



Growth and metabolism enhancement in microalgae co-cultured in suspension with the bacterium *Azospirillum brasilense* under heterotrophic conditions

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Abstract

The mutualistic interactions of microalgae with other microorganisms can be altered by different culture conditions or environment factors. The mutualistic interaction of the bacterium *Azospirillum brasilense* co-cultured in suspension with the microalgae *Chlorella* sp. and *Scenedesmus* sp. under heterotrophy was evaluated in this study. The results demonstrated that the production of indole-3-acetic acid (IAA) and tryptophan (Trp) by the bacterium and microalgae, respectively, allowed maintain their affinity and mutualistic association under a heterotrophic regime. However, the glucose uptake of the consortium depends on the culture system, immobilized, or in suspension. Co-cultured in suspension with the bacterium, the biomass production of *Chlorella* sp. ($0.8 \pm 0.1 \text{ g L}^{-1}$) and *Scenedesmus* sp. ($0.9 \pm 0.1 \text{ g L}^{-1}$) and cell compound accumulation—mostly carbohydrates and proteins—were higher than when each microalga was cultured alone. Overall, these results demonstrated that, co-cultured in suspension, *A. brasilense* can be a suitable partner to *Chlorella* sp. and *Scenedesmus* sp., highlighting that the compatibility and mutualistic interaction of this consortium does not change under different culture systems and growth conditions. Also, this study expands the biotechnological potential of this consortium microalgal-*Azospirillum* as well as its incidence in different bioprocesses supported by microalgae.

Keywords *Chlorella* · Microbial consortia · Mutualism · Phytohormone · *Scenedesmus* · Tryptophan

Introduction

Microbial consortia—either natural or artificial—are considered a meaningful strategy in industrial activities, since metabolism complementation enhances the efficiency of different bioprocesses, such as bioremediation, biorefinery, and bioenergy (Yong et al. 2021). Particularly, microalgae improve their physiological performance by synergically interacting with other microorganisms such as bacteria, yeast, or another microalga (Rosero-Chasoy et al. 2021). However, they can establish distinct kinds of interactions, such as parasitism, commensalism, competition, and mutualism (Ramanan et al. 2016; Zhang et al. 2020). Mutualistic interactions are exploited in biotechnological applications to carry out complex tasks (Rosero-Chasoy et al. 2021). These mutualistic associations are supported by the affinity between the microorganisms involved through the exchange of metabolites, such as amino acids, vitamins, sugars, and hormones (Ramanan et al. 2016; Zhang et al. 2020). Nonetheless, environmental factors or culture conditions (pH,

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light intensity, temperature, nutrient availability, and growth regime) can alter the affinity of consortium and modify the type interaction established (Zhang et al. 2020). For instance, the mutualistic interaction between the microalgae *Chlorella vulgaris* and the bacterium *Pseudomonas* sp. changed to competition when the growth conditions were switched from photoautotrophic to photoheterotrophic (Guo and Tong 2013). Accordingly, it is crucial to determine the capacity of a consortium to maintain its mutualistic association under the different conditions of each biotechnological application (Zhang et al. 2020; Yong et al. 2021).

In this regard, the microalgae genera *Chlorella*, *Scenedesmus*, *Chlamydomonas*, and the cyanobacterium *Synechococcus* have established a mutualistic association co-immobilized in alginate beads with the bacterium *Azospirillum*—the most representative microalgae growth promoting bacteria (MGPB)—under different bioprocesses, such as wastewater bioremediation (Ruiz-Güerica and Sanchez-Saavedra 2016) and CO₂ fixation from biogas Barbosa-Núñez et al., 2022a, b). This mutualistic relationship is mainly sustained by metabolite exchange, meaning the microalga exudates Trp and vitamin thiamine, which are involved in IAA biosynthesis by *Azospirillum brasilense*. Subsequently, this phytohormone (IAA) is consumed by microalga, altering its metabolism (Palacios et al. 2016b) such as enhancing its nutrient uptake, high-valuable metabolite accumulation, and cell density (de-Bashan et al. 2016; Choix et al. 2018; Ramos-Ibarra et al. 2019). This microalga-*Azospirillum* consortium has mainly been evaluated when both microorganisms are forced to interact co-immobilized in alginate beads to ensure its physical contact (González-González and de-Bashan 2021). Nevertheless, the mutualistic interaction of this consortium co-cultured in suspension (without forcing them to interact) has been poorly evaluated.

On the other hand, a heterotrophic growth regime is considered an important culture strategy for enhancing the physiological performance of microalga (Perez-García et al. 2011; Carone et al. 2019). Under heterotrophy, microalgae assimilate simple organic compounds, such as glucose, acetate, or glycerol, as carbon and energy sources. Since the energy density of organic compounds (e.g. glucose, $\Delta H = 2801 \text{ kJ mol}^{-1}$) is higher than that of CO₂ ($\Delta H = 395 \text{ kJ mol}^{-1}$) some microalgae such as *Chlorella* and *Scenedesmus* record higher biomass and valuable compound production than respect to their autotrophic growth (Nirmalakhandan et al. 2019). However, the biochemical composition and biomass production vary depending on the microalga strain and carbon source used (Patel et al. 2020). Earlier, Palacios et al. (2016a) demonstrated that co-immobilized in alginate beads, *A. brasilense* enhanced the starch production of *Chlorella sorokiniana* cultured under heterotrophy using

D-glucose as a carbon source. Notwithstanding that the use of alginate beads during microalga–bacteria interactions is proposed to facilitate the recovery of microalgal biomass after wastewater bioremediation (Covarrubias et al. 2012), suspended microalgae cultures show different physicochemical properties regarding immobilized microalgae cultures owing to the different growing environments (Zhuang et al. 2020). Thus, the mutualistic interaction of *A. brasilense* on microalgae co-cultured in suspension under a heterotrophic regime should be evaluated to determine its robustness and to expand the biotechnological potential of this consortium.

Considering the above, our work hypothesis was that *A. brasilense* could maintain its mutualistic interaction with microalgae independently of the culture system (immobilized or in suspension) under a heterotrophic regime. Thus, our aims were to evaluate the effect of *A. brasilense* co-cultured in suspension and co-immobilized in alginate beads on physiological performance—glucose uptake, biomass production, and high-valuable metabolite production—of two microalgae, *Chlorella* sp. and *Scenedesmus* sp. Moreover, the signal molecule production, IAA and Trp, by the bacterium and both microalgae under heterotrophy was also evaluated.

Materials and methods

Microorganisms and culture conditions

The green microalgae *Chlorella* sp. and *Scenedesmus* sp. isolated from Lago de Chapala (Jalisco, Mexico; 20° 15' 27" N, 103° 02' 33" W) according to the methodology described by Smith et al. (1996) and the bacterium *Azospirillum brasilense* Cd (ATCC 29,710) were used in this study. Both microalgae were maintained in medium C30 + M (Choix et al. 2017) at $28 \pm 2 \text{ }^\circ\text{C}$, $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and stirred at 140 rpm for eight days. The bacterium *A. brasilense* was maintained in medium BTB-2 (de-Bashan et al. 2011); pH was adjusted to 7 with 1 M KOH, incubated at $30 \pm 2 \text{ }^\circ\text{C}$ and stirred at 140 rpm for 24 h.

Microalga–bacteria association in suspension and co-immobilized

To associate microorganisms under suspension culture, 10 mL of *A. brasilense* with a concentration of $1 \times 10^9 \text{ CFU mL}^{-1}$ were added to 100 mL of each microalga culture (*Chlorella* sp. and *Scenedesmus* sp.) with a cell density of $1.5 \times 10^6 \text{ cells mL}^{-1}$ (0.35 g L^{-1}). To induce co-immobilization conditions, microorganisms were immobilized using the procedure established by de-Bashan et al. (2004). Briefly, the same volume and concentration aforementioned for each microalga culture

was centrifuged at $6800 \times g$ for 5 min, and the pellet was washed thrice with 20 mL of 0.85% sterile saline solution. To immobilize the microorganisms, each pellet was resuspended in 20 mL of saline solution and mixed individually with 80 mL of 2% alginate solution (#180,947, Sigma, U.S.A.). To co-immobilize the microorganisms (one microalga and *A. brasilense*) in the same bead, each pellet was resuspended in 10 mL of saline solution and then mixed in the alginate solution. The solution was dripped from a sterile syringe into 3% CaCl_2 solution using a peristaltic pump. Since the immobilization process reduces the population of *A. brasilense* in the beads, a second incubation in dilute nutrient broth 10% v/v (#70,122, Sigma, U.S.A.) incubated at 28 ± 2 °C, 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and stirred at 120 rpm for 24 h was carried out.

