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Growth promoter effect on the *Botryococcus braunii* Kutzing 1849 culture by several different bacterial strains

Efecto de promotores de crecimiento en *Botryococcus braunii* Kutzing 1849 a partir de diferentes cepas bacterianas

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Abstract

There are several strategies to improve the growth of microalgae in industrial processes. In recent years, one of them has gained strength to achieve this goal: the co-culture with bacteria. Using growth-promoting substances, producer bacteria enhance microalgae biology activity, similar to how they have been used to promote the successful production of crops. The aim of this study was to evaluate the promoter capacity of strains *Bacillus subtilis, Corynebacterium aquatile* and *Flavobacterium aquatile,* evaluate their ability to improve the growth rate of microalgae *Botryococcus braunii* and to optimize the process derived from its cultivation. This study showed that the tested bacteria were able to increase up to 1.7 times the *B. braunii* growth rate and this promoting ability remained present in cell lysate preparations from the same bacterial strains.

Key words: *Botryococcus braunii, Flavobacterium aquatile, Corynobacterium aquatile, Bacillus subtilis,* microbial elicitation, co-cultivation

Resumen

El empleo de bacterias productoras de sustancias promotoras de crecimiento, para mejorar la eficiencia en el crecimiento de las microalgas y así potenciar su actividad en procesos industriales es una práctica que ha tomado fuerza durante los últimos años, de manera análoga a como han sido utilizadas las bacterias para favorecer la producción exitosa de cultivos vegetales. El objetivo de este trabajo fue evaluar la capacidad de las cepas *Flavobacterium aquatile, Corynobacterium aquatile* y *Bacillus subtilis* de actuar como promotoras en el crecimiento de la microalga *Botryococcus braunii* con el fin de mejorar su velocidad de crecimiento y optimizar los procesos derivados de su cultivo. Este estudio muestra que las bacterias evaluadas tienen la capacidad de aumentar hasta 1,7 veces el crecimiento de *B. braunii* y esta capacidad promotora continúa presente en preparaciones de lisados celulares procedentes de estas mismas cepas bacterianas.

Palabras clave: Botryococcus braunii, Flavobacterium aquatile, Corynobacterium aquatile, Bacillus subtilis, elicitación microbiana, cocultivo

INTRODUCTION

The consortium of algae and bacteria is widely present in nature. For this reason, some researchers have undertaken the task of revealing the relationship among these microorganisms. A study conducted on 326 different species of algae revealed that about 52% of them require vitamin B12 exogenously, which seems to be provided by the bacteria in a symbiotic way (Croft et al. 2005).

Regarding *Chlorella* sp, it is known that this microalga naturally forms consortiums with a variety of bacteria belonging to the genera *Acinetobacter, Bacillus, Flavobacterium* and *Pseudomonas. Chlorella* sp. have been found in association with some fungi too, which can be

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attached through the membrane because of the presence of polysaccharides (also, this situation could be present with bacteria). Several studies have demonstrated the promotion of growth of *C. pyrenoidosa* and *C. vulgaris* by strains of bacteria such as *Brevundimonas* sp., *Leptolyngbya* sp. and *Ralstonia* sp. through the production of phytohormones, which also provide resistance to the culture, against the attack of crop contaminating fungi (Tate et al. 2013).

