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

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ABSTRACT

The effect of the microalgae-growth promoting bacterium *Azospirillum brasilense* on accumulation of total carbohydrates and starch in two species of *Chlorella* (*Chlorella vulgaris* and *Chlorella sorokiniana*), when the bacterium and each microalga were jointly immobilized in alginate beads was studied under autotrophic conditions for 144 h in synthetic medium. The interaction of the bacterium with the microalgae enhanced accumulation of total carbohydrate and starch. Cells of *Chlorella* accumulated the highest amounts of carbohydrate after incubation for 24 h. Yet, this did not coincide with the highest affinity and volumetric productivity measured in these cultures. However, after incubation for 72 h, mainly in jointly immobilized treatments of both microalgae species, the cultures reached their highest total carbohydrate content (mainly as starch) and also the highest affinity and volumetric productivity. These results demonstrate the potential of *A. brasilense* to affect carbohydrates and starch accumulation in *Chlorella* spp. when both microorganisms are co-cultured, which can be an important tool for applications of microalgae.

1. Introduction

Chlorella spp. are simple, immotile, unicellular, aquatic green microalgae having the potential to produce a large variety of useful compounds, such as polysaccharides, lipids, proteins, pigments, vitamins, enzymes, and pharmaceutical compounds [1,2].

Similar to any plant, *Chlorella* spp. accumulates carbohydrates as an assimilatory product of photosynthesis and intracellular storage material in several forms, such as starch, several sugars including glucose, and polysaccharides [3–8]. Specifically, under autotrophic conditions, *Chlorella vulgaris* P12 had a high capacity to accumulate starch, up to 41% of cellular dry weight [4] and another strain of *C. vulgaris* accumulated up to 55% under conditions of limited nitrogen in the growth medium [9]. The net accumulation of carbohydrates and starch can be affected by several chemical and physical factors, such as the type of light and light intensity, medium composition, and growth conditions, autotrophic and heterotrophic [3,4,10,11]. Recently, studies have concentrated on increasing the starch content in microalgae, mainly to obtain ethanol [4,9,12,13].

Azospirillum is a rhizosphere-dwelling, free-living plant growth-promoting bacterium (PGPB), capable of enhancing growth and yield of numerous plant species of agronomic and ecological significance [14,15]; including *Chlorella* spp. [16,17]. Joint immobilization (co-immobilization) of *Azospirillum* and *Chlorella* spp. in alginate beads is proposed to remove nutrients (nitrogen and phosphorus) from synthetic and real wastewater under normal [18–20] and extreme conditions of temperature and light intensity [21]. *Azospirillum brasilense* influences major enzymatic activities of the nitrogen cycle in several strains of *Chlorella* [22]. de-Bashan et al. [18] demonstrated that joint immobilization also increased the content of pigments, such as chlorophyll *a* and *b*, lutein, and violaxanthin and caused a

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significant increase in lipid content and variety of fatty acids in the cells. Considering all the benefits to the microalgae by this bacterium, the bacterium was designated as a microalgae growth-promoting bacterium (MGPB) [19]. Finally, joint immobilization was proposed as a general model to study plant–bacteria interactions [23].

The intricate ways that *Azospirillum* spp. influence several metabolic pathways of *Chlorella* spp. led to the conclusion that this bacterium might affect the formation of carbohydrates in microalgae. Consequently, we tested the hypothesis that, similar to the beneficial effects of *A. brasilense* on the metabolism of lipids and pigments in *Chlorella* spp., the interaction with this bacterium will also affect the carbohydrate and starch content in two strains of *Chlorella* spp. In this investigation, we studied the effect of *A. brasilense* on carbohydrate and starch accumulation in *Chlorella* spp. when jointly immobilized in alginate beads under autotrophic (this manuscript) and heterotrophic growth conditions (Part II, submitted simultaneously to this journal) in synthetic growth medium.

