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 brasilense*

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Abstract: Three strains of the freshwater microalgae used for wastewater treatment, *Chlorella vulgaris* and *Chlorella sorokiniana* co-immobilized separately in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense* Cd, resulted in significant changes in microalgal-population size, cell size, cell cytology, pigment, lipid content, and the variety of fatty acids produced in comparison with microalgae immobilized in alginate without the bacterium. Cells of *C. vulgaris* UTEX 2714 did not change in size, but the population size within the beads significantly increased. On the other hand, *C. vulgaris* UTEX 395 cells grew 62% larger, but their numbers did not increase. The population of *C. sorokiniana* UTEX 1602 increased, but not their cell size. The content of pigments chlorophyll *a* and *b*, lutein, and violoaxanthin increased in all microalgal species. The lipid content also significantly increased in all three strains, and the number of different fatty acids in the microalgae increased from four to eight. This study indicates that the microalgae-growth-promoting bacterium induced significant changes in the metabolism of the microalgae.

Key words: alginate, *Azospirillum, Chlorella,* bacterial immobilization, microalgae, wastewater.

Resume : Trois souches d'algues microscopiques d'eau douce utilisées pour le traitement des eaux usées, *Chlorella vulgaris* et *Chlorella sorokiniana,* ont été co-immobilisées dans des Mlles d'alginates avec *Azospirillum brasilense* Cd, une bactérie favorisant la croissances des algues. Ceci a entraîné des changements importants dans la taille de la population des algues microscopiques et des cellules, dans la cytologie cellulaire, dans les contenus en pigments et en lipides, et dans la diversité des acides gras produits, comparativement à des algues immobilisées dans l'alginate sans la bactérie. La taille des cellules de *C. vulgaris* UTEX 2714 n'a pas changé, mais la population à l'intérieur des bille a augmenté significativement. D'autre part, les cellules de C. *vulgaris* UTEX 395 ont grossi de 62%, mais leurs nombres n'ont pas augmentés. La population de *C. sorokiniana* UTEX 1602 a augmenté, mais non la taille de ses cellules. Les quantités de pigments de chlorophylle *a* et *b*, de lutéine et de violoaxanthine ont augmenté dans toutes les espèces d'algues microscopiques. Les quantité de lipides contenus dans les trois souches ont également significativement augmenté, et le nombre d'acides gras différents dans les algues est passé de quatre à huit. Cette étude indique que les bactéries favorisant la croissance des algues microscopiques sont à l'origine de changements importants dans le métabolisme algal.

Mots clés : alginate, *Azospirillum, Chlorella,* immobilisation de bactéries, algues microscopiques, eaux usées.

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Introduction

The freshwater unicellular microalga *Chlorella vulgaris* is used for tertiary wastewater treatment, mainly for removal of nitrogen and phosphorus compounds and heavy metals (Aksu et al. 1992; Gonzalez et al. 1997; Oh-Hama and Miyachi 1992; Tam et al. 1994, 1998; Tam and Wong 2000). It has also been proposed for use in industrial processes unrelated to wastewater treatment (Kayano et al. 1981; Wikstrom et al. 1982).

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The interactions of *Chlorella* spp. with bacteria are poorly understood. Two obligate aerobes isolated from laboratory algal cultures, *Pseudomonas diminuta* and *Pseudomonas vesicularis*, stimulated the growth of the green microalgae *Scenedesmus bicellularis* and *Chlorella* sp., without releasing any growth-promoting substance (Mouget et al. 1995). *Pseudomonas aeruginosa* produced anti-algal substances when it associated with several microalgal species (Dakhama et al. 1993). Recent studies have shown that co-immobilization of *C. vulgaris* with the plant growth-promoting bacterium *Azospirillum brasilense* Cd increased growth and prolonged the life span of the microalga (Gonzalez and Bashan 2000; Lebsky et al. 2001). In a similar manner, this bacterium stimulates the growth of numerous terrestrial plants (Bashan and Holguin 1997) and is used as an inoculant in agriculture (Bashan 1998). Co-immobilization of the two microorganisms also significantly improved the removal of ammonium and phosphorus from wastewater (de-Bashan et al. 2002). Therefore, *A. brasilense* may be considered a "microalgae-growth-promoting bacterium" (MGPB). On the other hand, although association of *C. vulgaris* with its natural associative bacterium *Phyllobacterium myrsinacearum* changed the metabolism of the microalga, it also caused senescence and death (Gonzalez-Bashan et al. 2000; Lebsky et al. 2001).

