
Vol. 45 • issue 1 • January-February 2009 • ISSN 1164-5563
ELSEVIER
EUROPEAN JOURNAL OF
SOIL
BIOLOGY
Ecology and Application of Azospirillum and other plant growth promoting bacteria (PGPB)
Special Issue
Guest Editors: Yoav Bashan and Anton Hartmann

EUROPEAN JOURNAL OF SOIL BIOLOGY 45 (2009) 88–93

available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

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

Juan-Pablo Hernandez[a], *Luz E. de-Bashan*[a,b], *D. Johana Rodriguez*[c], *Yaneth Rodriguez*[d], *Yoav Bashan*[a,b,*]

[a]*Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), La Paz, B.C.S., Mexico*
[b]*Department of Soil, Water and Environmental Science, University of Arizona, Tucson, Arizona, USA*
[c]*Department of Biology, Universidad del Tolima, Ibagué, Colombia*
[d]*Faculty of Agronomy, Universidad Nacional de Colombia, Bogota, Colombia*

ARTICLE INFO

Article history:
Received 24 January 2008
Received in revised form
22 August 2008
Accepted 27 August 2008
Published online 29 September 2008

Keywords:
Bacillus
Chlorella
Growth promotion
Microalgae
Nitrogen fixation

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,

* *Corresponding author*. Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, BCS 23090, Mexico. Tel.: +52 612 123 8484x3668; fax: +52 612 125 4710.
E-mail addresses: bashan@cibnor.mx, bashan@cals.arizona.edu (Y. Bashan).
1164-5563/$ – see front matter © 2008 Published by Elsevier Masson SAS.
doi:10.1016/j.ejsobi.2008.08.004

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

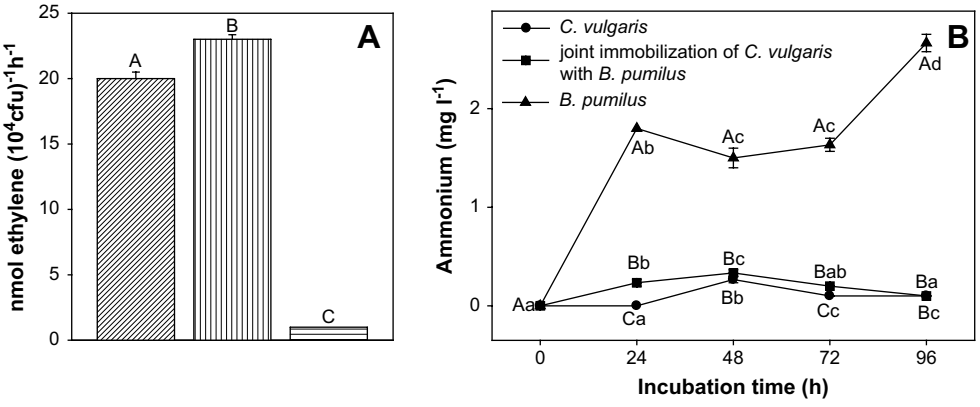

Fig. 1 – Nitrogen fixation (A) and accumulation of ammonium (B) in nitrogen-free synthetic growth medium during the growth of *Bacillus pumilus* and *Chlorella vulgaris* cultured alone and jointly in immobilized alginate beads. Joint immobilization without nitrogen (diagonal filling); *B. pumilus* immobilized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

observed and counted with an episcopic fluorescent microscope (Leitz Laborlux-S, Wetzlar, Germany) and *C. vulgaris* was counted with a Neubauer hemocytometer.

2.4. Ammonium and phosphorus analyses

Removal and accumulation of ammonium ions were measured by the salicylate method and removal of phosphorus by the molybdovanadate method, both standard water analysis techniques using special kits (Hach, Loveland, CO, USA) and spectrophotometer (Hach, Model DR 4000).

2.5. Nitrogen-fixing measurement

To measure the nitrogen-fixing activity of bacteria, 10% acetylene was injected into flasks showing bacterial growth. Acetylene reduction assay was done by gas chromatography as described by Holguin et al. [26]. Results are expressed as nmol ethylene $(10^4 \text{ cfu})^{-1}\ h^{-1}$.