The strong relationship between the growth of photosynthetic organisms and bacterial presence has long been known. This phenomenon also applies to higher plants. Generally, microbial cytokinins produce pathological symptoms in higher plants (Greene 1980). An example of this is *Corynebacterium fascians*, who is responsible for fasciation disease in plants, due to the presence of cytokines ribosyl type Z (Einset and Skoog 1977). Treatment of pea seedlings with exogenous CKs showed similar symptoms as those caused by this illness. This is clear evidence of a direct action of cytokines of microbial origin on plant cells, with these substances being fully responsible for the pathogenic effects. On the other hand, it is known that with virulence generation by *Agrobacterium tumefaciens* in plant cells, transfection with oncogenes is necessary; these genes have been related to the increase in the production of plant hormones, as they induce a high expression of tryptophan monooxygenase enzymes and indole-3-acetamide hydrolase (iaaM and IAAH); both of them intervene in the conversion of tryptophan in indole acetic acid (IAA) and the condensation of adenosine into monophosphate (AMP) by catalysis of protein isopentyl transferase (ipt). AMP is an essential precursor for the production of isoprenoid Cks (Britton et al. 2008). While these two examples in higher plants refer to pathogenic associations, the knowledge about this process has been exploited to elicit growth in plants and to confer resistance to crops against various stress conditions (Mayak et al. 2004, Nie et al. 2002).

Thus, an understanding of the events present in interaction networks is necessary and useful to improve existing biotechnology processes, based on plant cell cultivation; and this background is what motivated this study. The objective was to evaluate the promoter growth factor of reported cell on *B. braunii*. In this case, *Corynobacterium aquatile* and *Flavobacterium aquatile* (Chirac et al. 1985) were tested; but also other Gram-positive *Bacillus subtilis* ubiquitous consortia were analyzed.

MATERIALS AND METHODS

The activation of the strains *C. aquatile* DSM 20146 and *F. aquatile* DSM 1132 was performed, according to

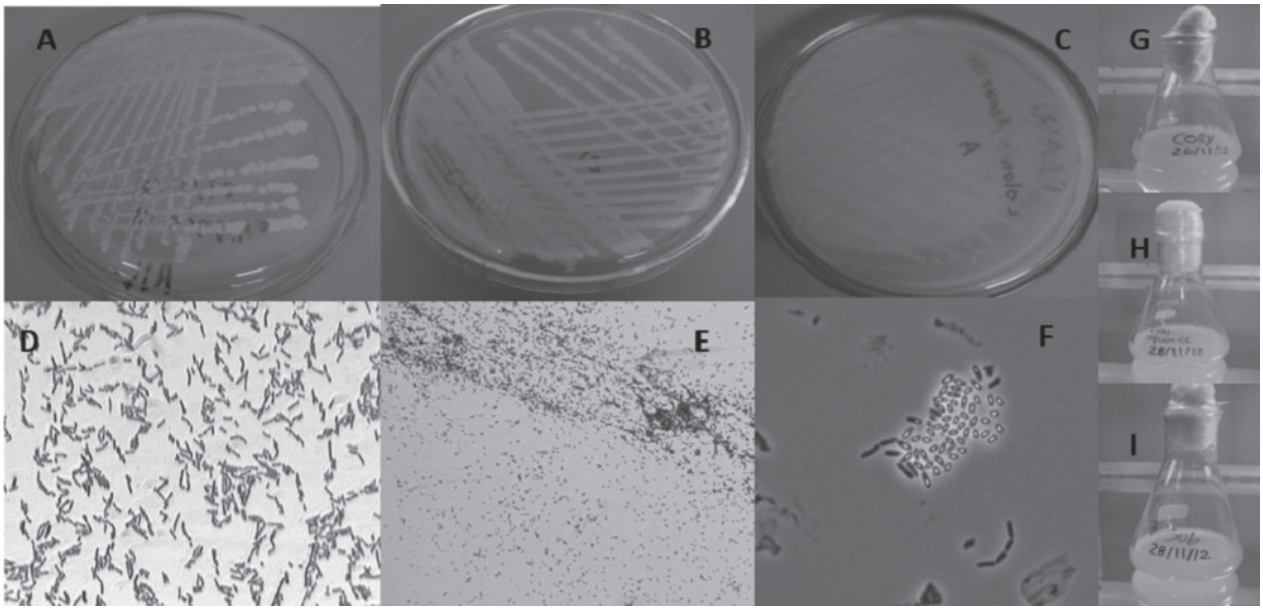

Figure 1. A- C. Photographs of the cultures of *B. subtilis*, *C. aquatile* and *F. aquatile* in solid medium, respectively**. D and E.** Microscopic photography with Gram stain of *B. subtili* and *C. aquatile* in bright field. **F**. Microscope photography with Gram stain of *F aquatile* in phase contrast for the observation of the endospore. **G- I**. Photographs of the cultures of *B. subtilis*, *C. aquatile*, *F. aquatile* in liquid medium, respectively.

