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Original article

Influence of tryptophan and indole-3-acetic acid on starch accumulation in the synthetic mutualistic *Chlorella sorokiniana–Azospirillum brasilense* system under heterotrophic conditions

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This study is dedicated to the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1964–2011) of CSIC, Spain.

Abstract

This study measured the relations between tryptophan production, the phytohormone indole-3-acetic acid (IAA) and the metabolism and accumulation of starch during synthetic mutualism between the microalgae *Chlorella sorokiniana* and the microalgae growth-promoting bacteria *Azospirillum brasilense*, created by co-immobilization in alginate beads. Experiments used two wild-type *A. brasilense* strains (Cd and Sp6) and an IAA-attenuated mutant (SpM7918) grown under nitrogen-replete and nitrogen-starved conditions tested under dark, heterotrophic and aerobic growth conditions. Under all incubating conditions, *C. sorokiniana*, but not *A. brasilense*, produced tryptophan. A significant correlation between IAA-production by *A. brasilense* and starch accumulation in *C. sorokiniana* was found, since the IAA-attenuated mutant was not producing increased starch levels. The highest ADP-glucose pyrophosphorylase (AGPase) activity, starch content and glucose uptake were found during the interaction of *A. brasilense* wild type strains with the microalgae. When the microalgae were grown alone, they produced only small amounts of starch. Supplementation with synthetic IAA to *C. sorokiniana* grown alone enhanced the above parameters, but only transiently. Activity of α-amylase decreased under nitrogen-replete conditions, but increased under nitrogen-starved conditions. In summary, this study demonstrated that, during synthetic mutualism, the exchange of tryptophan and IAA between the partners is a mechanism that governs several changes in starch metabolism of *C. sorokiniana*, yielding an increase in starch content.

Keywords: *Azospirillum*; *Chlorella*; Indole-3-acetic acid; Plant growth-promoting bacteria; Synthetic mutualism; Tryptophan

1. Introduction

Mutualistic interactions, either naturally occurring or man-made (synthetic mutualism [1,2]), among different species of either microorganisms or macro-organisms, can improve the performance of the partners and are based on the exchange of resources and services [3–5]. Synthetic mutualism was artificially created between the fresh water microalgae *Chlorella vulgaris* and the microalgae growth-promoting bacterium *Azospirillum brasilense* that were co-immobilized in alginate beads [6].

Azospirillum spp. are rhizosphere-dwelling, free-living, aerobic and micro-aerophylic root-associated bacteria [7] that are highly motile in rhizosphere soil [8] and efficiently colonize roots of many plant species [9]. For decades, many

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studies have demonstrated that these bacteria induce various beneficial effects in crops, mainly on yield [for review: Ref. [7]]. *Azospirillum* employs diverse mechanisms that positively affect the performance of many plants, including the microalgae *Chlorella* spp. [10]. *Chlorella* are green, unicellular microalgae having the ability to grow autotrophically or heterotrophically [11,12]. They grow better and produce more biomass under heterotrophic conditions [13], where D-glucose and Na-acetate are the main carbon sources [14].

Each partner in this synthetic mutualism has metabolic capabilities that may prove beneficial to the other. Several studies specifically claimed that the main mechanism by which *Azospirillum* induces its beneficial effects on microalgae and higher plants is hormonal [15–19], mainly indole-3-acetic acid (IAA), that most strains produce in abundance [17,18,20]. These bacteria have three pathways for producing IAA, using tryptophan as a precursor [21]. The IAA can increase metabolism and change cell physiology and biochemistry in plants and microalgae [20,22].

Under autotrophic and heterotrophic aerobic growth conditions, this microalga accumulates large quantities of starch [23,24]. This feature has several industrial applications as thickeners and sweeteners in food [25] and production of bioethanol [26]. For these biotechnological niches, different strategies to increase starch content in microalgae were tested. These include: (a) increasing ADP-glucose pyrophosphorylase (AGPase) activity (starch synthesis regulatory enzyme) [25,27–29]; (b) obtaining overproducing mutants by genetic engineering [30]; (c) growing microalgae under nutritional stress, such as nitrogen starvation [23,24,31]; or (d) reducing the activity of the cellular starch degradation enzyme, α-amylase [25].

During the mutualistic interaction of *Chlorella* spp. and *A. brasilense*, apart from increasing the population of the microalgae [6], *Azospirillum* alters several metabolic pathways in *Chlorella* spp. These involve: (a) increasing accumulation of cell components, including pigments, lipids and a variety of fatty acids [32–34]; (b) increasing enzymatic activity of the nitrogen assimilation enzymes [35]; and (c) increasing total carbohydrates and starch content [36,37] due to an increase in activity of the starch synthesis regulatory enzyme AGPase of the microalgae [38].

To demonstrate the creation of true mutualism between the microorganisms, it is essential to show that both partners obtain benefits from the association. We specifically chose to explore the possible interaction between IAA and starch metabolism that we had previously studied [20,38], in this case, concentrating on the exchange of resources between the partners. Our hypothesis was that, during this eukaryotic–prokaryotic synthetic mutualism, tryptophan produced by the microalga induces production of IAA by the bacterium, and this IAA alters the activity of the main enzymes (AGPase and α-amylase) of starch metabolism in *Chlorella* spp. Additionally, this study was intended to provide a metabolic mechanism for earlier observations on starch metabolism and accumulation in the microalgae [36–38]. To test this hypothesis, our objectives were to: (a) measure the exchange of tryptophan and IAA when *Chlorella sorokiniana* and *A. brasilense* are co-immobilized in alginate beads; and (b) assess whether IAA is responsible for increasing the activity of AGPase and starch content in *C. sorokiniana* by testing an IAA-attenuated mutant of *A. brasilense* Sp6 as a mutualistic partner. This was tested under nitrogen-replete or nitrogen-starved conditions, when grown under aerobic, heterotrophic conditions.

