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Microalgae growth-promoting bacteria as "helpers" for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater

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Abstract

A combination of microalgae (*Chlorella vulgaris* or *C. sorokiniana*) and a microalgae growth-promoting bacterium (MGPB, *Azospirillum brasilense* strain Cd), co-immobilized in small alginate beads, was developed to remove nutrients (*P* and *N*) from municipal wastewater. This paper describes the most recent technical details necessary for successful co-immobilization of the two microorganisms, and the usefulness of the approach in cleaning the municipal wastewater of the city of La Paz, Mexico. *A. brasilense* Cd significantly enhanced the growth of both *Chlorella* species when the co-immobilized microorganisms were grown in wastewater. *A. brasilense* is incapable of significant removal of nutrients from the wastewater, whereas both microalgae can. Co-immobilization of the two microorganisms was superior to removal by the microalgae alone, reaching removal of up to 100% ammonium, 15% nitrate, and 36% phosphorus within 6 days (varied with the source of the wastewater), compared to 75% ammonium, 6% nitrate, and 19% phosphorus by the microalgae alone. This study shows the potential of co-immobilization of microorganisms in small beads to serve as a treatment for wastewater in tropical areas.

Keywords: Microbial immobilization; Microalgae; Plant growth-promoting bacteria; Wastewater treatment; Nitrogen removal; Phosphorus removal

1. Introduction

Plant growth-promoting bacteria (PGPB) used as inoculants in agricultural experiments are common place for control of phytopathogens and for plant growth promotion [1]. Bacteria of the genus *Azospirillum* are well-known as PGPB for numerous crop plants [2]. Recently, one common strain, the type strain *A. brasilense* Cd was found to be capable of promoting many growth parameters of the unicellular microalgae *Chlorella vulgaris* [3], and change the cytology, lipid, and pigment production by the microalgae [4–6]. Therefore, it may be considered as a microalgae growth-promoting bacterium (MGPB). *C. vulgaris* is commonly used for tertiary wastewater treatment [7–10], yet it had not been demonstrated that the observed growth promotion might also yield improve capabilities of microalgae to remove nutrients from natural wastewater. The microbial carrier chosen in this study were alginate beads. Immobilization of microalgae in polysaccharide gels is an experimental way to use these microorganisms for wastewater treatment [11,12] because it ameliorates the major difficulty of collecting enormous populations of cells developed during the treatment, hampering regular microalgae treatments [9].

This study describes laboratory methods used to co-immobilize the two microorganisms in small alginate beads and to show that this artificial biological

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association, enforced by close proximities of the microorganisms inside small polymer beads, enhanced the population of microalgae in the wastewater and improved the capacity of the microalgae in its main practical task—removing nitrogen and phosphorus from municipal wastewater.

2. Materials and methods

2.1. Microorganisms

Two species of unicellular microalgae, *Chlorella vulgaris* Beijerinck (UTEX 2714) and *C. sorokiniana* Shih. et Krauss (UTEX 1602) were used. The MGPB *Azospirillum brasilense* Cd (DMS 1843) was used for co-immobilization experiments with each of the microalgal species.

2.2. Immobilization procedures

Various modifications of immobilizing microorganisms in alginate beads and counting and growing them in cultures were published as the research evolved [3,13,14]. To clarify contradictory details, the current procedures are summarized in detail in Fig. 1. *A. brasilense* was cultivated with prior immobilization, by standard techniques for this species [15].

2.3. Design and construction of a device for production of alginate beads

A device to manufacture spherical alginate beads of suitable diameter (~2 mm) for the experiments was constructed from aluminum sheet (0.079 cm). The device is essentially an open box with an array of 64 specially shaped nozzles formed in the surface of the bottom plate. Each nozzle consists of three parts: a dimple, a shoulder, and an orifice (Fig. 2). Liquid alginate is placed in the box and beads are allowed to form under the force of gravity, and to drip into a receptacle containing a hardening solution. As the liquid level in the device decreases, the flow rate slows. Liquid alginate has high surface tension, hence, during their fall and subsequent hardening, the beads are spherical. The showerhead satisfies the following set of constraints: no need for pressurized air; beads form under the force of gravity only; beads are of suitably small diameter; bead diameter is consistent; rate of formation is suitably rapid; apparatus can be autoclaved, and an adequate amount of alginate solution can be processed at one time.

The main design problems were to establish the appropriate diameter of the holes to control flow rate and to prevent the alginate from bridging the space between the adjacent orifices, thereby forming beads of inconsistent diameter. The diameter of the holes (0.137 cm) is small enough that, when the liquid level is 15 cm above the surface of the showerhead, the flow of liquid alginate forms individual beads, but is large enough that the container drains completely in a few minutes.

