Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: II. Heterotrophic conditions

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This study is dedicated to the memory of the Italian microbiologist Prof. Franco Favilli (1933–2012) of the University of Florence, Italy, one of the pioneers of *Azospirillum* studies.

ABSTRACT

The effect of the bacterium *Azospirillum brasilense* jointly immobilized with *Chlorella vulgaris* or *C. sorokiniana* in alginate beads on total carbohydrates and starch was studied under dark and heterotrophic conditions for 144 h in synthetic growth medium supplemented with either D-glucose or Na-acetate as carbon sources. In all treatments, enhanced total carbohydrates and starch content per culture and per cell was obtained after 24 h; only jointly immobilized *C. vulgaris* growing on D-glucose significantly increased total carbohydrates and starch content after 96 h. Enhanced accumulation of carbohydrate and starch under jointly immobilized conditions was variable with time of sampling and substrate used. Similar results occurred when the microalgae was immobilized alone. In both microalgae growing on either carbon sources, the bacterium promoted accumulation of carbohydrates and starch; when the microalgae were immobilized alone, they used the carbon sources for cell multiplication. In jointly immobilized conditions with *Chlorella* spp., affinity to carbon source and volumetric productivity and yield were higher than when *Chlorella* spp. were immobilized alone; however, the growth rate was higher in microalgae immobilized alone. This study demonstrates that under heterotrophic conditions, *A. brasilense* promotes the accumulation of carbohydrates in two strains *Chlorella* spp. under certain time–substrate combinations, producing mainly starch. As such, this bacterium is a biological factor that can change the composition of compounds in microalgae in dark, heterotrophic conditions.

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1. Introduction

Microalgae are simple, autotrophic microorganisms capable of generating complex carbon compounds from CO_2 and light energy, many of economic importance [1,2]. However, several microalgae can grow heterotrophically in the dark [3–5]. As a substitute for CO_2 and light, they use small organic molecules as carbon and energy sources and convert them into the building blocks of their cellular components, lipids, proteins, and complex carbohydrates [3,6,7], the latter mainly as starch. Starch is a major storage carbohydrate and energy in many algae and terrestrial higher plants [8–10]. Under heterotrophic cultures of microalgae, starch serves as long-term carbon storage that can be used in reproductive growth [11]. Several strains of *Chlorella* spp. and *C. vulgaris* can grow heterotrophically [4], while others cannot [12,13]; their main intracellular storage material is starch [14]. Heterotrophic growth of *Chlorella* spp. in the dark requires supplementation of the growth medium with a carbon source, D-glucose or sodium acetate, known to support heterotrophic growth of the studied species [7]. Under these conditions, growth rate, dry biomass, ATP generated by the supplied energy, and effect on ATP yield (mg biomass generated by mg of consumed ATP) are significantly higher than under autotrophic cultivation [5,7,15]. Although heterotrophic cultivation of microalgae is usually cheaper at a large scale [7,15,16], the superior performance of the microalgae is the most important driving force to grow microalgae under heterotrophic conditions [5,7]. Similar to autotrophic growth, where cell composition of microalgae can be affected by chemical, physical, and environmental factors [17–19] and growth conditions [18–20], under heterotrophic cultivation, the carbon source can also influence the biochemical composition of microalgae [21,22].

The bacterium *Azospirillum brasilense*, of rhizosphere origin, is a plant growth-promoting bacteria (PGPB) that enhances growth

and yield of numerous terrestrial plants [23,24], as well as fresh-water microalgae, including *Chlorella* spp. [25,26]. *Chlorella* spp. and *A. brasilense* that are jointly immobilized in calcium alginate beads in laboratory-scale experiments remove nitrogen and phosphorus from synthetic and real municipal wastewater [27–30] and serve as a model for eukaryotic–prokaryotic interaction [31,32]. This model was proposed because this specific interaction induces, under autotrophic conditions, significant changes in the microalgae including major enzymatic activities of the nitrogen cycle [33], increases content of pigments, lipids [27], and carbohydrates and starch (Choix et al. submitted simultaneously with this paper to this journal, as Part I). The hypothesis of this study is similar to the previous study (Choix et al., submitted as Part I), with the sole major modification that the study was performed in the dark using carbon sources (D-glucose or Na-acetate) for growth under heterotrophic conditions.

2. Materials and methods

2.1. Microorganisms, growth media, immobilization, and counting

Microalgae *Chlorella vulgaris* Beijerinck (UTEX 2714, Austin, TX) and *Chlorella sorokiniana* Shihira and Krauss (UTEX 2805) and the bacterium *A. brasilense* Cd (DSM 1843, Braunschweig, Germany) were used as test models. Media for initial cultivation of microorganisms, immobilization, and counting of microorganisms are described in Part I ([28,34] and Choix et al., submitted, Part I).

2.2. Experimental culturing conditions

After secondary incubation, the beads were washed three times with sterile saline solution (0.85% NaCl). For experiments, 8 g of beads with microorganisms immobilized or jointly-immobilized were inoculated in 100 mL of synthetic growth medium (SGM) [32] containing (in mg L^{-1}): NaCl (7), CaCl$_2$ (4), MgSO$_4$·7H$_2$O (2), K$_2$HPO$_4$ (217), KH$_2$PO$_4$ (8.5), Na$_2$HPO$_4$ (33.4), NH$_4$Cl; (191). This medium was supplemented with 10 g L^{-1} of D-glucose or sodium acetate (Sigma, St. Louis, MO, USA) as carbon sources. Both carbon sources were sterilized by filtration through a 0.2 μm Acrodisc syringe filter (Pall Corp., Port Washington, NY, USA). Both strains of the microalgae grow on these substances under heterotrophic conditions [15,16]. All experiments were performed under heterotrophic conditions in complete darkness, stirred at 140 rpm at 27 ± 2 °C (*C. vulgaris*) or 37 ± 2 °C (*C. sorokiniana*) for 144 h.

2.3. Analytical methods

One-gram samples (per replicate and per treatment, $n = 9$) of alginate beads from each treatment were taken every 24 h, washed in distilled water, dried at 80 °C for 12 h, and ground with a mortar and pestle. Extraction of total carbohydrates and quantification of carbohydrates and starch was described in Part I (Choix et al., submitted).

Uptake of D-glucose or sodium acetate from SGM by microorganisms was analyzed using kits: the Megazyme D-glucose (glucose oxidase/peroxidase) assay kit (catalog #K-GLUC, gopod format, Megazyme International, Bray, Ireland), and a kit to measure acetic acid (catalog #K-ACETAF 12/07, acetyl-coA synthetase format; Megazyme International). These tests were performed according to the manufacturer's instruction.