2. Materials and methods

2.1. Microorganisms and initial growth conditions

The unicellular microalga *C. sorokiniana* Shih. et Krauss (formerly *C. vulgaris* UTEX 2714, University of Texas, Austin, TX; [39]) and three strains of *Azospirillum* spp. that produce different amounts of IAA were used when L-tryptophan was added. These included wild-type strain *A. brasilense* Sp6, which produces 51.18 ± 0.81 ng μL^{-1} IAA in pure culture [40], its derivative IAA-attenuated mutant *A. brasilense* SpM7918 (IAA = 15.53 ± 0.4 ng μL^{-1}) [40] and an additional wild-type strain *A. brasilense* Cd (IAA = 44.01 ± 0.17 ng μL^{-1}, DSM 1843) (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), serving as a positive control.

For cultivation of the microalgae, 10 mL pure culture of *C. sorokiniana* was inoculated into 90 mL sterile tryptophan-free mineral medium (C30), composed of (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) and (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), incubated at 27 ± 2 °C with stirring at 140 rpm under 60 μmol photon m^{-2} s^{-1} light intensity for 7 days [41]. For separate cultivation of the bacteria, BTB-2 medium was used [42], composed of (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), yeast extract (5) and glycerol (8 mL). The culture was adjusted to pH 7 with 1 M KOH and incubated at 32 ± 2 °C with stirring at 120 rpm for 16 h. These *Azospirillum* strains grow within the range of 25–42 °C.

2.2. Immobilization of microorganisms

All microorganisms were immobilized in alginate using the procedure described in de-Bashan et al. [43]. Briefly, 20 mL pure cultures (*C. sorokiniana* or *A. brasilense*) were mixed separately with 2% alginate solution. Beads (3–4 mm diameter) were formed using automated equipment [44]. To co-immobilize the two microorganisms in the same bead, after washing the cultures, each was re-suspended in 10 mL 0.85% saline solution and then mixed in the liquid alginate before forming the beads. Because immobilization normally reduces the number of *A. brasilense* in the beads [45], a second 24 h incubation was necessary, depending on the type of inoculation performed later in the experiments. This second incubation was done in diluted nutrient broth (10% v/v; N7519,

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Sigma–Aldrich, St. Louis, MO) or N-free OAB medium composed of (in g L^{-1}): KOH (4.80), malic acid (5.00), NaCl (1.20), $MgSO_4 \cdot 7H_2O$ (0.25), K_2HPO_4 (0.13), $CaCl_2$ (0.22), K_2SO_4 (0.17), Na_2SO_4 (2.40), $NaHCO_3$ (0.50), Na_2CO_3 (0.09), Fe_{III} EDTA (0.07) and (in μg L^{-1}): H_3BO_3 (0.2), $MnCl_2 \cdot 4H_2O$ (0.2), $ZnCl_2$ (0.15), $CuCl_2 \cdot 2H_2O$ (0.2), $NaMoO_4 \cdot 2H_2O$ (20) [46].

2.3. Experimental culture conditions

After secondary incubation, the beads were washed three times with sterile 0.85% saline solution. For experiments, 12 g of beads with microorganisms, either immobilized separately or co-immobilized, were inoculated into 150 mL synthetic tryptophan-free growth medium (SGM) containing (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) [47] under nitrogen-replete (sufficient N; adding 191 mg L^{-1} NH_4Cl) or nitrogen-starved conditions (without addition of NH_4Cl).

SGM was supplemented with 10 g L^{-1} D-glucose (G5767, Sigma–Aldrich) as the carbon source. This carbon source was sterilized by filtration through a 0.2 μm syringe filter (Acrodisc 28145-477, Pall Corp., Port Washington, NY). *C. sorokiniana* can grow and use D-glucose under heterotrophic conditions [14,48]. For specific treatments, SGM was supplemented with 47.5 ng μL^{-1} IAA. This amount represents the average production of IAA by the two wild-type *A. brasilense* strains that were used. All experiments were performed under heterotrophic conditions in complete darkness and stirred at 140 rpm at 27 ± 2 °C for 96 h, a comfortable temperature for both microorganisms.

2.4. Determination of microbial numbers in the beads

In each experiment, three beads from 250 mL flasks were each solubilized separately 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–Aldrich), as described in Chrzanowski et al. [49] and then directly counted under a fluorescent microscope (BX41, Olympus, Tokyo). *C. sorokiniana* cells were counted under the light microscope using a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD). Growth rate (μ) of *C. sorokiniana* was defined as: $\mu = (\ln Nt_1 - \ln Nt_0)/(t_1 - t_0)$, 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, t_1 is sampling time and t_0 the beginning of the experiment [50].

2.5. Analytical methods

2.5.1. Determination of tryptophan and IAA in exudates

Samples of 1 mL of culture media from each treatment (per replicate and per treatment, $n = 27$) were taken at intervals of 24 h. These were later filtered through a 0.22 μm membrane (GSWP02500, EMD Millipore, Billerica, MA) and analyzed by high-performance liquid chromatography (HPLC).

2.5.2. HPLC analysis

The chromatograph (Agilent 1100; Agilent Technologies, Santa Clara, CA) analyzed for tryptophan and IAA [51]. Chromatograms were analyzed and recorded with HPCHEM integrating software (G2170BA; Hewlett-Packard, Wilmington, DE).

2.5.3. Chromatographic conditions

The HPLC system was equipped with a reversed phase column (TSKgel ODS-120A, 5 μm particle size, 150 × 46 mm; Supelco, Bellefonte, PA) and was run isocratically (all analyses used the same ratio of solvents), using methanol:water:acetic acid (36:64:1 v/v) as the mobile phase. The injection volume was 100 μL and the flow rate was 0.5 mL min^{-1}. The wave length for detection was 290 nm. The standards were: tryptophan (PHR1176, Sigma–Aldrich) and IAA (I5148, Sigma–Aldrich).

2.5.4. Determination of starch content in the beads

One gram (per replicate and per treatment, $n = 27$) of alginate beads from each treatment was sampled at intervals of 24 h, washed in distilled water, dried at 80 °C for 12 h and ground with a mortar and pestle, yielding 10 mg samples. Starch was quantified by the method described by Brányiková et al. [23], which is a modification of the method described by McCready et al. [52]. The method is based on total hydrolysis of starch with 30% perchloric acid and quantification of the liberated glucose, which was measured as described below.