the described procedure in the lyophilized vials insert. The content of the medium for activating the bacteria *F. aquatile* had a concentration of 5 g/l of KH_2PO_4, 13 g/l of K_2HPO_4, 0.2 g/l of $MgCl_2$, 2 g/l of $(NH_4)_2SO_4$ and 5 g/l of glucose. For *C. aquatile* the concentration in medium was 10 g/l of glucose, 15 g/l of peptone, 3 g/l of K_2HPO_4, 1 g/l of yeast extract, 2 g/l of NaCl and 0.2g/l of $MgSO_4 \cdot 7H_2O$. Both mediums were stabilized at pH 7 (Chohnan et al. 1997, Wada et al. 1999). The strain of *Bacillus subtilis* was already active when donated by the Biocontrol and Environmental Microbiology (BIOME) group of the University of Antioquia. The culture of *C. aquatile* and *F. aquatile* bacteria was developed by using a specific medium for their growth (Wada et al. 1999). For *Bacillus subtilis* both nutrients were used as a growth medium (figure 1). The growth curve for each bacterium was determined by tracking bacterial growth every two hours by spectrophotometric measurements at 450 nm; which was related to cell concentration expressed in terms of number of cells/mL, quantified in a Neubauer chamber, through linear regression (Bainbridge 2000).

The *B. braunii* strain UTEX LB 572 was kept active by weekly culture in 100ml Erlenmeyer flasks containing 50ml of BG11 modified medium (Rao et al. 2007). *B. braunii* cultures were performed for 16 days in the same medium; its conditions were 200 rpm orbital shaking, 17.6 µmol/m^2s intensity of light for 24 hours, room temperature of 20 °C and daily blowing by 60 seconds with flows of 70 ml/min of CO_2 to achieve 20% saturation in the atmosphere; which were the control culture conditions.

For the co-culture of *B. braunii* with three bacteria, three factors that could affect the response variable (g/l of dry biomass at the end of culture) were evaluated: 1) Viability of the bacteria, 2) Species of bacteria (*B. subtilis*, *C. aquatile* and *F. aquatile*) and 3) the presence or absence of *B. braunii* cells in the culture medium, as well as the interactions among these factors (figure 2 and 3). Co-culture assays were settled at control culture conditions; we performed four control cultures, one for each strain. It is important to highlight that the bacterial species needed two sets of culture, alive and lysed cells.