Experimental growth conditions

Under co-immobilization, 10 g beads with immobilized or co-immobilized microorganisms were inoculated in 100 mL of C30 + M medium employing a flask of 250 mL. In suspension, each microorganism alone and both microalga-bacterium cultures were also maintained in a 250 mL flask with a working volume of 110 mL of C30 + M culture medium. In both conditions, 5 g L^{-1} of D-glucose was added to each culture, incubated in complete darkness at 28 °C, and stirred at 140 rpm for six days.

Counting microorganisms

Microalgal cell density was determined each two days by cell count with a Neubauer hemocytometer. In co-immobilized culture, three beads per treatment were taken and dissolved in one mL of 2% NaHCO_3 solution at room temperature; whilst under suspension samples of one mL were directly taken of culture. Growth rate (μ) was calculated during the exponential growth phase by the formula:

$$\mu = (\ln N_1 - \ln N_0) / (t_1 - t_0) \quad (1)$$

where N is the number of cells at initial (t_0) and final (t_1) sampling time. Biomass production (X ; g L^{-1}) was measured by dry weight at the beginning and end of experimental time (6 days); 20 mL of suspension culture were centrifuged at $6800 \times g$ for 10 min; or 10 g of beads were dissolved and centrifuged as aforementioned. Then the pellet was washed thrice with 20 mL of distilled water and dried at 80 °C for 24 h. Biomass productivity (P ; $\text{g L}^{-1} \text{ day}^{-1}$); was calculated according to Eq. 2:

$$P = (X_f - X_i) / (t_f - t_i) \quad (2)$$

where X is biomass concentration at the beginning (t_i) and end (t_f) of experimental time. The bacterium *A. brasilense* was counted after serial dilution by the plate count method on Congo Red solid medium (Puente et al. 2020).

Microalgal biomass characterization

At the end of experimental time, the biochemical characterization of dry microalgal biomass (carbohydrates, proteins, and lipids) was carried out by Fourier transform infrared spectroscopy (FTIR). Cultured in suspension, 50 mL of each microalga culture alone or associated with the bacterium was centrifuged at $6800 \times g$ for 10 min; or 5 g of alginate beads of each microalga immobilized alone or co-immobilized with *A. brasilense* were washed twice with distilled water and dried at 80 °C for 12 h. FTIR spectra from dry biomass were collected using FTIR spectrometer CARY 630 (Agilent, U.S.A.) equipped with an attenuated total reflection (ATR) accessory; 20 scans per sample were carried out with a spectrum range from 4000 to 650 cm^{-1} at a spectral resolution of four cm^{-1} . FTIR spectra were recorded in transmittance units (a.u) versus wavenumber (cm^{-1}), and data were assessed with Resolution-pro software (Agilent). Subsequently, quantitative determination of carbohydrates, lipid and protein content was carried out by the phenol–sulfuric (Dubois et al. 1956) and Lowry (Lowry et al. 1951) methods, respectively. Lipids were extracted by the procedure established by Bligh and Dyer (1959) and quantified by gravimetry. Proteins, lipids and carbohydrate productivity ($\text{mg L}^{-1} \text{ day}^{-1}$) were determined according to Guldhe et al. (2017).

$$\text{Protein productivity} = \text{Biomass productivity} * \frac{\text{Protein content}}{100} \quad (3)$$

$$\text{Carbohydrate productivity} = \text{Biomass productivity} * \frac{\text{Carbohydrate content}}{100} \quad (4)$$

$$\text{Lipid productivity} = \text{Biomass productivity} * \frac{\text{Lipid content}}{100} \quad (5)$$

where, biomass productivity is in $\text{mg L}^{-1} \text{ day}^{-1}$ and protein, lipid and carbohydrate content in percentage per dry biomass weight.

Quantification of tryptophan and indole-3-acetic acid

Samples of one mL of culture medium from each treatment were taken each two days and filtered through a 0.22 μm membrane (#GSWP01300; Millipore, USA) and analyzed by HPLC (Waters Alliance e2695, USA) according to Palacios et al. (2016b). The HPLC-system was equipped with a reversed phase column TSKgelTM ODS-120A,

5 μm particle size, 150 \times 46 mm (Supelco, USA) and was run isocratically using methanol:water:acetic acid (36:64:1 v/v) as the mobile phase. The injection volume was 100 μL and the flow rate 0.5 mL min. The wavelength used for detection was 290 nm. The standards were tryptophan (#T0254, Sigma) and indole-3-acetic acid (#I3750, Sigma).

Determination of glucose uptake by microalgae

At the end of the experimental time, 30 mL of culture medium of each treatment were centrifuged at $6800 \times g$ and filtered through a 0.22- μm membrane filter (GSWP02500 EMD Millipore). The filtered samples were concentrated at 60 $^{\circ}\text{C}$ for 24 h, and subsequently, the total carbohydrates were quantified by spectrometry using the phenol–sulfuric method adapted for microplates using D-glucose as a standard (Choix et al. 2012). The percentage of glucose uptake by microalgae was determined with respect to 5 g L^{-1} of D-glucose added to each microalga culture.

Identification of residual glucose in culture medium by nuclear magnetic resonance (NMR)

At the end of the experimental time, 30 mL of culture medium of each treatment were centrifuged at $6800 \times g$. Then, 0.540 μL of supernatant (cell free) were filtered using an NMR tube adding 60 μL of a solution containing 10 mM TMSP- d_4 (3-(trimethylsilyl) propionic-2,2,3,3- d_4 acid sodium salt, Sigma-Aldrich) as an internal reference and 20 mM NaN_3 (Sigma-Aldrich) in D_2O . Similarly, 30 mL of sterile culture medium was used as a control. Then, each sample was analyzed using the WATERSUP program with the pulse sequence *noesygppr1d* using 16 scans and $\text{D1} = 10$. All spectra were processed using the MestReNova 12.0 program.

Visualization by scanning electron microscopy (SEM)

At the end of experimentation time (six days), 20 mL of each culture in suspension were centrifuged at $6800 \times g$ for 5 min; the pellet was washed twice with distilled water and then lyophilized. In immobilized cultures, 5 g of spheres were washed twice with distilled water, lyophilized, and subsequently pulverized in a mortar and pestle. The samples were visualized in a high resolution (1 nm for high vacuum) scanning electron microscope: TESCAN-MIRA 3 LMU (Czech Republic). Each sample was exposed to gold for 30 s with a power of 10 kV and then analyzed with a working plan of 15 mm and magnifications of 5.000–20.000X.

Experimental designs and statistical analyses

To evaluate the effect of *A. brasilense* on microalgae cultured in suspension, the setup of experiments was a separate culture of *Chlorella* sp., *Scenedesmus* sp., and the bacterium *A. brasilense* (controls), as well as microalga–bacteria associations *Chlorella* sp.–*A. brasilense* and *Scenedesmus* sp.–*A. brasilense* (treatments). To compare the positive effect in microalgae co-immobilized in alginate beads with the bacterium, beads with microalgae (*Chlorella* sp., *Scenedesmus* sp.) and the bacterium (*A. brasilense*) immobilized alone and beads with each microalga co-immobilized with the bacterium, *Chlorella* sp.–*A. brasilense* and *Scenedesmus* sp.–*A. brasilense* were also used as controls. Each experiment was carried out in triplicate and repeated three times ($n = 9$). The data from each treatment from the two repetitions were combined for analysis. For data of growth, the homoscedasticity of the data was tested and then analyzed first by one-way ANOVA and then by least significant difference (LSD) post-hoc analysis, with significance set at $P < 0.05$. Data on glucose uptake were analyzed by a T-student test for independent samples, with a significance set at $P < 0.05$. The statistical analyses were performed using Statistica 6.0 software (StatSoft, USA).

