ENHANCED REMOVAL OF NITROGEN AND PHOSPHORUS FROM WASTEWATER BY THE MICROALGAE *Chlorella* sp., USING THE MICROALGAE GROWTH-PROMOTING BACTERIUM *Azospirillum brasilense*

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ABSTRACT

A combination of the freshwater 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 P and N from wastewater. This paper describes the usefulness of the approach in removing P and N from synthetic and domestic 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 synthetic and domestic wastewater. While *A. brasilense* is incapable of significant removal of N and P from the wastewater, both microalgae can. Co-immobilization of the bacterium and the microalgae was superior to removal by the microalgae alone, removing up to 100% ammonium, and 92% phosphorus within six days (varied with the source of the wastewater), compared to 75% ammonium and 89% phosphorus by the microalgae alone. Removal of ammonium from the wastewater was better with the co-immobilized microorganisms when the level of ammonium in the wastewater was increased from 3 to 7 mg/L. This study shows the potential of co-immobilization of microorganisms (microalgae and PGPB) in small beads to serve as a tertiary treatment for wastewater.

INTRODUCTION

Plant growth-promoting bacteria (PGPB), used as inoculants in agricultural experiments, are commonplace, both for control of phytopathogens and for promoting plant growth. Bacteria of the genus *Azospirillum* are well known as PGPB for numerous crop plants. Recently, it was observed that one common strain, *A. brasilense* Cd, is also capable of promoting many growth characteristics of the unicellular microalgae *Chlorella vulgaris* (Gonzalez and Bashan, 2000), and change the cytology, lipid, and pigment production by the microalgae (Gonzalez-Bashan et al., 2000; Lebsky et al., 2001; de Bashan et al., 2002a). Therefore, *A. brasilense* Cd may be considered a microalga growth-promoting bacterium (MGPB). *C. vulgaris* is commonly used for tertiary wastewater treatment (Gonzalez et al., 1997; De la Noue and De Pauw, 1988; Valderrama et al., 2002), yet it had not been demonstrated that the observed promotion of growth might also 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 (Lau et al., 1997) because it facilitates collecting an enormous population of cells developed during the treatment, which is a problem hampering regular microalgae treatments (De la Noue and De Pauw, 1988).

This study describes how co-immobilization of the two microorganisms, form an artificial biological association, enforced by close physical proximity inside small polymer beads, improves the capacity of the microalgae to remove N and P from wastewater.

MATERIALS AND METHODS

Microorganisms

Two species of unicellular microalgae *Chlorella vulgaris* Beijerinck (UTEX 2714) and *C. sorokiniana* Shih. et Krauss (UTEX 1602) were used. The microalgae growth-promoting bacterium *Azospirillum brasilense* Cd (DMS 1843) was used in co-immobilization experiments with each of the microalgae species. Co-immobilization procedures were performed as described by Gonzalez and Bashan (2000).

Wastewater sources

Synthetic wastewater was prepared as described in a previous report (Gonzalez and Bashan, 2000). 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 transported to the laboratory. If necessary, debris in the wastewater was filtered through a gauze-cotton filter in a funnel. All wastewater was used as it arrived from the treatment plant. 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: (mg/L) suspended solids, 0.978 to 80; BOD, 53.5 to 113; dissolved solids, 0.001; total nitrogen, up to 55; nitrates, 4 to 5.18; ammonium, 0.1 to 4.26; total phosphates, up to 5; orthophosphate, 4.1; NaCl 1.1; conductivity 1633 µS/cm; arsenic, 0.0013; cadmium, 0.005; cupper, 0.018; chrome, 0.004 to 0.018; mercury, 0.0013; nickel, 0.031; lead, 0.064, zinc, 0.118 and pH 6.3 to 7.9. The most notable variations observed among samples were the presence of different nitrogen ions (ammonia or nitrate) and their concentration. Standard water analyses techniques (APHA, AWWA, WPCF, 1992) were performed with a spectrophotometer (DR/2000, Hach Co., Loveland, CO, USA) and N and P test kits (Hach).