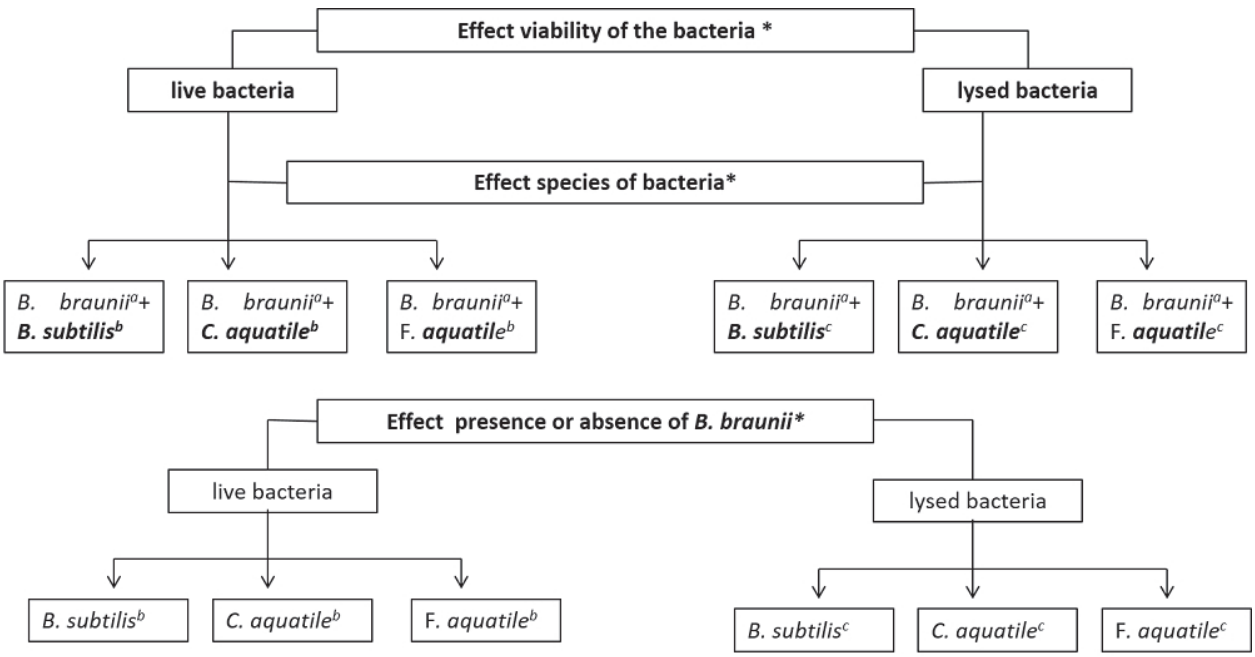

Figure 2. Schematic experimental design. for inoculation, 5 ml obtained from one-week pre-cultures were used.▫ For inoculation a corresponding volume was used, in concentrations between 1×10^1 and 1×10 cells/ml (Sakami et al. 1999, Kim, Kim, and Lee 2007). For inoculation a volume corresponding to a concentration between 1×10^1 and 1×10 cells/ml was used and taken later on to the lysis process. * All treatments were conducted in 200ml Erlenmeyer flasks containing 50ml of BG11 medium; likewise, all treatments were carried out in triplicate.