Results

Physical interaction of the *Azospirillum*–microalga consortium

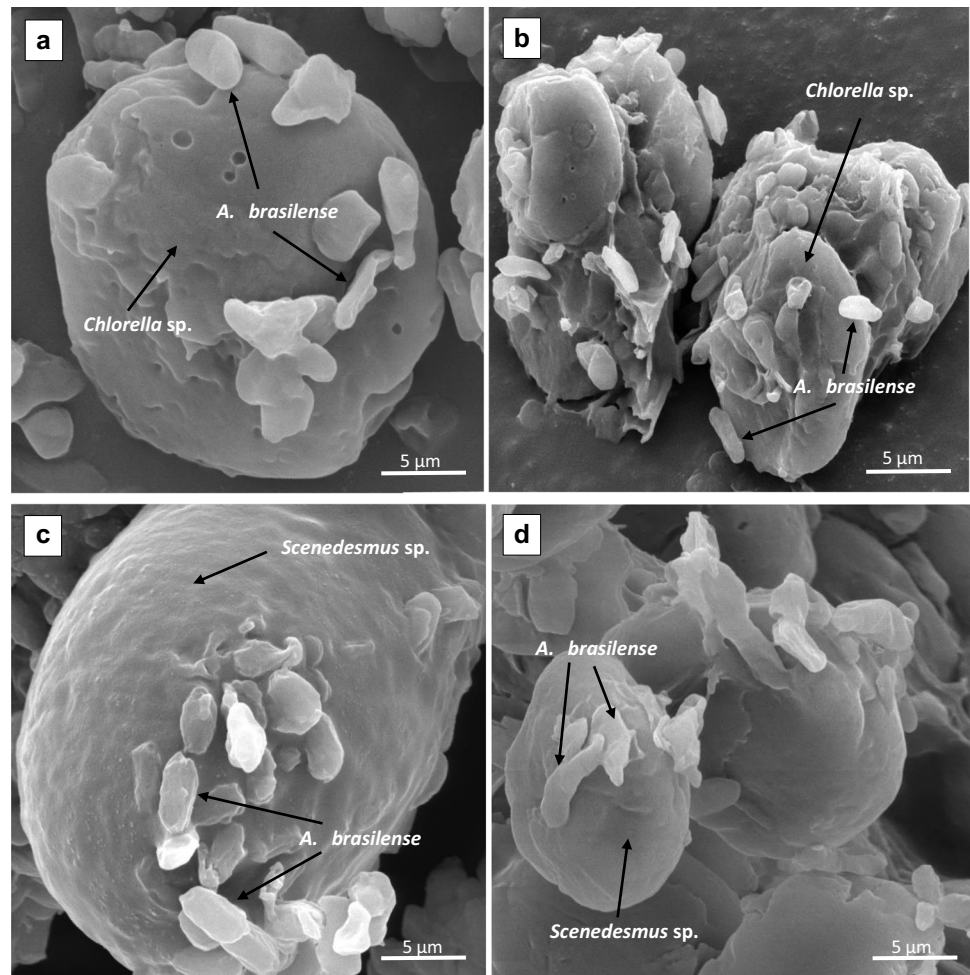
At the end of experimental time the bacterium *A. brasilense* showed physical attachment in the phycosphere of *Chlorella* sp. and *Scenedesmus* sp. growing under a heterotrophic regime, either co-immobilized in alginate beads or co-cultured in suspension (Fig. 1).

Tryptophan production by microalgae

Immobilized alone in alginate beads *Chlorella* sp. and *Scenedesmus* sp. produced Trp during all experimental times, attaining the highest production at six days, 29.1 ± 5.3 and 25.7 ± 4.6 $\mu\text{g mL}^{-1}$, respectively; although there were no significant differences with respect to four days (Fig. 2a, capital letters analysis). Meanwhile, the production of this metabolite when each microalga was co-immobilized with *A. brasilense* was not detected at any interval time, showing significant differences compared to cultured alone (Fig. 2a, lowercase letters analysis).

Cultured in suspension, the highest production of Trp was 43.4 ± 5.9 $\mu\text{g mL}^{-1}$ (*Chlorella* sp.) and 34.0 ± 2.2 $\mu\text{g mL}^{-1}$ (*Scenedesmus* sp.) at 6 days (Fig. 2b, capital letters analysis). Whilst 12.8 ± 6.4 $\mu\text{g mL}^{-1}$ and

Fig. 1 Photograph by scanning electron microscope of consortium *Chlorella* sp.-*A. brasilense* and *Scenedesmus* sp.-*A. brasilense* co-cultured in suspension (**a, b**) and co-immobilized in alginate beads (**c, d**) after six days of culture under heterotrophic regime



$11.7 \pm 2.2 \mu\text{g mL}^{-1}$, respectively, were quantified at six days when each microalga was co-cultured with the bacterium; in both microalgae, the Trp production showed significant differences when cultured alone than co-cultured with the bacterium in each interval time (Fig. 2b, lowercase letters analysis).

Indole-3-acetic acid production by *A. brasilense*

The highest IAA production by *A. brasilense* immobilized alone was $17.4 \pm 6.4 \mu\text{g mL}^{-1}$ at 6 days (Fig. 2c, capital letters analysis). While cultured in suspension, the highest production was $31.6 \pm 3.2 \mu\text{g mL}^{-1}$ at four days and decreased at six days, but there were no significant differences between the intervals (Fig. 2d, capital letters analysis). In contrast, this compound was not detected at any interval when this bacterium interacted with each microalga, either co-immobilized in alginate beads or co-cultured in suspension. Thus, the IAA production by this bacterium showed significant differences when it was cultured alone or associated with each microalga in both culture systems (Fig. 2c and d, lowercase letters analysis).

Likewise, the cell density (Log CFU mL^{-1}) of *A. brasilense* in both culture systems was significantly similar when it was grown alone or associated with each microalga (Table 1).

Cell density and biomass production by microalgae

At the end of experimental time (six days), the cell density of *Chlorella* sp. ($2.1 \times 10^6 \pm 0.2 \text{ cells mL}^{-1}$) and *Scenedesmus* sp. ($3.0 \times 10^6 \pm 0.3 \text{ cells mL}^{-1}$) interacting co-immobilized in alginate beads with the bacterium was significantly higher than when immobilized alone (Fig. 3a, lowercase analysis). A similar pattern was found when each microalga was co-cultured in suspension with *A. brasilense*. The population reached $3.2 \times 10^6 \pm 0.2$ (*Chlorella* sp.) and $3.4 \times 10^6 \pm 0.2 \text{ cells mL}^{-1}$ (*Scenedesmus* sp.), respectively, showing significant differences compared to when were growing alone (Fig. 3a, b lowercase analysis).

Likewise, the biomass production of each microalga associated with the bacterium, either co-immobilized or in suspension, was higher than that cultured alone. Interacting co-immobilized with *A. brasilense*, *Chlorella* sp., and