This study evaluates the effect of the MGPB *A. brasilense* Cd on lipid content and variety, pigment production, cell cytology, and population size of three strains of *Chlorella* spp., as these parameters are indicators of competence and function of the microalgal cell. This was done when both microorganisms were co-immobilized in alginate beads, an increasingly popular way of using microorganisms for environmental applications (Cassidy et al. 1996; Tam et al. 1998; Trevors et al. 1993).

Materials and methods

Microorganisms and axenic growth conditions

Chlorella vulgaris Beijerinck UTEX 2714 was isolated from a secondary effluent of a wastewater-treatment stabilization pond near Bogota (Gonzalez et al. 1997). Culture collection strains, C. *vulgaris* UTEX 395 and *C. sorokiniana* Shih. et Krauss UTEX 1602, were also used. Before immobilization in alginate beads, the microalgae were cultivated in a sterile mineral medium (C30), as previously described (Gonzalez et al. 1997), for 5 days. *Azospirillum brasilense* was grown in liquid nutrient broth (Difco, Detroit, Mich.) at 30 ± 2°C for 48 h in a rotary shaker.

Immobilization of microalgae and bacteria in alginate beads

Microorganisms were immobilized using the method described by Bashan (1986) and Gonzalez and Bashan (2000). Briefly, 20 mL of axenically grown cultures of *C. vulgaris* containing 6.0 x 10^6 cells/mL were mixed with 80 mL of a 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. *Azospirillum brasilense* cultures (approximately 10^9 CFU/mL) 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 OAB nitrogen-free medium (Bashan et al. 1993) for beads containing *A. brasilense* and 18 h in 6.5 mM phosphate buffer for beads containing C. *vulgaris*. The low concentration of phosphate and the short incubation period were insufficient to dissolve the beads. Where co-cultures of *A. brasilense* and the microalga were used, the same concentration of each microorganism 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.

Culture conditions for co-immobilized microorganisms or organisms alone

Co-immobilized microorganisms or C. *vulgaris* organisms alone were grown in the mineral salts of residual water medium containing the following (in mg/L): 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, 10. The level of phosphate in the medium was insufficient to dissolve the constructed beads. Batch cultures (500 mL) were incubated in nonbaffled Erlenmeyer flasks at 22 ± 2°C and 150 rpm with a light intensity of 60 μmol $m^{-2} \cdot s^{-1}$ 1 for 10 days. Samples for analysis were taken aseptically.

Synthetic wastewater composition, bead solubilization and count of microorganisms, scanning and transmission electron microscopy, and pigment analysis were performed, as described in detail by Bashan 1986, Bashan et al. 1993, Gonzalez and Bashan 2000, Gonzalez et al. 1997, Lebsky et al. 2001, and Vidussi et al. 1996.

Lipid and fatty acid analyses

Total lipids were analyzed according to the method described by Bligh and Dyer (1959). Fatty acids composition was analyzed by gas chromatography of methyl esters of cell fatty acids. These analyses were done by a commercial service (Microbial ID, Inc., Newark, Del.).

Experimental design and statistical analysis

Cultures of either co-immobilized microorganisms or microalgae immobilized alone in alginate beads were prepared in triplicate (a single flask was considered one replicate), and each experiment was repeated two to five times. Controls were prepared similarly, but without microorganisms in the beads. Five beads were taken randomly from each culture and dissolved for counting the total number of cells. Pigment and lipid contents were both also analyzed in triplicate; the level of pigments in five dissolved beads was one replicate; and for lipid analyses, 1 g of bead was used per replicate. Over 200 electron microscope photographs were taken during this study. Cell size was measured directly on the photographs in 20 replicates (a photo containing approximately 250 cells served as one replicate). Pigments were analyzed after 0, 1, 3, and 6 days. We observed the greatest differences on the third day of incubation, and present results from the third day. Electron microscope photographs were taken after 5 and 10 days because they showed large lipid accumulation at this time. Lipid contents and variety were analyzed after