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 at 60 µmole/m^2/s. 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 (de-Bashan et al., 2002). Controls (beads without microorganisms, wastewater alone, and microalgae 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 3 slightly 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 $P \leq 0.05$, using Statistica software (Statsoft, Inc. Tulsa, OK).

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, as was shown earlier for a bacteria-microalgae association (Mouget et al., 1995; Suminto and Hirayama, 1997; Gonzalez and Bashan, 2000; de-Bashan et al., 2002a), 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 in either synthetic or "natural" municipal wastewater, the N and P absorption capacity of the microalgae increases from the association, and the treated effluent wastewater is poorer in N and P.

Semi-continuous treatments were performed for 4 to 5 cycles with both microalgae species, where wastewater, but not co-immobilized microorganisms were replaced every 48 h. Three measurements were evaluated: promotion of growth of the two microalgal species affected by the MGPB and removal of NH_4^+ and P from the wastewater.

A. brasilense Cd continuously and significantly enhanced the growth of both *Chlorella* species when the co-immobilized microorganisms were grown in wastewater at 2.1 $x10^6$ to 4.8 $x10^6$ cells/mL (*C. vulgaris*) and at 2.8 $x10^6$ to 4.0 $x10^6$ cells/mL (*C. sorokiniana*) after 5 cycles. Addition of beads without microorganisms (control) did not affect ammonium removal (data not shown), while incubation of the non-sterile wastewater in biorectors removed some ammonium (from 0.08 to 0.07 mg/L after 3 to 5 cycles). *A. brasilense* Cd alone did not remove measurable quantities of NH_4^+ or P (data not shown). However, co-immobilization of *C. vulgaris* with *A. brasilense* Cd significantly enhanced NH_4^+ removal (Fig. 1). Increasing NH_4^+ from 3 mg/L to 7 mg/L improved removal by co-immobilization (Fig 2a, b). Removal of P from wastewater was always better when microalgae were co-immobilized with *A. brasilense* Cd (Fig. 3a, b). Controls were incapable of removing any P.

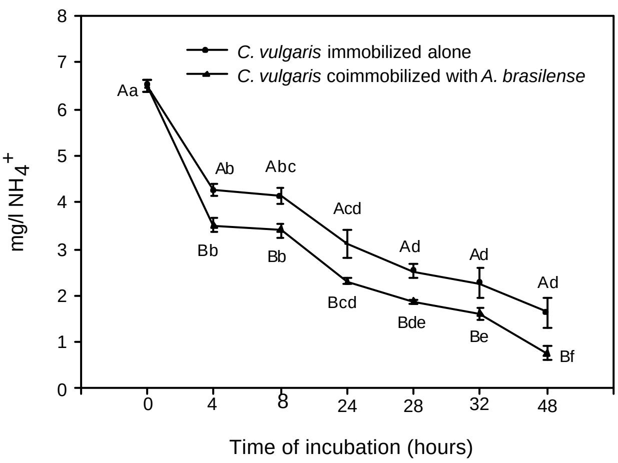

Fig. 1. Removal of ammonium ions from synthetic wastewater by *Chlorella vulgaris* co-immobilized with *A. brasilense* Cd during a 48 hours period. Points denoted by a different lower case letter, separately, differ significantly by ANOVA at $P \leq 0.05$. Points for each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P \leq 0.05$. Bars represent standard error.