2.6. Experimental design and statistical analysis

All experiments were carried out in batch culture in Erlenmeyer flasks using SGM with and without nitrogen for 5 days under the cultivation conditions described in Section 2.3. Each treatment was done in triplicate using one Erlenmeyer flask as a replicate. Each experiment was repeated three or four times in full. Beads without microorganisms were prepared similarly to serve as controls. Results of all experiments, showing normal distribution, were analyzed by one-way ANOVA and then by Tukey's HSD post-hoc analysis at the $P \leq 0.05$ significance level or by Student's *t*-test at $P \leq 0.05$. STATISTICA software was used (Statsoft, Tulsa, OK, USA).

3. Results

When *B. pumilus* was immobilized in alginate beads, it fixed nitrogen at an intermediate level of 58 ± 0.1 nmol ethylene $\text{flask}^{-1}\ h^{-1}$. When immobilized with *C. vulgaris*, the level of detected nitrogen fixation in the medium was reduced. In the presence of combined nitrogen in the medium, *B. pumilus* did not fix any nitrogen from the air (Fig. 1A). The level of ammonium in microalgae-free medium (*B. pumilus* growing alone) accumulated with time. *C. vulgaris*, by itself, did not contribute ammonium to the medium. Immobilizing the two microorganisms together slightly increased ammonium accumulation, but at very low levels (Fig. 1B).

In the presence of combined nitrogen in the medium, *B. pumilus* did not induce any growth promotion in *C. vulgaris* and both systems (microorganisms immobilized alone and jointly immobilized) developed similar high populations at levels of about 7×10^6 after incubation for 96 h (Fig. 2A). In the absence of combined nitrogen in the medium, *C. vulgaris* grew slowly. Joint immobilization of *B. pumilus* with the microalga enhanced the growth of the microalgae after 48 h and afterwards (Fig. 2B). *B. pumilus* also grew in this medium (Fig. 2C).

C. vulgaris was capable of removing ammonium and phosphorus from the medium, but *B. pumilus* was a poor remover of both nutrients. Joint immobilization of *C. vulgaris* with *B. pumilus* did not enhance removal of combined nitrogen and phosphorus by the microalgae cultures (Fig. 3A,B). However, by calculating removal of ammonium and phosphorus on a per-cell basis of the microalgae, removal of these two nutrients significantly increased during joint immobilization with the PGPB (Tables 1 and 2).

4. Discussion

The mechanisms by which PGPB (nonbiocontrol-PGPB) [3] affect plant growth varied greatly. PGPB directly affect the metabolism of plants by providing substances that are usually in short supply. These bacteria are capable of fixing atmospheric nitrogen, solubilizing phosphorus and iron, and producing plant hormones, such as auxins, gibberelins, cytokinins, and ethylene and nitrite and nitric oxide. Additionally,

