Plant growth promotion abilities and formulation of *Bacillus megaterium* strain B 388 (MTCC6521) isolated from a temperate Himalayan location

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Abstract *Bacillus megaterium* strain B388, isolated from rhizosphere soil of pine belonging to a temperate Himalayan location has been characterized. The plant growth promotion and biocontrol properties of the bacterium have been evaluated through petridish and broth based assays. The isolate solubilized tricalcium phosphate under in vitro conditions; maximum activity (166 µg/ml) was recorded at 28°C after 15 days of incubation. Production of indole acetic acid demonstrated in broth assays was another important plant growth promoting character. The bacterium produced diffusible and volatile compounds that inhibited the growth of two phytopathogens viz. *Alternaria alternata* and *Fusarium oxysporum*. The carrier based formulations of the bacterium resulted in increased plant growth in bioassays. The rhizosphere colonization and the viability of the cells entrapped in alginate beads were greater in comparison to coal or broth based formulations. The bacterium showed maximum similarity with *Bacillus megaterium* by 16S rRNA analysis.

Keywords Plant growth promoting rhizobacteria · *Bacillus megaterium* · P-solubilization · Biocontrol · Formulation · Viability · Storage

Introduction

Bacteria able to colonize plant root system and promote plant growth are referred as plant growth promoting rhizobacteria (PGPR) [1]. PGPR include a diverse group of free-living soil bacteria that can stimulate plant growth either by direct or indirect mechanisms [2]. While direct stimulation provides plants with compounds such as fixed nitrogen, phytohormones, or solubilized iron and phosphorus, [3] indirect stimulation includes preventing phytopathogens from inhibiting plant growth and development [4].

Significant enhancement in plant growth or control of pathogens by PGPR has been demonstrated under laboratory and greenhouse conditions, whereas results in the field have been less consistent. Screening and selection of efficient strains and their development in suitable formulation for a particular environment are the major challenges for feasibility of this microbe based technology. While a number of Gram negative bacteria, e.g., *Azotobacter*, *Azospirillum*, *Pseudomonas* have received considerable attention as potential PGPR(s) [5], the bacilli are getting attention mainly due to their capability of survival under extreme environments by virtue of spore formation [6]. In the present study an efficient strain of *Bacillus megaterium* B388, isolated from soil samples collected from rhizosphere of pine of a temperate location in Uttarakhand Himalaya has been characterized with special reference to its plant growth promotion abilities. Development of the bacterium in carrier based formulation and its viability under storage are also assessed.

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Material and methods

Isolation, characterization and identification of bacterial isolate

The rhizosphere soil of pine (*Pinus roxburghii* Sarg.) was collected from a temperate site at Jageshwar, District Almora, Uttarakhand (altitude 2020 m above mean sea level) in Indian Himalayan Region (IHR). The bacterium was isolated by diluting the soil, plating the appropriate dilution on Tryptone yeast extract agar (TYA) and incubating the plates at 25°C for 5 days. The pure culture, obtained through sub-culturing was characterized for its morphological, cultural, physiological and biochemical properties following standard methods. The species level was confirmed by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh and The Energy and Resource Institute (TERI), New Delhi (following 16S rRNA gene sequence).

Plant growth promotion properties of the bacterium

Solubilization of tricalcium phosphate (TCP) and production of Indoleacetic acid (IAA)

Quantitative estimation of the phosphate solubilization of bacterium was carried out in 250.0 ml Erlenmeyer flasks containing 100.0 ml of NBRIP medium [7] at four temperatures (21, 28, 35 and 42°C) upto 30 days at 3 days interval. P_2O_5 content of the supernatant was determined by chlorostannous reduced molybdo-phosphoric acid blue method [8]. The pH of the supernatant was recorded in each case. Quantitative estimation of IAA production was performed by inoculating a loopful of bacterium culture in 25.0 ml Luria Bertani broth amended with varying amount of tryptophan (0.0, 100, 500 µg/ml). The broth was incubated for 24 h at 28°C on a rotary shaker. IAA production was quantified periodically [9].