To prevent liquid alginate from bridging, dimples were formed in the surface of the aluminum sheet metal. The dimples are deep enough to prevent alginate from climbing up the dimple surface to an adjacent dimple. Dimples were formed by deforming 0.079 cm aluminum sheet metal between a hemispherical 0.635 cm diameter punch and a steel plate with a concentric 0.673-cm hole through a distance of 0.216 cm. Dimples were then perforated in the center with a 0.137-cm diameter needle from the inside. The perforation operation formed a drip-edge shoulder of torn aluminum approximately 0.064 cm deep. The low-area drip edge is necessary to combat the high adhesion of the alginate and to form beads that separate from the nozzle under gravity when suitably small. The device produces half-liter of alginate beads in about 5 min. Larger devises with similar nozzle design can produce larger quantities of beads.

2.4. Municipal wastewater source

Wastewater was collected periodically for every separate run of the bioreactors at the municipal wastewater treatment plant of the city of La Paz, Baja California Sur, Mexico. Samples were collected from a stream of wastewater after the initial aerobic activated sludge treatment and immediately transferred to the laboratory. Aerobic activated sludge removes organic matter but not nutrients, resulting in high levels of *N* in the wastewater. If necessary, debris in the wastewater was filtered through a gauze-cotton filter in a funnel. All wastewater were used as they arrived from the treatment plant. We stored wastewater at 4°C for several days only as a precaution following the run of the bioreactors. Analyses of the wastewater content done by the Analytical Service Unit of CIB and by the municipal wastewater treatment plant of La Paz showed that the average content of the wastewater is: (in $mg\,l^{-1}$) suspended solids, 0.978–80; BOD, 53.5–113; total nitrogen, up to 55; nitrates, 4–5.18; ammonium, 0.1–4.26; total phosphates, up to 5; orthophosphate, 4.1; NaCl 1.1; arsenic, 0.0013; cadmium, <0.005; copper, 0.018; chromium, 0.004–0.018; mercury, 0.0013; nickel, 0.031; lead, 0.064, zinc, 0.118; conductivity, $1633\,\mu S\,cm^{-1}$ and pH, 6.3–7.9. The most notable variations among samples were the presence of different nitrogen ions (ammonium or nitrate) and their concentration. Therefore, the values of the initial ion concentration are given in each figure.