2. Materials and methods

2.1. Microorganisms and initial growth conditions

The unicellular microalgae, *C. vulgaris* Beijerinck (UTEX 2714, Austin, TX) and *Chlorella sorokiniana* Shihira et Krauss (UTEX 2805) [19,24,25] and the bacterium *A. brasilense* Cd (DSM 1843, Braunschweig, Germany) are used in wastewater treatment studies. To produce pre-cultures of the microalgae, 10 mL of axenic culture from each microalgae was separately inoculated into 90 mL sterile mineral medium (C30), which has the following composition (in g L^{-1}): KNO_3 (25), $MgSO_4 \cdot 7H_2O$ (10), KH_2PO_4 (4), K_2HPO_4 (1), $FeSO_4 \cdot 7H_2O$ (1) or (in μg L^{-1}): H_3BO_3 (2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.11), $CuSO_4 \cdot 5H_2O$ (0.09), $NaMoO_4$ (0.021) and incubated at 27 ± 2 °C, and stirred at 140 rpm under light intensity 60 μmol photon m^{-2} s^{-1} for 7 days [26]. The bacterium grew in medium BTB-2 [27] having the following composition (in g L^{-1}): NaCl (1.2), $MgSO_4 \cdot 7H_2O$ (0.25), K_2HPO_4 (0.13), $CaCl_2$ (0.22), K_2SO_4 (0.17), NH_4Cl (1), Na_2SO_4 (2.4), $NaHCO_3$ (0.5), Na_2CO_3 (0.09), Fe_{III}EDTA (0.07), tryptone (5), glycerol (8 mL), and yeast extract (5). The pH was adjusted to 7 with 1 M KOH, incubated at 32 ± 2 °C, and stirred at 120 rpm for 16 h.

2.2. Immobilization of microorganisms

Microorganisms were immobilized according to the procedure established by de-Bashan et al. [19]. Briefly, 20 mL of axenic cultures (*C. vulgaris*, *C. sorokiniana* or *A. brasilense*) were mixed separately with 2% alginate solution. Beads (3–4 mm diameter) were formed using automated equipment [28]. For joint immobilization of the two microorganisms in the same bead, after washing the cultures, each of them was resuspended in 10 mL 0.85% saline solution and then mixed in the alginate before forming the beads. Because immobilization normally reduces the number of *A. brasilense* in the beads, a second overnight incubation in diluted nutrient broth (Sigma–Aldrich, 10%, v/v) was necessary.

2.3. Experimental culture conditions

After secondary incubation, the beads were washed three times with sterile saline solution (0.85% NaCl). For experiments, 8 g beads with microorganisms immobilized and jointly immobilized were inoculated in 100 mL synthetic growth medium (SGM) containing the following (in mg L^{-1}): NaCl (7), $CaCl_2$ (4), $MgSO_4 \cdot 7H_2O$ (2), K_2HPO_4 (217), KH_2PO_4 (8.5), Na_2HPO_4 (33.4), $NaCl_4$ (191) and incubated under autotrophic conditions under light intensity of 60 μmol photon m^{-2} s^{-1}, stirred at 140 rpm, at 27 ± 2 °C (*C. vulgaris*) or 37 ± 2 °C (*C. sorokiniana*) for 144 h.

2.4. Counting microorganisms

In each experiment, three beads from flask of 250 mL were counted. Each bead was solubilized by immersion in 1 mL 4% $NaHCO_3$ solution for 30 min at ambient temperature of 25 ± 4 °C. *A. brasilense* cells were first stained with fluorescein diacetate (Sigma), as described in Chrzanowski et al. [29] and then directly counted under a fluorescent microscope (BX41, Olympus, Tokyo). *Chlorella* was counted under a light microscopy with a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD) [16]. Growth rate (μ) was defined by: $\mu = (\ln Nt_1 - \ln Nt_0)/(t_1 - t_0)$, where Nt_1 is the number of cells at sampling time and Nt_0 is the number of cells at the beginning of the experiment [30].

2.5. Analytical methods

Samples of 1 g (per replicate and per treatment, $n = 9$) of alginate beads from each treatment were taken at intervals of 24 h, washed in distilled water, dried at 80 °C for 12 h, and ground with a mortar and pestle, which yielded 10 mg samples. These 10 mg ground samples were re-suspended in 5 mL 1 M H_2SO_4 and sonicated for 4 min at 22.5 kHz with an ultrasonic cell disruptor (Misonix, Farmingdale, NY). Carbohydrates were extracted by acid hydrolysis of the slurry for 60 min at 100 °C. Total carbohydrates were quantified by the phenol–sulfuric method [31] adapted to microplates [32,33], using glucose as the standard.

Starch was quantified by the method described by Brányiková et al. [13], which is a modification of the method of McCready et al. [34], based on total hydrolysis of starch by 30% perchloric acid and quantification of liberated glucose by colorimetry.