2.5.5. Determination of D-glucose uptake

Uptake of D-glucose from SGM by microorganisms was analyzed using the Megazyme D-glucose (glucose oxidase/peroxidase) assay kit (K-GLUC, gopod format, Megazyme International, Bray, Ireland).

2.5.6. Enzymatic activity of AGPase and α-amylase

To determine enzymatic activity of AGPase (E.C.2.7.7.27) and α-amylase (EC 3.2.11), 6 g of alginate beads from each treatment were sampled at intervals of 24 h. The beads were dissolved in 30 mL 4% $NaHCO_3$ solution and centrifuged at 2000 × *g* for 6 min. The supernatant was discarded and the pellet containing the cells was washed three times with sterile 0.85% saline solution. Three milliliters of extraction buffer was added to the pellet. The buffer contained: 50 mM HEPES (H3375, Sigma–Aldrich) at pH 7.4, 10 mM $MgCl_2$ (M2670, Sigma–Aldrich), 2 mM EDTA (E9884, Sigma–Aldrich), 20 mM β-mercaptoethanol (M6250, Sigma–Aldrich), 12.5% (v/v) glycerol (G7893, Sigma–Aldrich) and 5% (w/v) insoluble polyvinylpolypyrrolidone-40 (93H0317, Sigma–Aldrich) and maintained at 4 °C [53]. The pellet was then ground in liquid nitrogen with pestle and mortar. The extract was centrifuged at 10,500 × *g* for 10 min. The pellet was discarded and the supernatant was used for enzymatic activity analysis.

Enzymatic activity of AGPase was measured as described by Li et al. [54]. The reaction mixture contained: 100 mM HEPES (H3375, Sigma–Aldrich) at pH 7.4, 1.2 mM ADP-glucose (A0627, Sigma–Aldrich), 3 mM sodium pyrophosphate

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(S1876, Sigma–Aldrich), 5 mM $MgCl_2$ (M2670, Sigma–Aldrich) and 4 mM dithiothreitol (D0632, Sigma–Aldrich) in a final volume of 500 μL. A quantity of 500 μL of the extracted enzyme was added to the reaction mixture.

A 1 mL reaction mixture was incubated at room temperature (26 ± 2 °C) for 20 min. The reaction was stopped by heating in boiling water for 2 min. Then, 600 μL distilled water was added and the mixture was centrifuged at 13,000 × *g* for 10 min. Then, 1000 μL supernatant was mixed with 0.3 mg $NADP^+$. The activity was recorded as the increase in A_{340} after adding 2 μL of each of the two enzymes: phosphoglucomutase (0.8 U; P4109, Sigma–Aldrich) and glucose-6-phosphate dehydrogenase (1 U; G6378, Sigma–Aldrich). The enzymatic activity of AGPase is expressed as U mg^{-1} protein, where one unit is 1 nmol ADP mg^{-1} protein min^{-1}. Proteins in the mixture were determined by the method of Bradford [55]. Activity of α-amylase was determined with the amylase assay kit (E33651, Invitrogen, Carlsbad, CA), using a fluorescence imaging scanner (FMBIO, Hitachi Solutions of America, San Bruno, CA).

2.6. Experimental design and statistical analysis

The setup of all experiments was by batch cultures. Each experiment was performed in triplicate per treatment, where each 250 mL Erlenmeyer flask containing 150 mL SGM served as a replicate. Each setup contained nine treatments: (1) beads without microorganisms, (2) beads containing *C. sorokiniana*, (3) beads containing *C. sorokiniana* supplemented with synthetic IAA (48 ng μL^{-1}) in the SGM, (4) beads containing wild type *A. brasilense* Cd, (5) beads containing *A. brasilense* Sp6 and (6) beads containing IAA-attenuated mutant *A. brasilense* SpM7918. These six treatments served as controls for the three mutualistic interaction couples containing *C. sorokiniana* with one of the strains of *A. brasilense* ($n = 27$). The following variables were analyzed: volumetric productivity $(Yp) = P_1 - P_0$, where P_1 and P_0 are grams of product as cells or biomass in 100 mL measured between initial and final sampling. Affinity of the microalgal cells during a specific time interval was calculated as $(Sa) = S_t/N_t$, where S_t are grams of product formed in 24 h and N_t is the number of microalgae cells at this time [56]. Starch yield (quantity of starch produced per gram carbon source uptake of culture) was calculated as $Qs = [(P_1 - P_0)/(S_1 - S_0)]/V$, where P_1 is the quantity of starch after 24 h, P_0 is the quantity at the beginning of the time interval, S_1 is the substrate concentration (D-glucose) after 24 h, S_0 is the substrate at the beginning of the time interval and V is 100 mL of the medium [36]. Each experiment was repeated twice, each as separate experiments. Data from each treatment from the two independent repetitions ($n = 54$; $n = 6$ per each data point) were combined for analysis, first by one-way ANOVA and then by LSD post-hoc analysis. Significance was set at $P < 0.05$, using Statistica 6.0 software (StatSoft, Tulsa, OK). The Pearson correlation ($P = 0.05$) during tryptophan and IAA production by *C. sorokiniana*, co-immobilized with one of the three *A. brasilense* strains, was carried out using SAS 9.0 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Production of tryptophan by C. sorokiniana when immobilized with A. brasilense

Under nitrogen-replete or nitrogen-starved conditions, the three strains of *A. brasilense* (Cd, Sp6 and SpM7918) did not produce measurable amounts of tryptophan at any duration of incubation. Under nitrogen-replete conditions, after 96 h, all treatments of *C. sorokiniana* showed similar levels of production of tryptophan (Fig. 1a, lower case letter analysis). After 120 h, only in *C. sorokiniana* supplemented with synthetic IAA or when immobilized with the mutant *A. brasilense* SpM7918 strain did production of tryptophan increase (Fig. 1a, capital letter analysis). Tryptophan was significantly higher in the microalgae interacting with this mutant strain

