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Original article

Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils

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ABSTRACT

Immobilization of *Bacillus pumilus* ES4 from arid land soils, a plant growth-promoting bacterium and the freshwater, green microalga *Chlorella vulgaris* enhanced microalgal growth only in the absence of combined nitrogen in synthetic growth medium (SGM), but not in medium with combined nitrogen. *B. pumilus* was able to fix nitrogen in N-free SGM and its growth yielded an accumulation of ammonium in the medium. On its own, *B. pumilus* is a poor agent for removing nitrogen and phosphorus from wastewater, while *C. vulgaris* is a capable microorganism. By jointly immobilizing the two microorganisms, the capacity to remove nitrogen and phosphorus from the medium by the microalgae culture was not enhanced, but, at the cell level, removal of these nutrients was significantly enhanced. It appears that growth promotion induced by *B. pumilus* on *C. vulgaris* is related to nitrogen fixation.

1. Introduction

Microalgae are a very large group of microscopic algae, primary producers on a global scale, and involved in all marine and freshwater ecosystems, wastewater treatment, and some soil processes. Growth promotion of microalgae by microalgae growth-promoting bacteria has been reported for a few strains of two species of the microalgae *Chlorella vulgaris* and *C. sorokiniana* and several strains of terrestrial *Azospirillum* spp. [13,15,21], as well as for few aquatic bacteria and microalgae, mainly phytoplankton [1,20,32,38,42,43]. Consequently, it has not been established whether growth promotion of *Chlorella* is a unique characteristic of species of *Azospirillum* or if this is a wider phenomenon.

Growth promotion of agricultural and wild plants by plant growth-promoting bacteria (PGPB) [3] is commonplace,

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involving different plant–bacteria mechanisms in which the end product of these numerous associations is a better plant feature, usually depending on the usefulness of the plant for human consumption [2]. Promotion of aquatic microalgae by bacteria, although revealed initially decades ago [44], is an emerging field in which almost all studies have been conducted in recent years [14,15,21,45]. The main interest in this artificial association and in joint associations of microalgae and bacteria in general, so far, has been because the community associations were better at removing pollutants from wastewater [11,12,25,33] than microalgae alone [8,9,16] or the microalgae grew better when they were used in aquaculture [20].

The hypotheses of this exploratory study were that: (1) there are other PGPB than *Azospirillum*, a common PGPB for crop plants [5], capable of promoting the growth of the microalga and these do not necessarily originate from the aquatic natural habitat of the microalgae; (2) the interaction of microalgae and PGPB are not specific; this study employed a nitrogen-fixing PGPB, *Bacillus pumilus* ES4, originally isolated from the rhizoplane of an arid land cactus; and (3) the mechanism by which this operates relates to its nitrogen-fixing ability.

2. Material and methods

2.1. Microorganisms and initial growth conditions

Prior to immobilization in beads, 10 ml of axenic *Chlorella vulgaris* Beijerinck UTEX 2714 were inoculated into 100 ml of sterile mineral medium C30 and incubated at 27 ± 2 °C and stirred at 140 rpm under light intensity of 60 μmol photon m^{-2} s^{-1} for 7 days [22].

Bacillus pumilus ES4 [35] (FJ032017, NBCR) was used in these experiments. The bacteria were stored in liquid nitrogen and, for daily use, were kept on tryptic soy slants (Sigma, St. Louis, MO). Two days before immobilization, a loop of *B. pumilus* was transferred to 25 ml of liquid tryptic soy broth (Sigma) and incubated overnight at 30 ± 1 °C and agitated at 120 rpm. The day before immobilization, 3–4 ml of pre-inoculum were introduced into 50 ml of fresh tryptic soy broth and incubated at 30 ± 1 °C for 18 h at 120 rpm. Cells were harvested by centrifugation at $1000 \times g$ for 20 min. The pellet was suspended in 0.85% saline solution to a final concentration of 10^6 colony-forming units (cfu) ml^{-1}.

2.2. Immobilization of *C. vulgaris* and *B. pumilus* in alginate beads

Microorganisms were immobilized according to the method described by de-Bashan et al. [12]. Briefly, axenic cultures (either *C. vulgaris* or the PGPB *B. pumilus*) were mixed with 2% alginate solution. The solution was dripped from a sterile syringe into 2% $CaCl_2$ solution, with periodic mixing of the solution. For joint immobilization of the two microorganisms in the same bead, after washing the cultures, each of them was re-suspended in 10 ml of 0.85% saline solution and then mixed together in the alginate before forming the beads. Because immobilization normally reduces the number of *B. pumilus* cells in the beads, a second, overnight incubation in diluted nutrient broth was necessary.

2.3. Culturing conditions for joint immobilization of microorganisms, solubilization of beads, and cell counts

Initial concentration of ammonium was 10 mg l^{-1} NH_4Cl; initial concentration of phosphorus was 35.5 mg l^{-1} PO_4^{-3}. Experiments were carried out in SGM [21] with and without dissolved nitrogen. The medium did not contain tryptophan. After secondary multiplication of the microorganisms inside the beads, the beads were washed twice with saline solution (0.85% NaCl) and beads weighing 40 g were added to 200 ml of SGM. Batch cultures were incubated for 5 days in Erlenmeyer flasks at 28 °C with continuous stirring at 140 rpm under light intensity of 60 μmol m^{-2} s^{-1}. Cells were released from the beads and counted, using five beads solubilized by immersion in 5 ml of 4% sodium bicarbonate for 30 min at room temperature (24–26 °C). *B. pumilus* was counted using fluorescein diacetate (FDA) stain [27]. The slides were

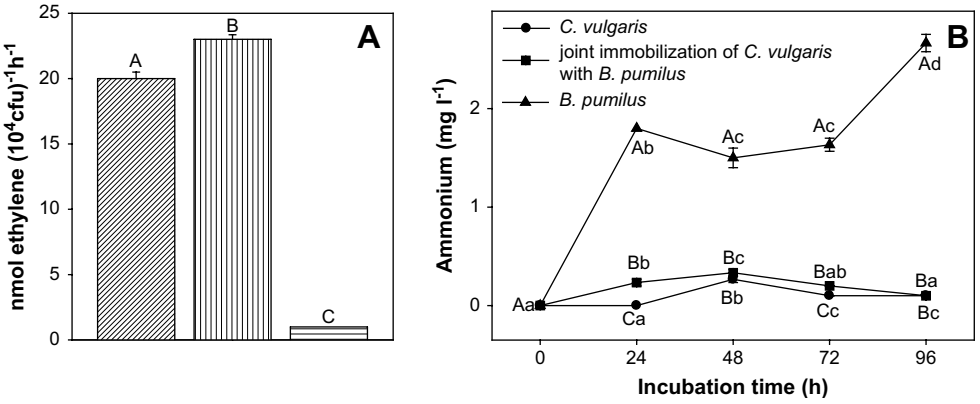

Fig. 1 – Nitrogen fixation (A) and accumulation of ammonium (B) in nitrogen-free synthetic growth medium during the growth of *Bacillus pumilus* and *Chlorella vulgaris* cultured alone and jointly in immobilized alginate beads. Joint immobilization without nitrogen (diagonal filling); *B. pumilus* immobilized without nitrogen (vertical filling); *B. pumilus* immobilized with nitrogen (horizontal filling). Bar whiskers represent SE; their absence indicates negligible SE.