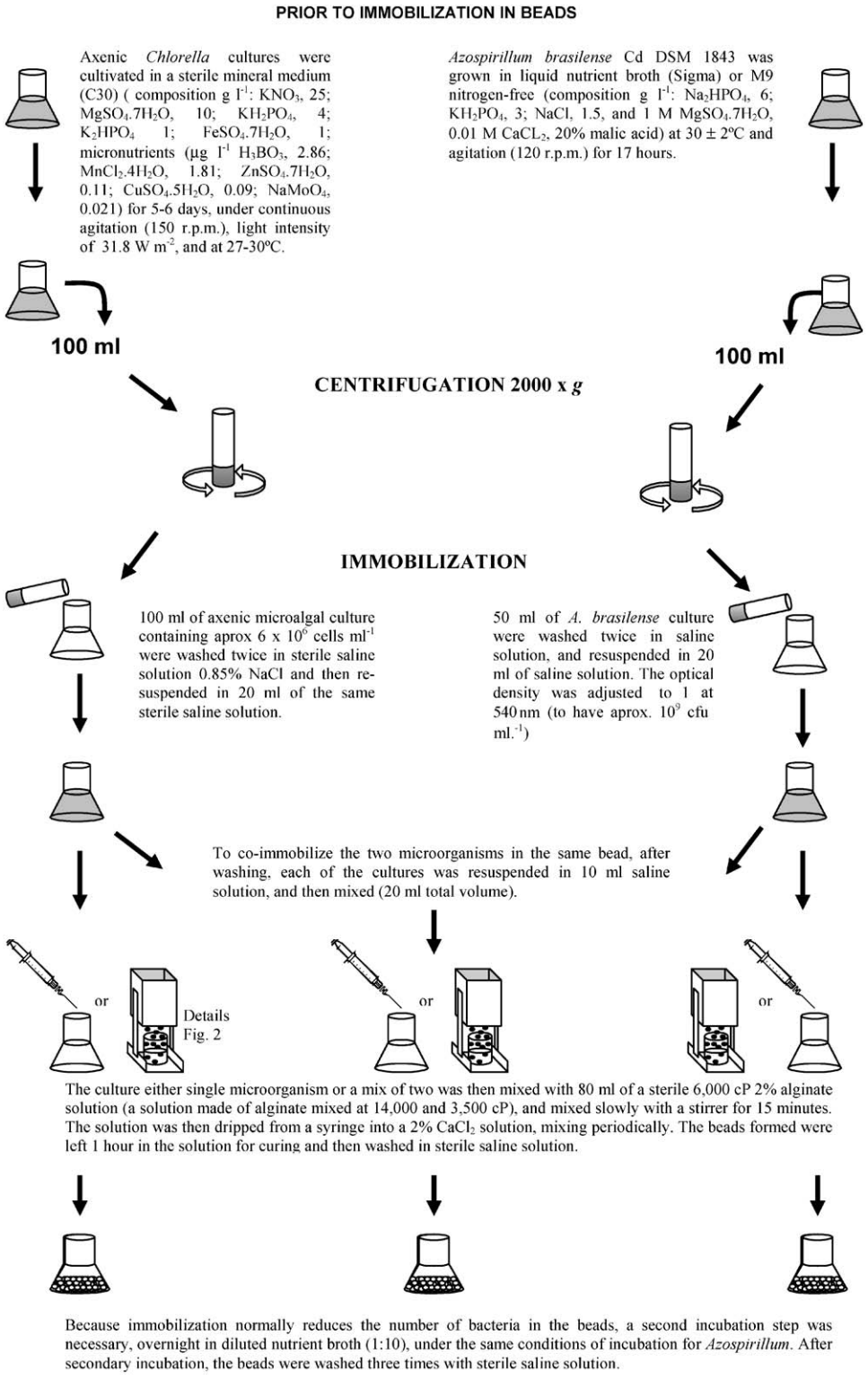

Fig. 1. Flow chart showing methods and techniques used to immobilize, co-immobilize, count, and cultivate microalgae and MGPB for wastewater treatment.

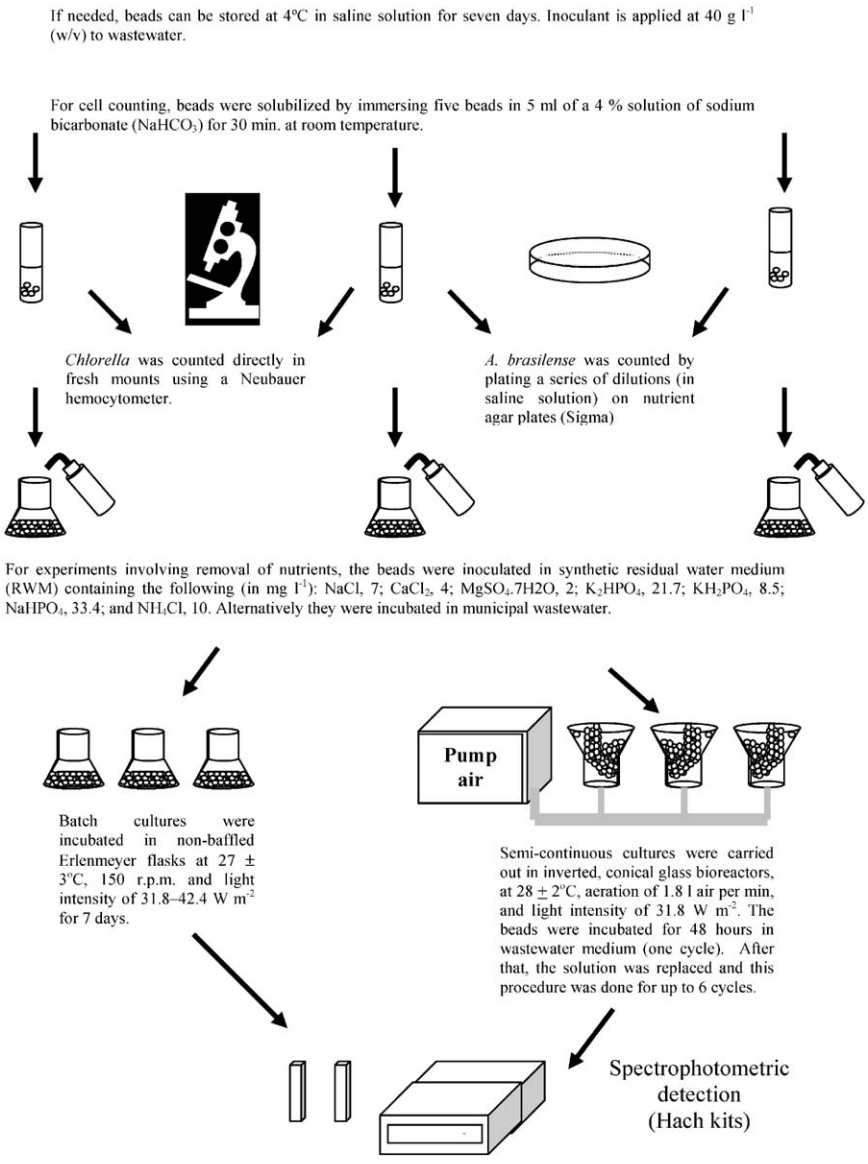

Fig. 1 (*continued*).