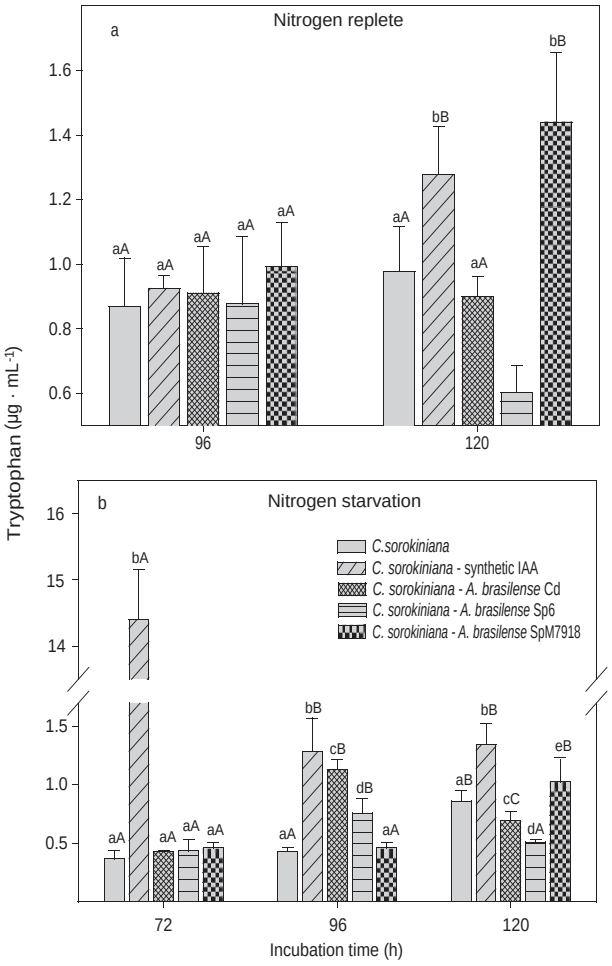

Fig. 1. Tryptophan production by *Chlorella sorokiniana*, immobilized alone or with different *Azospirillum* strains, using D-glucose as the carbon source, in alginate beads under nitrogen-replete conditions (a) and nitrogen starvation conditions (b). Columns in panel (a) denoted by different capital letters differ significantly at various sampling times, using *t*-test analysis at $P < 0.05$. Columns in panel (b) denoted by different capital letters differ significantly in different intervals at various sampling times, using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$, where values at each sampling time denoted by different lower case letters differ significantly, using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$. Whiskers represent SE.

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(Fig. 1a, lower case analysis). Within the first two sampling periods (48 h and 72 h), only microalgae supplemented with synthetic IAA or immobilized with the mutant *A. brasilense* SpM7918 produced tryptophan, reaching 1.42 ± 0.35 in the first case and 0.33 ± 0.02 μg mL^{-1} in the second case at 72 h.

Under nitrogen-starved conditions, treatments with *C. sorokiniana* produced tryptophan at 72 h (Fig. 1b); microalgae immobilized alone and supplemented with synthetic IAA had peak production of tryptophan and then declined (Fig. 1b, capital letter analysis). This amount of tryptophan was significantly higher than the tryptophan produced in all other treatments and at all time intervals (Fig. 1b, lower case letter analysis). When *C. sorokiniana* was co-immobilized with either of the wild-type strains, *A. brasilense* Cd or *A. brasilense* Sp6, it reached peak production of tryptophan at 96 h (Fig. 1b, capital letter analysis), being significantly higher when interacting with *A. brasilense* Cd (Fig. 1b, lower case letter analysis). When *C. sorokiniana* was immobilized alone or co-immobilized with the mutant *A. brasilense* SpM7918, it reached peak production of tryptophan after 120 h (Fig. 1b, capital letter analysis) and was significantly higher when the microalgae was interacting with the mutant *A. brasilense* SpM7918 strain (Fig. 1b, lower case letter analysis).

3.2. Production of IAA by A. brasilense when co-immobilized with C. sorokiniana

Under nitrogen-replete conditions, *A. brasilense* Cd co-immobilized with *C. sorokiniana* produced 0.55 ± 0.14 μg IAA mL^{-1} after 96 h; later production was lower (Fig. 2a). Under the same conditions, *A. brasilense* Sp6 reached peak production of 0.37 ± 0.04 μg IAA mL^{-1} after 120 h (Fig. 2b). *A. brasilense* Cd, when immobilized alone, reached peak production of IAA at 24 h (2.68 ± 0.47 μg IAA mL^{-1}); *A. brasilense* Sp6 reached peak production of IAA at 48 h (2.48 ± 0.53 μg IAA mL^{-1}). The IAA-attenuated *A. brasilense* SpM7918 strain did not produce IAA when grown alone or when co-immobilized with *C. sorokiniana* (Fig. 2c).

Under nitrogen-starved conditions, *A. brasilense* Cd or *A. brasilense* Sp6, co-immobilized with *C. sorokiniana*, produced IAA that peaked after 48 h. At 72 h, the treatment with co-immobilized *A. brasilense* Cd reached peak production of IAA followed by a decline in production of IAA (Fig. 2d). Similarly, at 96 h, co-immobilized *A. brasilense* Sp6 reached peak production, followed by a decline in production (Fig. 2e). With or without nitrogen, the mutant *A. brasilense* SpM7918, co-immobilized with *C. sorokiniana*, did not produce any measurable quantity of IAA by HPLC analysis at any sample time (Fig. 2c and f). Similar results occurred when *C. sorokiniana* was immobilized alone or when supplemented with synthetic IAA at time zero (data not shown). After incubation for 24 h, HPLC analysis did not detect IAA in the culture of *C. sorokiniana* that was immobilized alone or when supplemented with synthetic IAA. Presumably, *C. sorokiniana* consumed the synthetic IAA and did not produce any.

Independently of the nitrogen condition or wild type strains, IAA production by *A. brasilense* was highly correlated with tryptophan production by *C. sorokiniana*. Under nitrogen-replete conditions, production of IAA by *A. brasilense* Cd or Sp6 was significant ($P = 0.007$ and 0.032, respectively) with production of tryptophan by *C. sorokiniana* (Fig. 2a and b). When cultured without nitrogen, the correlation was highly significant for *A. brasilense* Sp6 ($P = 0.007$), but not for *A. brasilense* Cd ($P = 0.388$). However, similar patterns in production of tryptophan and IAA production were found (Fig. 2d and e). Under either nitrogen condition, IAA production of the IAA-attenuated mutant *A. brasilense* SpM7918 did not correlate with tryptophan production by *Chlorella* because of its incapacity for efficient IAA biosynthesis (Fig. 2c and f).

