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FULL-LENGTH RESEARCH ARTICLE

Phosphorus Solubilization by Thermotolerant *Bacillus subtilis* Isolated from Cow Dung Microflora

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Abstract Soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus (P). Cow dung (CD) is normally used as an organic manure for enhancing soil fertility in tropical agriculture. Thermotolerant (<50 °C) *Bacillus subtilis* strains isolated from CD solubilized tricalcium phosphate (CP) to available (soluble) phosphate in culture and in autoclaved soil amended with 1 % CP. The solubilization of CP was associated with the phosphatase activity of the bacterium, particularly acid phosphatase (AcP). Autoclaved soil amended with CD (10 %) showed 25.3 and 12.6 % higher solubilization of P and AcP activity, respectively, than autoclaved soil inoculated with *B. subtilis*. Also, *B. subtilis* inoculation and CD amendment resulted in higher P solubilization and phosphatase activity in both rhizosphere and nonrhizosphere of soil planted with cowpea (*Vigna unguiculata* L.) than without these two treatments. Similarly, root length, plant height, and plant biomass of cowpea seedlings were higher in bacterium- or CD-treated soil than in untreated soil. These results suggest that these thermotolerant *B. subtilis* strains as bio-inoculant or CD amendment can be successfully employed in tropical agriculture for solubilization of P and maintaining soil health, being useful in the context of global warming.

Keywords Cow dung · *Bacillus subtilis* · Phosphorus · Tricalcium phosphate · Acid phosphatase · Alkaline phosphatase

Introduction

Phosphorus (P) is one of the major nutrients limiting plant growth [1]. However, essential plant nutrients such as P remain insoluble in soil systems [10]. Phosphorus is present in the soil in both inorganic and organic compounds. Microorganisms bring about a number of transformations of soil P, which include: (a) increasing the solubility of inorganic compounds of P, (b) mineralizing organic compounds with release of inorganic phosphate, (c) converting inorganic, available anion (phosphate) into cell components, and (d) oxidation or reduction of organic P compounds [2].

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As far as P availability to plants is concerned, mineral soil P may be divided into three categories: P soluble in the soil solution (P available to plant uptake), labile P in the solid phase (P ready to solubilize in soil solution), and insoluble (fixed) P in the solid phase [1]. Only a small soil P stock contained in soil solution serves as an immediate source of P for plants. When this pool is depleted, the soil system balance is disturbed and some of the labile P is released into soil solution. Also, the phosphate concentration in soil solution may continuously change due to phosphate sorption and desorption by both biotic (microorganisms) and abiotic (pH, temperature, etc.) factors [10]. The dominant process determines the net changes in P concentration.

Regular, high amounts of mineral P, i.e., calcium hydrogen phosphate and tricalcium phosphate, are added to agricultural soils to achieve high crop yield [10, 12]. Phosphate ions are extremely reactive, and a large portion of applied inorganic P is converted to insoluble P through

immobilization and chelation with metal ligands such as Ca^{2+} in neutral and alkaline soils, and with Fe^{2+} and Al^{3+} in acidic soils, becoming unavailable for plant uptake [4]. One of the options for transforming these insoluble forms of P into a (soluble) form available to plants is by solubilization by microorganisms possessing acid and alkaline phosphatase activity [3, 17]. Strains from the genera *Alcaligenes*, *Burkholderia*, *Enterobactor*, *Pseudomonas*, and *Bacillus* (bacteria) and *Aspergillus*, *Fusarium*, *Penicillium*, and *Rhizopus* (fungi) are among the most powerful phosphate-solubilizing microorganisms isolated from soil and rhizosphere [6, 18].

In our earlier studies, *Bacillus subtilis* strains were found to be predominant bacteria isolated from cow dung (CD). These strains are thermotolerant (<50 °C), and their role in biotransformations of minerals such as P and sulfur in soil have been demonstrated in vitro [22]. The present investigation was carried out to study P solubilization by *B. subtilis* in culture medium and in autoclaved soil inoculated with *B. subtilis* or amended with CD, and study the growth of cow pea (*Vigna unguiculata* L.) in a soil system treated with *B. subtilis* or CD.