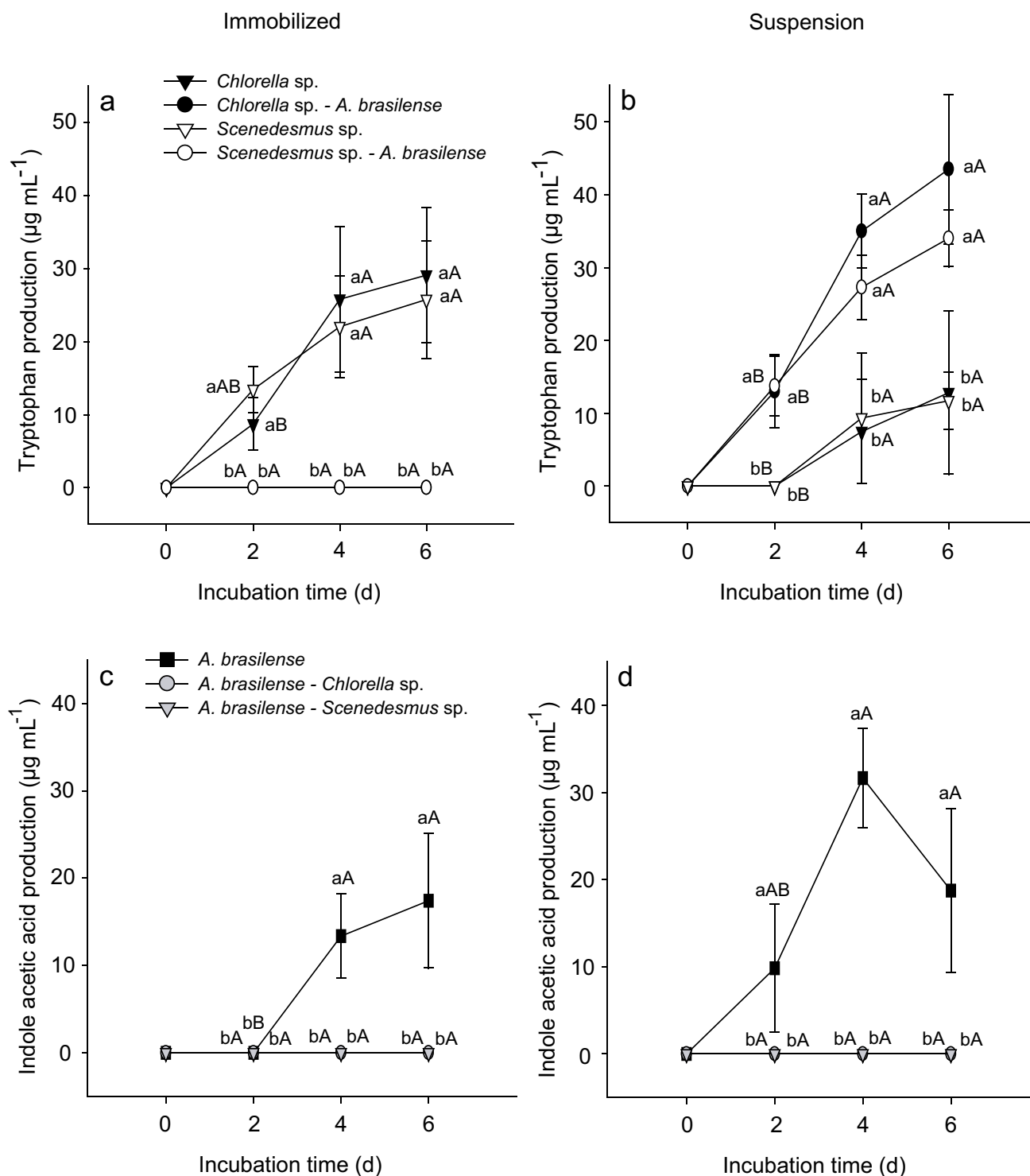


Fig. 2 Tryptophan production of microalgae (a, b) and indole-3-acetic acid production of *A. brasilense* (c, d) under heterotrophy. Points at each time interval denoted by different lowercase letters differ significantly when each microalga was growing alone or associated with the bacterium ($n=9$). Values denoted by different capital letters dif-

fer significantly in the different time intervals in the same treatment (microalga alone or associated with the bacterium) ($n=9$). Statistical analyses were performed using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) post hoc analysis at $P < 0.05$. Bars represent standard error

Table 1 Cell density of *A. brasilense* growing alone or co-immobilized in alginate beads or co-cultured in suspension with *Scenedesmus* sp. or *Chlorella* sp

Condition	Bacterium-microalga	Log FCU mL ⁻¹
Immobilized	<i>A. brasilense</i>	5.90a
	<i>A. brasilense</i> — <i>Chlorella</i>	5.45a
	<i>A. brasilense</i> — <i>Scenedesmus</i>	4.50a
Suspension	<i>A. brasilense</i>	6.72a
	<i>A. brasilense</i> — <i>Chlorella</i>	5.15a
	<i>A. brasilense</i> — <i>Scenedesmus</i>	4.86a

Values denoted by different lowercase letters differ significantly when *A. brasilense* was growing alone or associated with each microalga co-immobilized in alginate beads or co-cultured in suspension. Statistical analyses were performed using ANOVA and LSD post hoc analysis at $P < 0.05$. \pm represents standard error ($n = 9$)

Scenedesmus sp. recorded 0.7 ± 0.1 and 0.6 ± 0.1 g L⁻¹, respectively, although only the former showed a significant difference (Fig. 3c, lowercase analysis). Meanwhile, co-cultured in suspension with the bacterium, the biomass production of *Chlorella* sp. was 0.8 ± 0.1 g L⁻¹, whilst *Scenedesmus* sp. attained 0.9 ± 0.1 g L⁻¹ being significantly higher than when cultured alone (Fig. 3d, lowercase analysis).

Similarly, the highest growth rates and biomass productivity were also recorded when each microalga interacted with the bacterium rather than growing alone, either co-cultured in suspension or co-immobilized in alginate beads (Table 2).

Qualitative and quantitative microalgae biomass characterization

At the end of experimental time (six days), the qualitative *Chlorella* sp. biomass characterization by FTIR showed that the protein and carbohydrate content was similar when this microalga was immobilized alone or co-immobilized with *A. brasilense* since the high of peaks shown at 1645 and 1530 cm⁻¹ belonged to vibrations of C=O and N–H bonds of amide II and I, respectively, associated to proteins (Pistorius et al. 2009), as well as at 1020 cm⁻¹ attributed to vibration of C–O–C bond related with carbohydrates (Suart 2004) were similar in both treatments (Fig. 4a). However, *Scenedesmus* sp. co-immobilized with the bacterium only showed an increase in the high peaks of carbohydrates (Fig. 4c). In contrast, when both microalgae were associated with the bacterium co-cultured suspension, the high peaks of the two cell components were clearly higher than when they were cultured alone (Fig. 4b, d). Nonetheless, it is worth noting that under our experimental conditions, peaks corresponding to lipids were not detected.

Similarly, the quantitative characterization of *Chlorella* sp. and *Scenedesmus* sp. co-immobilized with the bacterium recorded $17.0 \pm 2.9\%$ and $24.6 \pm 3.4\%$ carbohydrates, respectively (Fig. 5a). While co-cultured in suspension with

A. brasilense, *Chlorella* sp. reached a carbohydrate content of $25.6 \pm 2.6\%$, and *Scenedesmus* sp. attained $30.1 \pm 3.1\%$ (Fig. 5b). In both culture systems, the accumulation of this compound was significantly higher when both microalgae interacted with the bacterium than when they grew alone (Fig. 5a, b lowercase analysis).

Similarly, the protein content of *Chlorella* sp. and *Scenedesmus* sp. co-immobilized with *A. brasilense* reached 23.5 ± 4.2 and $27.4 \pm 1.7\%$, respectively, showing significant differences when growing alone. Meanwhile, co-cultured in suspension, the protein content reached by *Chlorella* sp. ($30.6 \pm 2.9\%$) and *Scenedesmus* sp. ($33.4 \pm 2.1\%$) also showed significant differences when associated with the bacterium compared to growing alone (Fig. 5c, d lowercase analysis).

On the other hand, in both culture systems, carbohydrate, and protein productivities were also significantly higher when each microalga interacted with the bacterium than when growing alone (Table 3).

Glucose uptake by microalgae

Immobilized in alginate beads, *Chlorella* sp. cultured alone recorded a glucose uptake of $14.05 \pm 3.52\%$, while co-immobilized with the bacterium attained $30.91 \pm 6.24\%$ (Fig. 6a).

Meanwhile, *Scenedesmus* sp. only recorded glucose uptake when co-immobilized with *A. brasilense*, consuming $14.52 \pm 2.80\%$ and showing significant differences compared to when immobilized alone (Fig. 6a). Similarly cultured in suspension, the glucose uptake of *Chlorella* sp. growing alone ($28.77 \pm 2.98\%$) was significantly similar when co-cultured with *A. brasilense* ($24.15 \pm 1.60\%$). In contrast, the glucose uptake by *Scenedesmus* sp. growing alone in suspension was $43.46 \pm 1.04\%$, which was statistically higher than when it was co-cultured with the bacterium, $12.41 \pm 1.27\%$ (Fig. 6b). In both culture systems, *A. brasilense* growing alone did not consume glucose (Table S1).

