

Characterization of Plant Growth Promoting *Bacillus subtilis* (VBKT5) Isolated from Vermicompost

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Abstract

Vermicompost is a rich source of nutrients and beneficial soil bacteria which can promote plant growth in various ways. Ten prevalent bacterial strains were isolated from the cow dung vermicompost and assessed for growth promotion of paddy. Bacterial suspensions (3×10^8 CFU/ml) were individually applied to the pots planted with paddy variety and vegetative growth parameters and yield were recorded. Among the bacterial treatments, better shoot length, root length and yield were recorded for *Bacillus subtilis* (VBKT5). Average yield in untreated control plants was less than a half of the yield for *B. subtilis* (VBKT5) treated plant. This strain was Gram positive, rod shaped, endospore forming bacillus and identified as *B. subtilis*. The results of the present study clearly indicated that *B. subtilis* isolated from vermicompost significantly boosted up growth and production of paddy plants and the strain may be explored as a plant growth promoting bacteria (PGPB) for rice improvement.

Keywords: *Bacillus subtilis*, paddy plant, plant growth, PGPR, vermicompost

INTRODUCTION

Use of chemical fertilizer is not encouraged due to environmental and health hazards and therefore, application of biofertilizers is becoming more relevant [1]. Vermicompost is an important biofertilizer originated from animal and microbial interaction [2]. It is absolutely ecofriendly, inexpensive and acts as a massive store house of nutrients and beneficial microorganisms [3]. It is rich in NKP, beneficial

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Received Date: December 14, 2020

Accepted Date: January 15, 2021

Published Date: April 7, 2021

Citation: Sucharita Ghosh, Tilak Nayak, Paltu Kumar Dhal, Tushar Kanti Dangar, Soumendranath Chatterjee. Characterization of Plant Growth Promoting *Bacillus subtilis* (VBKT5) Isolated from Vermicompost. Research & Reviews: A Journal of Life Sciences. 2021; 11(1): 12–21p.

soil microbes and various plant growth hormones and enzymes [4]. They also possess good amount of calcium, magnesium, zinc and manganese [5]. Additionally, vermicompost contains many enzymes such as cellulase, amylase, chitinase, lipase, etc. which promote release of soil nutrients by breaking down complex organic molecules and making them accessible to the plant [6]. Several studies showed that application of vermicompost itself could increase the growth and yield of many plants such as strawberry, basil, chamomile, garlic, barley, plantain, turmeric, black peepers, etc. [7]. Various kinds of plant growth-promoting rhizospheric bacteria (PGPR) such as, *Azotobacter* spp., *Rhizobium* spp., *Bacillus* spp., *Pseudomonas* spp., *Azospirillum* spp. are ingested by earthworm along with organic debris and their population is enhanced in the earthworm gut due to perfect micro-environment [4]. It has been reported that PGPR promotes plant growth in various way, such

as biological nitrogen fixation, phosphate solubilization, production of phytohormones, secretion of specific enzymes, lessening of abiotic stresses, activation of defence mechanisms against phytopathogens, etc. [8]. Due to their fascinating advantageous property, there is a huge demand of PGPR biofertilizers [9].

During the present investigation, the most abundant bacterial strains were isolated from cow dung vermicompost and assessed against paddy variety in pot culture to check their effect on growth and yield to reveal the superior plant growth promoting bacteria (PGPB) for exploitation in field conditions in future.

MATERIALS AND METHODS

Collection of Vermicompost

Vermicompost (prepared from cow dung and kitchen wastes) was collected in sterile plastic zipper bag from Kalna vermicompost farm (23°22' N, 88°34' E), Purba Burdwan district of West Bengal, India. After collection, soil samples were brought to the Parasitology and Microbiology Research Laboratory of the Department of Zoology, University of Burdwan, Purba Burdwan, India for further microbial and biochemical analysis.