Materials and Methods

B. subtilis Strains

Five *Bacillus* isolates (CM1, CM2, CM3, CM4, and CM5) were isolated from CD [22]. These isolates were confirmed as *B. subtilis* strains based on morphological, biochemical, and molecular [16S DNA sequence and random amplification of polymorphic DNA (RAPD) analysis] characteristics and comparison with *B. subtilis* type strain MTCC 441 [22, 23]. The isolates were maintained on nutrient agar slants at 4 °C, and the same were used in the present investigation.

Cow dung (fresh, and 6- and 12-month aged) was collected from lactating cows and brought in sterile polyethylene bags to the Microbiology Laboratory of Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar, Orissa, India. The collected CD was diluted to 10^2–10^6 dilutions with sterile distilled water, and 1 ml of each dilution was added on the nutrient agar for enumerating bacteria. Three replicates (Petri plates) were maintained for each dilution and incubated at 30–50 °C until colonies were observed [22]. The bacterial strains CM1, CM2, CM3, CM4, and CM5 were finally selected because of their thermotolerance (<50 °C), antagonistic activity against plant pathogens such as *Fusarium oxysporum* and *Botryodiplodia theobromae*, and other beneficial activities such as sulfur oxidation and indole-3-acetic acid production [23].

P Solubilization

Preliminary studies (qualitative testing) had shown that all five of these strains could solubilize inorganic P to PO_4^{3-} at ambient (30 ± 2 °C) to 50 °C in National Botanical Research Institute's phosphate (NBRIP) growth medium [13]. However, further experiments were conducted only at ambient temperature. To select the most efficient P-solubilizing strain, qualitative estimation of P solubilization was carried out using modified NBRIP medium in Erlenmeyer flasks (250 ml) in triplicates containing 100 ml medium [(g/l): glucose, 10.00; CP, 5.00; $MgCl_2$, 5.00; $MgSO_4$, 0.5; $(NH_4)_2SO_4$, 0.1; bromophenol blue, 0.025] inoculated with 1 ml [2×10^8 colony-forming units (CFU)/ml] of *B. subtilis* (CM1–CM5). Uninoculated flasks were kept as control. Inoculated and control flasks were incubated at room temperature (30 ± 2 °C) for 48 h. The decrease in dark-blue color of bromophenol blue due to acidification of the medium (indicating P solubilization) into light-yellow color [13] was observed visually using a color chart.

For quantitative estimation of P solubilization, NBRIP medium minus bromophenol blue (to eliminate color interference) was used to determine available (soluble) phosphate. One hundred milliliters of medium taken in 250-ml Erlenmeyer flasks (in triplicates) was inoculated with bacterial (CM1–CM5) culture (2×10^8 CFU/ml) and incubated for 48 h. After incubation, the culture filtrates (filtered through Whatman no. 42 filter paper) were analyzed for available phosphate, pH, and phosphatase (acid and alkaline) activity. In another experiment, only strain CM1 was used to study the progress of P solubilization (phosphate availability and phosphatase activity) at 12-h intervals up to 48 h.

Phosphatase Activity in the Soil System

A neutral sandy loam soil (Entisol, pH 7.2; organic C, 5.6 g/kg; total N, 0.8 g/kg; total P, 0.045 g/kg) was used for this study. Soil samples (20 g sieved to particle size of 2 mm) taken in 250-ml Erlenmeyer flasks were autoclaved for 1 h at 121 °C for three successive days, and on cooling, the soil moisture content was adjusted to 60 % (v/w) (moisture holding capacity) with sterile, deionized water. The sterilized soil was then amended with CP (1 %) and inoculated with 1 ml bacterial starter culture (2×10^8 CFU/ml). The flasks were wrapped with aluminum foil, perforated with fine holes to allow gas exchange. Available phosphate, pH, and phosphatase (acid and alkaline) activity were determined at 5-day intervals up to 25 days.