Biocontrol properties of the bacterium

Production of diffusible and volatile antifungal compounds by the bacterium was evaluated following dual cultures using standard methods as described in Chaurasia et al. [10] using *Alternaria alternata* (ITCC 4215) and *Fusarium oxysporum* (ITCC 4219) as test isolates. Reduction in fungal biomass was determined in broth assays [11]. 1.0 ml of bacterial culture grown in TY broth for 24 h (containing 10^6 cfu/ml) and a disc of test fungus (5 mm) from well grown fungal colony on Potato Dextrose agar plate were inoculat-

ed onto 50 ml broth of six different medium in 250 ml conical flask at 25°C on a rotary shaker. Broth inoculated only with respective fungi served as control. The differences in the dry weights between the fungus and the bacteria or the control culture (without bacteria) were recorded by passing 48 h grown dual cultures through the preweighed filter paper (Whatman No. 1). The filter papers were dried for 24 h at 70°C and weights were measured. The percent reduction in weight of the test fungus was calculated using the formula: $(w_1 - w_2 / w_1) \times 100$ where w_1 (control value) represents the weight of the test fungus in control flasks (i.e., without bacteria) and w_2 represents the weight of the fungus in sets inoculated with the bacteria.

Formulations and plant based bioassays

Three formulations (broth, coal and alginate beads) of the bacterium were prepared following the procedure described in Trivedi et al. [12]. TY broth containing 10^9 cfu/ml was directly used as broth formulation. The coal based formulation was raised by growing the bacterial culture in TY agar and harvesting the cells in sterile water (10^9 cfu/ml). Sterile charcoal (150.0 g) was mixed with 150.0 ml bacterial suspension and 10.0 g of *gur* (jaggery). The slurry was mixed properly under aseptic conditions and allowed to air dry overnight, at 28°C, in a laminar flow hood. Alginate beads were prepared by thoroughly mixing 50 ml of TY broth (10^9 cfu/ml) with 100 ml sterile solution of sodium alginate (2.0%, w/v). The mixture was added, drop-wise using a micropipette, into 0.1 M calcium chloride ($CaCl_2$). The resulting alginate beads (mean dia. 2.0 mm) contained entrapped bacterial cells. The beads were maintained in the solution at room temperature for 1 to 3 h to obtain firm and uniform beads. The $CaCl_2$ solution was then pumped out and the beads were twice washed with sterile tap water. Afterwards these beads were further incubated in fresh TY broth for 24 h on a rotary shaker at 28°C to allow multiplication of bacteria within the beads. Finally the beads were twice washed with sterile distilled water and allowed to dry overnight in a laminar flow hood. The formulations were stored at 4 °C in refrigerator in hermetically sealed flasks. Viability of bacterium in various formulations following storage was determined by dissolving 1.0 g in case of solid formulations or by adding 1.0 ml of liquid formulation in 9.0 ml potassium phosphate buffer (0.25 M, pH 6.8) in a test tube for 16–24 h at 28°C. Further enumeration was carried out by dilution plate technique using TY agar.

The bioassays were conducted using maize (*Zea mays*) and wheat (*Triticum aestivum*) as test species. The seeds were grown in trays (32 × 32 × 10 cm) containing 16 cups (6.5 cm dia., 10 cm ht). A total of four treatments were used

for each plant species (three with various inoculant carriers and one control). For each treatment three trays were used and a single seedling was raised per cup filled with local soil (soil per cup = 175g). The bacterial inoculations were carried out by adding freshly prepared 1.0 g alginate and coal based formulations or 1.0 ml of broth based preparation in the soil around each seed at the time of sowing. The trays were kept inside a greenhouse in the Institute's nursery. The plants were uprooted and the observations were recorded for increment in various growth parameters and P-content. For determining the rhizosphere colonization by the introduced bacteria, soil samples were collected from inoculated treatments at weekly intervals, upto a period of 6 weeks. The enumerations were carried out on TY agar supplemented with ampicillin (150.0 µg/ml) using serial dilution technique. Each experiment was conducted in triplicate. Data were statistically analyzed using standard methods.