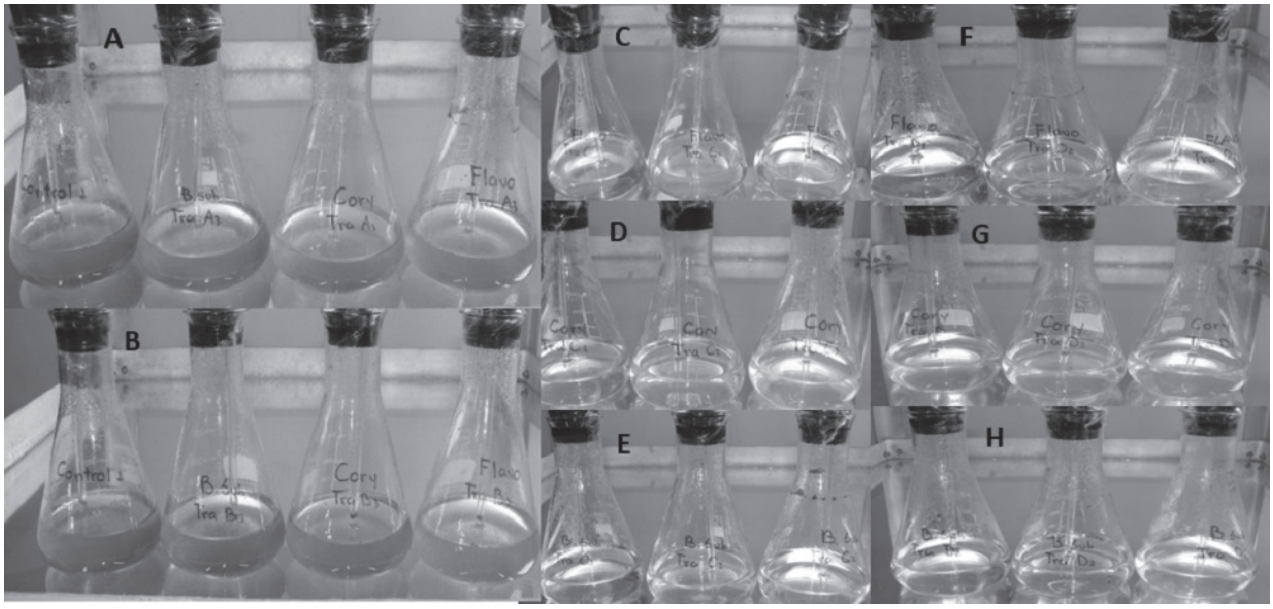

Figure 3. Photographs of culture of bacteria and *B. braunii*. **A.** Representative sample at the end of the crop control and co-culture of *B. braunii* with live cells of bacteria *B. subtilis*, *C. aquatile* and *F. aquatile* **B.** Representation at the end of control culture and co-culture of *B. braunii* with lysed cells of bacteria *B. subtilis*, *C. aquatile* and *F. aquatile*. **C-E.** Culture of live bacteria *B. subtilis*, *C. aquatile* and *F. aquatile* on BG11 medium. **F-H.** Culture of lysed bacteria *B. subtilis*, *C. aquatile* and *F. aquatile* on BG11 medium.

To test co-culture with dead bacteria it was necessary to lyse the bacterial cells, submitting them to an autoclave cycle of 121 °C, 15 psi for 20 minutes. Cell viability was verified by all bacteria cultures with a diacetate fluorescein (FDA) test (Barer and Harwood 1999). After the contact by 10 minutes of 1 mL of culture with 20 µl of 0.5% acetone FDA solution (Gaurav 2011), performance was estimated by fluorescence microscopy of cells. The absent of fluorescence in green filter meant complete cell death.

Final biomass of the cultures was collected by centrifugation at 11090 RCF (relative centrifugal force) and was dried in a convection oven. The dry weight was determined by gravimetric measurement and was expressed in terms of g/l (Dayananda et al. 2005). The increase in cell biomass was compared with the dry weight of the culture of each alive-and-lysed bacteria strain independently grown, as well as the dry weight of the culture of *B. braunii*.

The statistical analysis was performed using the program StatGraphics XVI version 16.1.17 and graphics were performed in Prism 6.0 for Windows version 6.03. In all cases the basic assumptions of the model (normality, homoscedasticity and independence of the data) were verified. To assess the statistical significance of differences between means, Multiple Range Tests were applied via the method of maximum significant difference (LSD) of Fisher; where a $p \geq 0.05$ value was considered not significant, and a value of $p < 0.05$ was considered as significant.

RESULTS

Initially, the data were analyzed by a 3×2×2 multifactorial design (The strain used: *B. subtilis*, *C. aquatile* and *F. aquatil* × intact or lysed state of bacterial cells × absent or presence of *B. braunii* cells) to determine the promoting effect of bacteria-microalgae co-culture. The response variable was the amount of biomass obtained at the end of cultivation defined in terms of dry weight (g/l). The analysis of variance conducted indicated that the type of bacterial species used and the cell state had no effect on the amount of final biomass in dry weight (g/l). The presence or absence of *B. braunii* cells in the culture medium showed an effect on the amount of final biomass obtained. There is no interaction among factors (table 1).

It was verified that the effect of bacteria was only shown in the presence of cells of *B. braunii.* Then, an analysis was conducted using a single categorical individual factor design,

with the intention of checking whether there was a statistical difference in the final biomass obtained among control cultures, containing only *B. braunii* cells and the co-cultivation with bacteria. A multiple range test showed that final culture biomass with any of the bacterial treatment was statistically superior to that obtained in the control cultures. However, there was no statistical difference on the average final biomass obtained among the different co-cultures (figure 4).