In another experiment, 2 g CD (g/kg dry weight: organic C, 121 ± 5.60; total N, 11.6 ± 0.11; total P, 4.9 ± 2.40, containing total bacteria 8.0×10^7 CFU/g, including

2×10^7 CFU/g of *Bacillus* spp.; fungi, 4.6×10^5 CFU/g; actinomycetes, 2×10^5 CFU/g) along with 1 % CP was added to autoclaved soil sample (1:10 ratio), taken in Erlenmeyer flasks (in triplicate). Autoclaved soil (20 g) (in triplicate) without amendment of CD but with 1 % CP served as the control. Moisture content in control and soil plus CD was adjusted to 60 % (moisture holding capacity). Available phosphate, pH, and phosphatase activity of the soil samples ($n = 3$) were determined at 5-day intervals up to 25 days. The control and CD-amended soils ($n = 3$) were analyzed for pH (soil:water, 1:2) using glass electrode.

Available P was determined by shaking the soil (20 g) with 80 ml Olsen's reagent (0.5 M $NaHCO_3$, pH 8.5) followed by filtration through Whatman no. 42 filter paper, using the aliquots for P estimation [15].

Phosphate Estimation and Phosphatase Assay

Phosphate concentrations in soil supernatants and bacterial cultures (in triplicate) were determined spectrophotometrically by ascorbic acid method [26]. The absorbance (nm) values of the phosphate concentrations were extrapolated on a standard curve determined by using serially diluted standard P (KH_2PO_4) solution. Phosphate was expressed as µg/ml (in liquid medium) or µg/g soil (in soil samples).

Phosphatase activity in soil supernatants and bacterial cultures (in triplicate) was determined spectrophotometrically by the method of Tabatabai and Bremner [24]. One unit of phosphatase activity was defined as the amount of enzyme required to release 1 µmol *p*-nitrophenol/min/ml from disodium *p*-nitrophenyl phosphate (tetrahydrate) under the assay condition. For soil, units of enzyme activity were calculated as units/g dry soil.

In Vivo Application

Cow pea (*Vigna unguiculata* L.) seeds were surface-sterilized with 1 % NaOCl solution for 10 min and divided into three sets. In the first set, the seeds were placed in planting holes (5 mm each) of presterilized (autoclaved at 121 °C for 1 h for three successive days) soil (1 kg) amended with CP (1 %) contained in polyethylene bags. Three milliliters of bacterial spore suspensions was applied to the individual polyethylene bags in planting holes. In the second set, seeds were similarly placed in polyethylene bags containing presterilized soil (1 kg) but amended with 100 g dry and powdered CD (=10 %) instead of bacterial inoculum. The third set (control) was maintained by just planting the seeds in presterilized soil but without bacterial inoculum or CD. Two seedlings were allowed to grow in each polyethylene bag. The experiment was laid out in completely randomized block design with four replicates each for control, CD, and *B. subtilis*-treated soils. Twenty days after seedling emergence, rhizosphere and nonrhizosphere soil samples were collected from all three sets by standard procedure [25] and assayed for phosphate concentration and phosphatase activity. Root length, plant height, and plant biomass were also examined by the standard method as described by Reyes et al. [17].

Statistical Analysis

Data for phosphatase and available P (Tables 1, 2, 3) were analyzed using one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) comparison test was applied to compare the mean level difference where ANOVA showed significant variation. The analysis was performed using TMTAT-C (version 2.0; Michigan State University, Michigan, USA).

