microbiology*, vol. 49, no. 2, pp. 128–133, 2009.
- [20] K. Kumar, N. Amaresan, S. Bhagat, K. Madhuri, and R. C. Srivastava, "Isolation and characterization of rhizobacteria associated with coastal agricultural ecosystem of rhizosphere soils of cultivated vegetable crops," *World Journal of Microbiology and Biotechnology*, vol. 27, no. 7, pp. 1625–1632, 2011.
- [21] J. A. Smoker and S. R. Barnum, "Rapid small scale DNA isolation from filamentous cyanobacteria," *FEMS Microbiology Letters*, vol. 56, no. 1, pp. 119–122, 1988.
- [22] U. Edwards, T. Rogall, H. Blocker, M. Emde, and E. C. Bottger, "Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA," *Nucleic Acids Research*, vol. 17, no. 19, pp. 7843–7853, 1989.
- [23] D. G. Higgins, A. J. Bleasby, and R. Fuchs, "CLUSTAL V: improved software for multiple sequence alignment," *Computer Applications in the Biosciences*, vol. 8, no. 2, pp. 189–191, 1992.
- [24] M. Kimura, "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences," *Journal of Molecular Evolution*, vol. 16, no. 2, pp. 111–120, 1980.
- [25] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Molecular Biology and Evolution*, vol. 4, no. 4, pp. 406–425, 1987.
- [26] E. Barbu, K. Y. Lee, and R. Wahl, "Content of purine and pyrimidine base in desoxyribonucleic acid of bacteria," *Annales de l'Institut Pasteur*, vol. 91, no. 2, pp. 212–224, 1956.
- [27] N. Sueoka, "Variation and heterogeneity of base composition of deoxyribonucleic acids: a compilation of old and new data," *Journal of Molecular Biology*, vol. 3, no. 1, pp. 31–40, 1961.
- [28] H. Hori and S. Osawa, "Evolutionary change in 5S rRNA secondary structure and a phylogenetic tree of 352 5S rRNA species," *BioSystems*, vol. 19, no. 3, pp. 163–172, 1986.
- [29] V. Bess, "Evaluating microbiology of compost: microbial content of composting is helping the producers and growers to understand its role as a soil inoculant and plant," *BioCycle Magazine*, p. 62, 1999.
- [30] O. Graff, "Preliminary experiments of vermin composting of different waste materials using *Eudrilus eugeniae* Kinberg," in *Workshop on the Role of Earthworms in the Stabilization of Organic Residues*, M. Happelhof, Ed., pp. 178–191, Beech Leaf Press, Kalamazoo, Mich, USA, 1981.
- [31] J. E. Satchell, *Earthworm Ecology from Darwin to Vermiculture*, Chapman and Hall, 1983.
- [32] G. H. Xie, M. Y. Cai, G. C. Tao, and Y. Steinberger, "Cultivable heterotrophic N₂-fixing bacterial diversity in rice fields in the Yangtze River Plain," *Biology and Fertility of Soils*, vol. 37, no. 1, pp. 29–38, 2003.
- [33] B. B. McSpadden Gardener, "Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems," *Phytopathology*, vol. 94, no. 11, pp. 1252–1258, 2004.
- [34] J. Dobereiner, "Biological nitrogen fixation in the tropics: social and economic contributions," *Soil Biology and Biochemistry*, vol. 29, no. 5-6, pp. 771–774, 1997.
- [35] V. M. Reis, F. L. Olivares, and J. Dobereiner, "Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat," *World Journal of Microbiology and Biotechnology*, vol. 10, no. 4, pp. 401–405, 1994.
- [36] S. Khalil, N. Ayub, S. Alam, and F. Latif, "Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under in vitro conditions," *Pakistan Journal of Biological Sciences*, vol. 7, no. 2, pp. 187–196, 2004.
- [37] H. M. A. El-Komy, "Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants," *Food Technology and Biotechnology*, vol. 43, no. 1, pp. 19–27, 2005.
- [38] N. B. Paul and W. V. B. Sundara Rao, "Phosphate-dissolving bacteria in the rhizosphere of some cultivated legumes," *Plant and Soil*, vol. 35, no. 1, pp. 127–132, 1971.
- [39] A. Beneduzi, D. Peres, L. K. Vargas, M. H. Bodanese-Zanettini, and L. M. P. Passaglia, "Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil," *Applied Soil Ecology*, vol. 39, no. 3, pp. 311–320, 2008.
- [40] M. P. Raimam, U. Albino, M. F. Cruz et al., "Interaction among free-living N-fixing bacteria isolated from *Drosera villosa* var. *villosa* and AM fungi (*Glomus clarum*) in rice (*Oryza sativa*)," *Applied Soil Ecology*, vol. 35, no. 1, pp. 25–34, 2007.
- [41] M. R. Swain, S. K. Naskar, and R. C. Ray, "Indole-3-acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisets by *Bacillus subtilis* isolated from culturable cowdung microflora," *Polish Journal of Microbiology*, vol. 56, no. 2, pp. 103–110, 2007.
- [42] T. M. Tien, M. H. Garkins, and D. H. Hubbell, "Plant growth substances produced by *Azospirillum brasilense* and their effect

- on the growth of pearl Millet *Pennisetum americanum* (L.),” *Applied and Environmental Microbiology*, vol. 37, no. 5, pp. 1016–1024, 1979.
- [43] A. Esitken, L. Pirlak, M. Turan, and F. Sahin, “Effects of floral and foliar application of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrition of sweet cherry,” *Scientia Horticulturae*, vol. 110, no. 4, pp. 324–327, 2006.
- [44] H. Karlidag, A. Esitken, M. Turan, and F. Sahin, “Effects of root inoculation of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient element contents of leaves of apple,” *Scientia Horticulturae*, vol. 114, no. 1, pp. 16–20, 2007.
- [45] C. S. Nautiyal, S. Bhadauria, P. Kumar, H. Lal, R. Mondal, and D. Verma, “Stress induced phosphate solubilization in bacteria isolated from alkaline soils,” *FEMS Microbiology Letters*, vol. 182, no. 2, pp. 291–296, 2000.
- [46] A. K. Halder, A. K. Mishra, P. Bhattacharya, and P. K. Chakrabarthy, “Solubilization of inorganic phosphates by *Bradyrhizobium*,” *Indian Journal of Experimental Biology*, vol. 29, pp. 28–31, 1991.