2.4. Experimental designs and statistical analysis

The setup of all experiments was as described in Part I (Choix et al., submitted). The following variables were analyzed: Volumetric productivity and growth rate were calculated, as described in Part I. Affinity of the microalgal cells to the substrate during 24 h was calculated as: $Affinity = S_t/N_t$, where S_t is in grams of substrate S consumed in that time, and N_t is the number of microalgae cells after 24 h. Carbohydrate yield (quantity of carbohydrates produced per gram of carbon source uptake of culture during 24 h) was calculated as: $Carbohydrate\ yield = (P_1 - P_0)/(S_1 - S_0)/V$, where P_1 is the quantity of carbohydrates after 24 h, P_0 at the beginning of this time interval, S_1 is the substrate concentration (D-glucose or sodium acetate) after 24 h, and S_0 at the beginning of this time interval. V is the volume of the medium used (100 mL). Each experiment was repeated twice. The data from the two repetitions of each treatment ($n = 36$) were combined for analysis, first by one-way ANOVA and then by LSD post hoc analysis, with significance set at $P < 0.05$, using Statistica 6.0 software (StatSoft, Tulsa, OK).

3. Results

3.1. Accumulation of carbohydrates during incubation of Chlorella spp. jointly immobilized with A. brasilense, using D-glucose as the carbon source

3.1.1. Chlorella vulgaris

The total carbohydrate content in *A. brasilense* cells was constant during 144 h of incubation, within the range of 15.9 ± 7.1 mg g^{-1} (Fig. 1a, capital letter analysis). *C. vulgaris* had increased total carbohydrate content at 24 h, either immobilized (113.4 ± 18.9 mg g^{-1}) or jointly immobilized (133 ± 20 mg g^{-1}). Significant differences between immobilized and jointly immobilized conditions were not found at this time (Fig. 1a, lower case analysis). Carbohydrate content then decreased up to 72 h (Fig. 1a, capital letter analysis), reaching the same level of carbohydrates either immobilized alone or jointly immobilized (Fig. 1a, lower case analysis). The highest total carbohydrate content was reached at 96 h of culturing. At this time, the total carbohydrate content was significantly higher in the jointly immobilized *C. vulgaris* (200 ± 20 mg g^{-1}) than in solely immobilized cultures (113 ± 7.1 mg g^{-1}) (Fig. 1a, lower case analysis). This carbohydrate content remained constant until the end of incubation time at 144 h.

There was an increase of 143 ± 5% in *C. vulgaris* immobilized alone and 333 ± 8% in jointly immobilized cultures after 96 h of incubation. However, at this time, contents of carbohydrates per cell were not the highest (58 ± 3.2 when immobilized alone and 366 ± 145 ng cell^{-1} when jointly immobilized). The highest content per cell occurred at 24 h (310 ± 130 when immobilized alone and unchanged from time 0 and 468 ± 92 ng cell^{-1} when jointly immobilized) (Fig. 1d and e).

A similar pattern for accumulation of starch occurred. After 24 h, content increased (46 ± 14 mg g^{-1}) when solely immobilized and was significantly higher (65 ± 11 mg g^{-1}) when jointly immobilized (Fig. 1b, lower case analysis). As with total carbohydrates, highest starch production occurred at 96 h, with significant differences when immobilized alone (81 ± 14 mg g^{-1}) and jointly immobilized (126 ± 23 mg g^{-1}) (Fig. 1b, lower case analysis). After this time, starch content decreased (Fig. 1b, capital letter analysis). Of the total carbohydrates accumulated after 96 h, starch content was 71.2 ± 1.5% when incubated alone and 62.2 ± 2% when jointly incubated (Fig. 1f). Although starch content at 96 h was higher than after 24 h (Fig. 1b, capital letter analysis), starch content per cell in *C. vulgaris* immobilized alone and jointly immobilized at 24 h and 96 h was almost the same; 125 ± 47 ng cell^{-1} versus 123 ± 26 ng g^{-1} when solely incubated and 229 ± 46 ng cell^{-1} versus 228 ± 51 ng cell^{-1} when jointly immobilized (detailed data not shown).

3.1.2. Chlorella sorokiniana

Under heterotrophic conditions, *C. sorokiniana* immobilized alone or jointly immobilized with *A. brasilense* had similar amounts of total carbohydrates for the entire incubation period (Fig. 2a, capital letter analysis). A constant level of carbohydrates in *A. brasilense*, when immobilized alone, was measured. After 24 h of incubation *C. sorokiniana* reached a peak carbohydrate production of 96 ± 40 mg g^{-1} when immobilized alone and 115 ± 38 mg g^{-1} when immobilized with *A. brasilense*. No significant differences between treatments occurred at any time interval (Fig. 2a, lower case analysis). Increase in carbohydrate content after 24 h was 96 ± 5.09% when incubated alone and 135 ± 6.79% when jointly immobilized, compared to the initial level. At 24 h, the highest carbohydrate content per cell was 332 ± 175 mg g^{-1} when immobilized alone and 504 ± 298 ng cell^{-1} when immobilized with *A. brasilense*. Lower contents per cell occurred for the rest of the incubation period (Fig. 2d and e).
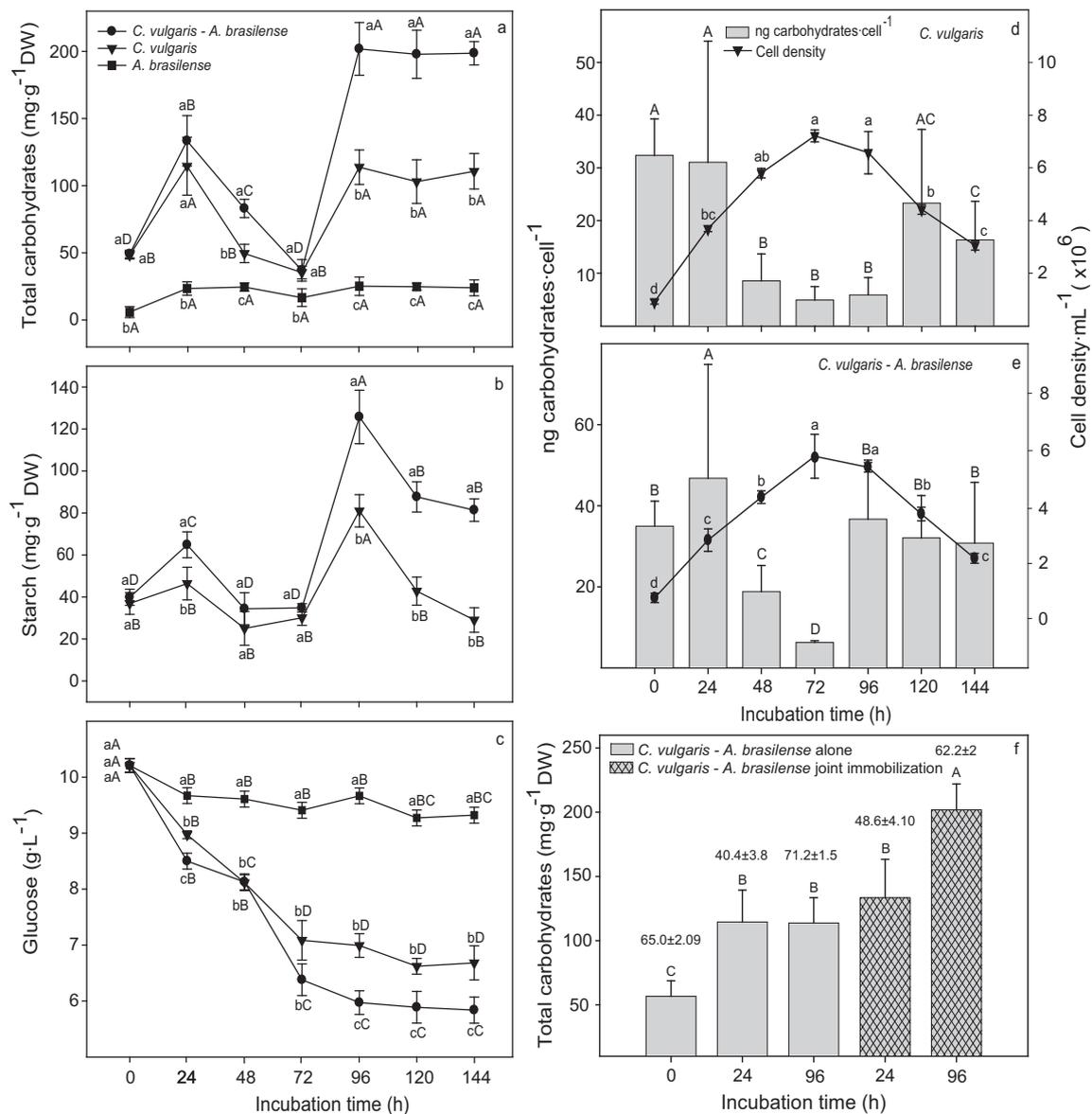
Fig. 1. (a) Accumulation of total carbohydrates, (b) accumulation of starch, (c) glucose uptake, (d) growth (solid line) and accumulation of carbohydrates per cell (columns) by *Chlorella vulgaris* immobilized alone in alginate beads. (e) Cell density (solid line) and accumulation of carbohydrates per cell when jointly immobilized in alginate beads with *A. brasilense*. (f) Total carbohydrates (columns) and percentage of starch (values above columns) after 24 h and 96 h of incubation. All experiments performed under heterotrophic conditions using glucose as the carbon source. Values on curves denoted by different capital letters differ significantly using one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different lowercase letters differ significantly at $P < 0.05$ in (a and c) using ANOVA, in (b) using Student's *t*-test. In (d–f), columns denoted by different capital letters differ significantly by one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Bars represent SE.