The changes in pH, phosphatase, and available P over time (incubation period) in *B. subtilis*-inoculated Entisol and cow dung-amended Entisol (Figs. 1, 2) were analyzed using one-way repeated-measures ANOVA. Polynomial contrasts were computed to compare with mean difference of pH, phosphatase, and phosphate production at different incubation periods. These statistical methods were performed using SPSS software (for Windows, release 13.0; SPSS Inc., Chicago, IL, USA).

Survival of *B. subtilis*

Survival of *B. subtilis* was determined after each experiment in soil samples. One gram of soil was resuspended in sterile water (100 ml), shaken thoroughly for 30 min, and serially diluted up to 10^6 dilution. Each 1 ml of diluted suspension (10^5–10^8) was spread-plated on nutrient agar and incubated for 48 h in an incubator at 30 ± 2 °C. Bacterial colonies developed on nutrient agar plates and were counted as CFU/g dry soil using a colony counter.

Results and Discussion

Several studies had showed that inorganic phosphate is solubilized by soil and rhizosphere microorganisms by the production of organic acids and chelating oxo-acids from sugar [10, 14]. Therefore, most quantitative tests to assay the reactive efficiency of phosphate-solubilizing bacteria are based on lowering of pH owing to production of organic acids into the surrounding medium [7]. Bromophenol blue is a pH indicator dye whose color changes from dark blue to faint yellow when pH drops. Incorporation of bromophenol blue into culture medium has been studied by several workers for qualitative determination of P solubilization by microorganisms in culture media [7, 13]. Phosphate-solubilizing bacteria can be routinely

screened by a plate assay method using Pikovskaya agar [16]. Testing of the relative efficiency of isolated strains is carried out by selecting microorganisms that are capable of producing a halo/clear zone on a plate owing to the production of organic acids. However, the reliability of this halo-based technique is very often questioned, as many isolates did not produce any visible halo/zone on agar plates but could solubilize various types of insoluble inorganic phosphates in liquid medium [7]. Using NBRIP medium with bromophenol blue, the five bacterial strains (CM1–CM5) were screened initially by observing the change in color from blue to faint yellow using a color chart; CM1 was found to be the most efficient P-solubilizing strain based on the visual change of blue color to faintish yellow.

For phosphate solubilization, phosphobacteria produce AcP and alkaline phosphatase (AlP) enzymes. Quantitative estimation of P solubilization and AcP and AlP enzyme activities also confirmed that CM1 was the most efficient strain (Table 1). CM1 produced the highest phosphatase [AcP ($p = 0.001$, LSD = 3.26); AlP ($p = 0.001$, LSD = 1.96)] activity in NBRIP medium. The rate of P solubilization was associated with decease of the pH of the growth medium from 6.9 (initial) to 3.5, 4.3, 4.1, 3.8, and 5.2 (final) for strains CM1, CM2, CM3, CM4, and CM5, respectively. This pH decrease was possibly due to production of organic acids by *B. subtilis*. There are reports that various P-solubilizing species of *Bacillus* (i.e., *B. licheniformis*, *B. amyloliquefaciens*, *B. firmus*, etc.) produce a mixture of lactic, oxalic, glycolic, 2-ketoguconic, malonic, and succinic acid [18]. There was a positive significant relationship (r) between P solubilization (P concentration in soil solution after incubation) and phosphatase activity [AcP ($r = 0.44$, $p = 0.05$); AlP ($r = 0.89$, $p = 0.01$] and a negative significant relationship between P solubilization and pH ($r = -0.83$, $p = 0.01$). Further, there was a steady increase in phosphatase production during the 48-h course of study. AcP production was 30.2 units at 12 h, which increased to 45.2, 57.2, and 78.1 units at 24, 36, and 48 h, respectively. Similarly, AlP activity was 37.2, 53.0, 71.2, and 94.6 units at 12, 24, 36, and 48 h, respectively. Available P (μg/ml) in the medium also increased from 10.0 (12 h) to 30.1 (48 h) during the corresponding period; simultaneously, a gradual decrease in pH from 6.1 (12 h) to 3.5 (48 h) was observed.