2.5. Water analyses of treated wastewater

Standard water analyses techniques [16] were performed with a Hach DR/2000 spectrophotometer and Hach kits (Hach CO., Loveland, CO, USA) for nitrogen and phosphorus.

2.6. Experimental design and statistical analysis

The experiments were performed in inverted, 1000-ml conical, glass bioreactors containing 600 ml wastewater, equipped with bottom aeration controlled by a peristaltic pump (1.8 l air per min) at 26 + 2°C, with constant illumination of 31.8 W m^{-2}. Each experiment was performed in triplicate, where one bioreactor served as a replicate. The setup was of semi-continuous cultures, where wastewater was replaced every 48 h, as described earlier [17]. Controls (beads without microorganisms, wastewater alone, microalgae alone, and bacteria alone) were routinely used. Three 50 ml samples were taken for each water analysis at each sampling time. Each experiment was repeated 3 times using three slightly

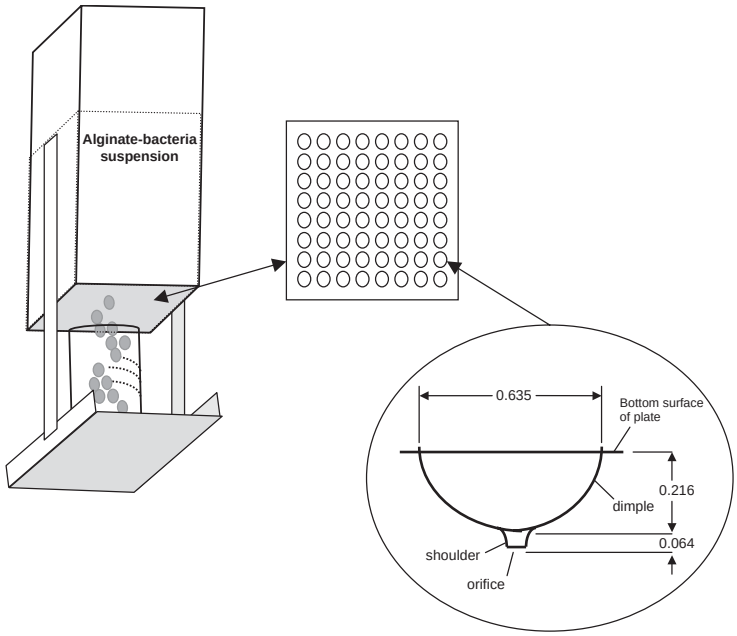

Fig. 2. Design of the alginate bead production devise.

different natural municipal wastewater samples, since we could not control the output effluent of the municipal wastewater treatment facility. Results were analyzed by ANOVA and Student's *t*-test, with significance at $P \leqslant 0.05$, using Statistica software (Statsoft, Inc. Tulsa, OK). As results were similar, only one representative experiment is presented.

3. Results and discussion

To define a practical, useful association between two microorganisms, it is essential to demonstrate that one (or both) microorganism affects the main practical function of the other. Demonstration of improved growth parameters, as was shown earlier for a bacteria–microalgae association [3,4,18,19], is insufficient to that end, since these growth promotions occurred under defined in vitro mixed cultivation. Therefore, the main purpose of this study was to show that when a microalgae is co-immobilized and co-cultured with a MGPB, with both submerged in "natural" municipal wastewater, the nutrient absorption capacity of the microalgae increases from the association, and the treated effluent wastewater is poorer in nitrogen and phosphorus.

Semi-continuous treatments performed for 3–4 cycles each, where the municipal wastewater but not co-immobilized microorganisms were replaced every 48 h, using both microalgae species. Four parameters were evaluated: growth promotion of the two microalgal species affected by the MGPB and removal of ammonium, nitrate, and phosphorus from the wastewater.