Although significant differences in the total carbohydrates content in *C. sorokiniana* were not found when immobilized alone or jointly, starch was the main carbohydrate detected. In jointly immobilized treatments, starch content was significantly higher at several incubation intervals (Fig. 2b, lower case analysis). The highest starch content occurred 24 h; 52 ± 15 mg g^{-1} when immobilized alone and 73 ± 8 mg g^{-1} when jointly immobilized. Thereafter, starch content was decreased slightly with time (Fig. 2b, capital letter analysis). Starch content was $54.1 \pm 2.5\%$ alone and $63.3 \pm 1.4\%$ jointly, of total carbohydrates (Fig. 2f). At 24 h, the highest starch content per cell was 181 ± 64 ng cell^{-1}, when immobilized alone, and 318 ± 53 ng cell^{-1}, when jointly immobilized (detailed data not shown).

3.2. Uptake of ᴅ-glucose

A. brasilense did not consume ᴅ-glucose during incubation (Fig. 1c, capital letter analysis), as is known for this species [35]. *C. vulgaris* immobilized alone and jointly immobilized showed a continuous uptake of ᴅ-glucose during the first 72 h, but without significant differences (Fig. 1c, lower case analysis). Uptake of ᴅ-glucose continued to the end of the incubation (Fig. 1c, capital letter analysis), but significant differences were found between immobilized alone and jointly immobilized treatments (Fig. 1c, lower case analysis).

C. sorokiniana immobilized alone gradually increased ᴅ-glucose uptake to 96 h and then uptake of ᴅ-glucose ceased (Fig. 2c, capital
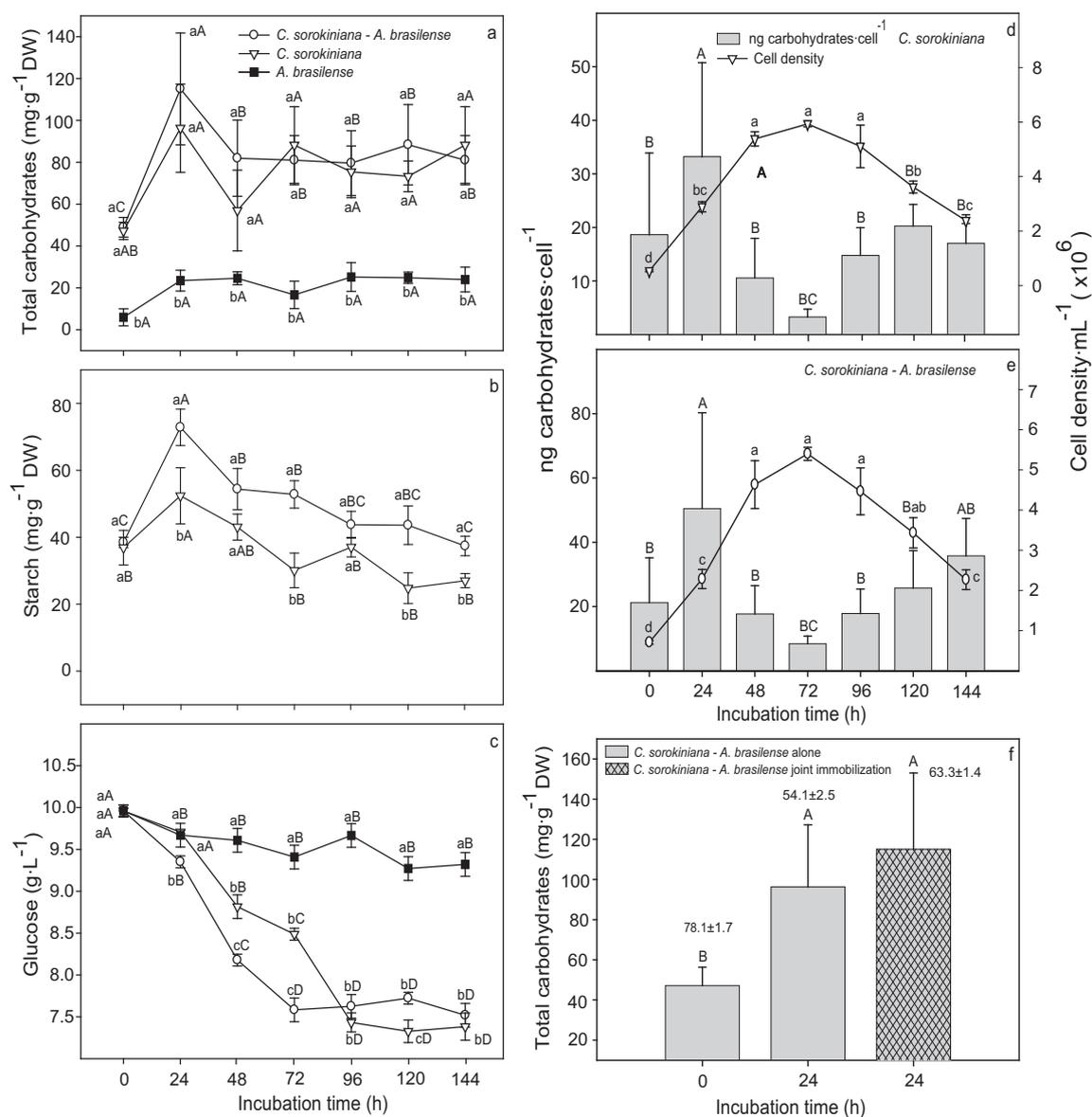
Fig. 2. (a) Accumulation of total carbohydrates, (b) accumulation of starch, (c) glucose uptake, (d) growth (solid line) and accumulation of carbohydrates per cell (columns) by *Chorella sorokiniana* immobilized alone, (e) cell density (solid line) and accumulation of carbohydrates per cell when jointly immobilized in alginate beads with *Azospirillum brasilense*. (f) Total carbohydrates (columns) and percentage of starch (values above columns) after 24 h of incubation. All experiments performed under heterotrophic conditions using glucose as the carbon source. Values on curves denoted by different capital letters differ significantly using one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different lowercase letters differ significantly at $P < 0.05$ in (a and c) using ANOVA, in (b) using Student's t-test. In (d–f), columns denoted by different capital letters differ significantly by one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Bars represent SE.