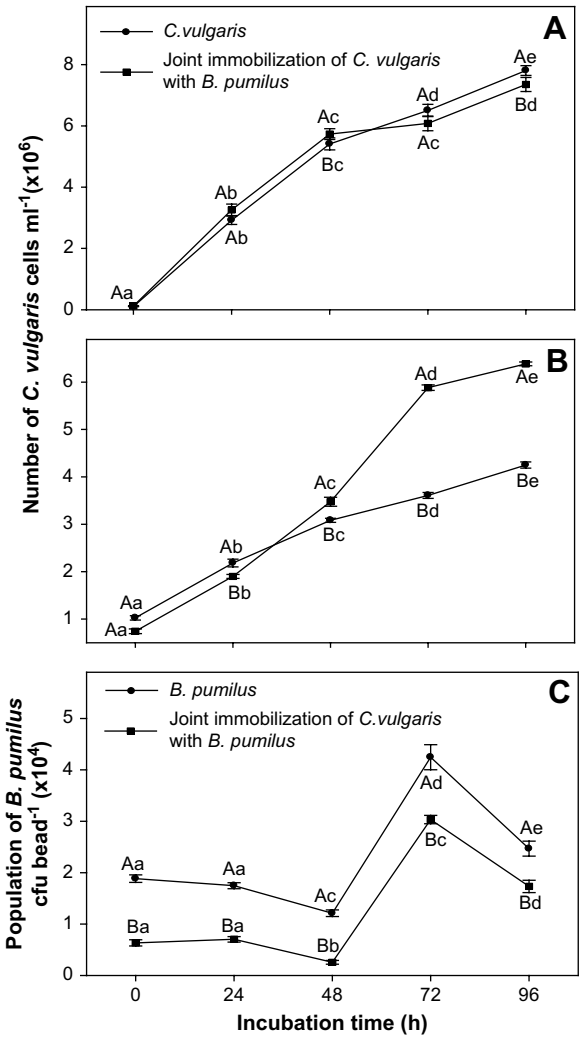

Fig. 2 – (A) Growth of *Chlorella vulgaris* alone and jointly immobilized with *Bacillus pumilus* in alginate beads in synthetic growth medium supplemented with 10 mg ml^{-1} NH_4Cl as a source of nitrogen. (B) Similar growth in nitrogen-free synthetic growth medium. Bar whiskers represent SE; their absence indicates negligible SE.

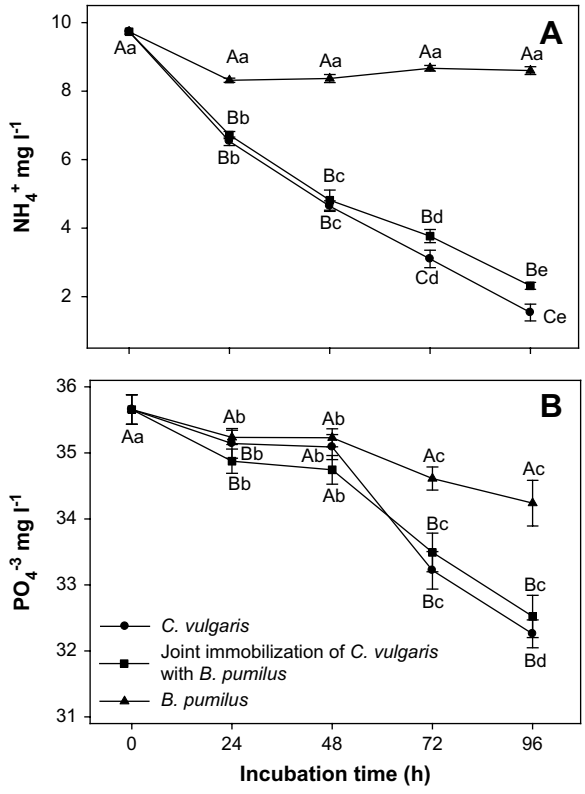

Fig. 3 – Removal of ammonium (A) and phosphorus (B) from synthetic wastewater by *Bacillus pumilus* and *Chlorella vulgaris* when immobilized alone and jointly in alginate beads. Bar whiskers represent SE; their absence indicates negligible SE.

they improve a plant's tolerance to stress, such as drought, high salinity, metal toxicity, and pesticide load. One or more of these mechanisms may contribute to the increases in plant growth and development that are higher than normal for plants grown under conditions of standard cultivation [2,5,30,40]. Most PGPB *Bacillus* spp. operate via control of soil-borne diseases [28], but some bacilli promote plant well-being directly in the absence of a disease [4,6,7,19,24,34,36,37,41]. The possible interactions of *Bacillus* spp. with microalgae are unknown.

So far, *Azospirillum* is the only genus of bacteria known as to promote microalgae growth (MGPB) [12,21]. *Azospirillum* spp. significantly alter the metabolism of microalgae [10] mainly by producing indole-3-acetic acid (IAA) [15] and enhancing enzymes of the nitrogen cycle in the microalgae [16]. Although inoculation of freshwater and marine phytoplankton with bacteria sometimes enhances their productivity [20,43,44], these studies are descriptive and exploratory and no mechanisms for the phenomenon have been demonstrated. Microalgal growth promotion by bacteria notwithstanding, not all interactions are positive; the interaction of *C. vulgaris* with its naturally occurring, associative bacteria *Phyllobacterium myrsinacearum* brings senescence to the culture [23,29].