DISCUSSION

An example of artificial linkages is given between bacteria and *Chlorella* sp., which has been evaluated with the aim to improve its growth and incidentally also improve the wastewater treatment, whose process has the greatest application of the microalgae, taking advantage of nitrogen fixation properties of some bacteria. Another example is given by *C. sorokiniana* culture and *C. vulgaris* conducted in partnership with an immobilized manner *Azospirillum brasilense*, in which an increase in dry weight was found, in the number of cells, in algal cluster size and in the pigment levels (chlorophyll *a* and *b*, violaxanthin, lutein, and β-carotene), as well as an increase in the amount and the profile of produced lipid by the appearance of four new fatty acids (De-Bashan et al. 2002), demonstrating thereby that the presence of this bacterium induced significant changes in metabolism of the microalgae. This promoter

Table 1. Results of multivariate analysis of variance for number of final biomass dry weight (g/l), obtained by co-culturing *B. braunii* and the bacteria *B. subtilis*, *C.aquatile* and *F. aquatile*

Reponse Factor	*Main effects (p value)*			*Effects of interaction (p value)*		
	Factor A: Bacterial Species	Factor B: Live or lysate state	Factor C: Presence or absence of *B. braunii*	Factor A and B	Factor A and C	Factor B and C
Dry weight g/L	0,0863	0,7865	0,0000	0,9219	0,1142	0,7453

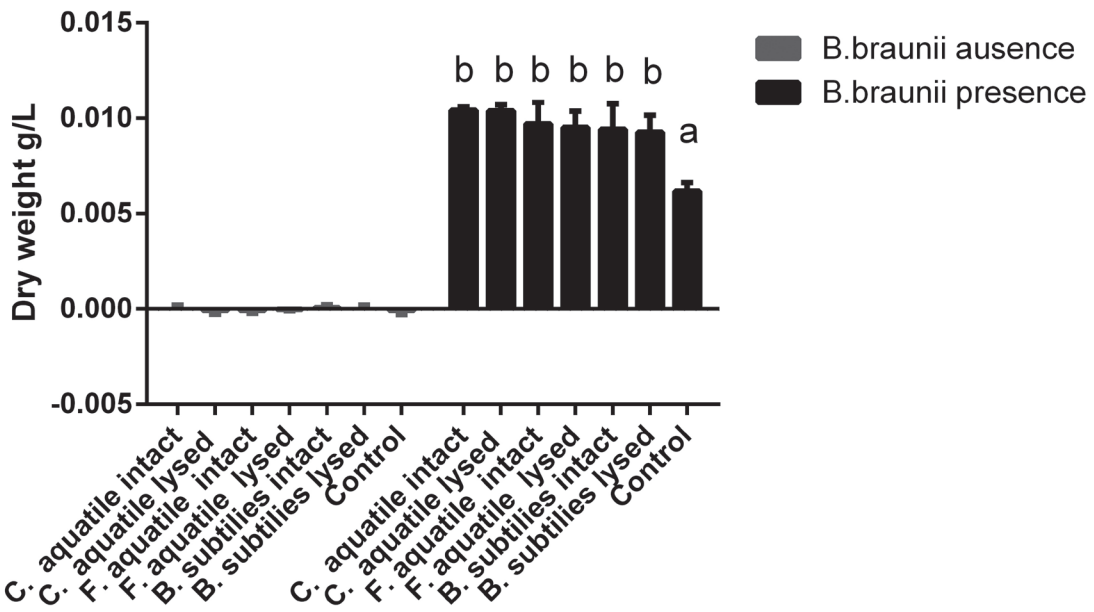

Figure 4. Behavior of the effect on the amount of biomass g/l by treatment with bacterial cells in the presence and absence of *B. braunii*. Comparison with the control culture.