A. brasilense Cd continuously and significantly enhanced the growth of both *Chlorella* species when the co-immobilized microorganisms were grown in the wastewater at 2.1×10^6–4.8×10^6 cells ml^{-1} (*C. vulgaris*) after 4 cycles and at 2.8×10^6–4.0×10^6 cells ml^{-1} (*C. sorokiniana*) after 3 cycles (Fig. 3A and B). Addition of beads without microorganisms (control) did not affect ammonium removal (data not shown), while incubation of the non-sterile wastewater in bioreactors removed some ammonium (from 0.08 to 0.07 mg l^{-1} after 3–4 cycles). *A. brasilense* Cd alone did not remove measurable quantities of ammonium or phosphorus (data not shown). However, co-immobilization of *C. vulgaris* or *C. sorokiniana* with *A. brasilense* Cd significantly enhanced ammonium removal (Fig. 4A and B), although both *Chlorella* species were capable of eliminating most of the ammonium when immobilized alone. Removal of nitrates were tested under two conditions—(1) when the wastewater arrived from the treatment plant were loaded with nitrates, and (2) during the incubation cycles in the bioreactors when the natural resident microflora of the wastewater converted ammonium to nitrate, increasing nitrate concentration (Fig. 5E). In general, removal of high initial nitrate concentrations by co-immobilization with both microalgae species was superior to the removal of nitrate

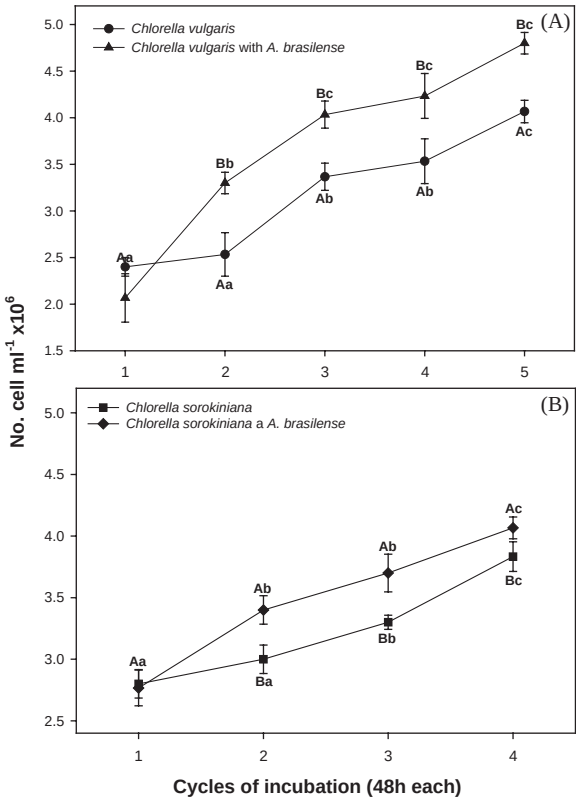

Fig. 3. Promotion of *C. vulgaris* and *C. sorokiniana* growth by *A. brasilense* Cd in municipal wastewater. Points on curves denoted by a different lower case letter differ significance by ANOVA at $P \leqslant 0.05$. Points at each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P \leqslant 0.05$. Bars represent standard error.

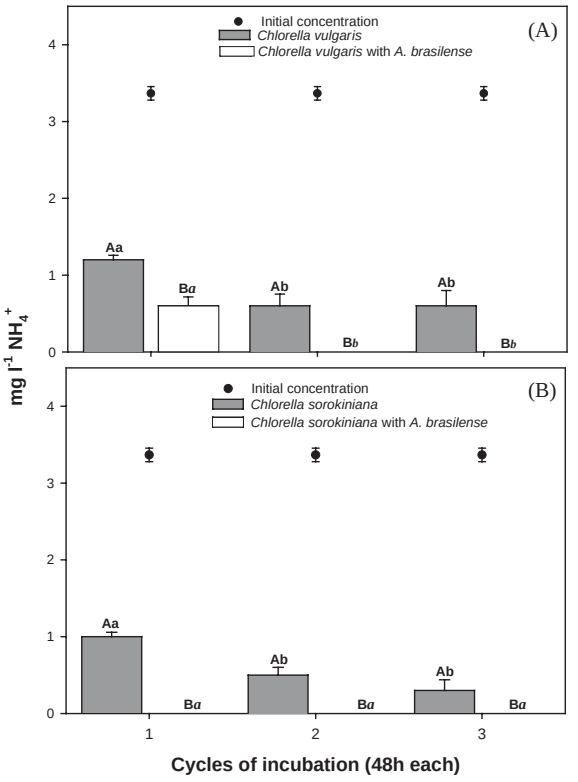

Fig. 4. Removal of ammonium ions from municipal wastewater by *Chlorella* spp. co-immobilized with *A. brasilense* Cd: (A) *C. vulgaris*; (B) *C. sorokiniana*. Columns denoted by a different lower case letter (for immobilized *Chlorella* spp.) or an italics lower case letter (for co-immobilized *Chlorella* spp. and *A. brasilense* Cd), separately, differ significantly by ANOVA at $P \leqslant 0.05$. Pairs of columns for each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P \leqslant 0.05$. Bars represent standard error.