To extend the view that other potential genera with PGPB capacity, even from unlikely origins, contain species of bacteria that might serve as MGPB, the PGPB *Bacillus pumilus*

Table 1 – Removal of ammonium per cell of *Chlorella vulgaris* when jointly immobilized with *Bacillus pumilus* in synthetic wastewater.

Incubation period (h)	Removal of NH_4^+ (μg $cell^{-1}$)	
	Alone	Jointly immobilized
24	0.87 ± 0.05 Aa	1.01 ± 0.05 Aa
48	3.00 ± 0.37 Ab	3.26 ± 0.43 Bb
72	4.62 ± 0.56 Ac	6.17 ± 0.69 Bc
96	5.05 ± 0.27 Ad	5.48 ± 0.76 Bd

Points for each incubation period denoted by a different capital letter differ significantly by Student's *t*-test at $P \leq 0.05$. Columns for each treatment, denoted by a different lower case letter, differ significantly by one-way ANOVA and Tukey's post-hoc analysis at $P \leq 0.05$. ± represents standard error.

Es4, originally isolated from the rhizoplane of a cactus was tested. This PGPB fixed atmospheric nitrogen [35], produced IAA *in vitro* in the presence of tryptophan, produced siderophores, and efficiently enhanced growth of cardon cactus seedlings over prolonged periods [36].

The PGPB *Bacillus pumilus* Es4 proved to be an MGPB capable of promoting the growth of microalga and enhancing the capacity of individual microalgae cells to absorb nitrogen and phosphorus. However, the jointly-immobilized culture was not useful for the common use of *C. vulgaris* in wastewater treatment [17]. It did not enhance the capacity of the joint culture to remove pollutants from wastewater as does *Azospirillum* spp. [11,12,25].

Growth promotion, in the case of *B. pumilus*, was restricted to the absence of nitrogen, a condition that largely prohibited the growth of the microalga. *Chlorella* spp. are capable of growing without combined nitrogen for a limited time, as ammonium can be produced and recycled inside the organism by a variety of metabolic pathways, such as photorespiration, phenylpropanoid metabolism, use of nitrogen transport compounds, and amino acid catabolism [18]. In this regard, the growth of *Chlorella* in the absence of other microorganisms can be explained by the dissimilatory activity of the enzyme glutamate dehydrogenase. This enzyme serves as a link between carbon and nitrogen metabolism because it is

Table 2 – Removal of phosphorus per cell of *Chlorella vulgaris* when jointly immobilized with *Bacillus pumilus* in synthetic wastewater.

Incubation period (h)	Removal of PO_4^{-3} (μg $cell^{-1}$)	
	Alone	Jointly immobilized
24	1.261 ± 0.393 Aa	2.495 ± 0.423Ba
48	0.684 ± 0.169 Ad	0.421 ± 0.190 Bd
72	1.882 ± 0.259 Ab	2.065 ± 0.184 Bb
96	1.384 ± 0.145 Ac	1.338 ± 0.168 Ac

Points for each incubation period denoted by a different capital letter differ significantly by Student's *t*-test at $P \leq 0.05$. Columns for each treatment, denoted by a different lower case letter, differ significantly by one-way ANOVA and Tukey's post-hoc analysis at $P \leq 0.05$. ± represents standard error.