2.6. Experimental designs and statistical analysis

The setup of all experiments was with batch cultures. Each experiment was performed in triplicate, where each 250 mL Erlenmeyer flask served as a replicate. Each setup contained 6 treatments: beads without microorganisms, beads containing *C. vulgaris*, beads containing *C. sorokiniana*, beads containing *A. brasilense* (all serving as controls) and beads containing the two jointly immobilized microorganisms (*C. vulgaris–A. brasilense* and *C. sorokiniana–A. brasilense*) ($n = 18$). The following variables were analyzed: *Volumetric productivity* = $P_1 - P_0$, where P_1 and P_0 are grams of product (as cells or biomass) in a defined volume (100 mL) between initial and final sampling. Affinity of the microalgal cells in a specific time interval was calculated as: *affinity* = S_t/N_t, where S_t is grams of product formed in 24 h and N_t is the number of microalgae cells at this time [35]. Each experiment was repeated twice. The data from each treatment from the two repetitions ($n = 36$) 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

The average content of carbohydrates in *Azospirillum* cells immobilized alone was constant and within the range of 9.7 ± 1 mg g^{-1}, regardless of the length of incubation (Fig. 1a, capital letter analysis). *C. vulgaris* immobilized alone did not accumulate a significant amount of carbohydrates. The highest content of carbohydrates was obtained after 72 h of incubation at a level of 57 ± 14 mg g^{-1}; afterward, carbohydrate content decreased with time (Fig. 1a, capital letter analysis). In joint immobilization of *C. vulgaris* and *A. brasilense*, total carbohydrates increased with time, yielding the highest content of carbohydrates after 72 h (Fig. 1a, capital letter analysis) and was significantly higher than in *C. vulgaris* immobilized alone (Fig. 1a, lower case analysis). The highest carbohydrate content was 94 ± 5.7 mg g^{-1}. Similar to microalgae immobilized alone, the content of carbohydrates from jointly immobilized experiments began to decrease, reaching the same level as in *C. vulgaris* immobilized alone after 144 h (Fig. 1a, lower case analysis). The increase in carbohydrates in both treatments after 72 h was 18 ± 1.68% in immobilized cultures and a 96 ± 1.75% increase in jointly immobilized cultures.

At the time of highest production of carbohydrates (72 h), the content of carbohydrates per cell was not the highest (17.9 ± 0.44 ng $cell^{-1}$ when immobilized alone and 27.7 ± 0.16 ng $cell^{-1}$, when jointly immobilized). The highest level of carbohydrates per cell was recorded after incubation for 24 h (37.9 ± 1.3 when immobilized alone and 43.1 ± 0.92 ng $cell^{-1}$ when jointly immobilized; Fig. 2a and b). At 24 h, accumulation of carbohydrates per culture was 47 ± 16 mg g^{-1} when immobilized alone and 72 ± 15 mg g^{-1} when jointly immobilized (Fig. 1a). No carbohydrates were detected in control alginate beads without microorganisms in any experiment. Therefore, this fact is not mentioned further.

Most carbohydrates in the microalgae were starch. Consequently, a similar pattern of accumulation of starch, over time, as was the case for total carbohydrates, was measured. Highest starch content was detected after 72 h. There were significant differences between immobilization and joint immobilization