3.3. Production of starch under nitrogen-replete condition

3.3.1. Enzymatic activity of AGPase in C. sorokiniana when co-immobilized with A. brasilense

Whether growing under either nitrogen condition, AGPase activity was not detected in the three *Azospirillum* strains (Cd, Sp6, SpM7918) at any sampling time. Under nitrogen-replete conditions, *C. sorokiniana*, immobilized alone, reached peak AGPase activity after 96 h and remained at this level (Fig. 3a, capital letter analysis). When supplemented with synthetic IAA, peak activity of *C. sorokiniana* occurred at 48 h and then declined and remained at the lower level (Fig. 3a, capital letter analysis). At 48 h, AGPase activity in microalgae supplemented with synthetic IAA was significantly higher than in microalgae that were not supplemented. At longer incubation times, the opposite occurred, indicating a transient effect of supplemental IAA (Fig. 3a, lower case analysis).

Peak AGPase activity in *C. sorokiniana*, co-immobilized with the IAA-attenuated mutant *A. brasilense* SpM7918, occurred after 72 h. This activity was significantly higher than when *C. sorokiniana* was cultivated alone or supplemented with synthetic IAA (Fig. 3a, lower case letter analysis); then this activity decreased (Fig. 3a, capital letter analysis). Peak activity of AGPase in *C. sorokiniana* was obtained when it was co-immobilized with either of the two wild-type strains (*A. brasilense* Cd or Sp6), which was significantly higher than the other treatments at all sampling times after 48 h (Fig. 3a, lower case letter analysis). Peak AGPase activity in *C. sorokiniana*, when co-immobilized with *A. brasilense* Cd, was after 48 h and remained in this range for up to 96 h; activity then declined (Fig. 3a, capital letter analysis). When interacting with *A. brasilense* Sp6, AGPase activity peaked after 48 h, decreased at 72 h and remained at the lower level (Fig. 3a, capital letter analysis). At most sampling times, AGPase activity in *C. sorokiniana*, when immobilized with *A. brasilense* Cd, was higher than when immobilized with *A. brasilense* Sp6. There were significant differences between the strains of *A. brasilense* at most sampling times (Fig. 3a, lower case analysis).

3.3.2. Enzymatic activity of α-amylase of C. sorokiniana when co-immobilized with A. brasilense

Under nitrogen-replete conditions, peak α-amylase activity occurred at 24 h in all treatments (Fig. 5a, capital letter

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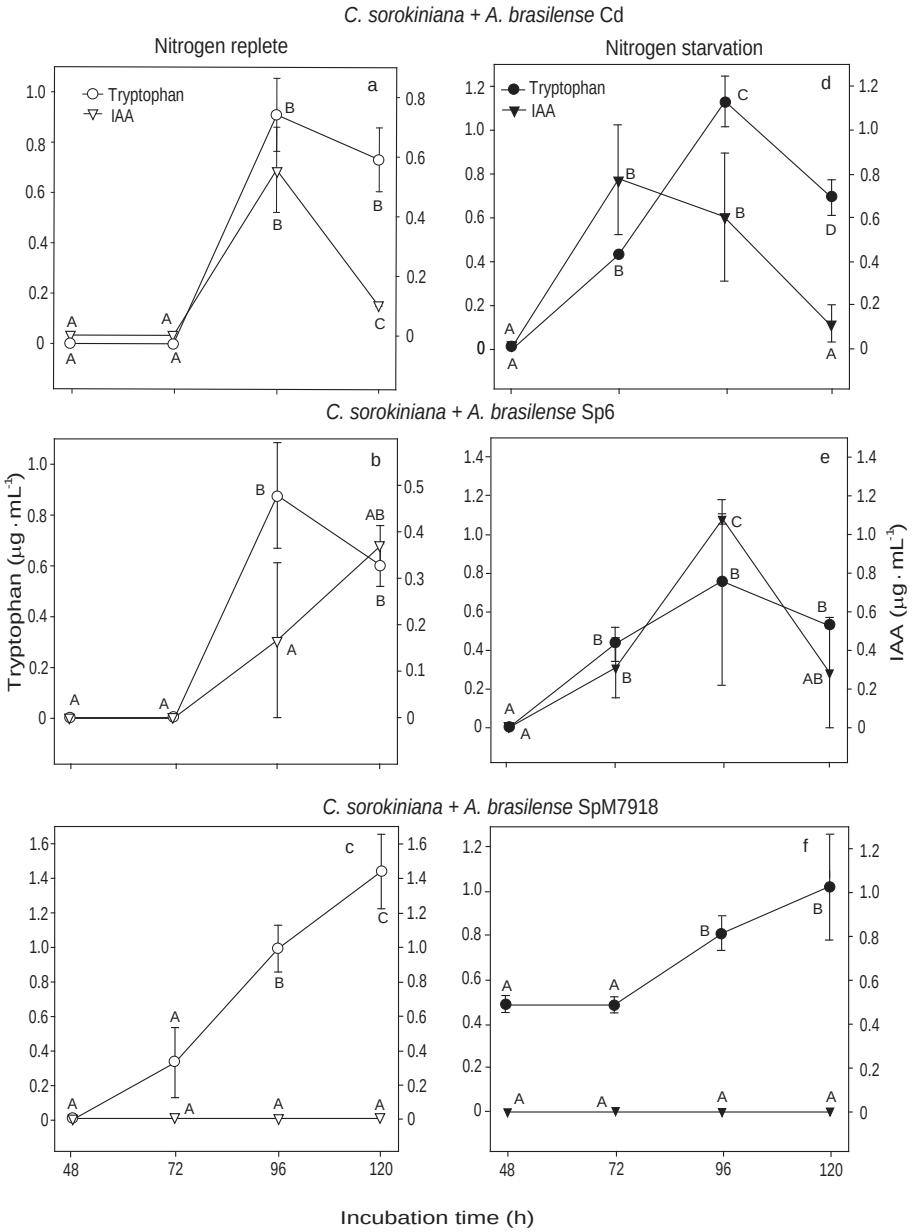