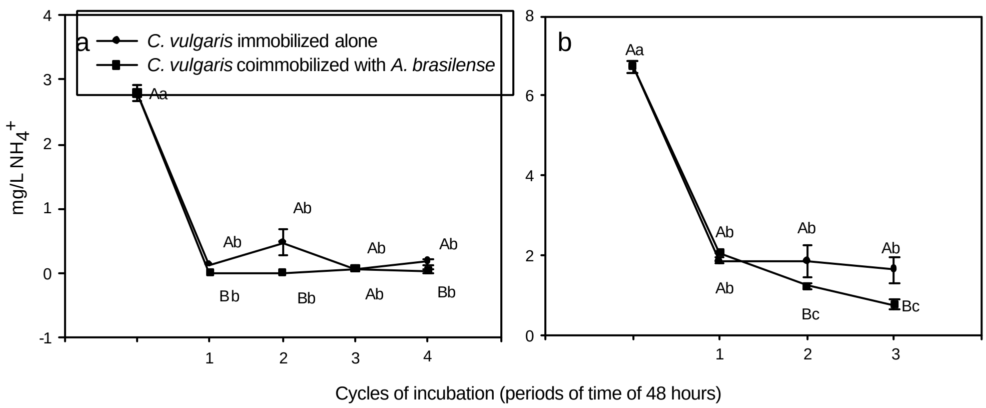

Fig. 2. Differential removal of ammonium ions from synthetic wastewater by *Chlorella vulgaris* co-immobilized with *A. brasilense* Cd, depending on the initial concentration of ammonium. a) Initial concentration of 3 mg/L NH_4^+, b) Initial concentration of 7 mg/L NH_4^+. Points denoted by a different lower case letter, separately, differ significantly by ANOVA at $P \leq 0.05$. Points for each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P \leq 0.05$. Bars represent standard error.

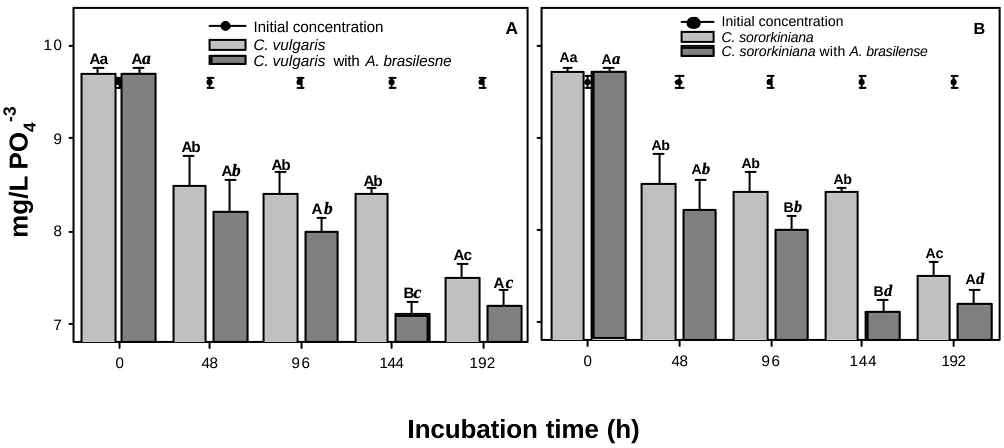

Fig. 3 Removal of phosphate ions from municipal wastewater by *Chlorella* spp. co-immobilized with *A. brasilense* Cd. a) *C. vulgaris*; b) *C. sorokiniana*. Columns (of each gray scale) denoted by a different lower case letter or italics lower case letter, separately, differ significantly by ANOVA at $P \leq 0.05$. Pairs of columns for each cycle denoted by a different capital letter differ significantly with Student's *t*-test at $P < 0.05$. Bars represent standard error.

No report on removal of nutrients from wastewater by co-immobilized *Chlorella* spp. with other bacterial species is available. This study demonstrates that new co-immobilization technology is capable of reducing nutrients from municipal wastewater and has potential for new approaches to biologically removing nitrogen and phosphorus contaminants from wastewater.

ACKNOWLEDGEMENTS

We thank Ivan Murillo and Baudilio Acosta of CIB for analyzing wastewater, and Ira Fogel at CIB modifying the English text. This study was partially supported by Secretaria de Medio Ambiente y Recursos Naturales (SEMARNAT-2002-C01-0005) and by The Bashan Foundation.

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