Isolation of Bacterial Strains

Vermicompost was serially diluted with sterile distilled water up to 10^{-5} level and pour plated on nutrient agar (NA) medium (HiMedia, India). The plates were incubated at $30 \pm 1^\circ\text{C}$ for 24–48 h. At the end of incubation, bacterial colonies were picked from plates and streaked repeatedly on NA plates and maintained as pure culture at 4°C for further study.

Assessment of the Effect of Bacterial Strain on Growth Improvement of Paddy

Sun-dried soil was sieved through 200 mesh sieve. About 10 kg portions of soil were taken separately in autoclavable polybags and sterilized at 121°C for three consecutive days. Each bag of soil was distributed in fifty earthen pots [(50 cm × 30 cm × 35 cm)(l × w × h)]. Ten different bacterial isolates and a control were tested each in five replicated pots. The seeds of paddy (*Oryza sativa* L.) variety IET-4786 were collected from the University farmhouse. A total of ten plants that were fifteen days old were transplanted in each pot and the pots were watered with sterile water to keep the soil moist for the experimental period. The bacterial strains were inoculated on nutrient broth (NB; Composition: Peptone 5 g/l, Beef extract 3 g/l, Sodium Chloride 3 g/l; pH 7.00 ± 0.2) and incubated at $30 \pm 1^\circ\text{C}$ until the CFU reached $10^8/\text{ml}$. After one week of transplantation, 5 ml of each bacterial suspension (3×10^8 CFU/ml) was applied separately on rhizospheric soil of paddy plants at seven days intervals; 5 ml NB was applied in control. All the plants were kept under same environmental conditions (temperature $27\text{--}32^\circ\text{C}$, pH 6–7, soil moisture >80%, photoperiod of 14L:10D cycle). The morphological parameters (shoot length, root length, leaf count and seed yield) in relation to plant growth were recorded at ten days interval.

Characterization of Bacterial Isolate

The most effective PGPB was selected from the pot test and characterized. The morphological and biochemical characterization of bacterial strain was done by following the standard methods [10–13]. For scanning electron photomicrography, a thin smear was made on cover slip, fixed using 2.5% glutaraldehyde and gradually dehydrated through 10–100% ethanol. Finally the specimen was gold coated and observed under scanning electron microscope (ZEISS). Antibiotic sensitivity test was performed with standard antibiotics discs (HiMedia, India) following Brown and Smith [14] and sugar fermentation test was carried out with sugar fermentation discs (HiMedia, India). Multiple Antibiotic Resistance (MAR) index was calculated following Krumpnam [15].

For molecular characterization, genomic DNA of the bacteria was extracted using DNeasy UltraClean Microbial Kit of Qiagen. Fragment of ~1.5 kb rDNA was amplified by polymerase chain reaction (PCR) following the programme: first denaturation at 95°C for 5 min once followed by 35

cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, finally one additional extension at 72°C for 7 min and retention at 4°C for 10 min [16] using 27F forward (5'AGAGTTTGATCATGGCTCAG3') and 1492 reverse (5'GGT TAC CTT GTT ACG ACTT3') primers. The PCR product was purified with MinElute PCR Purification Kit, Qiagen. Purified PCR product was sequenced using universal bacterial primer in DNA sequences. Sequenced information was aligned by ClustalW (<http://www.ebi.ac.uk/clustalw>) and analyzed using MEGA 4.0.2 software [17]. Evolutionary distances of mostly related bacteria were calculated according to the scheme of Jukes and Cantor [18] and phylogenetic tree of the bacterial strain was constructed following Tamura *et al* [19]. Percentage of AT and GC was calculated by ACUA 1.0 software.

Statistical Analysis

Statistical analysis such as one-way ANOVA and Dunnett's multiple comparisons test were performed using GraphPad Prism 7.

RESULTS AND DISCUSSION

Screening and Selection of Potent Bacterial Isolate

Altogether ten bacterial strains were isolated and screened from mature cow dung vermicompost. During the study period the plant growth related morphological parameters were recorded which are enlisted in Table 1.