Fig. 2. IAA (triangle) and tryptophan (circle) production, using D-glucose as the carbon source by *Chlorella sorokiniana* and *Azospirillum* strains, co-immobilized in alginate beads under nitrogen-replete (a, b, c) and nitrogen-starved (d, e, f) conditions. Values along curves denoted by different capital letters differ significantly, using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$. Whisker lines represent SE. Absence of a whisker line indicates negligible SE.

analysis). At 24 h, the highest α-amylase activity was obtained with *C. sorokiniana* co-immobilized with *A. brasilense* Sp6. This was significantly higher than the other treatments (Fig. 5a, lower case analysis). Activity of α-amylase in *C. sorokiniana*, immobilized alone or supplemented with synthetic IAA, was similar and low (Fig. 5a, lower case letter analysis). When *C. sorokiniana* was immobilized with the mutant *A. brasilense* SpM7918, α-amylase activity was slightly lower. The lowest activity occurred when *C. sorokiniana* was co-immobilized with *A. brasilense* Cd. α-Amylase activity decreased in all treatments (Fig. 5a, capital letter analysis) after 24 h, except when co-immobilized with *A. brasilense* SpM7918. In this instance, it decreased at 48 h and then increased later (Fig. 5a, capital letter analysis).

3.3.3. Starch accumulation in *C. sorokiniana* when co-immobilized with *A. brasilense*

Under nitrogen-replete conditions and growing on minimal medium, starch content in *C. sorokiniana*, when immobilized alone, was the lowest of any treatment. When co-immobilized with the mutant *A. brasilense* SpM7918, it produced twice as much starch, but still in low quantities at all sampling times (Fig. 3b, capital letter analysis). Supplemented with exogenous synthetic IAA, *C. sorokiniana* recorded peak starch content at 48 h. This quantity was significantly higher than the two

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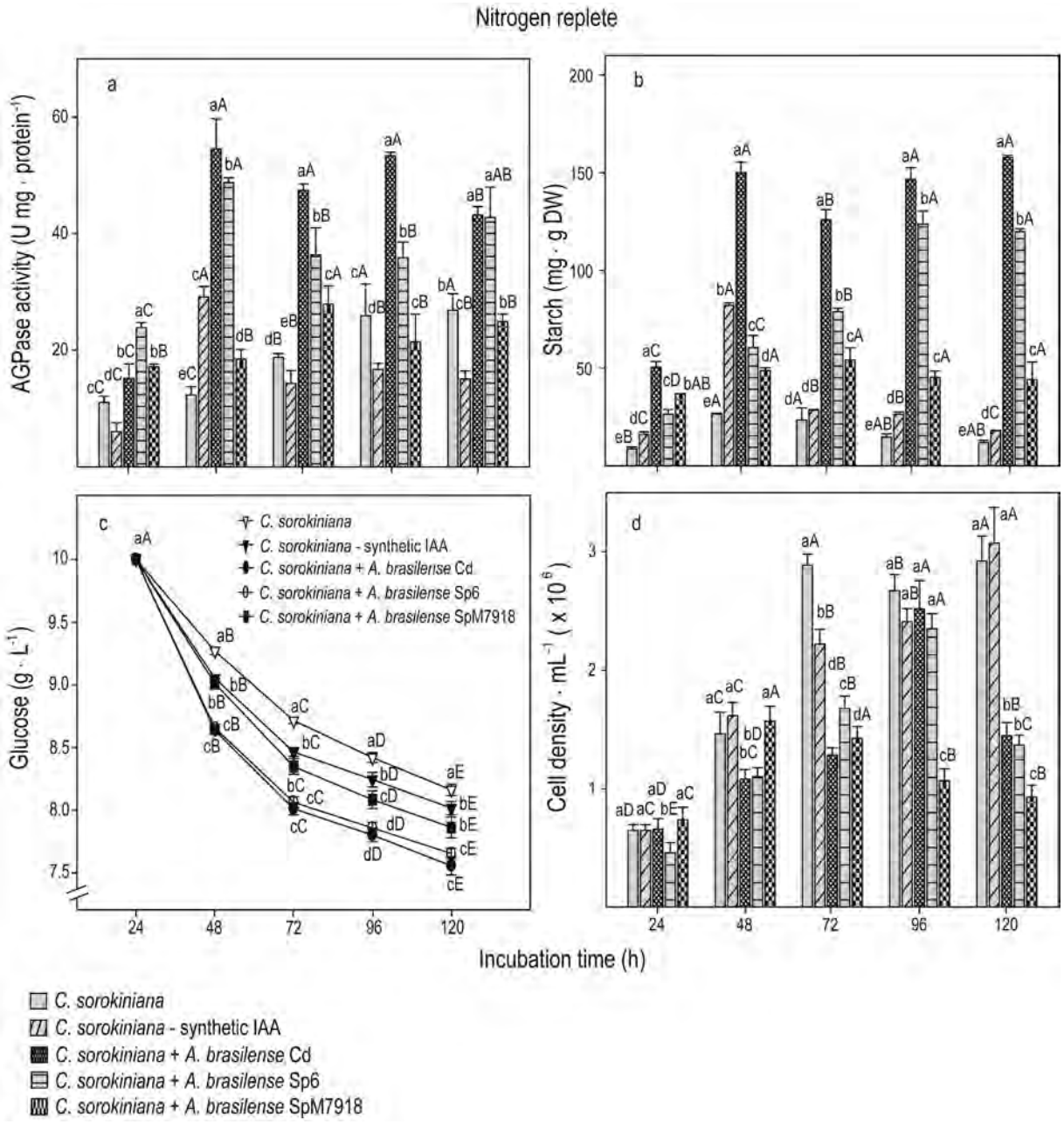