by the microalgae alone (Fig. 5A and B). However, in the case where nitrate increased during the process, only co-immobilization with *C. sorokiniana* was more efficient than the removal by the microalgae alone (Fig. 5C and D). The control treatments (beads without microorganisms and untreated wastewater incubated similarly) did not remove any nitrate during the 3 cycles tested (Fig. 5E). Removal of phosphate from the wastewater was always better when microalgae were co-immobilized with *A. brasilense* Cd (Fig. 6A and B). Controls were incapable of removing any phosphate.

Presently, the main commercial processes for removing phosphorus from wastewater effluents are chemical precipitation with iron, alum, or lime [20,21] achieving over 95% removal, and to a lesser extent biological treatment [22]. Practical biological methods of removal are far less efficient, ranging between 20% and 30% of P with various microorganisms, while up to 90% removal with some bacterial species has been recorded in laboratory tests [23–25]. The relatively low efficiency of biological phosphorus removal, and an attempt to increase it, is the aim of the new technology proposed in this study.

The indigenous microflora of the wastewater was not analyzed in detail in this study. There were a large number of bacteria and definitely a population of nitrifying bacteria in some wastewater samples. However, the semi-continuous treatment scheme performed in this study did not yield significant removal of nutrients by the native microflora.

Removal of ammonium and phosphate from culture medium (synthetic wastewater lacking a carbon source) showed similar tendencies as in this study, regarding the interaction of *C. vulgaris* and *A. brasilense* Cd [17]. No report of nutrient removal by co-immobilized