Upon completion of experiment it was found that all the bacterial suspension treated plants showed longer shoot and root length, greater leaf count and higher yields than the untreated plants. But the plants treated with bacterial isolate VBKT5 exhibited longer shoot and root lengths in comparison to other bacterial strains. After hundred day's growth, average shoot length of control plants was 76.45 cm whereas average shoot length of VBKT5 exposed plants was 101.60 cm (Figure 1). From one-way ANOVA and Dunnett's multiple comparisons test it was found that shoot lengths of all bacterial suspension treated plants increased significantly than control ($p < 0.0001$; $F = 109.80$). Similarly, the higher value of root length was depicted by VBKT5 applied plants (Figure 2). Root length of VBKT5 treated plants was 32.50 cm which significantly differed from control group plants ($p < 0.0001$; $F = 5.756$). From Dunnett's multiple comparisons test, it was found that root length of most of bacteria treated plants excluding plants treated with VBKT6 and VBKT9 increased significantly than control plants. The number of leaves per plant in bacteria challenged plants also differed significantly ($p < 0.0001$; $F = 132.8$) against control and higher number of leaves per plant was shown by VBKT3 exposed plants followed by VBKT5 treated plants (Figure 3). Average leaf number in control and VBKT3 exposed plants were 18.3 and 40.0, respectively. However, average width of the leaf was found maximum in the plant treated with VBKT1 rather than other plants treated with other strains.

Table 1: Different Plant Growth Parameters of Treated and Control Plants at the End of the Experiment.

Bacterial isolates	Shoot length (cm) $\pm$ SE	Number of leaves/plant $\pm$ SE	Root length (cm) $\pm$ SE	Weight of seeds/plant (g) $\pm$ SE
Control	76.45 $\pm$ 2.18	18.3 $\pm$ 0.90	26.2 $\pm$ 1.96	14.81 $\pm$ 0.56
VBKT 1	97.79 $\pm$ 3.55***	34.8 $\pm$ 0.86***	30.2 $\pm$ 1.46**	18.96 $\pm$ 0.45**
VBKT 2	97.53 $\pm$ 2.52***	29.4 $\pm$ 0.91***	29.8 $\pm$ 1.17***	15.28 $\pm$ 0.76 ns
VBKT 3	98.80 $\pm$ 2.05***	40.0 $\pm$ 1.21***	31.1 $\pm$ 1.46*	24.20 $\pm$ 0.62***
VBKT 4	91.69 $\pm$ 3.38***	36.2 $\pm$ 0.87***	29.8 $\pm$ 1.18*	18.78 $\pm$ 1.08*
VBKT 5	101.60 $\pm$ 3.79 ***	39.0 $\pm$ 1.05***	32.5 $\pm$ 1.14***	31.20 $\pm$ 0.82***
VBKT 6	87.88 $\pm$ 3.57***	19.8 $\pm$ 1.34 ns	28.7 $\pm$ 1.96 ns	17.61 $\pm$ 0.53 ns
VBKT 7	92.45 $\pm$ 4.86***	33.0 $\pm$ 0.99***	29.6 $\pm$ 1.34*	15.43 $\pm$ 0.82 ns
VBKT 8	93.98 $\pm$ 3.86***	23.3 $\pm$ 1.01***	29.1 $\pm$ 1.19*	19.50 $\pm$ 0.74**
VBKT 9	83.82 $\pm$ 2.46***	20.0 $\pm$ 1.06 ns	28.1 $\pm$ 1.14 ns	16.04 $\pm$ 1.15 ns
VBKT 10	96.52 $\pm$ 4.05***	23.6 $\pm$ 0.59***	30.8 $\pm$ 1.56**	22.81 $\pm$ 0.32***

Level of significance marked by ***, **, * and ns denotes Adjusted P value ≤ 0.0001 , $P \leq 0.001$, $P < 0.05$ and no significant difference, respectively. [Results are mean of five replications $\pm$ SE].