NMR analyses of residual culture medium

To confirm that carbohydrates analyzed spectrophotometrically correspond to residual glucose, the culture medium analysis (cell free) of each microalga either alone or associated with the bacterium was analyzed by NMR for each treatment (Fig. 7). At the end of the experimental time (six days), independent of the culture system (immobilized or suspension), the spectra of the residual culture media showed the absence of the characteristic signals for the D-glucose anomeric hydrogens, which were observed in the glucose standard at 5.24 ppm (d, $J = 3.8$ Hz) for H_α and 4.65 (d, $J = 7.8$ Hz) for H_β (Fig. 7a). Nevertheless, interestingly, in residual culture media of each microalga interacting with the bacterium either immobilized or suspension, the presence of metabolites signals different from glucose were observed (Fig. 7c, e–g).

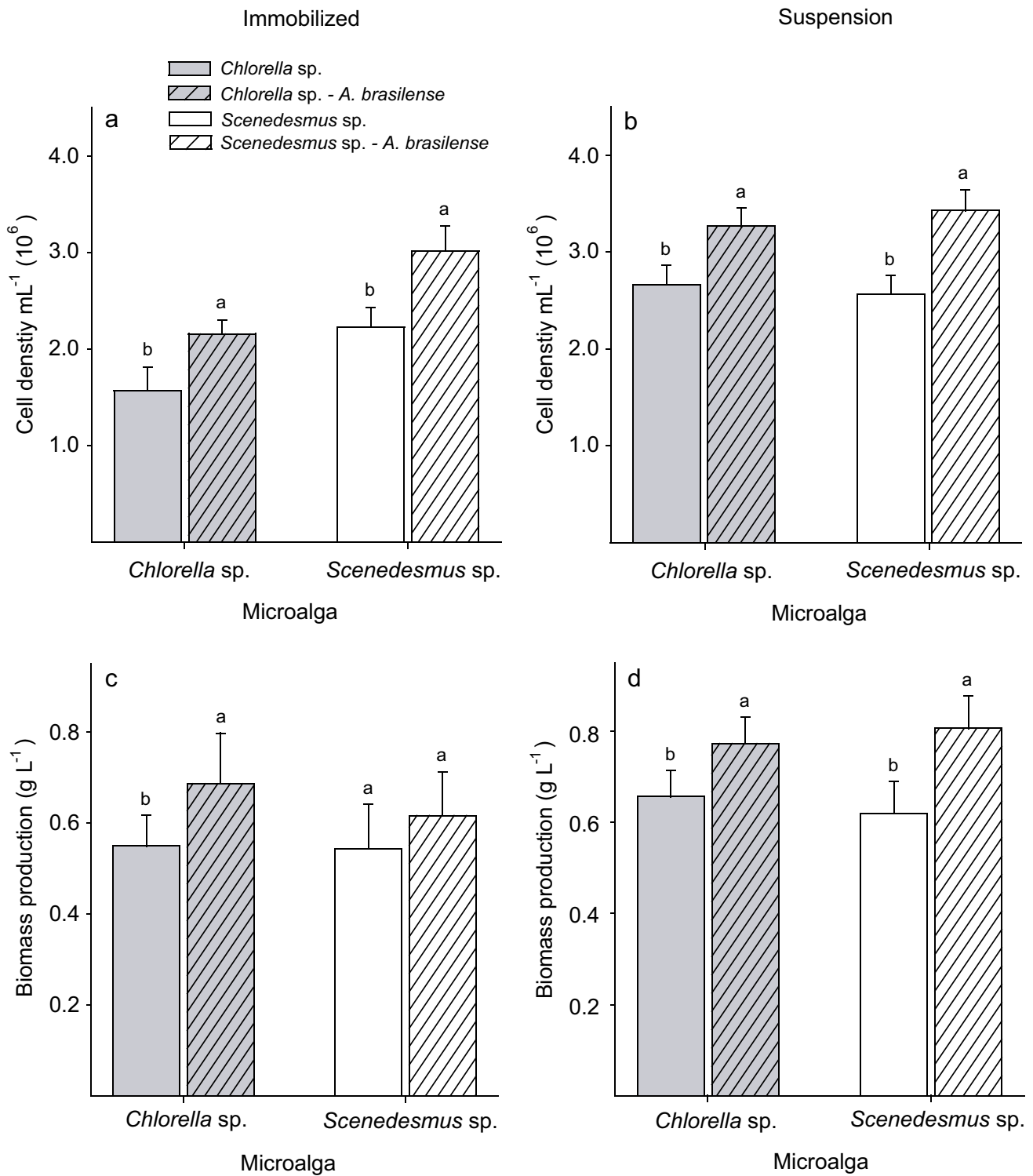


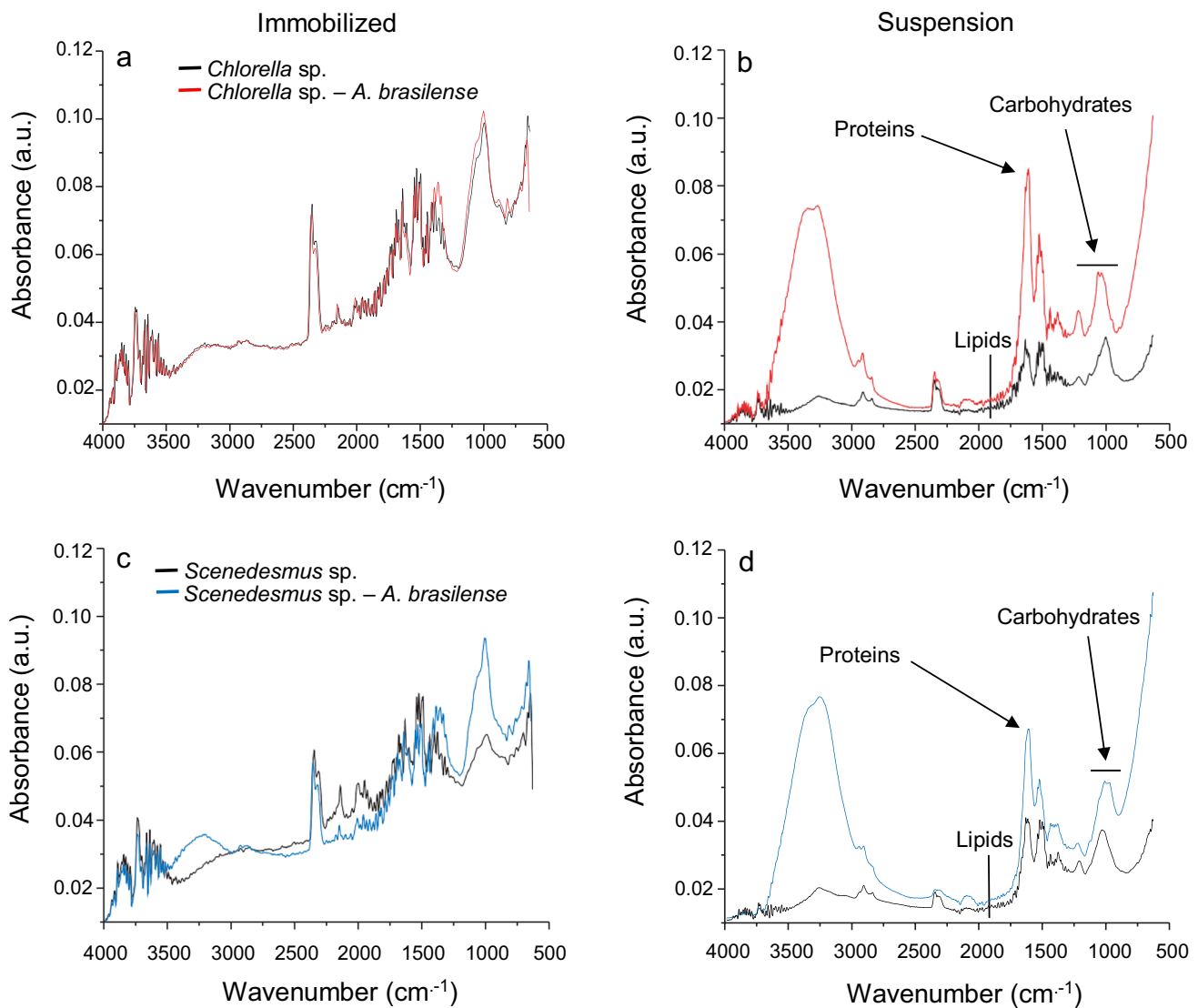
Fig. 3 Cell density (a, b), and biomass production (c, d) of *Chlorella* sp., and *Scenedesmus* sp. co-immobilized or co-cultured in suspension with *Azospirillum brasilense* under heterotrophy. Columns denoted by different lowercase letters differ significantly when

each microalga was growing alone or associated with the bacterium ($n=9$). Statistical analyses were performed using ANOVA and LSD post hoc analysis at $P < 0.05$. Bars represent standard error