capable of assimilating ammonia into glutamate or de-aminating glutamate into 2-oxoglutarate and ammonia under stress conditions [18,31,39]; thus, the ammonia can be re-assimilated by *Chlorella* and used for limited growth. In previous studies, we found that this enzyme plays a key role in the metabolism of nitrogen in *Chlorella* when immobilized with the PGPB *Azospirillum brasilense* [16]. In our present study, when combined nitrogen was present in the wastewater medium, there was no apparent promotion of growth by the MGPB. This might indicate that the bacteria's potential for producing IAA was not employed, probably because the synthetic wastewater did not contain tryptophan, the precursor of IAA in this species. However, in the absence of combined nitrogen, this species was capable of accumulating sufficient ammonium in the medium that, in the presence of the microalgae, was consumed, probably translating into enhanced microalgae mass. Therefore, the most likely mechanism by which *B. pumilus* Es4 promotes the growth of *C. vulgaris* is nitrogen fixation under conditions of severe nitrogen starvation.

In summary, this study shows that useful MGPB can be found even in the most unlikely habitats. Therefore, this artificial association may be tested for a variety of PGPB and not necessarily only aquatic PGPB. However, promoting the growth of microalgae does not always apply to common biotechnological applications of the microalgae.

Acknowledgments

Johana Rodriguez and Yaneth Rodriguez contributed equally to this study. Yoav Bashan participated in this study in memory of the late Mr Avner Bashan from Israel. We thank Esther Puente for providing many technical suggestions throughout this study and Ira Fogel for valuable editorial clarification. This study was mainly supported by Consejo Nacional de Ciencia y Tecnología (CONACYT, Investigacion Cientifica Basica 2005 contract #50560-Z), Secretaria de Medio Ambiente y Recursos Naturales of Mexico (SEMARNAT contract #23510), and partially by The Bashan Foundation, USA.

REFERENCES

[1] M. Adachi, T. Kanno, R. Okamoto, S. Itakura, M. Yamaguchi, T. Nishijima, Population structure of *Alexandrium* (Dinophyceae) cyst formation-promoting bacteria in Hiroshima Bay, Japan, Appl. Environ. Microbiol. 69 (2003) 6560–6568.

[2] Y. Bashan, L.E. de-Bashan, Bacteria/plant growth-promotion, in: D. Hillel (Ed.), Encyclopedia of Soils in the Environment, Vol. 1, Elsevier, Oxford, UK, 2005, pp. 103–115.

[3] Y. Bashan, G. Holguin, Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol–PGPB (plant growth-promoting bacteria) and PGPB, Soil Biol. Biochem. 30 (1998) 1225–1228.

[4] Y. Bashan, M. Moreno, E. Troyo, Growth promotion of the seawater-irrigated oilseed halophyte *Salicornia bigelovii* inoculated with mangrove rhizosphere bacteria and halotolerant *Azospirillum* spp, Biol. Fertil. Soils 32 (2000) 265–272.

[5] Y. Bashan, G. Holguin, L.E. de-Bashan, *Azospirillum*–plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003), Can. J. Microbiol. 50 (2004) 521–577.

[6] C.P. Chanway, F.B. Holl, R. Turkington, Genotypic coadaptation in plant growth promotion of forage species by *Bacillus polymyxa*, Plant Soil 106 (1988) 281–284.

[7] C.P. Chanway, M. Shishido, J. Nairn, S. Jungwirth, J. Markham, G. Xiao, F.B. Holl, Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria, For. Ecol. Manage 133 (2000) 81–88.

[8] L.E. de-Bashan, Y. Bashan, Microalgae growth-promoting bacteria: a novel approach in wastewater treatment, Rev. Colomb. Biotecnol. 5 (2003) 85–90.

[9] L.E. de-Bashan, Y. Bashan, Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997–2003), Water Res. 38 (2004) 4222–4246.

[10] L.E. de-Bashan, Y. Bashan, M. Moreno, V.K. Lebsky, J.J. Bustillos, Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilenses*, Can. J. Microbiol. 48 (2002) 514–521.

[11] L.E. de-Bashan, M. Moreno, J.-P. Hernandez, Y. Bashan, Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae *Chlorella vulgaris* coimmobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*, Water Res. 36 (2002) 2941–2948.

[12] L.E. de-Bashan, J.P. Hernandez, T. Morey, Y. Bashan, Microalgae growth-promoting bacteria as "helpers" for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater, Water Res. 38 (2004) 466–474.