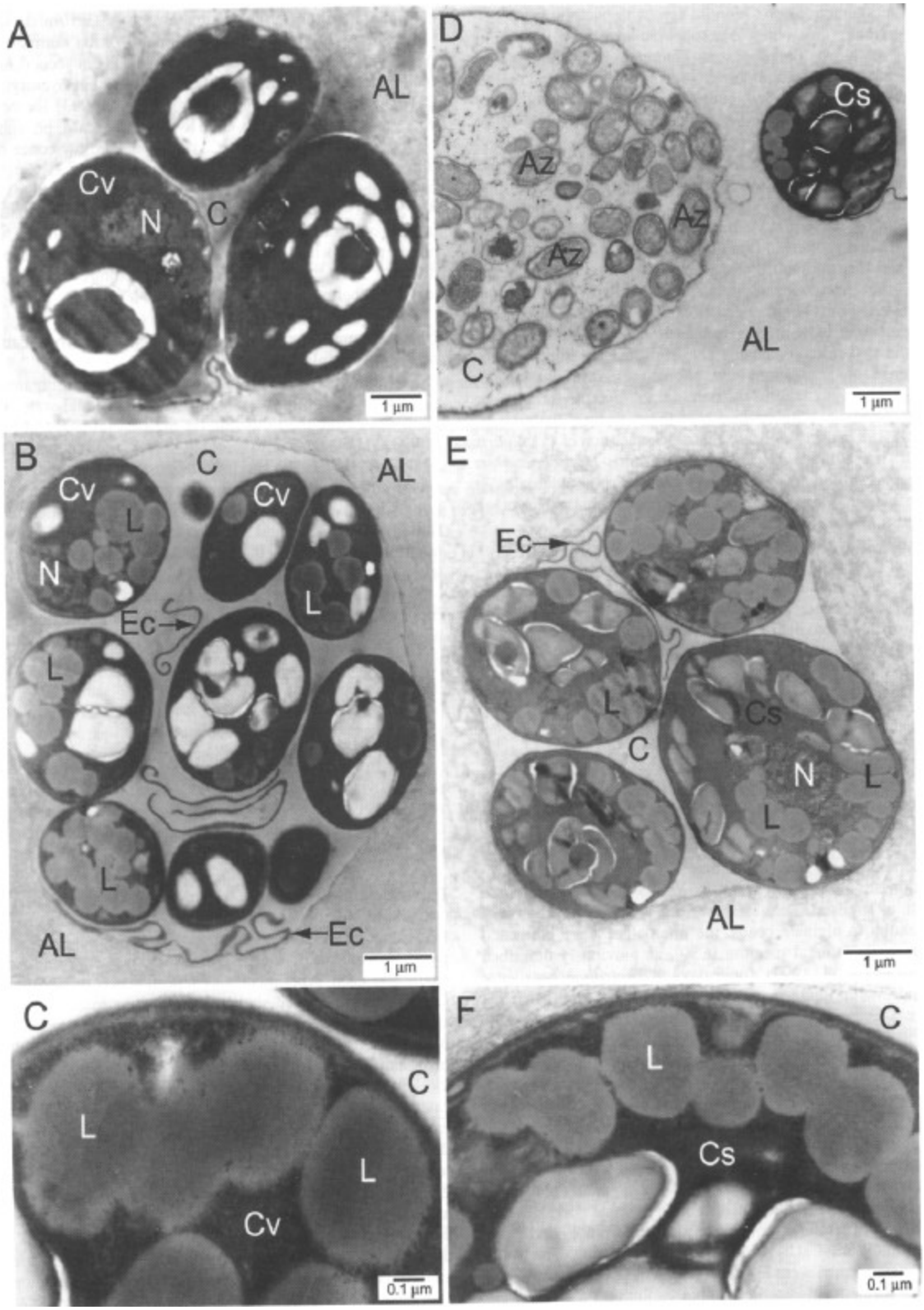


Fig. 1. Transmission electron microscopy of cross sections of cells of *Chlorella vulgaris* UTEX *395* and *Chlorella sorokiniana* UTEX 1602 with *Azospirillum brasilense* Cd in alginate beads 5 days (A, D) and 10 days (B, C, E, F) after co-immobilization. A-C, *Chlorella vulgaris;* D-F, *Chlorella sorokiniana.* (A) *C. vulgaris* immobilized alone in alginate beads in the absence of *A. brasilense.* (B) Cells of *C. vulgaris* after co-immobilization in alginate beads with *A. brasilense* (both species do not share at this stage the same cavity inside the bead - similar to Fig. 1D). (C) Higher magnification of a part of one *C. vulgaris* cell. (D) Co-immobilization of *C. sorokiniana* and *A. brasilense* in the same alginate bead. Both microorganisms colonized adjacent cavities within the bead. (E) Larger magnification of *C. sorokiniana* cells co-immobilized with *A. brasilense* in alginate beads. (F) Higher magnification of a part of one *C. sorokiniana* cell. Abbreviations: AL, alginate bead; Az, *Azospirillum brasilense*; C, cavity inside the bead; Cs, *Chlorella sorokiniana*; Cv, *Chlorella vulgaris;* EC, electron dense material of unknown nature; N, nucleus; and L, lipid droplets. Lipids were identified on the micrographs according to Van Etten et al. (1987).