letter analysis). When jointly immobilized, gradual, continuous D-glucose uptake occurred to 72 h and was significantly different from uptake when immobilized alone (Fig. 2c, lower case analysis). After 72 h, uptake of D-glucose ceased (Fig. 2c, capital letter analysis).

Highest affinity for immobilized alone and joint immobilized *C. vulgaris* occurred at 72 h, but was significantly higher when jointly immobilized at all time intervals (Table 1, lower case analysis). The lowest volumetric productivity and carbohydrate yield were calculated at 72 h (Table 1, capital letter analysis). The highest volumetric productivity and carbohydrate yield were calculated at 96 h (Table 1, capital letter analysis); at this time, the jointly immobilized treatment was significantly higher (Table 1, lower case analysis). At 96 h, the affinity for *C. vulgaris* immobilized alone was the lowest value calculated (Table 1, capital letter analysis).

After 72 h, *C. sorokiniana* also had peak affinity (Table 1, capital letter analysis), and similar to *C. vulgaris*, there was significant differences between treatments (Table 1, lower case analysis).

Volumetric productivity and carbohydrate yield were significantly lower when jointly immobilized. The highest volumetric productivity and carbohydrate yield were calculated at 24 h in both treatments (Table 1, capital letter analysis), with volumetric productivity and carbohydrate yield significantly higher when jointly immobilized (Table 1, lower case analysis). The affinity at 24 h was low in both treatments (Table 1, capital letter analysis).

3.3. Accumulation of carbohydrates in Chlorella spp. jointly immobilized with A. brasilense, using sodium acetate as carbon source

3.3.1. Chlorella vulgaris

Carbohydrate content of *A. brasilense* was constant during incubation when grown on sodium acetate, an average of $16 \pm 5.2 \, \text{mg g}^{-1}$ (Fig. 3a, capital letter analysis). Throughout the incubation, there were no significant differences between cultures

Table 1

General analysis of total carbohydrates in *Chlorella* spp. jointly immobilized with *Azospirillum brasilense* in alginate beads growing in synthetic growth medium under heterotrophic conditions using glucose as the carbon source.

	Affinity		Volumetric productivity		Carbohydrate yield	
	mg d^{-1}		mg 100 mL^{-1} d^{-1}		mg 100 mL^{-1} d^{-1}	
	Joint immobilization	Alone	Joint immobilization	Alone	Joint immobilization	Alone
C. vulgaris						
0–24 h	0.81 ± 0.08 aA	0.44 ± 0.04 bA	7.21 ± 1.11 aA	5.78 ± 0.42 bA	4.67 ± 1.7 aA	4.22 ± 0.4 aA
48–72 h	1.17 ± 0.06 aA	0.72 ± 0.24bA	−4.60 ± 1.51 aB	−1.42 ± 0.81 bB	−1.38 ± 0.07 aB	−2.63 ± 0.6 bB
C. sorokiniana						
0–24 h	0.44 ± 0.32 aA	0.12 ± 0.09 bA	6.61 ± 1.16 aA	4.90 ± 0.49 bA	19.4 ± 0.31 aA	10.8 ± 0.08 bA
48–72 h	0.97 ± 0.58 aA	0.68 ± 0.50 bB	−0.09 ± 8.2 aB	3.13 ± 0.37 bA	−0.15 ± 1.26 aB	9.52 ± 2.30 bA