[13] L.E. de-Bashan, H. Antoun, Y. Bashan, Cultivation factors and population size control uptake of nitrogen by the microalgae *Chlorella vulgaris* when interacting with the microalgae growth-promoting bacterium *Azospirillum brasilense*, FEMS Microbiol. Ecol. 54 (2005) 197–203.

[14] L.E. de-Bashan, A. Trejo, V.A.R. Huss, J.-P. Hernandez, Y. Bashan, *Chlorella sorokiniana* UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater, Bioresour. Technol. 99 (2008a) 4980–4989.

[15] L.E. de-Bashan, H. Antoun, Y. Bashan, Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*, J. Phycol. 44 (2008b) 938–947.

[16] L.E. de-Bashan, P. Magallon, H. Antoun, Y. Bashan, Role of glutamate dehydrogenase and glutamine synthetase in Chlorella vulgaris during assimilation of ammonium when jointly immobilized with the microalgae growth-promoting bacterium *Azospirillum brasilense*, J. Phycol 44 (2008c) 1188–1196.

[17] J. De la Noüe, N. De Pauw, The potential of microalgal biotechnology: a review of production and uses of microalgae, Biotechnol. Adv. 6 (1988) 725–770.

[18] F. Dubois, T. Terce-Laforgue, M.B. Gonzalez-Moro, J.M. Estavillo, R. Sangwan, A. Gallais, B. Hirel, Glutamate dehydrogenase in plants: is there a new story for an old enzyme? Plant Physiol. Biochem. 41 (2003) 565–576.

[19] S.A. Enebak, G. Wei, J.W. Kloepper, Effects of plant growth-promoting rhizobacteria on loblolly and slash pine seedlings, Forest Sci. 44 (1998) 139–144.

[20] S.K. Garg, A. Bhatnagar, Effect of *Azospirillum* and *Azotobacter* inoculation on pond productivity and fish growth under fresh water conditions, Indian J. Microbiol. 39 (1999) 227–233.

[21] L.E. Gonzalez, Y. Bashan, Growth promotion of the microalgae *Chlorella vulgaris* when coimmobilized and