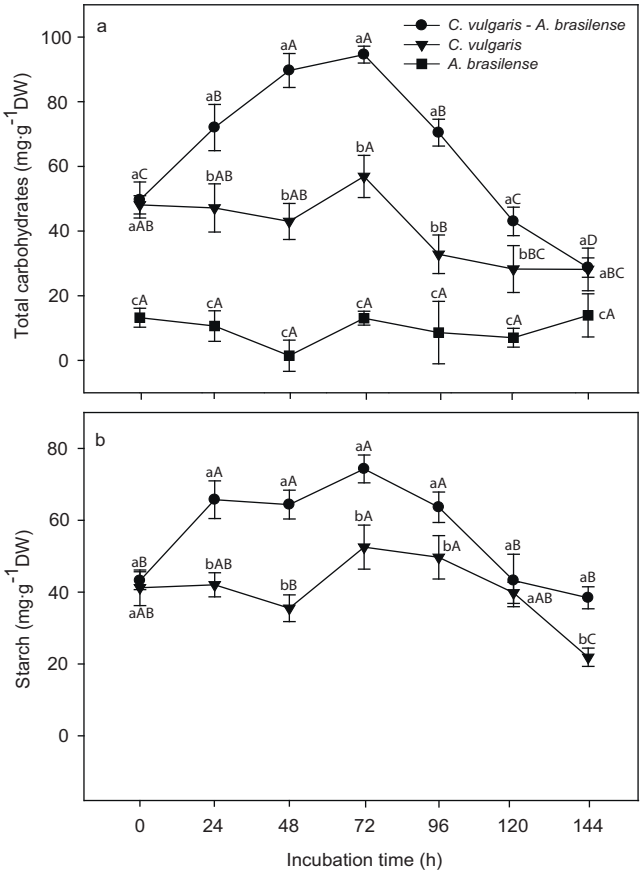

Fig. 1. Accumulation of (a) total carbohydrates and (b) starch in *C. vulgaris* immobilized singly and jointly immobilized in alginate beads with *A. brasilense* under autotrophic growth conditions. Values on curves denoted by different capital letters differ significantly using one-way ANOVA combined 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) ANOVA and in (b) *t*-test). Bars represent SE.

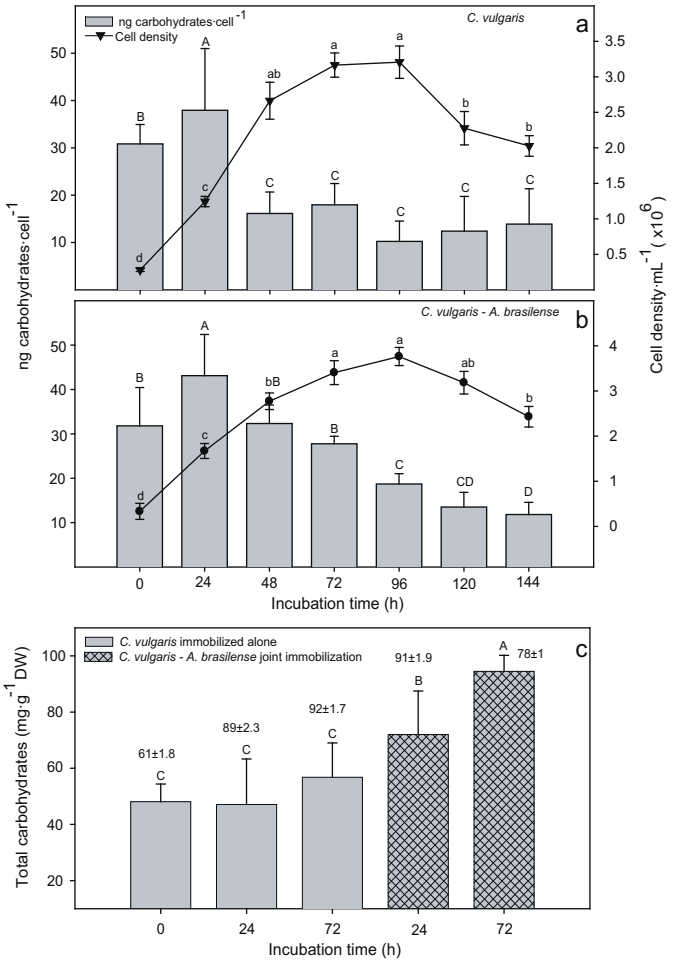

Fig. 2. Cell density and accumulation of carbohydrates per cell by *C. vulgaris* immobilized singly (a) and jointly immobilized (b) in alginate beads with *A. brasilense* under autotrophic growth conditions. (c) Total carbohydrates (columns) and percentage of starch (values above columns) after incubation for 24 h and 72 h. Values along curves denoted by different lowercase letters differ significantly using one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Columns denoted by different capital letters differ significantly by ANOVA combined with LSD post hoc analysis at $P < 0.05$. Bars represent SE.