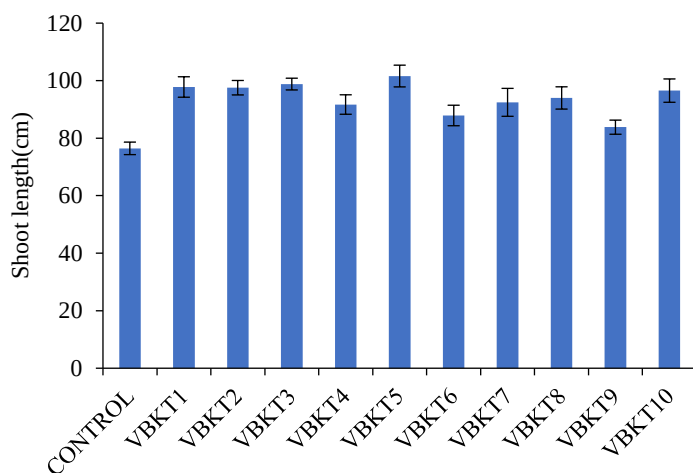


Figure 1: Shoot Length of the Bacteria-Treated Plants (Standard errors are represented by error bars).

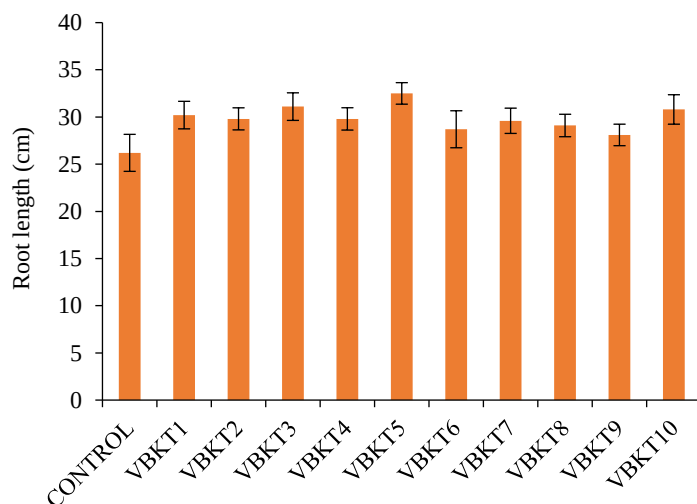


Figure 2: Root Length of the Bacteria-Treated Plants (Standard errors are represented by error bars).

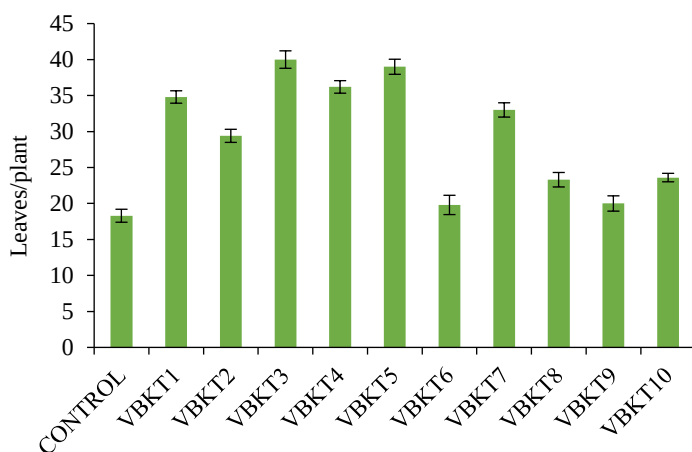


Figure 3: Number of Leaves of the Bacteria-Treated Plants (Standard errors are represented by error bars).

Foremost flowering was recorded in the plant treated with VBKT5 on 64th day of exposure, whereas in the control, initial flowering was recorded at 80th day of experiment. VBKT5 treated plants depicted comparatively higher yield than other treated and control plants (Figure 4). The average yield per plant for VBKT5 was 31.20 g whereas in control it was 14.81 g which is less than a half of the maximum yield. From one-way ANOVA and Dunnett's multiple comparisons test, it was found that production of most of the bacterial suspension treated plants improved significantly than the control ($p < 0.0001$; $F = 48.53$). So, among all ten isolates, VBKT5 has appeared as the most potent isolate which significantly enhanced plant growth and increased crop production which was characterized and identified.