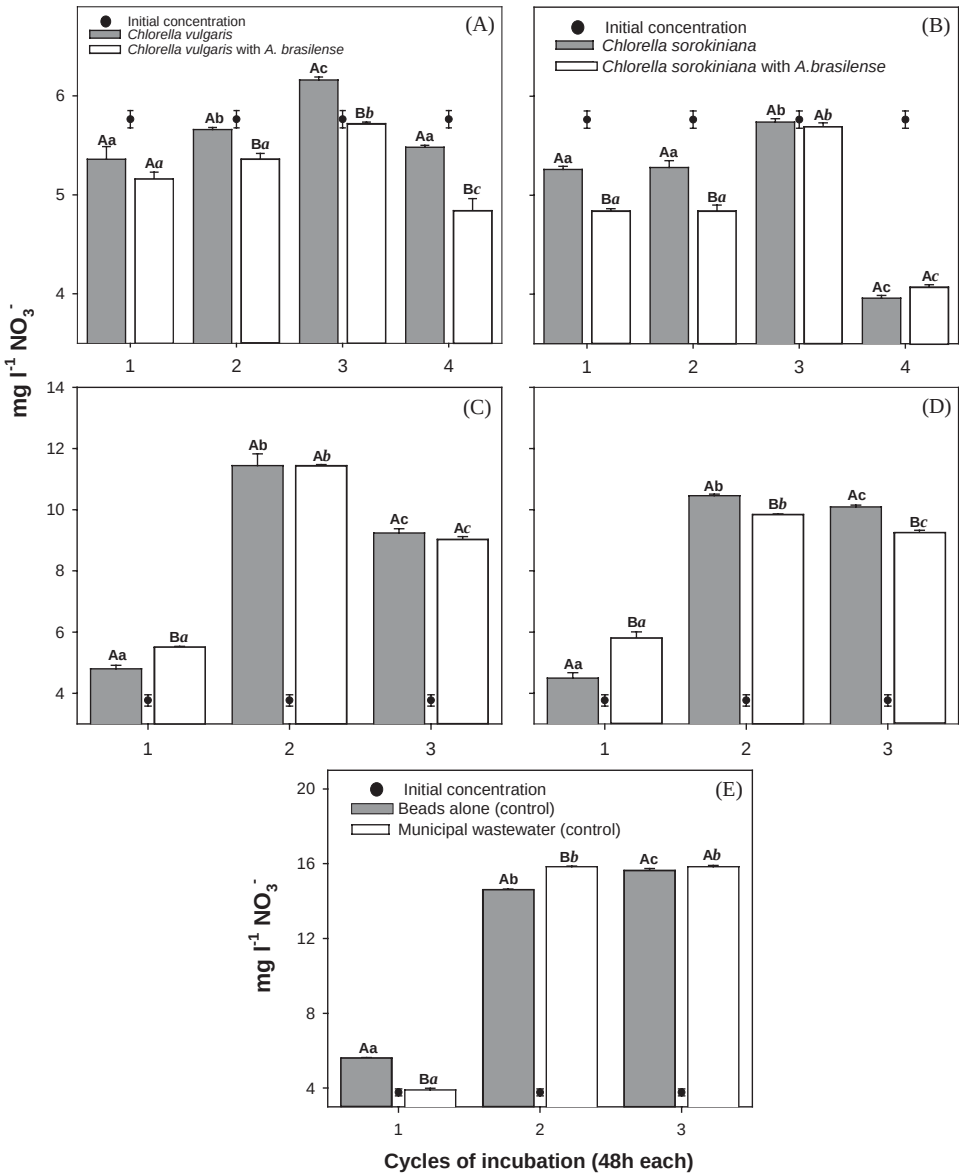

Fig. 5. Removal of nitrate ions from municipal wastewater by *Chlorella* spp. co-immobilized with *A. brasilense* Cd. Subfigures A and C represent *C. vulgaris*; Subfigures B and D represent *C. sorokiniana*. Subfigures A and B represent original high levels of nitrate; subfigures C and D represent elevation of nitrate during incubation. Subfigure E is the control without immobilized microorganisms. Columns denoted by a different lower case letter (for immobilized *Chlorella* spp.) or an italics lower case letter (for co-immobilized *Chlorella* spp. and *A. brasilense* Cd), separately, differ significantly by ANOVA at $P \leqslant 0.05$. Pairs of columns for each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P \leqslant 0.05$. Bars represent standard error.

C. sorokiniana and *A. brasilense* or with any other bacterial species is available. However, a mixed free suspended culture of *C. sorokiniana* and *Rhodobacter sphaeroides* could remove acetate, propionate, ammonia, nitrate and phosphate. When growing as a monoculture, none of the strains could accomplish this [26].

4. Conclusion

This study is the first report demonstrating that the new co-immobilization technology is capable of reducing nutrients (*N* and *P*) from regular municipal wastewater. Although the removal by the current co-

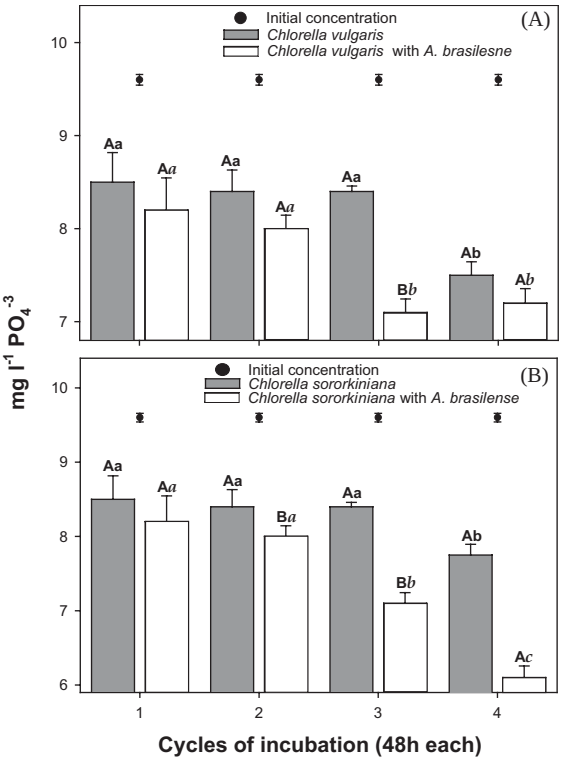

Fig. 6. Removal of phosphate ions from municipal wastewater by *Chlorella* spp. co-immobilized with *A. brasilense* Cd: (A) *C. vulgaris*; (B) *C. sorokiniana*. Columns denoted by a different lower case letter (for immobilized *Chlorella* spp.) or an italics lower case letter (for co-immobilized *Chlorella* spp. and *A. brasilense* Cd), separately, differ significantly by ANOVA at $P \leqslant 0.05$. Pairs of columns for each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P \leqslant 0.05$. Bars represent standard error.

immobilization system is still small (under $1\,mg\,l^{-1}$), it has potential in new approaches to biologically removing nitrogen and phosphorus from wastewater.

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References

[1] Bashan Y. Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv 1998;16: 729–70.

[2] Bashan Y, Holguin G. *Azospirillum*-plant relationships: environmental and physiological advances (1990–1996). Can J Microbiol 1997;43:103–21.