treatments at most time intervals (Fig. 1b, lower case analysis). *C. vulgaris*, immobilized alone, reached 52 ± 12 mg g^{-1} and, jointly immobilized, reached 74 ± 8.6 mg g^{-1} of starch (Fig. 1b). This indicates that, of the total carbohydrates accumulated after 72 h, 92 ± 1.7% and 78 ± 1% are starch, respectively (Fig. 2c). The contents of starch per cell likewise showed the same pattern of total carbohydrates. The highest starch content per cell was also obtained after 24 h, 33.8 ± 0.76 ng cell^{-1}, when immobilized alone, and 39.3 ± 0.88 ng cell^{-1}, when jointly immobilized (detailed data not shown). Still, accumulation of starch per culture at this time was only 42 ± 7.7 mg g^{-1} and 57 ± 12 mg g^{-1}, respectively (Fig. 1b). At the greatest accumulation of culture after 72 h of incubation, the content of starch per cell was not the highest, reaching 16.5 ± 0.54 ng cell^{-1} when immobilized alone and 21.8 ± 0.32 ng cell^{-1} when jointly immobilized (detailed data not shown).

A similar pattern of carbohydrate and starch accumulation was found with *C. sorokiniana* (Fig. 3a, capital letter analysis). After incubation for 72 h, the highest content of carbohydrates was 63 ± 18 mg g^{-1} in microalgae immobilized alone and 98 ± 29 mg g^{-1} in jointly immobilized microalgae, showing significant differences between both treatments (Fig. 3a, lower case analysis). This increase represents 62 ± 1.84% in immobilized and 141 ± 3.80% in jointly immobilized microalgae; after 96 h, the content of carbohydrates significantly decreased and remained at this level up to 144 h (Fig. 3a, capital letter analysis).

At 24 h, the highest content of carbohydrates per cell reached 83.7 ± 4.03 ng^{-1} when immobilized alone and 68.4 ± 2.97 ng cell^{-1} when jointly immobilized (Fig. 4a and b); accumulation of carbohydrates per culture at this time was only 42 ± 2 and 55 ± 2.4 mg g^{-1}, respectively (Fig. 3a). After 72 h, the content of carbohydrate per cell declined, reaching 38.2 ± 0.88 ng cell^{-1} when immobilized alone and 46.2 ± 1.37 ng cell^{-1} when jointly immobilized (Fig. 4a and b).

This increase also was mainly of starch, reaching significant differences at 72 h between the two treatments (Fig. 3b, lower case analysis). Immobilized *C. sorokiniana* reached 57 ± 8 and 71 ± 6.8 mg g^{-1} of starch when jointly immobilized, which represents 91 ± 1% and 72 ± 1%, respectively of total accumulated carbohydrates (Fig. 4c). At this time, the contents of starch per cell were 32.6 ± 0.45 ng cell^{-1} when immobilized alone and 44.1 ± 0.42 ng cell^{-1} when jointly immobilized. At 24 h of incubation, the highest starch content per cell reached was 60.2 ± 1.59 ng cell^{-1} and 49.6 ± 0.49 ng cell^{-1} (detailed data not shown). At this time, the production of starch per culture reached was 32 ± 3.6 mg g^{-1} and 30 ± 5.2 mg g^{-1}, respectively (Fig. 3b).

Both microalgae showed the highest affinity and volumetric productivity after 72 h. There were significant differences between microalgae immobilized alone (lower value), and jointly immobilized (higher value), although in all treatments, the highest content

Table 1
General analysis of total carbohydrates in *Chlorella* spp. grown singly or with *A. brasilense* in alginate beads.

	Affinity (mg day^{-1})		Volumetric productivity (mg 100 mL^{-1} day^{-1})	
	Joint immobilization	Alone	Joint immobilization	Alone
C. vulgaris				
0–24 h	1.67 ± 1.29 aA	0.26 ± 0.02 bA	61.5 ± 4.8 aA	2.6 ± 2.0 bA
48–72 h	7.09 ± 1.33 aB	1.75 ± 1.1 bB	123.6 ± 27.9 aB	24.1 ± 3.5 bB
C. sorokiniana				
0–24 h	2.14 ± 0.13 aA	1.02 ± 0.17 bA	40.6 ± 7.8 aA	9.6 ± 5.8 bA
48–72 h	7.38 ± 2.44 aB	2.41 ± 1.22 bB	157.8 ± 10.0 aB	66.4 ± 3.3 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 singly immobilized and jointly immobilized microorganisms, denoted by lowercase letters, differ significantly. Statistical analyses were performed using one-way ANOVA and post hoc analysis at $P < 0.05$ by Student's *t*-test. (±) Represents SE.