Table 2 Growth rate and biomass productivity of *Chlorella* sp. and *Scenedesmus* sp. interacting co-immobilized or co-cultured in suspension with *A. brasilense*

Microalga	Growth rate (μ ; day ⁻¹)		Biomass productivity (P ; g L ⁻¹ day ⁻¹)		pH	
	Immobilized	Suspension	Immobilized	Suspension	Immobilized	Suspension
<i>Chlorella</i> sp.	0.07 ± 0.02a	0.16 ± 0.02a	0.07 ± 0.04a	0.09 ± 0.04a	7.11 ± 0.15a	7.85 ± 0.27a
<i>Chlorella</i> sp.—Az	0.13 ± 0.01b	0.19 ± 0.02a	0.10 ± 0.02a	0.11 ± 0.04a	7.46 ± 0.32a	8.04 ± 0.11a
<i>Scenedesmus</i> sp.	0.13 ± 0.02a	0.15 ± 0.02a	0.06 ± 0.02a	0.05 ± 0.02a	7.34 ± 0.21a	7.92 ± 0.24a
<i>Scenedesmus</i> sp.—Az	0.18 ± 0.02b	0.20 ± 0.02b	0.15 ± 0.02b	0.09 ± 0.02b	7.60 ± 0.18a	8.30 ± 0.29a

Values denoted by different lowercase letters differ significantly when each microalga was growing alone or associated with *A. brasilense* co-immobilized in alginate beads or co-cultured in suspension. Statistical analyses were performed using ANOVA and LSD post hoc analysis at $P < 0.05$. \pm represents standard error ($n=9$)

**Fig. 4** Qualitative biomass characterization of *Chlorella* sp., and *Scenedesmus* co-immobilized (a, c) or co-cultured in suspension (b, d) with *Azospirillum brasilense* cultured under heterotrophic regime

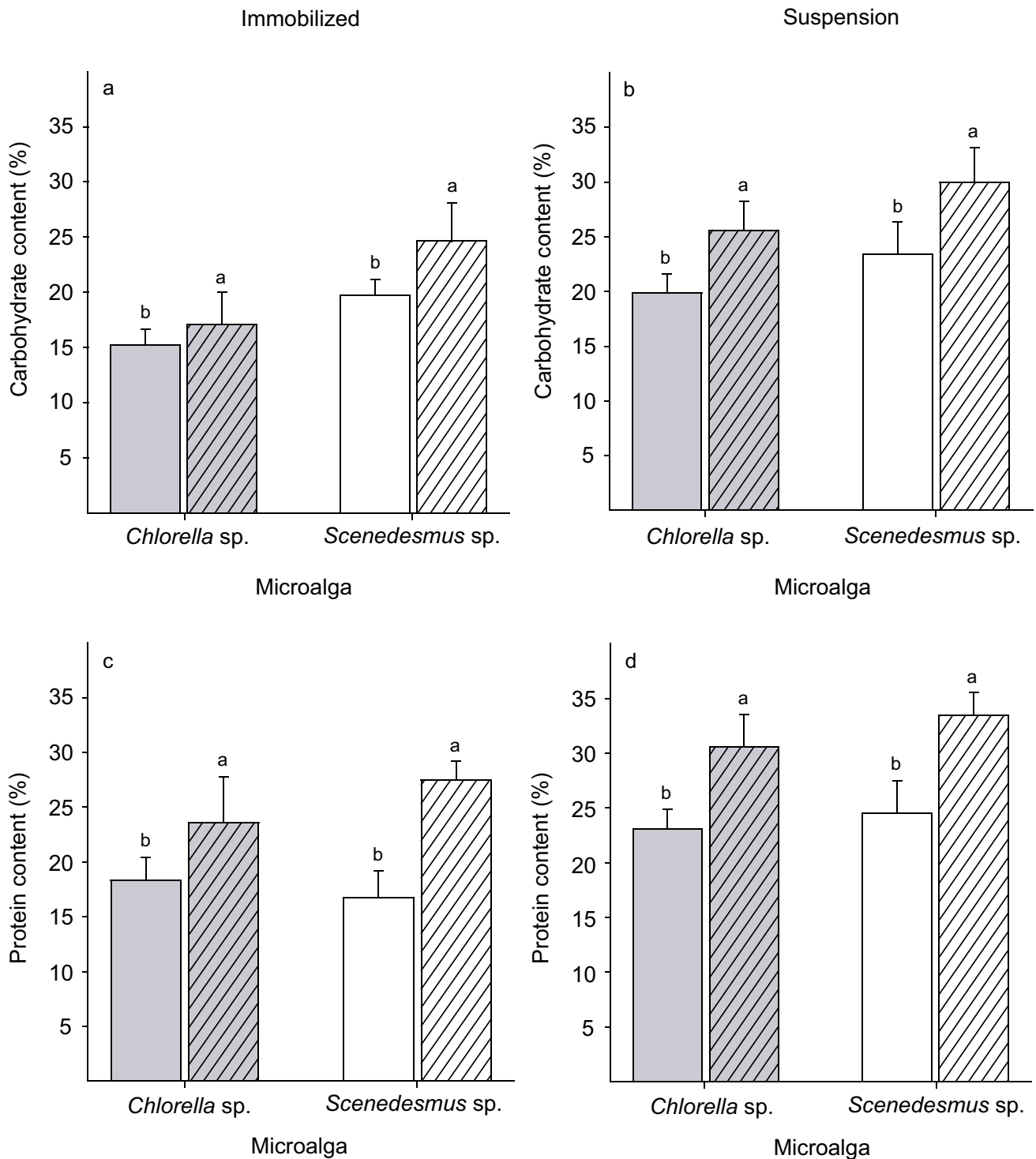


Fig. 5 Carbohydrate (a, b) and protein (c, d) content of *Chlorella sp.*, and *Scenedesmus* co-immobilized or co-cultured in suspension with *Azospirillum brasilense* under heterotrophy. Columns denoted by different lowercase letters differ significantly when each microalga was

growing alone or associated with the bacterium ($n=9$). Statistical analyses were performed using ANOVA and LSD post hoc analysis at $P < 0.05$. Bars represent standard error

Table 3 Carbohydrate and protein productivities of *Chlorella* sp. and *Scenedesmus* sp. interacting co-immobilized or co-cultured in suspension with *A. brasilense*