Table 1. Pigment production (μg/g cells) by the microalgae *Chlorella vulgaris* and *Chlorella sorokiniana* after co-immobilization with *Azospirillum brasilense* in alginate beads and after incubation in batch cultures for 3 days.

Pigment	*C. sorokiniana* UTEX 1602	*C. sorokiniana* UTEX 1602 + *A. brasilense*	*C. vulgaris* UTEX 395	*C. vulgaris* UTEX 395 + *A. Brasilense*	*C. vulgaris* UTEX 2714	*C. Vulgaris* UTEX 2714 + *A. brasilense*
Chlorophyll *a*	1061.3*a*	1646.17*b*	836.9*a*	1326.87*b*	250.6*a*	706.23*b*
Chlorophyll *b*	491.9*a*	807.2*b*	311.18*a*	558.45*a*	71.9*a*	180.4*b*
Lutein	116.1*a*	201.53*b*	117.3*a*	159.34*a*	52.0*a*	120.4*b*
Violoaxanthin	16.82*a*	41.66*b*	35.19*a*	37.16*a*	19.2*a*	28.3*b*

Note: Numbers denoted by a different letter in each pigment and in each microalgae species differ significantly at $P \leq 0.05$, using Student's *t* test.

Fig. 2. Total lipid content of *Chlorella vulgaris* UTEX *395* and UTEX 2714 and *Chlorella sorokiniana* UTEX 1602 immobilized alone and co-immobilized with *Azospirillum brasilense* Cd after 10 days of incubation. Columns denoted by a different lower case letter for each microalgae species differ significantly at $P \leq 0.05$ using Student's t test. Columns denoted by a different capital letter differ significantly at $P \leq 0.05$ using one-way ANOVA. Bars represent SE.

