FEMS Microbiology Ecology 54 (2005) 197–203

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Cultivation factors and population size control the uptake of nitrogen by the microalgae *Chlorella vulgaris* when interacting with the microalgae growth-promoting bacterium *Azospirillum brasilense*

Luz E. de-Bashan [a,b], Hani Antoun [b], Yoav Bashan [a,b,*]

[a] *Environmental Microbiology Group, Center for Biological Research of the Northwest (CIBNOR), Mar Col. Playa Palo de Santa Rita, Bermejo No. 195, La Paz, B.C.S., Mexico*

[b] *Départment des Sols et de Génie Agroalimentaire, Université Laval, Que., Canada*

Received 18 January 2005; received in revised form 28 March 2005; accepted 29 March 2005

First published online 6 May 2005

Abstract

Growth of and the capacity to take up nitrogen in the freshwater microalgae *Chlorella vulgaris* were studied while varying the concentrations of ammonium and nitrate, the pH and the source of carbon in a synthetic wastewater growth medium when co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*. Analyses of 29 independent experiments showed that co-immobilization of the microalgae with *A. brasilense* could result in two independent phenomena directly affected by cultivation factors, such as nitrogen species, pH and presence of a carbon source. First, growth of the microalgal population increased without an increase in the capacity of the single cells to take up nitrogen, or second, the capacity of cells to take up nitrogen increased without an increase of the total microalgal population. These phenomena were dependent on the population density of the microalgae, which was in turn affected by cultivation factors. This supports the conclusion that the size of the microalgal population controls the uptake of nitrogen in *C. vulgaris* cells – the higher the population (regardless the experimental parameters), the less nitrogen each cell takes up.

Keywords: Microalgae; Plant growth-promoting bacteria; *Azospirillum brasilense*; *Chlorella vulgaris*; Nitrogen uptake

1. Introduction

Microalgae are used in wastewater bio-treatments [1], as food for humans and animals [2], as feed in aquacultures [3,4], for the production of pigments [5] and in agriculture [6]. Naturally grown microalgae are always associated with bacteria [7,8]. The positive effects of bacteria on microalgae were reported decades ago [9,10]. More recently, the plant growth-promoting bacterium (PGPB) *Flavobacterium* sp. was found to promote the growth of a marine microalgae (the diatom *Chaetoceros gracilis*), which is used as feed in pearl oyster hatcheries [11,12]. Inoculation of freshwater aquaculture ponds with the PGPB *Azospirillum* sp. and *Azotobacter* sp. significantly increased the phytoplankton population, and consequently, fish yields [13]. When the two microorganisms (bacteria and microalgae) are growing together, there are mutually beneficial effects between them that can be explained in several ways. Microalgae are known to produce and release enough exogenous oxygen to

* Corresponding author. Tel.: +52 612 123 8484x3668; fax: +52 612 125 4710.

E-mail address: bashan@cibnor.mx (Y. Bashan).

0168-6496/$22.00 © 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.femsec.2005.03.014

fulfill most aerobic bacterial requirements, and they can also release large amounts of organic compounds that can be assimilated by bacteria [8]. In return, bacteria can stimulate algal growth by the release of vitamins and plant hormones [7,8,14], or they can be a source of CO_2, especially during periods of limited carbon. Some associations are not necessarily beneficial for microalgae; the natural associative bacterium *Phyllobacterium myrsinacearum* accelerates senescence of the microalgae *Chlorella vulgaris* when grown together [15].

Immobilized microalgae in alginate or carrageenan beads can remove N, P and heavy metals from wastewater [16–19]. In recent studies, we showed that co-immobilization in alginate beads of the freshwater microalgae *C. vulgaris* or *C. sorokiniana* with the microalgae growth-promoting bacterium (MGPB) *Azospirillum brasilense* strain Cd, commonly used as an inoculant in agriculture [20], significantly increases growth parameters over microalgae immobilized alone [14,15,21]. Furthermore, we have demonstrated that microalgae are able to take up higher amounts of nitrogen (ammonium and nitrate) and phosphorus when cultured together with the bacteria than when cultured alone under laboratory conditions in synthetic wastewater or municipal wastewater [22,23].