Values for each treatment and species of microalgae, separately denoted by different capital letters differ significantly at each incubation time. Values for each comparison between solely immobilized and jointly immobilized microorganisms denoted by lowercase letters differ significantly. Statistical analyses were performed using one-way ANOVA and LSD post hoc analysis at $P < 0.05$, and by Student's t-test. ± represents SE.
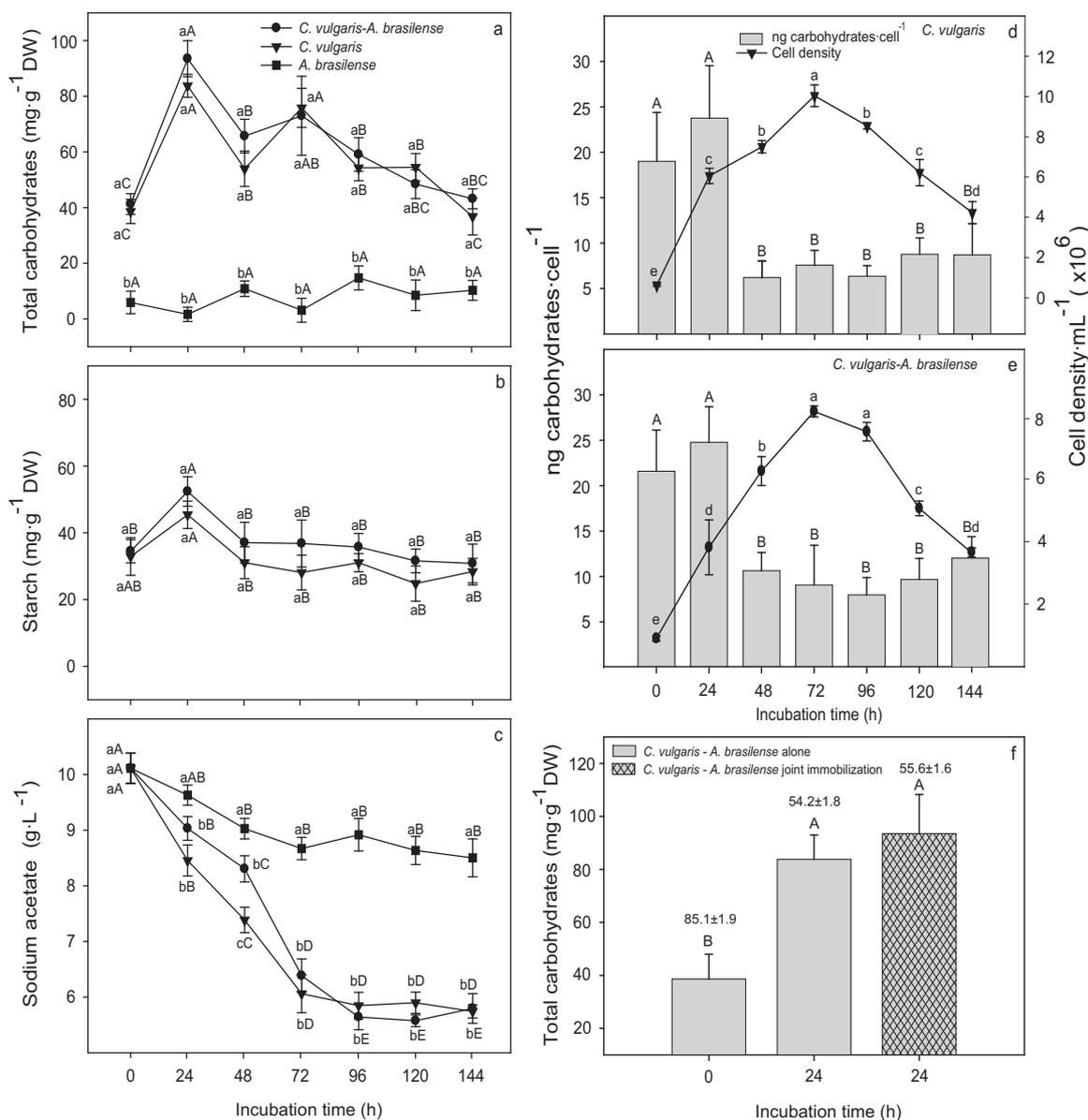

Fig. 3. (a) Accumulation of total carbohydrates, (b) accumulation of starch, (c) sodium acetate uptake, (d) growth (solid line) and accumulation of carbohydrates per cell (columns) by *Chorella vulgaris* immobilized alone, (e) cell density (solid line) and accumulation of carbohydrates when jointly immobilized in alginate beads with *Azospirillum brasilense*. (f) Total carbohydrates (columns) and percentage of starch (values above columns) after 24 h of incubation. All experiments performed under heterotrophic conditions using sodium acetate as the carbon source. Values on curves denoted by different capital letters differ significantly using one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different lowercase letters differ significantly at $P < 0.05$ in (a and c) using ANOVA and in (b) using Student's t-test. In (d–f), columns denoted by different lowercase letters differ significantly by one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Bars represent SE.

of *C. vulgaris* immobilized alone and jointly immobilized (Fig. 3a, lower case analysis). Both cultures reached peak total production of carbohydrates after 24 h, 83 ± 9 mg g^{-1} when immobilized alone and 93 ± 14 mg g^{-1}, when jointly immobilized. During incubation, the increase was $117 \pm 3.25\%$ when immobilized alone and $126 \pm 2.61\%$ when jointly immobilized, compared to carbohydrates present at initial growth. Carbohydrate content per cell was 237 ± 57 ng g^{-1} when immobilized alone and 247 ± 39 ng cell^{-1} when jointly immobilized (Fig. 3d and e), with lower levels at all other intervals of incubation. Carbohydrate content decreased with time (Fig. 3a, capital letter analysis).

Similar to content of carbohydrates, throughout the entire incubation there were no significant differences in the content of starch between cultures of *C. vulgaris* immobilized alone and jointly immobilized (Fig. 3b, lower case analysis). Peak starch content in *C. vulgaris* immobilized alone and jointly immobilized occurred at 24 h, reaching 45 ± 6.4 mg g^{-1} when immobilized alone and 52 ± 7.1 mg g^{-1} when jointly immobilized. Starch content at 24 h was $54.2 \pm 1.8\%$ of the carbohydrates, when immobilized alone, and $55.6 \pm 1.6\%$, when jointly immobilized (Fig. 3f). At 24 h, starch content per cell was 75 ± 15 ng cell^{-1}, when immobilized alone, and 138 ± 26 ng cell^{-1} when jointly immobilized (detailed data not shown). Later, starch declined and remained at the same range (Fig. 3b, capital letter analysis).

3.3.2. Chlorella sorokiniana

C. sorokiniana showed similar patterns for carbohydrate and starch accumulation as *C. vulgaris*, but accumulated, at certain intervals, greater quantities. Throughout the incubation, there were no significant differences regarding carbohydrate accumulation between cultures of *C. sorokiniana* immobilized alone or jointly immobilized (Fig. 4a, lower case analysis). Both cultures reached peak total carbohydrate content after 24 h; 123 ± 10 mg g^{-1}, when immobilized alone, and 102 ± 3.1 mg g^{-1}, when jointly immobilized. Total carbohydrates at 24 h increased $157 \pm 3.45\%$ and $114 \pm 5\%$, respectively, over the initial concentration of carbohydrates. At 24 h, the highest amount of carbohydrate per cell was similar: 341 ± 140 ng cell^{-1} and 341 ± 138 ng cell^{-1}, respectively (Fig. 4d and e), with the lesser amount at all other intervals and maintained at the lower level (Fig. 4a, capital letter analysis).