Fig. 3. AGPase activity (a), starch content (b), glucose uptake (c) and cell density (d) of *Chlorella sorokiniana* immobilized alone and co-immobilized with different *Azospirillum* strains under nitrogen-replete conditions. Columns denoted by different capital letters differ significantly at various sampling times, where values at each sampling time that is denoted by different lower case letters differ significantly, using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$. Values along 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 sampling time denoted by different lower case letters differ significantly at $P < 0.05$. Whisker lines represent SE. Absence of a whisker line indicates negligible SE.

experimental treatments mentioned previously. Starch production declined at later sampling times (Fig. 3b, capital letter analysis). The highest starch content occurred when *C. sorokiniana* was immobilized with the wild-type *A. brasilense* Cd or *A. brasilense* Sp6, reaching peak starch content at 48 h and 96 h, respectively, and then remained at these levels (Fig. 3b, capital letter analysis), which were significantly higher than for all the other treatments (Fig. 3b, lower case letter analysis for each cluster of columns). The highest volumetric productivity (*Yp*) and starch yield (*Qs*) occurred when *C. sorokiniana* was immobilized with *A. brasilense* Cd (Table 1).

3.4. Production of starch under nitrogen-starved condition

3.4.1. Activity of AGPase of C. sorokiniana when co-immobilized with A. brasilense

When grown under nitrogen-starved conditions, AGPase activity was significantly higher in *C. sorokiniana* receiving synthetic IAA than when grown alone after 48 h (Fig. 4a, lowercase analysis). With time, this activity decreased only in microalgae that were supplemented with IAA. In microalgae immobilized alone, AGPase activity continued to increase up

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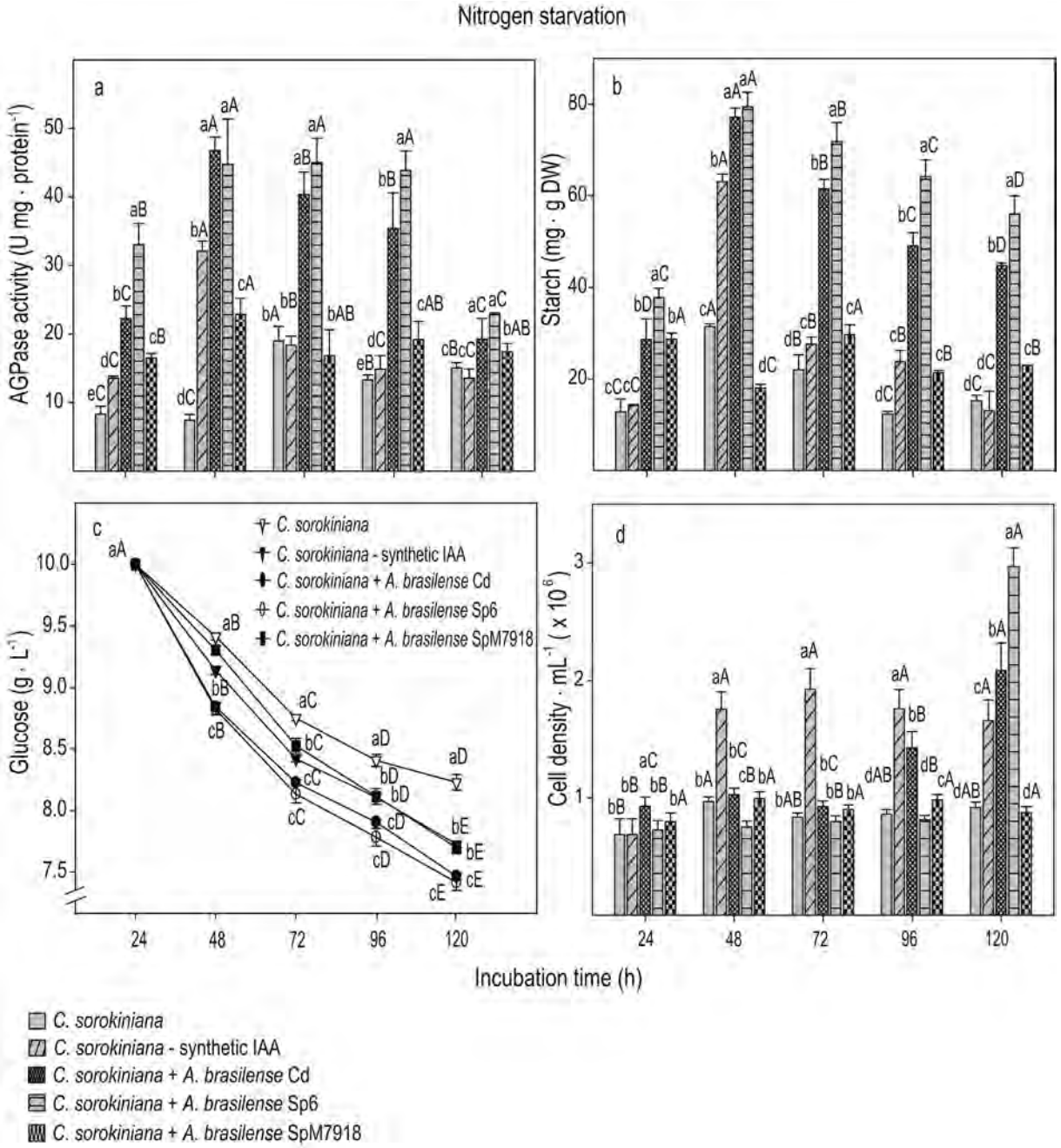

Fig. 4. AGPase activity (a), starch content (b), glucose uptake (c) and cell density (d) of *Chlorella sorokiniana*, when immobilized alone and when co-immobilized with different *Azospirillum* strains under nitrogen-starved conditions. Columns denoted by different capital letters differ significantly at various sampling times, where values at each time interval denoted by different lower case letters differ significantly, using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$. 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 sampling time denoted by different lower case letters differ significantly at $P < 0.05$. Whisker lines represent SE. Absence of a whisker line indicates negligible SE.

to 72 h and latter decreased. Yet, it was still higher than AGPase activity at 24 or 48 h (Fig. 4a, capital letters analysis). In *C. sorokiniana* that was co-immobilized with *A. brasilense* SpM7918, the AGPase activity was relatively low at 48 h and did not increase with time (Fig. 4a, capital letters analysis). Yet, when interacting with *A. brasilense* Cd or *A. brasilense* Sp6, the highest AGPase activity was detected at 48 h. Activity remained in this range up to 96 h for strain Sp6 (Fig. 4a, capital letters analysis) while, when co-immobilized with *A. brasilense* Cd, it decreased over time (Fig. 4a, capital letters analysis). After 120 h, the effect of the two wild-types diminished (Fig. 4a, lower case analysis).