Results and discussion

The bacterium developed undulate, spreading, rough, convex, whitish colonies with 2.0–3.0 mm dia. on TY agar after 48 h of incubation at 28°C. Microscopic observations revealed that the bacterium was gram positive having single thick rod(s) (size 1.6×2.4 µm) with a centrally located oval shaped endospore. On the basis of physiological properties, the isolate was classified as mesophilic, alkaliphilic and slightly halotolerant. While the bacterium was found to possess strong resistance against two antibiotics, viz. ampicillin and penicillin (≥ 100 µg/ml), it was sensitive to other antibiotics tested. The bacterium produced a wide range of extracellular and intracellular enzymes and fermented various sugars. Based on colony morphology, microscopic observations, cultural, biochemical, and physiological properties, the bacterium was given the genus *Bacillus*. Based on 16S rRNA partial gene sequence, the bacterium showed 99% similarity with *B. megaterium*. The bacterium has been deposited in MTCC, IMTECH, Chandigarh, India (Accession no. MTCC 6521). Prevalence of aerobic bacilli due to the formation of spores resistant to environmental stress under extreme conditions and their activation on getting favourable conditions is well known [13]. Dominance of species of *Bacillus* in soil samples of various temperate and alpine locations including the higher altitudes of IHR has been reported [14].

The bacterium under investigation exhibited two major properties directly associated with plant growth; solubilization of TCP and production of IAA. The bacterium developed a clear zone of solubilization on Pikovskaya's agar

after 7 days of incubation at 28°C. The phosphate solubilization activity of the isolate in liquid medium ranged from 59 to 102 µg/ml at 21°C, 62 to 166 µg/ml at 28°C, 60 to 155 µg/ml at 35°C and 56 to 99 µg/ml at 42°C. Maximum phosphate solubilization occurred after 15 days of incubation at all the temperatures tested. The pH of the medium turned acidic (initially set at 6.0) during the course of phosphate solubilization. The isolate produced 0.8, 6.1 and 12.32 µg/ml of IAA at OD₆₀₀ when the tryptophan concentration in the broth was 0, 100 and 500 µg/ml, respectively. The P-solubilization potential of the isolate was greater than some of the *Bacillus* species including *B. megaterium* isolated from the rhizosphere of rice grown in acidic soils [15]. Although the IAA production was lower yet it may be sufficient to stimulate plant growth.

Both the diffusible and volatile compounds produced by *B. megaterium* strain B388 inhibited the growth of test fungi viz. *A. alternata* and *F. oxysporum*. A clear zone of inhibition with sparse sporulation bound by a dense sporulation ring was observed near the bacterial growth for both the test fungi. The bacterial isolate showed reduction in biomass of both the test fungi in broth based dual cultures using six different growth media (Fig. 1). Potato dextrose and Potato carrot broth gave better results as far as the level of antagonism is considered. Antifungal activity of *B. megaterium* has been reported previously [16]. Effect of nutritional factors on the antagonistic activity of biocontrol agents have also been reported [11].

Fig. 1 Percent reduction in biomass of *A. alternata* and *F. oxysporum* by *B. megaterium*, different media. KB = Kings B broth, M9 = Miller 9 broth, PC = Potato carrot broth, PD = Potato dextrose broth, TY = Tryptone yeast extract broth, and SD = Sabouraud dextrose broth.