Starch content showed similar patterns as accumulated carbohydrates. After 24 h, the starch reached its peak amount: 56 ± 5 mg g^{-1} and the significantly different 77 ± 8.1 mg g^{-1}, respectively (Fig. 4b, lower case analysis), an accumulation of $45.7 \pm 1\%$ and $75.4 \pm 2\%$, respectively, of total carbohydrates (Fig. 4f). At 24 h, starch per cell was 156 ± 18 ng cell^{-1} and 258 ± 36 ng cell^{-1}, respectively. From 24 h to 144 h, starch content decreased and continued at the lower level, similar to the content of total carbohydrates (Fig. 4b, capital letter analysis).

3.4. Uptake of sodium acetate

A. brasilense showed increasing uptake of sodium acetate up to 72 h, but after this interval, uptake ceased (Fig. 3c, capital letter analysis). *C. vulgaris* immobilized alone and jointly increased uptake until 72 h. Afterward, uptake ceased (Fig. 3c, capital letter analysis). For the first 48 h, jointly immobilized organisms had significantly more uptake; at all other time intervals, no significant differences between treatments were detected (Fig. 3c, lower case analysis).

Sodium acetate uptake by *C. sorokiniana* was similar to that of *C. vulgaris*, with peak uptake after 72 h. During the entire incubation, there were no significant differences between immobilized along and jointly immobilized treatments (Fig. 4c, lower case analysis).

With sodium acetate as the carbon source, highest affinity in *C. vulgaris* and *C. sorokiniana* was calculated after 72 h (Table 2, capital letter analysis). Affinity was significantly higher in the jointly immobilized treatment (Table 2, lower case analysis). At 72 h, volumetric productivity and carbohydrate yield were lower (Table 2, capital letter analysis), with significant differences between the two treatments (Table 2, lower case analysis). After 24 h, immobilized alone and jointly immobilized treatments reached the highest volumetric productivity and carbohydrate yield (Table 2, capital letter analysis), but only *C. vulgaris* showed significant differences between the two treatments (Table 2, lower case analysis). Affinity at 24 h was low (Table 2, capital letter analysis).

3.5. Heterotrophic growth of Chlorella spp.

Generally, growth of *Chlorella* spp. immobilized alone was higher than when jointly immobilized with *A. brasilense*. When growth was based on D-glucose, *C. vulgaris* and *C. sorokiniana* reached peak population numbers and growth rate at 72 h. *C. vulgaris* reached a population of $7.2 \times 10^6 \pm 0.51$ cell mL^{-1} and growth rate of 0.83 ± 0.07 d^{-1} when immobilized alone. When jointly immobilized, the peak population occurred at 72 h; $5.5 \times 10^6 \pm 0.30$ cell mL^{-1} and a growth rate of 0.70 ± 0.08 d^{-1}. Differences between treatments were significant. After this interval, population size declined (Fig. 1d and e; Table S1 supplementary material). The same pattern occurred with *C. sorokiniana*. At 72 h, the cell population was significantly different between the treatments, reaching $6.0 \times 10^6 \pm 0.30$ cell mL^{-1} when immobilized alone and $5.4 \times 10^6 \pm 0.50$ cell mL^{-1} when jointly immobilized (Fig. 2d and e); growth rates were 0.80 ± 0.05 d^{-1} and 0.67 ± 0.05 d^{-1}, respectively (Table S1).

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C. vulgaris and *C. sorokiniana* growing on sodium acetate showed the same growth pattern, reaching peak population size and growth rate after 72 h, higher than when D-glucose was used as carbon source. At 72 h, *C. vulgaris* showed significant differences in both treatments, reaching a population of $10 \times 10^6 \pm 1.19$ cell mL^{-1} when immobilized alone and $8.5 \times 10^6 \pm 1.13$ cell mL^{-1} when jointly immobilized with *A. brasilense* (Fig. 3d and e); growth rates were 0.93 ± 0.09 d^{-1} and 0.73 ± 0.08 d^{-1}, respectively (Table S1). *C. sorokiniana* had a population of $6.6 \times 10^6 \pm 0.36$ mL^{-1} and $6.1 \times 10^6 \pm 0.56$ cell mL^{-1}, respectively; while the growth rate was 0.81 ± 0.05 d^{-1} and 0.72 ± 0.08 d^{-1} (Fig. 4d and e, Table S1). These growth rates were not significantly different between the two treatments. The growth rate and the population size of *C. vulgaris* were greater than *C. sorokiniana* when using either carbon sources (Table S1).