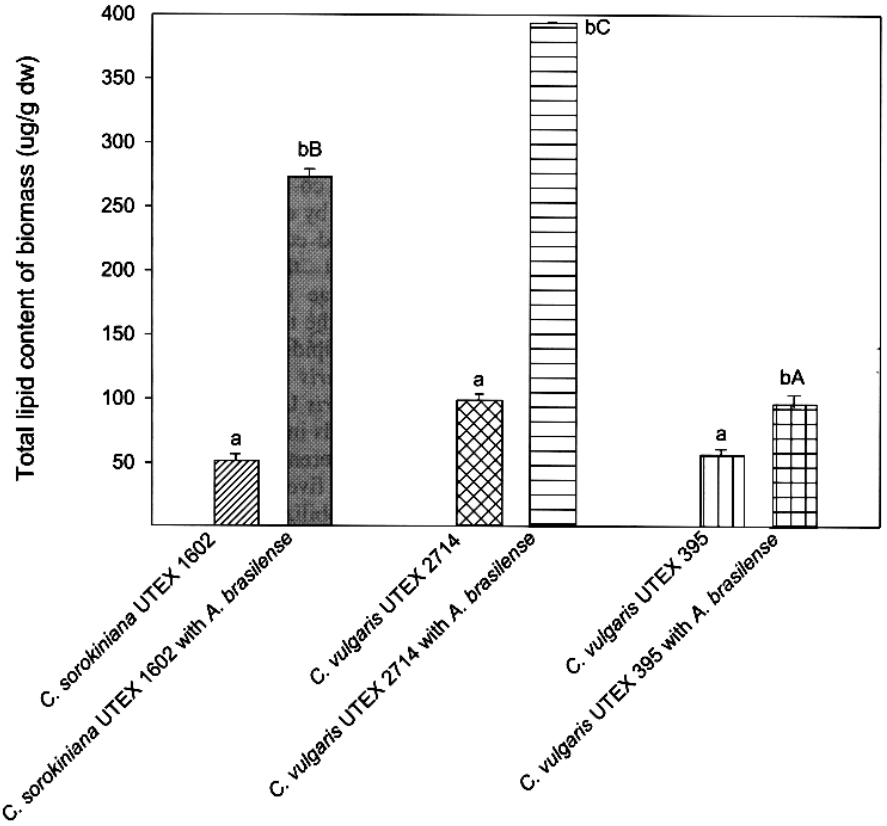


Table 2. Variety and concentration (µg/g cells) of fatty acids produced by *Chlorella vulgaris* UTEX 395 and UTEX 2714 and *Chlorella sorokiniana* UTEX 1602 immobilized alone and co-immobilized with *Azospirillum brasilense* Cd after 10 days of incubation.

Treatment	Dodecanoic acid 12:0	Tetradecanoic acid 14:0	Hexadecanoic acid 16:1	6,8-Hexadecadienoic acid 16:2ω6	Hexadecanoic acid 16:0	9,12-Octadecadienoic acid 18:2ω6	8-Octadecanoic – 9,12,15-octadecatrienoic acid 18:1ω8 – 18:3ω3	Octadecanoic acid 18:0
C. sorokiniana	—	4.53±1.05	7.34±0.99	—	18.28±0.98	6.14±0.55	13.51±0.27	—
C. sorokiniana + *A. brasilense*	—	7.78±0.04	31.94±1.1	20.87±0.76	74.81±0.27	48.07±1.7	78.15±0.9	4.15±1.5
C. vulgaris 2714	—	4.68±1.05	18.20±0.47	—	26.65±0.3	9.02±0.9	39.58±0.4	—
C. vulgaris 2714 + *A. brasilense*	7.70±1.9	9.41±0.23	73.06±0.88	15.97±0.4	85.9±0.21	44.41±0.11	150.05±0.38	7.12±0.18
C. vulgaris 395	—	5.49±0.9	5.6±0.9	—	19.71±0.34	5.71±0.1	20.38±0.35	—
C. vulgaris 395 + *A. brasilense*	—	—	12.5±0.12	4.26±0.44	21.49±0.22	12.75±0.13	45.39±0.47	—

Note: Values are means ± SE.

10 days. The mean of all repetitions was calculated and analyzed by one-way analysis of variance (ANOVA) ($P \leq 0.05$) or by Student's *t* test (for cell size measurement) using Statistica software (Statsoft, Tulsa, Okla.).

Results

Increase in pigment production in *C. vulgaris* and *C. sorokiniana* co-immobilized with *A. brasilense*

Pigment production in *C. vulgaris* and *C. sorokiniana*, co-immobilized with *A. brasilense* in alginate beads, was compared under axenic batch-culture conditions to the pigment content of microalgae immobilized alone in similar beads. Table 1 shows that the content of the four major microalgal pigments not produced by *A. brasilense* Cd significantly increased as a result of co-immobilization of the two microorganisms in the same bead. For *C. vulgaris* UTEX 395, only the production of chlorophyll *a* increased in the presence of *A. brasilense* Cd.

Changes in cell size cytology of *C. vulgaris* and *C. sorokiniana* co-immobilized with *A. brasilense*

Co-immobilization with *A. brasilense* significantly increased the cell diameter of *C. vulgaris* UTEX 395 from 2.8 ± 0.52 to 4.6 ± 1.1 µm. No effect on cell size was detected in *C. vulgaris* UTEX 2714 or *C. sorokiniana* UTEX 1602. More marked changes occurred at the cytological level, where large amounts of lipids were observed in the cytoplasm of the microalgae when co-immobilized with *A. brasilense* (Fig. 1).