Figure 1 shows the trend of P solubilization and phosphatase activity in autoclaved soils amended with 1 % CP (0.2 g CP/20 g soil) and inoculated with CM1 strain. One-way repeated-measures ANOVA showed that the concentration of available phosphate increased significantly [$F(1.56, 3.13) = 457.52$, $p = 0.000$, $\text{eta}^2 = 0.996$] over the incubation period. The polynomial contrasts confirmed that there was a significant linear trend [$F(1, 2) = 29{,}584.00$, $p = 0.000$, $\text{eta}^2 = 0.996$], concomitant with a decrease in pH from 6.9 (initial pH of soil after autoclaving) to 5.3 [$F(1.20, 2.39) = 27.13$, $p = 0.023$, $\text{eta}^2 = 0.931$]. Further, examination of means suggested that the decrease in pH with increasing incubation period and polynomial contrast confirmed that there was a significant linear trend [$F(1, 2) = 61.85$, $p = 0.016$, $\text{eta}^2 = 0.969$] after 25 days (Fig. 1a). The decrease in soil pH might be due to production of organic acids by *B. subtilis* CM1. AcP and AlP activity increased from zero (0th day) to 210 and 109 units (20th day), respectively. AcP activity showed significant difference from AlP activity [$F(1.11, 2.23) = 1{,}535.72$, $p = 0.000$, $\text{eta}^2 = 0.999$] after 25 days of incubation (Fig. 1b). Examination of means suggested that AcP activity increased until the 15th day and decreased thereafter [$F(1, 2) = 23{,}287.41$, $p = 0.000$, $\text{eta}^2 = 0.999$]. The increase in phosphatase activity in bacteria-inoculated soil was expected because of the increase in *B. subtilis* population during the incubation period [19]. In the present study, the *B. subtilis* population increased in inoculated soil from 1×10^7 CFU/g soil (0 days) to 3×10^7 CFU/g soil (5 days) to 5–7 $\times 10^7$ CFU/g soil during 10–25 days of incubation. Autoclaving removes competition and

Table 1 P solubilization and phosphatase production by *B. subtilis* strains in National Botanical Research Institute's phosphate culture medium after 48 h of incubation

Strain	Available phosphate (μg/ml)	Phosphatase (units/ml)		Final medium pH
		Acid	Alkaline	
CM1	30.1 ± 1.2	78.1 ± 2.8	94.6 ± 1.0	3.5 ± 0.1
CM2	25.8 ± 0.8	62.1 ± 1.6	68.3 ± 2.3	4.3 ± 0.2
CM3	23.8 ± 1.1	70.2 ± 2.6	82.3 ± 1.6	4.1 ± 0.0
CM4	26.3 ± 1.5	64.3 ± 1.2	72.1 ± 2.0	3.8 ± 0.1
CM5	20.0 ± 1.3	68.3 ± 1.4	90.2 ± 2.0	5.2 ± 0.2
LSD ($p = 0.05$)	2.36	3.26	1.91	0.19