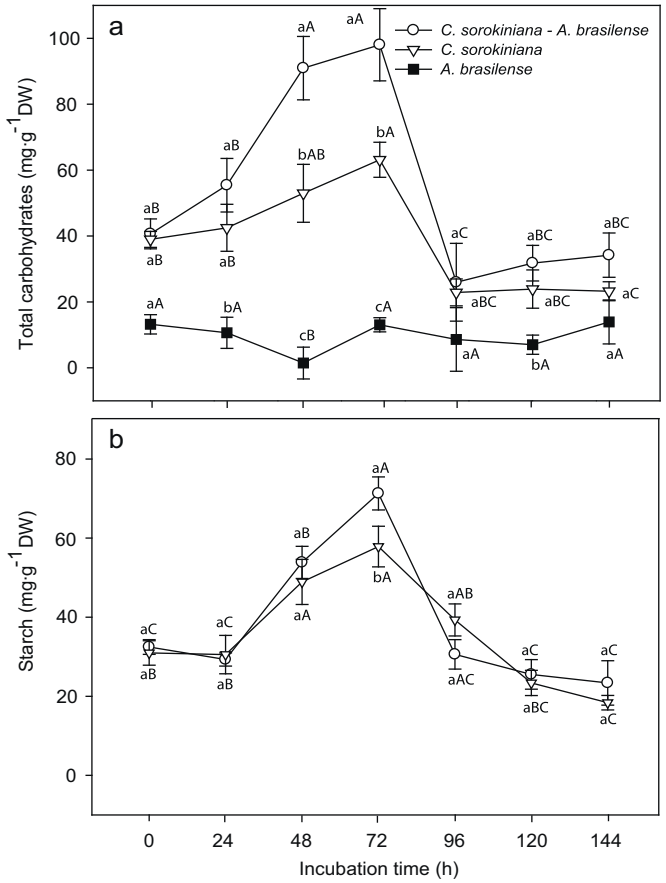

Fig. 3. Accumulation of (a) total carbohydrates and (b) starch by *C. sorokiniana* singly immobilized and jointly immobilized in alginate beads with *A. brasilense* under autotrophic growth conditions. Values on curves denoted by different differ capital letters significantly using one-way ANOVA combined 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) ANOVA and in (b) Student's *t*-test). Bars represent SE.

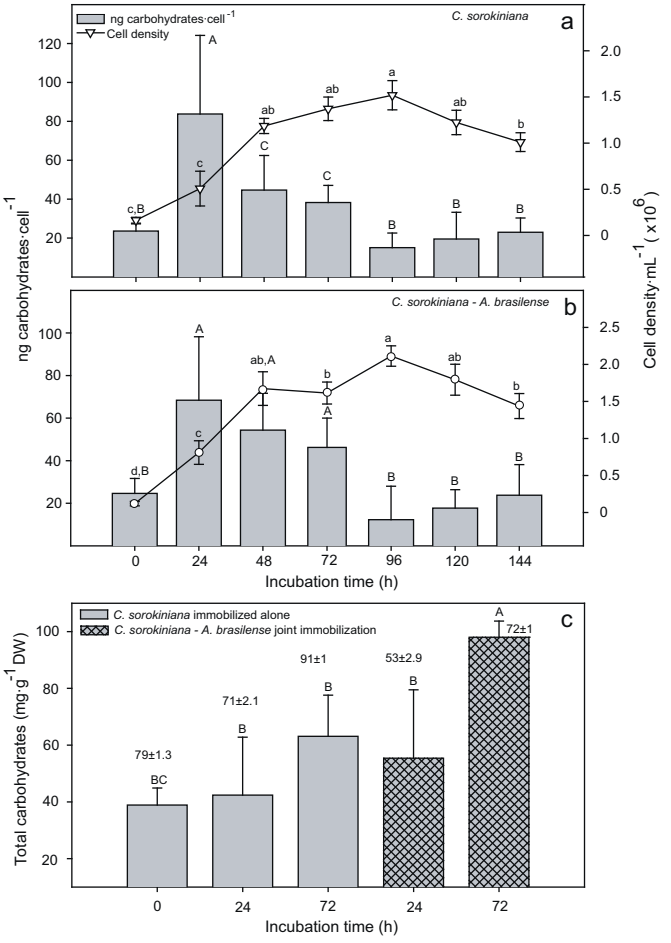

Fig. 4. Cell density and accumulation of carbohydrates per cell by *C. sorokiniana* singly immobilized (a) and jointly immobilized (b) in alginate beads with *A. brasilense* under autotrophic growth conditions. (c) Total carbohydrates (columns) and percentage of starch (values above columns) after incubation for 24 h and 72 h. Values along curves denoted by different lowercase letters differ significantly using one-way ANOVA combined with LSD post hoc analysis at $P < 0.05$. Columns denoted by different capital letters differ significantly by ANOVA combined with LSD post hoc analysis at $P < 0.05$. Bars represent SE.