Characterization of Potent Bacterial Isolate

Morphology

The colony of VBKT5 appeared on NA plate was more or less circular (4–5 mm in diameter), flat surfaced and off-white in colour. The margin of the colony was irregular. The bacterial isolate was Gram positive, endospore forming, rod-shaped bacilli form (Figures 5 and 6) and were about 3.664–4.834 micrometers (μm) in length and 853.6–912.6 nm in breadth.

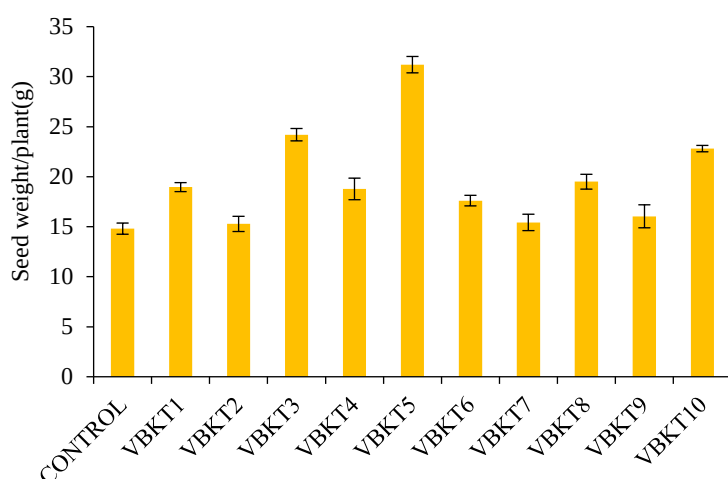


Figure 4: Seed Weight of the Bacteria-Treated Plants (Standard errors are represented by error bars).

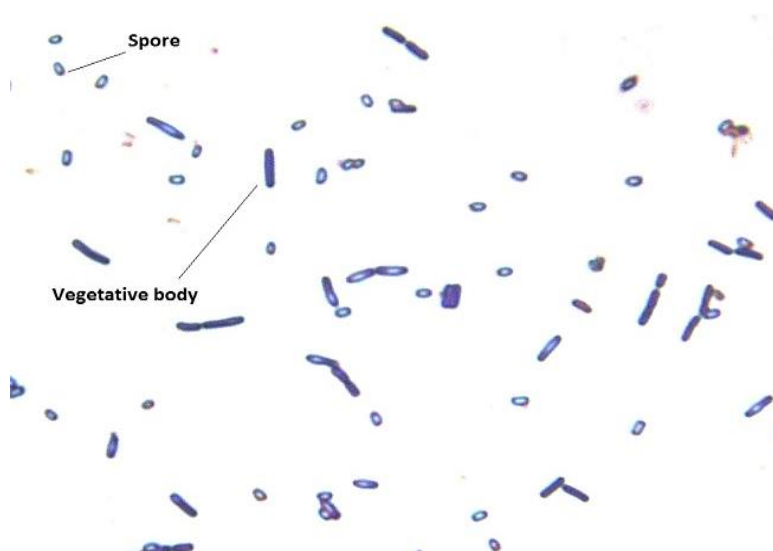


Figure 5: Gram-Stained Photomicrograph of the Isolate VBKT5 Showing Spores and Vegetative Cells Under 100X Light Microscope.