cocultured in alginate beads with the plant growth-promoting bacteria *Azospirillum brasilense*, Appl. Environ. Microbiol. 66 (2000) 1537–1541.
[22] L.E. Gonzalez, R.O. Cañizares, S. Baena, Efficiency of ammonia and phosphorus removal from Colombian agroindustrial wastewater by the microalgae *Chorella vulgaris* and *Scenedesmus dimorphus*, Bioresour. Technol. 60 (1997) 259–262.
[23] L.E. Gonzalez-Bashan, V. Lebsky, J.P. Hernandez, J.J. Bustillos, Y. Bashan, Changes in the metabolism of the microalgae *Chlorella vulgaris* when coimmobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum*, Can. J. Microbiol. 46 (2000) 653–659.
[24] F.J. Gutiérrez-Mañero, B. Ramos-Solano, A. Probanza, J. Mehouachi, F.R. Tadeo, M. Talon, The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins, Physiol. Plant. 111 (2001) 206–211.
[25] J.-P. Hernandez, L.E. de-Bashan, Y. Bashan, Starvation enhances phosphorus removal from wastewater by the microalga *Chlorella* spp. co-immobilized with *Azospirillum brasilense*, Enzyme Microb. Technol. 38 (2006) 190–198.
[26] G. Holguin, M.A. Guzman, Y. Bashan, Two new nitrogen-fixing bacteria from the rhizosphere of mangrove trees, isolation, identification and in vitro interaction with rhizosphere Staphylococcus sp, FEMS Microbiol. Ecol. 101 (1992) 207–216.
[27] E.R. Ingham, D.A. Klein, Soil fungi: relationships between hyphal activity and attaining with fluorescein diacetate, Soil Biol. Biochem. 16 (1984) 273–278.
[28] J.W. Kloepper, C.-M. Ryu, S. Zhang, Induced systemic resistance and promotion of plant growth by *Bacillus* spp, Phytopathology 94 (2004) 1259–1266.
[29] V.K. Lebsky, L.E. Gonzalez-Bashan, Y. Bashan, Ultrastructure of coimmobilization of the microalga *Chlorella vulgaris* with the plant growth-promoting bacterium *Azospirillum brasilense* and with its natural associative bacterium *Phyllobacterium myrsinacearum* in alginate beads, Can. J. Microbiol. 47 (2001) 1–8.
[30] M. Lucy, E. Reed, B.R. Glick, Applications of free living plant growth-promoting rhizobacteria, Anton. Leeuw. Int. J. G. 86 (2004) 1–25.
[31] R. Melo-Oliveira, I.C. Oliveira, G.M. Coruzzi, *Arabidopsis* mutant analysis and gene regulation define a nonreduntant role for glutamate dehydrogenase in nitrogen assimilation, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 4718–4723.
[32] J.L. Mouget, A. Dakhama, M.C. Lavoie, J. De la Noüe, Algal growth enhancement by bacteria: is consumption of photosynthetic oxygen involved? FEMS Microbiol. Ecol. 18 (1995) 35–44.
[33] J.C. Ogbonna, H. Yoshizawa, H. Tanaka, Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms, J. Appl. Phycol. 12 (2000) 277–284.
[34] A. Probanza, J.A. Lucas-Garcia, M. Ruiz-Palomino, B. Ramos, F.J. Gutiérrez-Mañero, *Pinus pinea* L. seedling growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus* (*B. licheniformis* CECT 5106 and *B. pumilus* CECT 5105), Appl. Soil Ecol. 20 (2002) 75–84.
[35] M.E. Puente, Y. Bashan, C.Y. Li, V.K. Lebsky, Microbial populations and activities in the rhizoplane of rock-weathering desert plants. I. Root colonization and weathering of igneous rocks, Plant Biol. 6 (2004) 629–642.
[36] M.E. Puente, C.Y. Li, Y. Bashan, Microbial populations and activities in the rhizoplane of rock-weathering desert plants. II. Growth promotion of cactus seedling, Plant Biol. 6 (2004) 643–650.
[37] N. Requena, I. Jimenez, M. Toro, J.M. Barea, Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in Mediterranean semi-arid ecosystems, New Phytol. 136 (1997) 667–677.
[38] C.E. Riquelm, K. Fukami, Y. Ishida, Effects of bacteria on the growth of a marine diatom, *Asterionella glacialis*, Bull. Jpn. Soc. Microb. Ecol. 3 (1988) 29–34.
[39] S.A. Robinson, A.P. Slade, G.G. Fox, R. Phillips, G. Ratcliffe, G.R. Stewart, The role of glutamate dehydrogenase in plant nitrogen metabolism, Plant Physiol. 95 (1991) 509–516.
[40] H. Rodriguez, R. Fraga, T. Gonzalez, Y. Bashan, Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria, Plant Soil 287 (2006) 15–21.
[41] M. Shishido, H.B. Massicotte, C.P. Chanway, Effect of plant growth promoting *Bacillus* strains on pine and spruce seedling growth and mycorrhizal infection, Ann. Bot. 77 (1996) 433–442.
[42] H. Suminto, K. Hirayama, Effects of bacterial coexistence on the growth of a marine diatom *Chaetoceros gracilis*, Fish Sci. 62 (1996) 40–43.
[43] H. Suminto, K. Hirayama, Application of a growth-promoting bacteria for stable mass culture of three marine microalgae, Hydrobiologia 358 (1997) 223–230.
[44] R. Ukeles, J. Bishop, Enhancement of phytoplankton growth by marine bacteria, J. Phycol. 11 (1975) 142–149.
[45] R. Yabur, Y. Bashan, G. Hernández-Carmona, Alginate from the macroalgae *Sargassum sinicola* as a novel source for microbial immobilization material in wastewater treatment and plant growth promotion, J. Appl. Phycol. 19 (2007) 43–53.