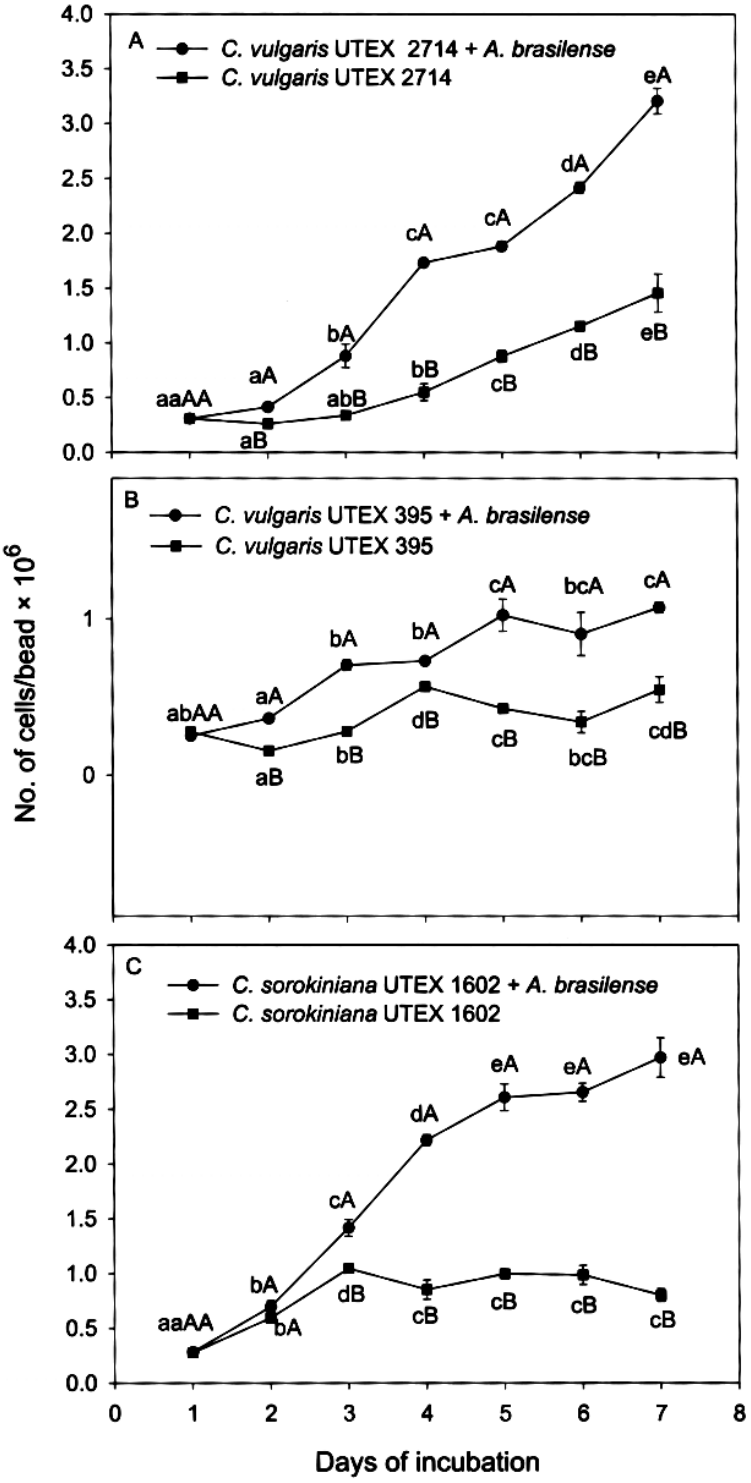
Increase in lipid content and in variety of fatty acids in *C. vulgaris* and *C. sorokiniana* co-immobilized with *A. brasilense*

Because an increase in the lipid content of the cells, as a result of co-immobilization, was the main cytological event detected by electron microscopy after 10 days of incubation, total lipid content and the variety of fatty acids were analyzed at this time. Co-immobilization of the three microalgae strains (separately) with *A. brasilense* Cd increased the total lipid contents in the cells. The net increase in total lipids among the microalgae species was as follows: *C. vulgaris* UTEX 395 < *C. sorokiniana* UTEX 1602 < *C. vulgaris* UTEX 2714 (Fig. 2). Additionally, the variety of fatty acids increased in these microalgae strains from four to five different fatty acids in microalgae-only immobilized cells to five to eight different fatty acids in microalgae co-immobilized with the MGPB (Table 2). As the methods used to quantify fatty acids are based on determination of weight, and the number of *A. brasilense* Cd cells in the association was relatively low, the lipid content and the profile of fatty acids of *A. brasilense* Cd was below the detection level of the methods.