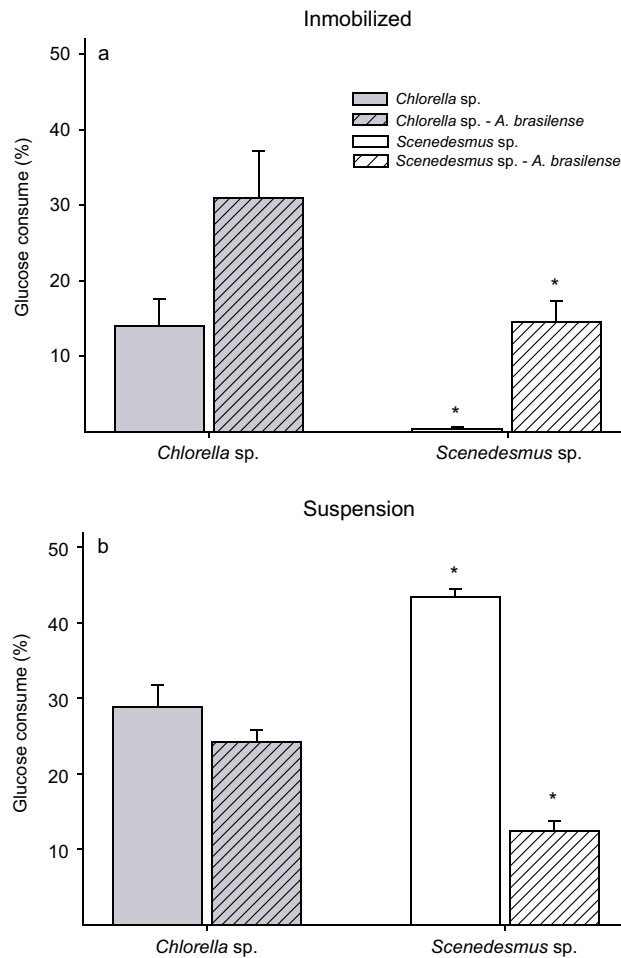
Microalga	Carbohydrate productivity (mg L ⁻¹ day ⁻¹)		Protein productivity (mg L ⁻¹ day ⁻¹)	
	Immobilized	Suspension	Immobilized	Suspension
<i>Chlorella</i> sp.	1.13 ± 0.21a	1.84 ± 0.31a	1.37 ± 0.15a	2.14 ± 0.27a
<i>Chlorella</i> sp.—Az	1.67 ± 0.13b	2.87 ± 0.16b	2.30 ± 0.32b	3.43 ± 0.09b
<i>Scenedesmus</i> sp.	1.30 ± 0.17a	1.24 ± 0.42a	1.23 ± 0.11a	1.30 ± 0.24a
<i>Scenedesmus</i> sp. – Az	1.45 ± 0.12a	2.53 ± 0.28b	1.44 ± 0.08b	2.82 ± 0.29b

Values denoted by different lowercase letters differ significantly when each microalga was growing alone or associated with *A. brasilense* co-immobilized in alginate beads or co-cultured in suspension. Statistical analyses were performed using ANOVA and LSD post hoc analysis at $P < 0.05$. ± represents standard error ($n = 9$)

Discussion

Considering that mutualistic interactions of microalgae with other microorganisms can be altered by different culture conditions (Zhang et al. 2020). The mutualistic effect of *A. brasilense* on the physiological performance of *Chlorella* sp. and *Scenedesmus* sp. co-cultured in suspension

under a heterotrophic regime was assessed in this study. Thus, the aims were to evaluate the signal molecules (Trp and IAA), biomass production, and metabolite accumulation of *Chlorella* sp. and *Scenedesmus* sp. interacting co-cultured in suspension and co-immobilized in alginate beads with the bacterium *A. brasilense* under a heterotrophic regime.

**Fig. 6** Glucose uptake of *Chlorella* sp., and *Scenedesmus* sp. co-immobilized or co-cultured in suspension with *Azospirillum brasilense*.

Columns denoted by an asterisk differ significantly when each microalga was growing alone or associated with the bacterium ($n = 9$). Statistical analyses were performed using ANOVA and LSD post hoc analysis at $P < 0.05$. Bars represent standard error

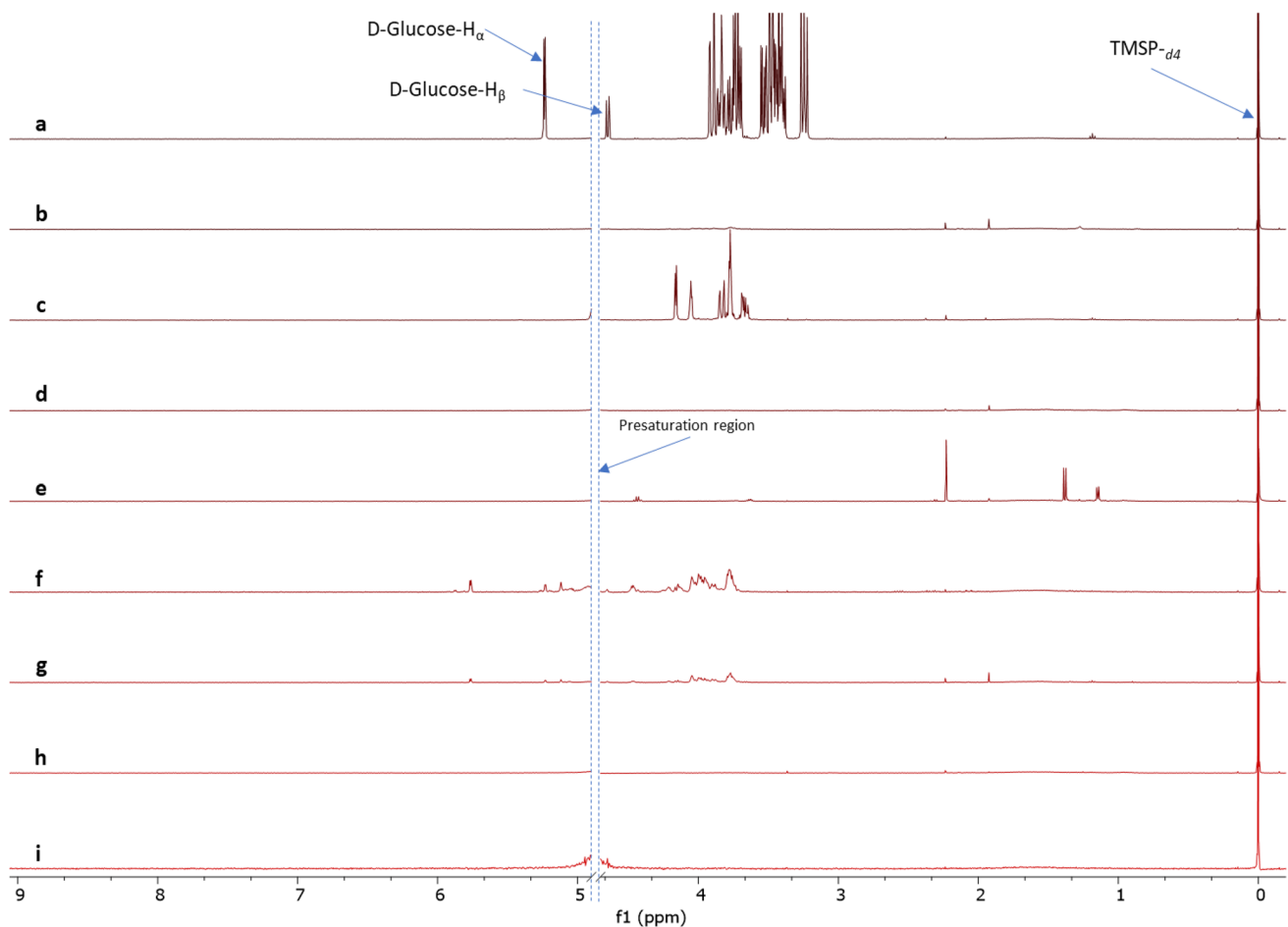


Fig. 7. ^1H NMR (400 MHz, D_2O) spectra of a) C30 culture media with 5 g L^{-1} of glucose; b) *Chlorella* sp. immobilized; c) Interaction *Chlorella* sp. – *Azospirillum brasilense* Co-immobilized; d) *Chlorella* sp. in suspension; e) Interaction *Chlorella* sp. – *Azospirillum* brasi-

lense in suspension; f) *Scenedesmus* sp. immobilized; g) Interaction *Scenedesmus* sp. – *Azospirillum brasilense* Co-immobilized; h) *Scenedesmus* sp. suspension; i) Interaction *Scenedesmus* sp. – *Azospirillum brasilense* in suspension