$n = 3$; ± standard error

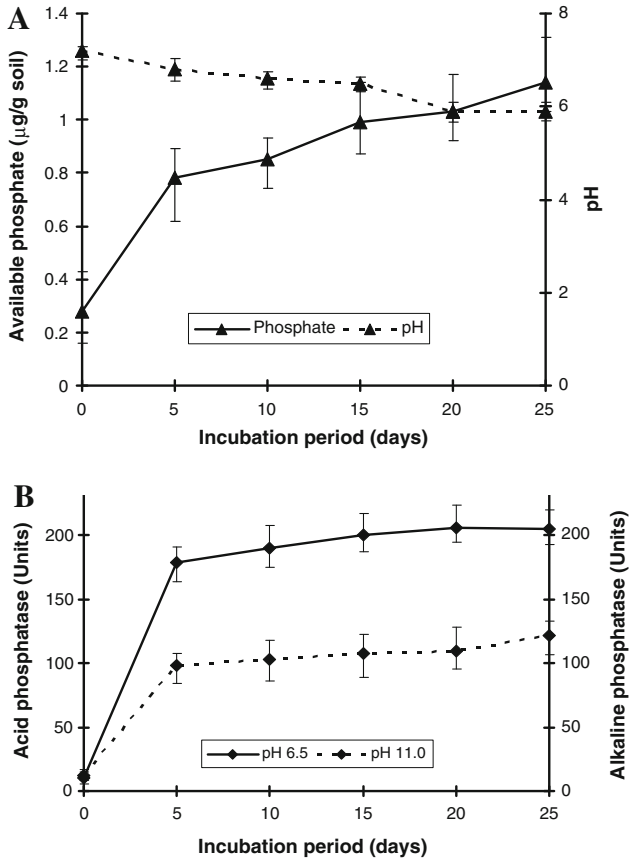

Fig. 1 Effect of *B. subtilis* CM1 inoculation on P solubilization and phosphatase activity in sterilized Entisol amended with CP (1 %): **a** available phosphate and pH, and **b** acid phosphatase and alkaline phosphatase

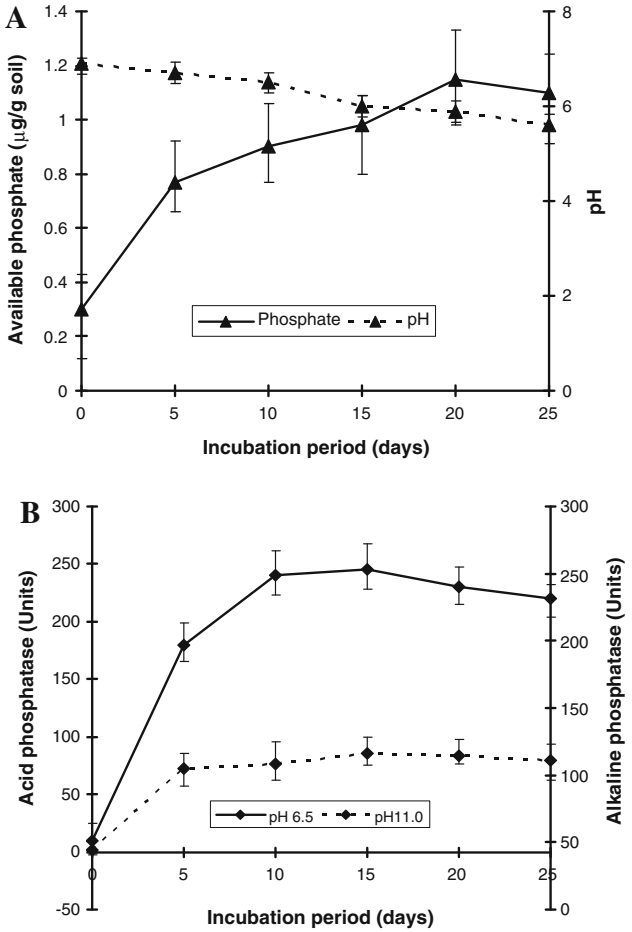

Fig. 2 Effect of added cow dung (10 %) on P solubilization and phosphatase activity in sterilized Entisol + 1 % CP: **a** available phosphate and pH, and **b** acid phosphatase and alkaline phosphatase

changes the chemical composition of the soil; *B. subtilis* in the present case could still grow under unnatural conditions. A bacterium that could solubilize CP in autoclaved soil can presumably do so in unsterilized soil. Further, the results of experiments carried out in sterile soils cannot always be extrapolated to natural soil conditions where the inoculant has to face tough competition from other microflora. Nevertheless, *B. subtilis* is a known stubborn organism that can compete with others in mediating microbial transformations such as those of S and P in natural conditions [8].