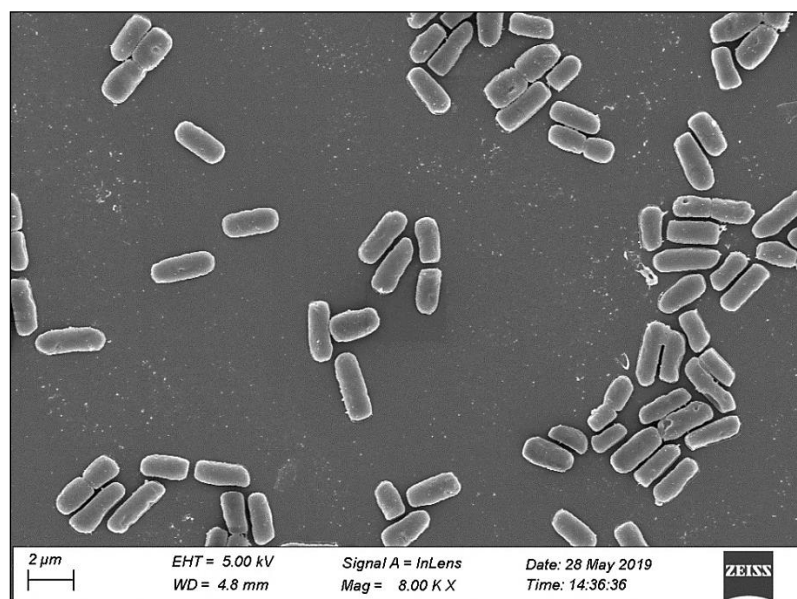


Figure 6: Scanning Electron Microscopic Photograph of Vegetative Cells of the Isolate VBKT5.

Biochemical Properties

The bacterial isolate (Table 2) was negative for MR, VP test and did not utilize urea as carbon source. VBKT5 contained catalase and oxidase enzyme and could utilize citrate as carbon source. Dry weight of the isolate was 1.25 mg/ml. Additionally, the isolate could hydrolyze starch, gelatin and lipid. The strain was negative for blood hemolysis and DNase tests.

Table 2: Morphological and Biochemical Characteristics of VBKT5.

Test	Response
Gram staining	Gram positive
Shape	Rod shaped
Dry weight	1.25 mg/ml
Length of bacterium	3.664–4.834 μm
Breadth of bacterium	853.6–912.6 nm
Endospore	Present
MR	Negative
VP	Negative
Catalase	Positive
Citrate	Positive
Urease	Negative
Oxidase	Positive
H ₂ S production	Negative
Blood hemolysis	No hemolysis
DNase test	Negative
Indole	Negative
Nitrate reduction to N ₂	Positive
Ammonia production	Positive
Starch hydrolysis	Positive
Protein (gelatin) hydrolysis	Positive
Fat hydrolysis	Positive

Bacterial strain VBKT5 showed sensitivity to the recommended doses of antibiotics tetracycline (30 µg/disc), rifampicin (5 µg/disc), ofloxacin (5 µg/disc), doxycycline (30 µg/disc), nalidixic acid (30 µg/disc), erythromycin (15 µg/disc), neomycin (30 µg/disc), levofloxacin (5 µg/disc), streptomycin (10 µg/disc), azithromycin (30 µg/disc), gentamycin (50 µg/disc), vancomycin (30 µg/disc), chloramphenicol (30 µg/disc), kanamycin (30 µg/disc) and norfloxacin (10 µg/disc). However, resistance was observed for amoxicillin (10 µg/disc), bacitracin (10 µg /disc), ampicillin (10 µg/disc) and penicillin (10 µg/disc) (Figure 7). MAR index of VBKT5 was 0.210. A variety of sugars were fermented by the particular bacterial strain. VBKT5 could ferment maltose, trehalose, salicin, fructose, dextrose, sucrose, xylose, dulcitol, cellobiose, lactose and mannose.

Phylogeny

Neighbour joining tree has been constructed from partial 16S rRNA sequence of the isolate using *Pseudomonas* sp. as an out-group (Figure 8). From neighbour joining tree it was found that the bacterium VBKT5 branched with *Bacillus subtilis* (JQ311992). The percentage of AT and GC content of 16S rRNA gene sequence of VBKT5 was 46.45% and 53.55%, respectively. Based on 16S rRNA sequence, phylogenetic analysis, biochemical properties and phenotypic characters of bacterium VBKT5 has been identified as *B. subtilis*. The partial 16S rRNA sequence of the strain was submitted to the NCBI and the isolate VBKT5 was provided with the accession number MK999406.

