IMPROVED GROWTH AND WATER BIOREMEDIATION CAPACITY OF THE MICROALGA *CHLORELLA VULGARIS* WHEN COIMMOBILIZED IN ALGINATE BEADS WITH THE PLANT GROWTH-PROMOTING BACTERIUM *AZOSPIRILLUM BRASILENSE*

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Microalgae have many uses. They can serve as water bioremediation agents, as feed for aquaculture, as food for humans and animals, in pigment production, in bioremoval of heavy metals, and in agriculture (3). For any application, it is usually desirable to establish large populations of microalgae, especially in aquatic environments where they are often employed (5). One means of increasing microalgal populations may be to inoculate them with other microorganisms, a strategy that is being tested for its potential to increase yields of agriculturally important plants (2).

One candidate microorganism for coinoculation with microalgae is *Azospirillum brasilense*, a known plant growth-promoting bacterium (PGPB). In our studies, we combined *A. brasilense* with the freshwater microalga *Chlorella vulgaris* (UTEX 2714), an important organism often used in wastewater treatment, in an effort to (i) increase microalgal cell growth and mass, (ii) improve the metabolism and pigment production of the microalgae, and (iii) enhance nitrogen and phosphorus removal from wastewater. To ensure the close proximity of the two microorganisms in the liquid medium essential for *C. vulgaris* (7), they were coimmobilized in alginate beads (1) and were cocultivated under controlled conditions suitable for both, in batch cultures and in continuous flow cultures in a chemostat (6). Alginate beads of various forms and shapes are convenient inoculant carriers for use in numerous industrial, environmental, and agricultural applications (4).

Coimmobilization of the freshwater microalga *C. vulgaris* UTEX 2714 and *A. brasilense* Cd in small alginate beads resulted in significant increased growth of the microalga. Dry and fresh weight, total number of cells, size of the microalgal clusters (colonies) within the bead, number of microalgal cells per cluster, and the levels of microalgal pigments (10) significantly increased. Light and transmission electron microscopy revealed that both microorganisms colonized the same cavities inside the beads, though the microalga tended to concentrate in the more aerated periphery, while the bacterium colonized the entire bead. The effect of indole-3-acetic acid addition to the microalgal culture prior to immobilization in alginate beads partially imitated the effect of *A. brasilense* inoculation (6).

Coimmobilization of *C. vulgaris* (UTEX 395) and *C. sorokiniana* (UTEX 1602), with *A. brasilense* Cd, resulted in significant changes in microalgal cell morphology and pigment content. The size of *C. vulgaris* UTEX 2714 and *C. sorokiniana* cells did not change, however, their population within the beads significantly increased (Fig 1). In contrast, *C. vulgaris* UTEX 395 cells grew larger (up to 7 μm), but their number did not increase. Over time, the production of several microalgal pigments (Fig 2) significantly increased as a result of coimmobilization.

The ability of the coimmobilized culture to clean wastewater (to remove ammonium ions and phosphorus) was analyzed in both continuous cultures and in multistep cultures where the wastewater in which the beads were suspended was replaced every 48 hours. In continuous cultures, nitrogen removal was moderate (36%) and phosphorus removal was poor (10%). In multistep cultures, almost all of the

ammonium ions were removed within 48 hours, although the removal of phosphorus was still low. This level of removal could be sustained for six consecutive 48-hour cycles until the bioremediation system was saturated and ammonium removal efficiency slightly decreased. In comparison, when the microalga was immobilized alone, the system was saturated after 3 cycles and the level of removal was lower (Fig 3).

The effect of coimmobilization and coincubation of *C. vulgaris* with *A. brasilense* was compared with the effect of coimmobilization of the microalga with its natural associative bacterium *Phyllobacterium myrsinacearum*, obtained from a wastewater treatment pool. The interactions between the microalga and each of the bacterial species were followed by transmission electron microscopy for 10 days. Initially, most of the small cavities within the beads were colonized by microcolonies of only one microorganism regardless of the bacterial species cocultured with the microalga. The bacterial and microalgal microcolonies later merged to form large, mixed colonies within the cavities. At this stage, the effect of bacterial association with the microalga differed depending on the bacterium present. The microalga entered a senescence phase in the presence of *P. myrsinacearum*, but remained in a growth phase in the presence of *A. brasilense* (8). This study suggests that there are commensal interactions between the microalga and the two plant associative bacteria and that with time the bacterial species determines whether the outcome for the microalga is senescence or continuous multiplication.

These studies are the first reports of deliberate inoculation of *Chlorella* sp. with a terrestrial plant growth-promoting bacteria (PGPB), perhaps because of the different origins of the two microorganisms. *C. vulgaris* is not known to harbor any beneficial associative bacteria, and *Azospirillum* sp. is rarely used for inoculation in aquatic environments (9). These studies indicate that the changes induced in the unicell microalga by the plant growth-promoting bacterium may improve the potential of the microalgae as a wastewater treatment agent. We propose that coimmobilization of microalgae and plant growth-promoting bacteria is an effective means of increasing microalgal populations within confined environments and of improving its wastewater cleaning capacity.

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Fig 1. Multiplication of *C. vulgaris* (UTEX 2714) and *C. sorokiniana* (UTEX 1602) within alginate beads under batch growth conditions. Points on each curve denoted by a different capital letter at the end of each line differ significantly ($P \leq 0.05$ in Student's *t*-test). The experiment was repeated 5 times and results presented are from a representative experiment. Error bars represent standard error. Where error bars are absent, the standard error is smaller than the point.

Fig 2. Pigment production by the microalgae *C. vulgaris* (UTEX 395) and *C. sorokiniana* (UTEX 1602) after coimmobilization with *A. brasilense* in alginate beads and incubation in batch cultures for 3 days

Fig. 3. Removal of ammonium ions from wastewater by the microalga *C. vulgaris* (UTEX 2714) immobilized alone and coimmobilized with *A. brasilense* in alginate beads. Inoculated beads were incubated in multistep cultures where the wastewater in which the beads were suspended was replaced every 48 hours. Error bars represent standard error. Where error bars are absent, the standard error is smaller than the point.