of carbohydrates per cell was recorded at 24 h (Table 1). At 24 h, affinity and volumetric productivity had not reached their highest levels.

3.2. Autotrophic growth of *Chlorella spp.*

The population of *C. vulgaris* immobilized with *A. brasilense* was only slightly higher than when the microalgae were grown alone. In both treatments, the microalgae populations reached the highest growth and growth rate (μ) after incubation for 96 h. At this time, populations of joint immobilized microalgae was $3.7 \pm 0.37 \times 10^6$ cell mL^{-1} and $\mu = 0.90 \pm 0.18$ (after 72 h), while cultures immobilized alone reached $3.2 \pm 0.5 \times 10^6$ cell mL^{-1} and $\mu = 0.81 \pm 0.05$ (after 72 h). After 96 h, populations decreased with time (Fig. 2a and b).

Cultures of *C. sorokiniana* showed similar pattern. They grew to higher population when jointly immobilized. The highest growth and rate of growth was reached after incubation for 96 h when jointly immobilized, *C. sorokiniana* reached $2.11 \pm 0.26 \times 10^6$ cell mL^{-1} and $\mu = 0.97 \pm 0.026$ (after 72 h), which was significantly more than populations of microalgae immobilized alone, which was $1.51 \pm 0.26 \times 10^6$ cell mL^{-1} and $\mu = 0.72 \pm 0.11$

(after 72 h) (Fig. 4a and b). The overall growth of *C. sorokiniana* was less than *C. vulgaris*.

4. Discussion

As in all green plants, one of the most important cell components of microalgae is carbohydrates. They are used for many cell functions and cellular structures and as storage of energy. Under autotrophic conditions, green microalgae use photosynthesis to produce and accumulate carbohydrates and ATP [36]. Because *A. brasilense* induces several changes in cells of several strains *Chlorella* spp. both of growth parameters and various physiological processes [16–18,22,23], our working hypothesis was that the bacterium will also affect accumulation of total carbohydrates and starch in the species of *Chlorella* that we tested. We demonstrated that cells of either *C. vulgaris* or *C. sorokiniana*, jointly immobilized in alginate beads with *A. brasilense*, increased their content of carbohydrates; and that both microalgae presented the same pattern for accumulation of carbohydrates, mostly comprised of starch. This may happen because this bacterium is known to enhance photosynthesis [28,37] and growth parameters of numerous crops and other plant species [14,15], including *Chlorella* spp. [16,18].

Similar to enhanced production and accumulation of lipids and pigments induced by *A. brasilense* in *Chlorella* spp., enhanced accumulation of carbohydrates in microalgae is attributed to major metabolic activities related to photosynthesis of *Chlorella* spp. For example, recent studies show that one of the major effects of *Azospirillum* on metabolism of *Chlorella* is from hormonal effects, mainly by bacteria-produced indole-3-acetic acid (IAA) [17]. In many plants IAA production by *Azospirillum* can enhance photosynthesis, increase production of pigments, and synthesis of various metabolites [15], such as 3-phosphoglycerate, a key metabolite in photosynthesis. Furthermore, increase in the production of photosynthetic pigments in *Chlorella* spp. induced by *Azospirillum*, such as chlorophyll *a*, *b*, lutein, and violaxanthin [18] are considered parameters that generally coincide with enhanced photosynthesis in plants [38].

In both species of microalgae that we tested, starch was the main stored carbohydrate. Green microalgae (Chlorophyta) use starch as the primary carbon storage compound and also as an energy storage product [5], similar to many other algae and higher plants [5,6]. This indicates a link between enhancement of photosynthesis and starch accumulation. As indicated earlier, photosynthesis produces 3-phosphoglycerate. This metabolite represents a signal for high carbon and energy content within the cell. It also activates the enzyme regulating starch synthesis; ADP-glucose pyrophosphorylase [39,40]. The increase in starch in joint immobilized *Chlorella* spp. may relate to an increase in the activity of ADP-glucose pyrophosphorylase during the starch biosynthesis and accumulation and is yet to be explored.