In the present study ten different bacterial suspensions were applied directly in the pot soil. The increased shoot length, root length, leaves per plant and yield were observed in all of the bacterial suspension treated plant (Table 1; Figures 1–4). This confirmed that direct application of the bacteria isolated from vermicompost could significantly increase crop production and plant growth several fold viz., application of *Bacillus* sp. and *Pseudomonas* sp. boosted development and production of fennel [20], lemon grass [21], black cumin, borage [22, 23], basil [24] and other crops [25]. Furthermore, in the present study, the optimum growth and yield were depicted by VBKT5 (Table 1; Figures 1–4). Cultural, morphological, physiological and biochemical characters of VBKT5 (Table 2; Figures 5–7) was confirmed to the characters of *B. subtilis* [10–12]. Besides, phenotypic identity, 16S rRNA sequence homology-based phylogenetic analysis of VBKT5 matched and identified it as *B. subtilis* which confirmed the phenotypic identity of the bacteria. The bacterium is a well known Gram

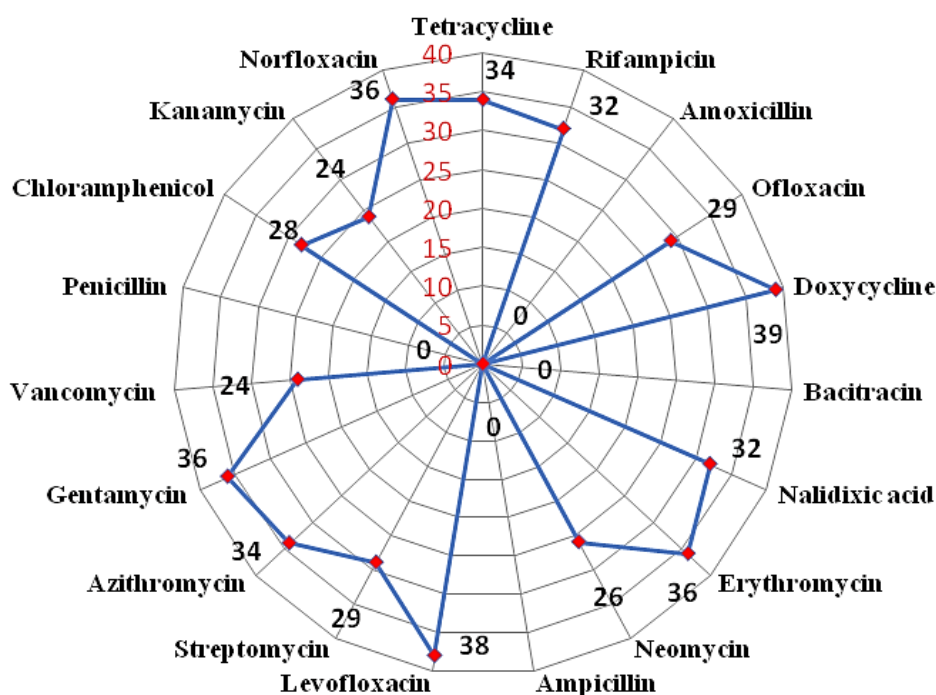


Figure 7: Sensitivity of the Isolate VBKT5 Against Standard Antibiotics.