Figure 2 shows the trend of P solubilization and phosphatase activity in autoclaved soil amended with 1 % CP and 10 % CD in lieu of *B. subtilis*. There was a steady increase in available P from the 5th day (0.77 µg/g soil) to 20th day (1.15 µg/g soil) of incubation [$F(1.06, 2.12) = 111.68$, $p = 0.000$, $\text{eta}^2 = 0.982$]; thereafter, a marginal decrease (4.34 %) in available phosphate was noticed at the 25th day. Examination of means suggested that the P solubilization increased as the incubation period increased up to the 20th day, and the polynomial contrasts indicated that there was a significant linear trend [$F(1, 2) = 1{,}521.33$, $p = 0.001$, $\text{eta}^2 = 0.999$]. This might be due to metabolism of a part of the available phosphate by *B. subtilis*. Further, there was a 15 % increase in P solubilization in terms of available PO_4^- on the 20th day in CD-amended soil (1.15 µg/g soil) (Fig. 2) in comparison with soil inoculated with *B. subtilis* (1 µg/g soil) (Fig. 1). Similarly, there was a 12.7 % increase in AcP activity in CD-treated soil (230 units) (Fig. 2) over *B. subtilis*-inoculated soil (204 units) (Fig. 1) after the 20th day of incubation. This presumably might be due to the presence of other microorganisms such as *Pseudomonas maltophila*, *Aspergillus* spp., etc. along with *B. subtilis* present in CD [9]. These microorganisms could have performed dual functions: solubilizing inorganic P (CP) as well as mineralizing organic P, i.e., orthophosphate acid esters (nucleic acid, nucleotides, phospholipids, etc.), present in CD into soluble P [10]. However, there was no significant variation [$F(1.15, 2.29) = 929.60$, $p = 0.000$, $\text{eta}^2 = 0.998$] in AlP activity between CD- and *B. subtilis*-treated soil on the same day (20th day) of incubation. Examination of means

suggested that the AlP activity increased as the incubation period increased. Polynomial contrasts also indicated that there was a significant linear trend between AlP activity and incubation period [$F(1, 2) = 6{,}909.43$, $p = 0.000$, $\text{eta}^2 = 0.999$].

Table 2 shows the impact of *B. subtilis* inoculation and CD amendment on phosphatase (acid and alkaline) activity and P solubilization (available P) in rhizosphere and non-rhizosphere samples from cowpea-planted soils. In general, there was higher phosphatase [AcP ($p = 0.001$, LSD = 3.86), AlP ($p = 0.001$, LSD = 7.91)] and P solubilization ($p = 0.001$, LSD = 0.042) activity in rhizosphere than nonrhizosphere soil. The values obtained (both in rhizosphere and in bulk soil) were higher in variants amended with *B. subtilis* than in variants amended with CD. The results appeared slightly different from data presented in Figs. 1 and 2, in which P solubilization and AcP activity were found higher in CD-treated soil than in *B. subtilis*-treated soil. This could be due to the two different sets of experimental conditions.

Cowpea plants grown in bacterial-inoculated or CD-amended presterilized soil showed a significant increase over those grown in nontreated soil in terms of plant height ($p = 0.001$, LSD = 1.00), root length ($p = 0.05$, LSD = 3.26), and dry matter ($p = 0.05$, LSD = 0.32) (Table 3). This might have been expected, because the considerable amount of nutrients (N, P, K, etc.) [11] and organic matter present in CD [5] contribute to greater plant growth. At present, in the Indian Subcontinent, CD, compost, green manure, etc. are usually added to agricultural soils at the rate of 15–20 tons/ha on dry matter basis [assuming soil depth of 15 cm (for agricultural crops) with 1.2 g/cc bulk density] [20, 21]. In the present experiment, CD was applied at 100 g (fresh weight)/kg soil in polyethylene bags (20×12 cm^2), equivalent to 45 tons/ha on dry matter basis. This is admittedly more than double the typical field application rate.