4. Discussion

A feasible alternative to the common phototrophic cultivation of microalgae, but restricted to only a few microalgae species, is to use their heterotrophic growth capacity in the absence of light. This approach is significantly less expensive than autotrophic growth [16]. Autotrophic joint immobilization in alginate beads of the microalgae growth-promoting bacterium (MGPB) *A. brasilense* with *Chlorella* spp. can significantly affect the physiology of several strains of the microalgae to enhance growth rate, population and lipid and pigment content [25–27,31,33] and accumulation of carbohydrates and starch (Choix et al., submitted, Part I). Because both *Chlorella* spp. and *Azospirillum* spp. can grow and interact under heterotrophic conditions [5], our study explored carbohydrate and
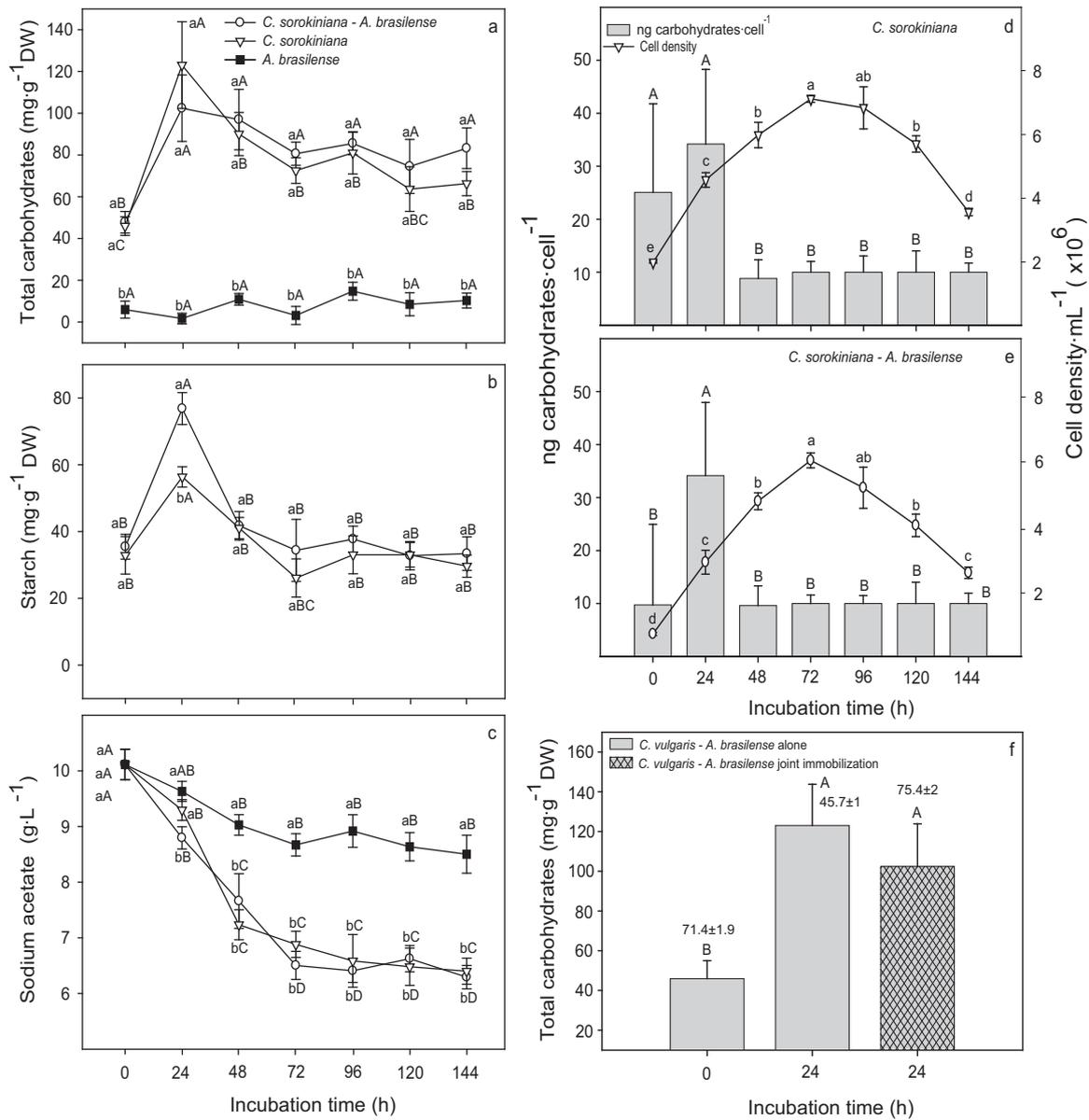
Fig. 4. (a) Accumulation of total carbohydrates, (b) accumulation of starch, (c) sodium acetate uptake, (d) growth (solid line) and accumulation of carbohydrates per cell (columns) by *Chlorella sorokiniana* immobilized alone, (e) cell density (solid line) and accumulation of carbohydrates per cell (columns) when jointly immobilized in alginate beads with *Azospirillum brasilense*. (f) Total carbohydrates (columns) and percentage of starch (values above columns) after 24 h of incubation. All experiments performed under heterotrophic conditions using sodium acetate as the carbon source. Values on curves denoted by different capital letters differ significantly using one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different lowercase letters differ significantly at $P < 0.05$ in (a and c) using ANOVA and in (b) using Student's t-test. In (d–f), columns denoted by different capital letters differ significantly by one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Bars represent SE.

Table 2
General analysis of total carbohydrates in *Chlorella* spp. jointly immobilized with *Azospirillum brasilense* in alginate beads growing in synthetic growth medium under heterotrophic conditions using sodium acetate as the carbon source.

	Affinity		Volumetric productivity		Carbohydrate yield	
	mg d^{-1}		mg 100 mL^{-1} d^{-1}		mg 100 mL^{-1} d^{-1}	
	Joint immobilization	Alone	Joint immobilization	Alone	Joint immobilization	Alone
C. vulgaris						
0–24 h	0.37 ± 0.15 aA	0.30 ± 0.09 aA	5.22 ± 0.63 aA	4.51 ± 0.47 bA	4.82 ± 0.40 aA	2.72 ± 0.97 bA
48–72 h	1.02 ± 0.18 aB	0.52 ± 0.06 bB	0.73 ± 0.17 aB	2.93 ± 0.51 bB	0.38 ± 0.07 aB	2.21 ± 0.31 bA
C. sorokiniana						
0–24 h	0.58 ± 0.13 aA	0.26 ± 0.05 aA	5.46 ± 1.09 aA	4.59 ± 0.22 aA	4.14 ± 1.03 aA	5.62 ± 1.14 aA
48–72 h	0.90 ± 0.22 aB	0.26 ± 0.19 bA	−1.63 ± 0.14 aB	−3.30 ± 0.36 bB	−1.41 ± 0.54 aB	−9.35 ± 0.49 bB

Values for each treatment and for each species of microalgae separately denoted by different capital letters differ significantly at each incubation time. Values for each comparison between solely immobilized and jointly immobilized microorganisms denoted by lowercase letters differ significantly. Statistical analyses were performed using one-way ANOVA and LSD post hoc analysis at $P < 0.05$ and Student's t-test. ± represents SE.

starch accumulation in the dark under heterotrophic condition, using D-glucose and sodium acetate as carbon sources.

We show that joint immobilization with *A. brasilense* promotes accumulation of starch in *Chlorella* spp. when grown heterotrophically. In theory, this happens because the bacterium changes several metabolic pathways of *Chlorella* spp. [26,27,31], which induces accumulation of carbohydrates. When the microalgae are grown alone, it uses the carbon source for cell multiplication, a topic that is discussed later. Additionally, de-Bashan and Bashan [31] suggested that *Chlorella* spp. responds to interaction with the MGPB *A. brasilense* in similar ways to the interactions of the bacterium with higher plants, where it enhances growth of microalga and changes its metabolism [24]. The effects of *A. brasilense* on *Chlorella* spp., either under autotrophic (Part I) and heterotrophic conditions (Part II, this study) possibly occurs from production of phytohormones by *Azospirillum* sp., such as indole-3-acetic acid (IAA). IAA in *Azospirillum* spp. is tryptophan-dependent [23] and *C. vulgaris* is known to synthesize tryptophan [36]. IAA is involved in biosynthesis of metabolites, numerous cellular mechanisms, and changes in plant and microalgae physiology, including carbohydrate metabolism [24,37]. Alternatively, there may be changes in enzymatic activity, yet to be discovered, in the metabolism of assimilation of carbon sources. The theoretical considerations are that IAA can alter the metabolism of several metabolic pathways, such as cell membrane activity [23,38] and accumulation and biosynthesis of carbohydrates (mainly starch), using D-glucose as the carbon source. The latter may occur because *C. vulgaris* incorporates D-glucose into starch without breakdown and resynthesis [39,40], as follows: oxidative assimilation of glucose yields glucose-6-phosphate, which reversibly forms glucose-1-phosphate, a molecule readily available for incorporation as starch [7,39].