Our results demonstrated that under the heterotrophic regime, *A. brasilense* maintained its mutualistic association co-cultured in suspension with *Chlorella* sp. and *Scenedesmus* sp., enhancing its biomass production and cell highly valuable compound accumulation. This happens due to the ability of *A. brasilense* and both microalgae to produce IAA and Trp, respectively, inducing their chemical and physical affinity in both culture systems, since these signal molecules activate the bacterial chemotaxis to associate with microalgae (Variem and Kizhakkedath 2021). According to Ramanan et al. (2016), the attached mechanism of *Azospirillum* to the microalgal phycosphere is like plant roots in that it is a microenvironment rich of compounds excreted by microalgae, such as organic carbon, macro and micronutrients. Although in this work only the Trp exuded by *Chlorella* sp. and *Scenedesmus* sp. was quantified, this metabolite is a precursor of IAA biosynthesis by *A. brasilense*, stimulating the chemical affinity with this bacterium. In this regard, Ganusova et al. (2021) demonstrated that *A. brasilense* uses two distinct chemotaxis pathways, Che1 and Che4,

and four different response regulators (CheY1, CheY4, CheY6, and CheY7) to control the swimming pattern during chemotaxis. Thus, co-cultured in suspension, this bacterium can freely swim toward each microalgae, since the physical attachment of *Azospirillum* with its partner is fundamental to establish its synergic relationship (Wheatley and Poole 2018). Conversely, co-immobilized inside alginate beads, this bacterium can also exude organic acids and dissolve alginate, allowing association with microalgae (de-Bashan et al. 2011). To date, several studies have demonstrated that the production and exchange of IAA and Trp support the beneficial effect of *A. brasilense* on the physiological performance of different microalgae genera (Pagnussat et al. 2020; Peng et al. 2020; 2021a; b). For instance, Palacios et al. (2016a) demonstrated that under heterotrophy, the exchange of Trp and IAA by *C. sorokiniana* and *A. brasilense* co-immobilized in alginate beads alters the metabolism of this microalgae. In another study, de-Bashan et al. (2016) demonstrated carbon and nitrogen exchange during the mutualistic association of *C. sorokiniana* and *A. brasilense* when

co-immobilized in alginate beads. The above could explain the lack of quantification of IAA and Trp in any interval when this consortium was maintained in a close association within the alginate beads, since these compounds were assimilated at the moment they were produced. While co-cultured in suspension, both metabolites could have dispersed in the culture medium, making them detectable. Because of the Trp production of *Chlorella* and *Scenedesmus* as well as the IAA production by *A. brasilense* recorded in this study were similar to previous works (de-Bashan et al. 2008; Masciarelli et al. 2013; Choix et al. 2018; Kargapolova et al. 2020; Barbosa-Núñez et al. 2022a, b; Pham et al. 2022). These results indicate that Trp and IAA production by microalgae and *A. brasilense* under heterotrophy allow for maintaining its chemical and physical association co-cultured in suspension, boosting the physiological performance of *Chlorella* sp. and *Scenedesmus* sp.

Under a heterotrophic regime, the carbon metabolism of microalgae is first supported by their transport or diffusion systems of organic carbon through the plasmatic membrane, and thereafter assimilate it through the metabolic pathways of aerobic respiration for energy generation, biomass, and cell compound biosynthesis (Perez-Garcia et al. 2011). In particular, the microalgae genera *Chlorella* and *Scenedesmus* possess a symport hexose transport in its cellular membrane to assimilate glucose (Morales-Sánchez et al. 2015), and the IAA production by *A. brasilense* can enhance the activity of hexose transport systems and increase the glucose assimilation of *Chlorella* (Palacios et al. 2016a). Interestingly, this study showed that both microalgae showed different patterns of glucose uptake in function to the culture system used. Immobilized in alginate beads, glucose uptake was higher in both microalgae interacting with *A. brasilense* than in cultures alone. Conversely, cells cultured in suspension showed a higher glucose uptake when growing alone than when associated with the bacterium. Nonetheless, at the end of experimental time, the glucose analysis by NMR demonstrated that in both culture systems, there was no evidence of glucose signals in the culture medium, suggesting that the two microalgae, either alone or associated with the bacterium, completely assimilated glucose. This analysis also revealed the presence of other compounds different from glucose in the culture medium, which could overestimate the glucose uptake determined by the spectrophotometric method used in this study. These results indicate that, under heterotrophy, this consortium could have stimulated the exudation of photosynthates or other compounds by microalgae or bacterium, although the identification of these compounds will be deeply studied later. These exudes could be a mechanism of consortium microalga–*A. brasilense* to maintain its compatibility under our experimental conditions since cell–cell interactions depend on signaling molecules diffusing and reaching cells to promote stability and robustness

to fluctuations in environments (Ren and Murray 2019). Recently, Palacios et al. (2022) demonstrated through RMN the ability of two strains of *C. sorokiniana* to exudate different metabolites during their association with *A. brasilense*. In another study, Shibl et al. (2020) found a change in transcriptional and metabolic reprogramming related to secondary metabolite production by the phytoplankton *Asterionellopsis glacialis* when interacting with the natural microbial community. However, in this study, the interval time of glucose depleted by each microalga, either alone or interacting with the bacterium, was not determined. This glucose depletion can be attributed to each microalga supporting the higher growth rates and biomass production attained by both microalgae in the two culture systems, since *A. brasilense* does not consume glucose (Goebel and Krieg 1984). Nonetheless, this bacterium cultured alone could use its reserve of poly-3-hydroxybutyrate (PHB) to maintain its viability during our experimental time (six days) as this compound allows *A. brasilense* survive long periods under nutrient stress (Okon and Itzigsohn 1992); whilst co-cultured it might have used exuded compounds by microalgae to grow and survive under heterotrophy. Nevertheless, these hypotheses need further investigation. These findings confirm that co-cultured in suspension *A. brasilense* can be a suitable partner to *Chlorella* sp. and *Scenedesmus* sp., highlighting the compatibility and stability of this consortium under a heterotrophic regime.

According to Ren and Murray (2019) a robust cell–cell interaction ensures the functionality of the consortium. In this regard, in this study, each microalga in both culture systems attained higher cell compound accumulation, mostly proteins and carbohydrates, when they were associated with *A. brasilense*. Although the biochemical composition is dependent on culture conditions and the microalgal strain (Bhattacharya and Goswami 2020), several studies demonstrate the direct effect of IAA improving physiological performance and increasing cell compound accumulation in different microalga genera (Stirk and van Staden 2020; González-González and de-Bashan 2021). In complete darkness, carbohydrate biosynthesis from glucose assimilation by microalgae is energetically more economical than lipid biosynthesis (Li et al. 2011). Moreover, the protein content indicates high metabolic activity (Li et al. 2020) explaining higher carbohydrate and protein productivities reached by both microalgae interacting with *A. brasilense*. Nevertheless, the FTIR spectra of both microalgae, either immobilized alone or co-immobilized with the bacterium, showed similar cell compound accumulation owing to alginate beads composed of sugars as mannuronic and guluronic interfering and inducing similar qualitative microalgal biochemical characterization. This latter can be supported by the distinct spectra in both microalgae cultured in suspension, as well as the low peaks of both metabolites in *A. brasilense* in both culture systems (Fig. S1). The above findings suggest that the use of microbial consortia

co-immobilized in different matrices or co-cultured in suspension depends on the specific objective of each biotechnological application. Besides, these results corroborate the robustness of mutualistic interaction of the consortium *A. brasilense*-microalga co-cultured in suspension under a heterotrophic regime, which could be proposed to enhance biomass and cell compound production by *Chlorella* sp. and *Scenedesmus* sp. in different culture conditions and bioprocess based in microalgae.

Conclusion

Overall, this study demonstrates that under heterotrophy, *A. brasilense* co-cultured in suspension maintains its mutualistic interaction with *Chlorella* sp. and *Scenedesmus* sp. because of signal molecule production, such as IAA and Trp. These results highlight the compatibility of the synergic association of this consortium in different culture systems and growth conditions. Finally, this study expands the biotechnological potential of this consortium microalgal-*Azospirillum*, as well as its incidence in different bioprocesses supported by microalgae.

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Author contribution FJC and OAP designed the research; JRTV, CACG and JCEH supervised the research; JRTV and CACG performed the research and statistical analysis; FJC, OAP, MCP and JCEH analyzed and interpreted the data; FJC, OAP, MCP and JCEH wrote the paper. All the authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information file.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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