Changes in the population of *C. vulgaris* and *C. sorokiniana* co-immobilized with *A. brasilense*

Co-immobilization initially increased the number of *C. vulgaris* UTEX 2714 and *C. sorokiniana* UTEX 1602 cells in the beads compared to immobilization of the microalgae alone (Fig. 3). Over time, the population of all microalgal species increased within the beads, regardless of

Fig. 3. Growth of *Chlorella vulgaris* UTEX 2714 (A) and UTEX 393 (B) and *Chlorella sorokiniana* UTEX 1602 (C) immobilized alone and co-immobilized with *Azospirillum brasilense* Cd and incubated in synthetic *wastewater*. Growth curves denoted by a different lower case letter for each microalgal species differ significantly at $P \leq 0.05$ using one-way ANOVA. Points at each incubation time for each microalgal species denoted by a different capital letter differ significantly at $P \leq 0.05$ using Student's *t* test. Bars represent SE. Absence of a bar means that the SE is smaller than the point.



treatment (Fig. 3). The relative final populations obtained after 7 days *of* incubation were as *follows: C. vulgaris* UTEX 2714 > *C. sorokiniana* UTEX 1602 > C. *vulgaris* UTEX 395. The population of *A. brasilense* Cd did not increase during this period, but it was within the range of 1.1-2.3 x 10^5 CFU/mL for the three co-immobilization treatments.

Discussion

The use of several different species of microalgae, including *Chlorella* spp., for tertiary wastewater treatment was suggested well over a decade ago (Chevalier and De la Noüe 1985; De la Noüe and De Pauw 1988) and continues to be evaluated today (Tam et al. 1998; Tang et al. 1997). The underlying assumption is that the microalgae will transform some of the contaminants in the wastewater to nonhazardous materials so that the water can be beneficially reused or safely discharged (Oswald 1992). Little is known about bacteria exclusively associating with unicellular aquatic microalgae (Gonzalez-Bashan et al. 2000; Lebsky et al. 2001; Mouget et al. 1995). Artificial inoculation of microalgae with MGPB, more commonly practiced in agriculture (Bashan 1998; Hallmann et al. 1997) and forestry (Chanway and Holl 1994) using plant growth - promoting bacteria, is in its infancy (Gonzalez and Bashan 2000).

This study shows that an MGPB profoundly changed the metabolism, cell size, and population density of three microalgal strains (separately), when both microorganisms were confined to small cavities inside alginate beads. In the beads, space is limited, microbial numbers are high, and there is no interference from other microorganisms. The most profound changes occurred at the cellular level with an accumulation of lipids. In addition, the lipids contained a greater variety of fatty acids. Increase in the pH of the medium where *Chlorella* sp. is growing interferes with the cell cycle and decreases the microalgal population. In high pH media, the cell cycle is reduced and the microalgal cells accumulate triglycerides (Guckert and Cooksey 1990). Although some modest pH increases (average Δ pH 0.5) (data not shown) were observed in most of our experiments, our microalgae-bacterium system differred, because the microalgal cell cycle in the presence of the MGPB increased and the microalgal population also increased (Gonzalez and Bashan 2000). Thus, the accumulation of lipids observed in this study are not stress lipids that have been induced by increased pH. Additionally, the cell diameter increased in one microalgal species. The physiological implications of the extra storage material may explain the prolonged life span of the microalgae when co-immobilized with an MGPB (Lebsky et al. 2001). Furthermore, the increase in pigment content, which may potentially improve photosynthesis, occurred in the population of two microalgal strains and the larger cell size of a third strain may account for the enhanced capacity of the microalgae to absorb ammonium and phosphorus ions from wastewater (de-Bashan et al. 2002). The differences in the effects of the MGPB on various microalgal strains points out the need for future optimization of the bacterial-microalgal combination. It is essential to find an efficient bacterium-microalgae combination for wastewater treatment, which is the long-term goal of these studies.

In summary, this paper demonstrates that artificial associations of the microalga *C. vulgaris* or *C. sorokiniana* and the MGPB *A. brasilense* affect the metabolism (pigment, lipid content, and variety of fatty acids produced), population size, and morphology (cell size and cell cytology) of the microalgae.

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