4.1. Heterotrophic conditions using D-glucose as the carbon source

For decades, glucose has been the most commonly used carbon source for heterotrophic cultivation of microalgae and many other microbial species [41–43]. Probably the greatest difference in glucose metabolism in heterotrophic versus autotrophic growth of microalgae is that, under darkness, glucose is mainly metabolized via the Pentose Phosphate Pathway (PPP), while the Embden–Meyerhof Pathway (EMP) is the main glycolytic process of cells under light cultivation. For example: in complete darkness and using D-glucose as the sole carbon source, the PPP pathway in *C. sorokiniana* (formerly *C. pyrenoidosa*) accounts for 90% of glucose metabolic flux distribution and the EMP is operative to a minor extent [42]. Glucose uptake in microalgae is carried out by specific transporters. Once inside the cell under heterotrophic conditions, glucose is assimilated via the PP Pathway [44].

Immobilized alone and jointly immobilized cultures of *C. vulgaris* show similar trends in total carbohydrate and starch accumulation. Yet, when the cultures were jointly immobilized with *A. brasilense*, the amount of starch was higher. Increased production could be attributed to higher assimilation of D-glucose and yet-to-be-measured higher enzymatic activity of ADP-glucose-pyrophosphorylase, the regulator enzyme in starch biosynthesis [8].

Although jointly immobilized *C. sorokiniana* and *A. brasilense* did not induce a major increase in total carbohydrate content using D-glucose as the carbon source, *A. brasilense* induced slightly higher accumulation and biosynthesis of starch than what occurred when *C. sorokiniana* was immobilized alone but different from what was obtained using *C. vulgaris*. This could be explained because D-glucose accumulation and its metabolisms in microalgae varies greatly and depends mainly on the species of microalgae [7]. For example, Griffiths et al. [12] show that oxidative assimilation of glucose yielded other polysaccharides beside starch, such as hemicelluloses in some strains of *Chlorella* and *Scenedesmus*. Olaitan and Northcote [45] purified three polysaccharides in *C. pyrenoidosa* (=*C. sorokiniana*) [7,40]; hemicellulose A and B, and starch in a ratio of 9:2:1, respectively. There is the possibility that, in our study, *C. sorokiniana* accumulated other compounds, such as lipids, proteins, or hemicelluloses after assimilation of D-glucose, as in the case of *C. prothecoides*, which incorporated D-glucose to form mainly lipids when grown heterotrophically [46].

4.2. Heterotrophic conditions using sodium acetate as the carbon source

Acetate is one of the most common carbon sources for many microbial species, including microalgae [47]. Acetate does not always promote growth. It could be toxic to many microorganisms at high concentrations [48]. In the dark, under aerobic conditions, eukaryotic cells take up acetate using the mono-carboxylic/proton transporter protein that aids transport of monocarboxylic molecules across the membrane to form acetyl Co-enzyme A. From there, it enters the glyoxylate cycle of the cells (for details, see Fig. 1 in [7]). When used as the carbon source, *A. brasilense* did not induce a significant increase in the total carbohydrate content of *C. sorokiniana* and *C. vulgaris*.

Studies show that assimilation and metabolism of sodium acetate in several microalgae species, even in *C. vulgaris*, promotes accumulation of fatty acids and lipids [49,50]. Chen and Chen [16] demonstrated that the superiority of acetate over D-glucose for fatty acid production occurs when D-glucose is converted into acetyl-CoA, the basic building block of fatty acids synthesis. Glucose needs several steps while acetate may be directly converted into acetyl-CoA. It is possible that when both microalgae grow on acetate, other compounds, such as fatty acids and lipids, accumulate. This was not tested in our study. Yet, *A. brasilense* slightly enhanced accumulation of starch. An indirect explanation for this is that, as *Azospirillum* alters the metabolism of *C. vulgaris*, it alters the profile of fatty acid [27] and might have done so also for carbohydrate, a point that needs further investigation.

For both species of microalgae, joint immobilization with *Azospirillum* sometimes enhanced accumulation of carbohydrates, but when immobilized alone, the carbon source was used mainly for growth. The evidence for that supposition came from calculating affinity; both microalgae, when immobilized with *A. brasilense*, had a higher affinity than when they were immobilized alone, while their growth rate was higher when grown alone. Over half a century ago, Samejima and Myers [51] similarly found that, under heterotrophic conditions, the substrate that generates low growth rates in *C. sorokiniana* are consumed to support maintenance of metabolism and not growth. This higher affinity did not coincide with the higher volumetric productivity and carbohydrate yield in jointly immobilized cultures. This finding is unlike what occurs under autotrophic conditions, where the affinity, volumetric productivity, and growth rate were higher only when *Chlorella* spp. was jointly immobilized with *A. brasilense* at the same interval of time (Choix et al., Part I). A similar phenomenon was obtained when uptake of ammonium occurred under jointly immobilized cultures under heterotrophic conditions. In this case, ammonium was stored. When the bacterium was not present, ammonium was used for growth [15]. This happens because carbon and nitrogen metabolism are linked by sharing organic carbon and energy supplied from the respiration of assimilated carbon [15].

In all treatments, the significant increase in total carbohydrates and starch after 24 h per culture and per cell could be attributed to storage phenomenon; the microalgae store carbon and energy as starch, which is later remobilized to support late phases of growth [9,11]. This is probably the reason that at this time we found the

lowest growth rate and high carbohydrate yield and volumetric productivity in *Chlorella* spp. The carbohydrates and starch were already consumed when the highest microalga population was obtained after 72 h, the same time when, in all treatments, the lowest total carbohydrates per cell were detected. Brányiková et al. [18] states that *C. vulgaris*, growing under heterotrophic conditions, uses the starch almost completely during cell division, decreasing cellular dry weight by 4%.

Many reports mention that total carbohydrate and starch content in several strains of microalgae can be increase by imposing nutrient limits of nitrogen, phosphorus, and sulfur ([18,19,52], and references therein). In our study, and as also shown when cultivating occurs under autotrophic conditions (Part I, Choix et al.), the culture medium used had enough nitrogen, phosphorus, and sulfur. This demonstrated that the increase in total carbohydrates and starch in jointly immobilized *Chlorella* spp. is not due to nitrogen and phosphorus limitation in the medium, but rather due to the influence of *A. brasilense.*

Under heterotrophic conditions, the two studied strains of *Chlorella* spp., when immobilized alone, had higher populations than under joint immobilization with *A. brasilense.* As discussed earlier, under these conditions, *C. vulgaris* growing without bacterial influence consumed the organic substrate for cell multiplication, when under joint immobilization, the microalgae stored carbohydrates. Although the D-glucose provides more energy per mol compared with other substrates, growth of both microalgae using sodium acetate was higher than using D-glucose as the carbon source. Our results are in agreement with several reports where sodium acetate generated a microalgae population higher than D-glucose ([15] and references therein).

In conclusion, taken together, the results under autotrophic conditions (Part I) and under heterotrophic conditions (this investigation) demonstrates that *A. brasilense* can promote accumulation of carbohydrates in two species of *Chlorella* spp., mostly producing starch. It demonstrates that *A. brasilense* is a biological factor that can change the composition of compounds in microalgae.

Acknowledgments

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