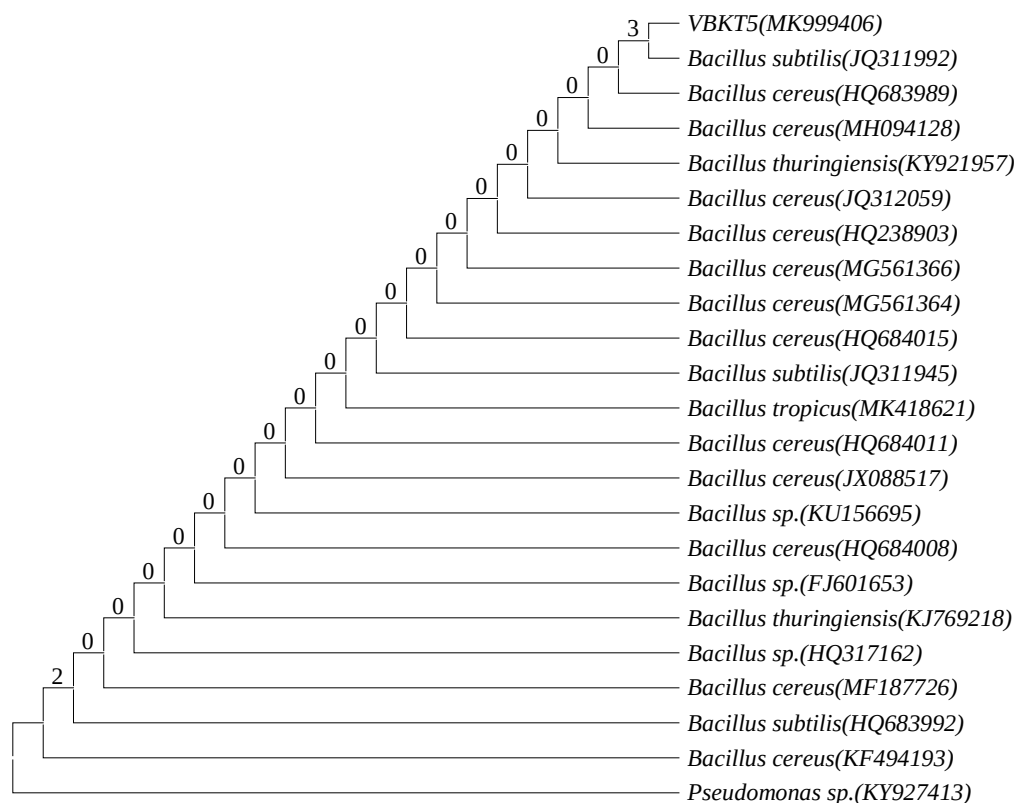


Figure 8: Partial 16S rDNA Sequence-Based Phylogram of the Isolate VBKT5 with Neighbouring 16S rDNA Gene Sequences Retrieved from NCBI and RDP Database.

positive, rod-shaped bacterium that can survive in extreme environmental conditions by forming endospores. It is commonly found in topmost layer of the soil [26] along with *Bacillus megaterium*, *Bacillus cereus*, *B. subtilis*, *Bacillus licheniformis*, *Bacillus benzoovorans*, *Bacillus pumilus*, *Bacillus macroides*, etc. in vermicompost [27] and many of them has been recognized as PGPR. *B. subtilis* was reported to increase shoot dry weight of lettuce [28], promote growth and act as control agents against *Macrophomina phaseolina*, *Rhizoctonia solani* [29] and root rot fungus in *Pinus roxburghii* [30]. Besides, it could produce the growth regulators such as auxins (IAA), gibberellins, cytokinins similar to other *Bacillus* spp., in vermicompost. It also increases the nutrient content in rhizospheric soil which ultimately leads to act as an effective PGPR and improve production and growth in paddy.

CONCLUSION

Preliminary study revealed that *B. subtilis* (VBKT5, MK999406) significantly increased plant growth and crop production several folds. It might directly or indirectly promote growth and production of paddy plant by increasing nutrient contents in soil or by controlling plant pathogens and stress elements or by producing plant growth promoting hormones. Further investigation is required to find out the exact mechanism and it can be exploited for more crop production and growth of paddy plant.

ACKNOWLEDGEMENT

The authors are thankful to the Head, Department of Zoology, The University of Burdwan, Purba Bardhaman, India and to the Head, Department of Life Science and Biotechnology, Jadavpur University, Kolkata, India for providing infrastructural facilities and supports to carry out the work. The authors are thankful to the UGC, WBDSTBT, DST-FIST and DST-PURSE for providing infrastructural facilities and supports to carry out the work.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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