[3] Gonzalez LE, Bashan Y. 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 2000;6:1537–41.

[4] de-Bashan LE, Bashan Y, Moreno M, Lebsky VK, Bustillos JJ. 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 2002;48: 514–21.

[5] Gonzalez-Bashan LE, Lebsky V, Hernandez JP, Bustillos JJ, Bashan Y. Changes in the metabolism of the microalgae *Chlorella vulgaris* when coimmobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum*. Can J Microbiol 2000;46:653–9.

[6] Lebsky VK, Gonzalez-Bashan LE, Bashan Y. 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 2001;47:1–8.

[7] Tam NFY, Wong YS. Effect of immobilized microalgal bead concentration on wastewater nutrient removal. Environ Pollut 2000;107:145–51.

[8] Valderrama LT, Del Campo CM, Rodriguez CM, de-Bashan LE, Bashan Y. Treatment of recalcitrant wastewater from ethanol and citric acid production using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscula*. Water Res 2002;36:4185–92.

[9] De la Noüe J, De Pauw N. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol Adv 1988;6:725–70.

[10] Gonzalez LE, Cañizares RO, Baena S. Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. Biores Technol 1997; 60:259–62.

[11] Chevalier P, De la Noüe J. Wastewater nutrient removal with microalgae immobilized in carrageenan. Enzyme Microbiol Technol 1985;7:621–4.

[12] Lau P, Tam NFY, Wong YS. Wastewater nutrients (*N* and *P*) removal by carrageenan and alginate immobilized *Chlorella vulgaris*. Environ Technol 1997;18:893–9.

[13] Bashan Y. Alginate beads as synthetic inoculant carriers for the slow release of bacteria that affect plant growth. Appl Environ Microbiol 1986;51:1089–98.

[14] Bashan Y, Hernandez J-P, Leyva LA, Bacilio M. Alginate microbeads as inoculant carrier for plant growth-promoting bacteria. Biol Fertil Soils 2002;35:359–68.

[15] Bashan Y, Holguin G, Lifshitz R. Isolation and characterization of plant growth-promoting rhizobacteria. In: Glick

BR, Thompson JE, editors. Methods in plant molecular biology and biotechnology. Boca Raton, FL: CRC Press; 1993. p. 331–45.

[16] APHA, AWWA, WPCF (American Public Health Association, American Waterworks Association. Water Pollution Control Federation) Standard methods for the examination of water and wastewater 17th ed. Madrid: Diaz de Santos, 1992. 1105 pp.

[17] de-Bashan LE, Moreno M, Hernandez J-P, Bashan Y. Ammonium and phosphorus removal from continuous and semi-continuous cultures by the microalgae *Chlorella vulgaris* coimmobilized in alginate beads with *Azospirillum brasilense*. Water Res 2002;36:2941–8.

[18] Mouget JL, Dakhama A, Lavoie MC, De la Noüe J. Algal growth enhancement by bacteria: is consumption of photosynthetic oxygen involved? FEMS Microbiol Ecol 1995;18:35–44.

[19] Suminto, Hirayama K. Application of a growth-promoting bacteria for stable mass culture of three marine microalgae. Hydrobiologia 1997;358:223-230.

[20] Donnert D, Salecker M. Elimination of phosphorus from municipal and industrial waste water. Water Sci Techol 1999;40:195–202.

[21] Penetra RG, Reali MAP, Foresti E, Campos JR. Post-treatment of effluents from anaerobic reactor treating domestic sewage by dissolved-air flotation. Water Sci Technol 1999;40:137–43.

[22] Stratful I, Brett S, Scrimshaw MB, Lester JN. Biological phosphorus removal, its role in phosphorus recycling. Environ Technol 1999;20:681–95.

[23] Sawayama S, Rao KK, Hall DO. Nitrate and phosphate ion removal from water by *Phormidium laminosum* immobilized on hollow fibres in a photobioreactor. Appl Microbiol Biot 1998;49:463–8.

[24] Choi SS, Yoo YJ. Removal of phosphate in a sequencing batch reactor by *Staphylococcus auricularis*. Biotechnol Lett 2000;22:1549–52.

[25] Nagadomi H, Kitamura T, Watanabe M, Sasaki K. Simultaneous removal of chemical oxygen demand (COD), phosphate, nitrate and H_2S in the synthetic sewage wastewater using porous ceramic immobilized photosynthetic bacteria. Biotechnol Lett 2000;22:1369–74.

[26] Ogbonna JC, Yoshizawa H, Tanaka H. Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms. J Appl Phycol 2000; 12:277–84.