Another reported explanation for accumulation of carbohydrates in microalgae is through depletion of nitrogen and phosphorus in the growth medium [4,9,13], where limitation of sulfur improves starch accumulation but growth of the culture stopped [13]. We did not find any of these effects in our study. Earlier studies by de-Bashan et al. [18,19,22] demonstrated that, under autotrophic conditions, jointly immobilized *Chlorella* spp. with *A. brasilense* removed all ammonium and nitrates from the medium only after ~96 h. About 30% of phosphorus was removed in most cases. In our study, the major carbohydrate content in cultures of *Chlorella* spp. was detected after a shorter period of incubation of 72 h. During this interval, the medium still contained some nitrogen and enough phosphorus. Consequently, it is more likely that accumulation of carbohydrates in our study results from the influence of *A. brasilense* and not from stress imposed by limits of nitrogen and phosphorus.

Optimum accumulation of carbohydrates by both microalgae cultures was incubation for 72 h, similar to 80 h found by Behrens et al. [9] in *C. vulgaris*. Yet, *Chlorella* spp., alone and jointly immobilized, showed the highest accumulation of carbohydrates per cell after 24 h when cell density in the culture is low; however, at this time, affinity and volumetric productivity of these cultures were less than the maximum. By comparison, incubation at 72 h, although total carbohydrates per cell were low, cultures had the highest affinity and volumetric productivity. This may happen because the density of microalgae is higher, in comparison with smaller populations at 24 h. This phenomena can also be explained in at least two other ways, probably influenced by the algal species or strain: (1) de-Bashan et al. [41] demonstrates that culturing conditions have a significant effect on the metabolism of *Chlorella* spp. Small populations can uptake large quantities of nitrogen, whereas larger populations were less efficient. Similarly, the cyanobacterium *Microcystis aeruginosa* is inversely correlates growth rate and cell content of carbohydrates [42]. (2) When microalgae cells are growing under optimal conditions, they normally perform at high photosynthetic efficiency to sustain growth and reproduction. Under these conditions, they can store reducing power in the form of starch rather than lipids. Synthesis of starch from 3-phosphoglycerate requires six molecules of NADPH and nine of ATP for each 18-carbon molecule. This is more energetically economical compared with the synthesis of lipids [5].

As demonstrated in several earlier studies and confirmed in our study, under autotrophic conditions, several strains of *C. vulgaris* and *C. sorokiniana*, reached denser populations and faster growth rates when jointly immobilized rather than immobilized alone [19,21,24,40]; however, in our study, regardless of their immobilization status, both microalgae had lower carbohydrate content after 96 h. At this time, all cultures had their densest populations. Population increase may explain the decline in carbohydrates. To sustain denser populations, the cells must synthesize others compounds, such as proteins, pigments, and lipids using the previously accumulated carbohydrates as precursors [42,43]. Li et al. [5] mention that the conversion of starch to neutral lipids in the microalgae *Pseudochlorococcum* sp. (Chlorophyceae) is one of the indispensible pathways for overall neutral lipid synthesis and accumulation in this alga. Furthermore, synthesis of cell walls, made of blocks of cellulose, is derived mainly from starch [44]. Brányiková et al. [13] showed that during cell division of autotrophic *C. vulgaris*, the starch content decrease up to 13% of its original dry weight. Therefore, it is plausible that although high cell density occurred after 96 h, it was accompanied by less total carbohydrates in each cell of this population.

It is common knowledge that different strains of *Chlorella* spp. display different behavior and accumulate compounds in variable quantities [2,30,36]. Comparisons among strains of the same species of *Chlorella* were not performed in this study or in the earlier ones done by our group. Consequently, the results of this study (parts I and II) should be attributed only to the tested strains.

In conclusion, this study demonstrates that under autotrophic conditions, *A. brasilense* enhances total carbohydrates, mainly starch, in two strains of *Chlorella* at certain times during cultivation. These results suggest that the bacterium *A. brasilense* is a biological factor that can change the composition of product compounds in microalgae.

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