To facilitate introduction of high cell numbers and increase survival of microorganisms in soil, carrier based preparation of microbial inoculants under right formulation is a prerequisite [17]. Inoculation of different carrier based formulations of the *B. megaterium* resulted in improvement of growth of both the test plants viz., maize and wheat (Table 1). Statistically significant increase (significant at $P < 0.05$) over the control was observed in many cases. Maximum increase in the growth parameters was observed in case of alginate based formulations followed by coal and broth based, respectively for both the plant species. Inoculation of wheat (*Triticum aestivum* L. cv. Beni Swif 1) with *B. megaterium* or *A. lipoferum* 137 as single or mixed inocula immobilized in alginate beads increased plant growth parameters and mineral contents [18]. Rhizosphere colonization of maize and wheat root by *B. megaterium* in different formulations is presented in Fig. 2. The population of *B. megaterium* in the rhizosphere of both the test plants was initially recorded low in case of alginate based formulations. This, however, increased with time. Contrary to this, the maximum number of bacteria was recorded shortly after inoculation in charcoal and coal based formulations and the numbers decreased drastically afterwards. Similar pattern of root colonization was observed in maize where inoculations were carried out with the similar carrier based formulations of *B. subtilis* and *P. corrugata* [16].

The rate of bacterial survival in the carrier based preparations over time was taken as a parameter for determining the capacity of the formulations to support the survival of *B. megaterium*. The viability of *B. megaterium* at 4°C in different carrier based formulations is shown in Fig. 3. The

Fig. 2 Colonization of rhizosphere of maize and wheat by various formulations of *B. megaterium*.

Table 1 The effect of inoculation in different formulations of *B. megaterium* on growth parameters of maize and wheat^a

Growth Parameter	Plant Species							
	Maize				Wheat			
	Control	Broth	Coal	Alginate	Control	Broth	Coal	Alginate
Root length (cm)	10.83	12.26*	13.10*	13.34*	09.32	12.21*	12.43*	13.88*
Shoot Length (cm)	69.86	70.66	71.34	75.43	40.66	46.81	48.70	52.19*
Root fresh weight (g)	0.26	0.30	0.31	0.38*	0.25	0.27	0.27	0.29
Shoot fresh weight (g)	3.29	3.45	3.43	3.98*	2.98	3.19	3.18	3.67*
Root dry weight (g)	0.07	0.14*	0.15*	0.18*	0.03	0.07*	0.08*	0.10*
Shoot dry weight (g)	0.41	0.43	0.51	0.63*	0.29	0.32	0.39*	0.48*
Root P (%)	0.03	0.04*	0.04*	0.05*	0.02	0.03	0.03	0.04*
Shoot P (%)	0.04	0.05*	0.05*	0.06*	0.05	0.05	0.06*	0.06*

*Significant at $P < 0.05$ as compared to respective control

^a For the evaluation of growth parameters, 25 plants were taken randomly from each treatment and the control, after 42 days of inoculation. Dry weight determined by placing the roots and shoots, separately into small, pre-weighed brown paper bags and drying them in an oven at 80°C for 48 h. For determination of plant P (%) samples were oven dried at 80°C for 48 h and grounded to a powder (2 mm). Plant samples (0.5 g) were digested in HCl, HClO₄ and H₂SO₄ and analyzed for P by molybdophosphoric blue color method [8].

Fig. 3 Recovery of bacterial cells from different formulations during storage at $4 \pm 2^\circ\text{C}$

number of bacteria in all the formulation decreased with time. The decrease in the formulations was minimum when alginate beads were used. Alginate has been known to preserve the beneficial properties and cell numbers of PGPR(s) for longer period of time [19] and its superiority over other carrier materials in maintaining the viability has been described previously [12].

Studies in various ecological niches have indicated that the relative proportion of different microbes and dominance of a particular group of microorganism (species or strains) depend upon various environmental factors. The soils of temperate and alpine climates have been reported dominated by species of *Bacillus* and *Pseudomonas* [10, 14]. Specific protocols must be followed to identify locally adapted beneficial microbes according to their tolerance to environmental stresses, soil types and crop under consideration. The findings of this study indicated that the native strain of *B. megaterium* B388 due to its various plant growth promotion abilities such as solubilization of phosphate, production of IAA and biocontrol can be developed as potential microbial inoculant for use in temperate climate conditions.

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