Similar plant growth parameters (biomass) were also obtained with *B. subtilis*-inoculated soil (Table 3). The increased growth attributes were probably due to synthesis of growth regulators such as indole-3-acetic acid by *B. subtilis* strain, as evident from our earlier study [23]. Further, in controlled environment such as in autoclaved soil, there could be faster growth of *B. subtilis* because of noncompetence of other microorganisms for utilizing available nutrients in the soil (apart from plants), which could have stimulated greater indole-3-acetic acid production. As a consequence, the effect of single bacterial (*B. subtilis*) inoculation was found to be on a par with the effect of CD application (Table 3). In our earlier study, *B. subtilis* CM1 was shown to produce indole-3-acetic acid that enhanced sprouting and root elongation of yam minisetts [23]. Besides, many phosphate-solubilizing bacteria, i.e., *Bacillus*, *Pseudomonas*, etc., were reported to enhance plant [mung bean (*Vigna radiata* L.), cluster bean (*Cyamopsis tetragonoloba* L.), wheat (*Triticum aestivum* L.), and chick pea (*Cicer arietinum* L.)] growth, owing to the production of growth regulators (indole-3-acetic acid and gibberellic acid) coupled with other beneficial activities such as biocontrol and P solubilization [27].

Table 2 Effect of *B. subtilis* CM1 and cow dung (10 %) on phosphatase (units = 1 μmol of *p*-nitrophenol/min/ml) and P solubilization activity (available P, μg/g soil) in rhizosphere and nonrhizosphere of cowpea-planted presterilized Entisol

	Rhizosphere				Nonrhizosphere			
	Soil	Soil + *B. subtilis*	Soil + CD	LSD ($p = 0.05$)	Soil	Soil + *B. subtilis*	Soil + CD	LSD ($p = 0.05$)
Acid phosphatase	108 ± 5.2	210 ± 11.2	176 ± 4.2	3.14	88 ± 4.2	180 ± 5.1	123 ± 4.2	4.17
Alkaline phosphatase	90 ± 6.4	128 ± 5.2	122 ± 2.1	2.47	63 ± 8.2	108 ± 2.1	98 ± 5.6	3.02
Available P	35 ± 2.0	42 ± 2.2	40 ± 3.1	3.23	28 ± 1.8	32 ± 2.1	38 ± 2.1	3.96

$n = 3$; ± standard deviation

Table 3 Effect of *B. subtilis* CM1 and cow dung (10 %) on plant growth parameters of cowpea in presterilized Entisol

	Soil	Soil + *B. subtilis*	Soil + CD	LSD ($p = 0.05$)
Plant height (cm)	15.3 ± 1.2	26.4 ± 2.0	24.6 ± 1.6	1.00
Root length (cm)	12.0 ± 1.0	19.5 ± 1.5	19.0 ± 1.2	3.26
Plant dry weight (g)	4.7 ± 0.6	6.8 ± 1.0	6.0 ± 0.5	0.32

$n = 3$; ± standard deviation

Conclusions

B. subtilis strains isolated from CD are proven to be a versatile bacteria having manifold beneficial activities such as biocontrol against plant pathogens, production of growth regulator (indole-3-acetic acid) (in our earlier studies), and P solubilization (as demonstrated in the present study). This bacterial strain is thermotolerant (<50 °C); therefore, in view of global climatic changes, it can be employed as a bio-inoculant in sustainable agricultural systems of the Tropics, where the temperature rises up to 42–45 °C during summer months (April–June) in countries such as India and Pakistan. Cow dung is traditionally applied in Indian Subcontinental agriculture to enhance soil fertility. In this context, organic farming using natural bioresources such as CD or an effective multifaceted bio-inoculant such as *B. subtilis* would not only enhance agricultural productivity but also maintain soil quality. Further research is in progress to validate the P-solubilizing activity of the bacterium in pot culture and field conditions.

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