Our working hypothesis was that the bioreactor cultivation factors (type of nitrogen, pH and carbon source) affect the microalgal population, in addition to the effects caused by the interaction with the MGPB. Consequently, the developing population of microalgae possibly controls the uptake of nitrogen from the wastewater at culture or at individual cell levels. Therefore, this study examined cultivation factors and population densities governing the uptake of nitrogen by *C. vulgaris*, when co-immobilized in alginate beads with the MGPB *A. brasilense* Cd.

2. Materials and methods

2.1. Microorganisms and axenic growth conditions

The unicellular microalgae *C. vulgaris* Beijerinck (UTEX 2714, University of Texas, Austin, TX) was used. Before immobilization in alginate beads, the microalgae were cultured in sterile mineral medium (C30) for 5 days, following methods described previously [24]. *A. brasilense* Cd (DMS 1843, Braunschweig, Germany) was used in co-immobilization experiments. The bacterium was grown in nutrient broth (Sigma) at 30 ± 2 °C for 18 h in a rotary shaker at 120 rpm.

2.2. Immobilization of microorganisms in alginate beads

Microorganisms were immobilized following methods described previously [23]. Briefly, 20 ml of axenically grown cultures of *C. vulgaris*, containing 6.0×10^6 cells ml^{-1}, were harvested by centrifugation at 2000*g* and washed twice with sterile saline solution (0.85% NaCl). Afterwards, the cells were mixed with 80 ml of sterile, 6000-cP (1 cP = 0.001 Pa s) 2% alginate solution (a solution made of alginate mixed at 14,000 and 3500 cP) and stirred for 15 min. The solution dripped from a sterile syringe into a 2% $CaCl_2$ solution with slow stirring. The beads that were formed were left for 1 h at 22 ± 2 °C for curing and then washed in sterile saline solution. *A. brasilense* cultures (approximately 10^9 cfu ml^{-1}) were immobilized similarly. Because immobilization normally reduces the number of organisms in the beads, a second incubation step was necessary. This was carried out overnight in diluted nutrient broth (1:10). Where co-cultures of *A. brasilense* and the microalgae were used, the same concentration of each microorganism as used in pure cultures was mixed prior to incorporation with alginate and bead formation, but the volume of each microbial culture was reduced to 10 ml before adding the alginate.

2.3. Growth medium

Synthetic wastewater growth medium (SGM) was prepared with the following components (in milligrams per liter): NaCl (7), $CaCl_2$ (4), $MgSO_4 \cdot 7H_2O$ (2), K_2HPO_4 (21.7), KH_2PO_4 (8.5), Na_2HPO_4 (33.4) and NH_4Cl (3). According to the experiment, the concentration of NH_4Cl was increased up to 25.2 mg l^{-1} to obtain a final maximum concentration of 13 mg l^{-1} NH_4^+. Nutrient broth, used by both microorganisms (2.7 mg l^{-1}), or arabinose (1 g l^{-1}) used by *A. brasilense*, but not by *Chlorella* spp. [25], were added when indicated. In some assays, the source of nitrogen was changed from ammonium to nitrate (KNO_3, 24 mg l^{-1}), or the pH was adjusted to 8.0 with a 0.2 M phosphate buffer solution.

2.4. Culture conditions and experiments

Microorganisms solitary immobilized or co-immobilized were grown under semi-continuous conditions during which changing the growth solution, but not the immobilized microorganism (every 48 h). The experiments were: (i) Growth of microorganisms in SGM at different concentrations of ammonium (3 and 13 mg l^{-1} NH_4^+), at pH 7.0; (ii) Growth of microorganisms in SGM at an initial 13 mg l^{-1} concentration of ammonium at pH 8.0; (iii) Growth of microorganisms in SGM supplemented with a source of carbon at an initial 13 mg l^{-1} concentration of ammonium; and (iv) Growth of microorganisms in SGM with nitrate as a source of nitrogen. All experiments lasted 8 days. The cultures were incubated in 250-ml unbaffled Erlenmeyer flasks (100 ml medium with 4 g beads) at 28 ± 2 °C, 120 rpm and constant light at 30 μmol m^{-2} s^{-1} photon flux den-