activity has been explained in part by the same authors, due to production of growth promoting substances like auxin, confirmed by performing co-cultures with mutated *A. brasilense* strains deficient in the production of IAA, presenting a clear decrease in promoter activity compared to cultures performed when using the wild strain, which is restored by the exogenous supplementation of auxin (De-Bashan et al. 2008). There are also associations between *Chlorella sp*. and fungi, an example is given with *Rhodotorula glutinis*, which shows that cultures in a mixed medium of molasses not only have the ability to increase the production of lipids by the microalgae, but also the production per se of characteristic fatty acids of plants (Cheirsilp et al. 2011). The evaluations conducted in this study showed that the eliciting effect of bacteria biomass may increase up to 1.7 times the final dry weight after 15 days of culture. Contrary to reports about the formation of consortia between species of microalgae and bacteria, the eliciting effect seemed to be unrelated to the viability and the active metabolite of bacteria, because the elicitation was statistically identical whether using cells lysed by an autoclaving process or viable bacterial cells. Similarly, elicitation was not peculiar to those bacterial species closely related to the formation of "blooms" by the green microalgae, but instead also was seen with *B. subtilis* which is not related in the same way to the microalgae. This lack of specificity in growth promoting activity could be caused by the previously reported growth inducing capacity of plants by *B. subtilis* (Zablotowicz et al. 1991), achieved by various strategies, including the production of phytohormones, especially of the type auxin IAA and gibberellins as AG3 (Chowdappa et al. 2013, López-Valdez et al. 2011).

Although reports relating to *C. aquatile* and *F. aquatile* bacteria with production of phytohormones are unknown, they could produce thermo-resistant phytohormones, such as the auxin indolebutyric acid (IBA) or K, BAP cytokinins, that remain unaltered even after the autoclave lysis process (Torres et al. 2010).

We could also think that growth promoting effects shown with the use of viable or dead bacteria on the cultivation of *B. braunii* may be due to other mechanisms such as those carried out by the group of plant growth promoting rhizobacteria; within them there are representatives of the genera *Bacillus* and *Flavobacterium*; these have the capacity to solubilize phosphate and other minerals, thereby enhancing their bioavailability. Therefore, the biomass of these bacteria is used as a fertilizer in agriculture (Rodríguez and Fraga 1999, Yao et al. 2006;).

Moreover, one recent compilation (Subashchandrabose et al. 2011) revealed that mixed cultures of bacteria and algae have an optimal degradation of organic and inorganic contaminants from bacterial activity. This degradation is carried out due to the large amount of dissolved oxygen. The increasing oxygen concentration has its origin in photosynthesis realized by the algae. Both factors improve the efficiency of CO_2 fixation, keeping the optimal dissolved CO_2/O_2 ratio constant. Also, this phenomenon reduces the algae inhibition growth due to accumulation of toxic metabolites during the period of culture (Subashchandrabose et al. 2011), this could contribute to the explanation of why these bacteria promote the growth of *B. braunii*.

Additionally, some studies of *B. braunii* have revealed its photo, mixo, or/and heterotrophic capacities, growing preferably by using glucose as a carbon source (Tanoi et al. 2011, Zhang et al. 2011). The possibility of development mixo or heterotrophic processes in the assays of the present study could explain the increased algae growth by using bacterial biomass as a carbon source. However, this would imply a superior competitive capacity in the case where the co-cultures were carried out, using viable bacteria solutions. Then it would be necessary to develop studies to confirm this behavior.

CONCLUSIONS

Bacterial cells of *C. aquatile* and *F. aquatile,* do not exhibit a particular interaction when they are grown with *B. braunii*, their biomass, as that of *B. subtilis* cells, have the ability to produce a growth promoting effect on the microalgae, reflected in the increase in the dry weight obtained at the end of the culture. The growth promoting activity obtained by the bacteria on the culture of *B. braunii* indicates an interest for the evaluation of its influence and eliciting effect on the production of valuable metabolites of microalga origin. The lack of specificity of this effect is remarkable for the production of *B. braunii* biomass, converting bacterial biomass into a low-cost item to promote growth in industrial processes associated with this microorganism, which at the moment represents a better cost benefit alternative, than the use of phytohormones.

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