3.4.2. Enzymatic activity of α-amylase of *C. sorokiniana* when co-immobilized with *A. brasilense*

A different pattern occurred when *C. sorokiniana* was cultured under nitrogen-starved conditions. In general, but not in all treatments at every sampling time, activity of α-amylase

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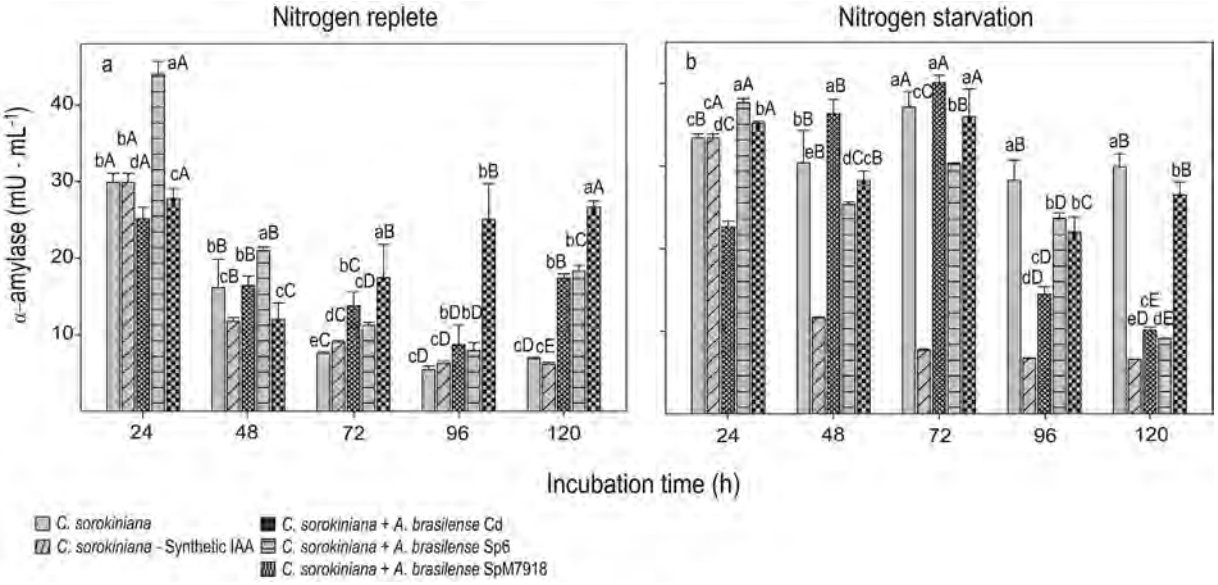

Fig. 5. α-Amylase activity in *Chlorella sorokiniana*, immobilized in alginate beads alone or co-immobilized with different *Azospirillum* strains under nitrogen-replete conditions (a) and nitrogen-starved (b) conditions, using D-glucose as the carbon source. Columns denoted by different capital letters differ significantly at various sampling times, where values at each time interval denoted by different lower case letters differ significantly, both using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$. Whisker lines represent SE. Absence of a whisker line indicates negligible SE.

Table 1
Production of starch by *C. sorokiniana* immobilized with *A. brasilense* wild-type strains and IAA-attenuated mutant strain in alginate beads after 24 h incubation, growing heterotrophically under nitrogen-replete or nitrogen-starved conditions and using D-glucose as the carbon source.

	Affinity (*Sa*) (mg h^{-1})		Volumetric productivity (*Yp*) (mg L^{-1} h^{-1})		Starch yield (*Qs*) (mg L^{-1})	
Alone						
	−N	+N	−N	+N	−N	+N
C. sorokiniana	0.02 ± 0.003^{d}	0.024 ± 0.002^{d}	6.67 ± 1.19^{d}	7.25 ± 1.29^{d}	6.89 ± 0.93^{d}	4.95 ± 0.52^{d}
C. sorokiniana with synthetic IAA	0.032 ± 0.004^{c}	0.026 ± 0.005^{c}	12.85 ± 2.4^{c}	27.66 ± 1.32^{b}	11.34 ± 0.23^{b}	7.92 ± 1.33^{c}
Co-immobilized						
C. sorokiniana with *A. brasilense* Cd	0.04 ± 0.017^{b}	0.052 ± 0.015^{a}	17.47 ± 1.3^{b}	42.01 ± 2.24^{a}	12.56 ± 2.18^{b}	38.83 ± 2.03^{a}
C. sorokiniana with *A. brasilense* Sp6	0.065 ± 0.023^{a}	0.05 ± 0.01^{b}	19.86 ± 5.79^{a}	18.62 ± 2.44^{c}	23.15 ± 1.65^{a}	17.09 ± 1.41^{b}
C. sorokiniana with *A. brasilense* SpM7918	0.035 ± 0.017^{c}	0.025 ± 0.007^{c}	4.97 ± 0.5^{e}	8.04 ± 1.71^{d}	5.51 ± 1.08^{d}	5.11 ± 1.61^{d}

Values in each column denoted by different letters significantly differ, using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$. ± denotes SE.

for the first 72 h was higher than activity obtained under nitrogen-replete conditions. *C. sorokiniana*, alone and co-immobilized with *A. brasilense* Cd or *A. brasilense* SpM7918, had peak activity at 72 h, although there were no significant differences among them at this sampling time (Fig. 5b, lower case letter analysis). These treatments had significantly higher production than the other treatments (Fig. 5b, lower case letter analysis) at this sampling time. Activity of α-amylase in *C. sorokiniana*, when supplemented with synthetic IAA or interacting with *A. brasilense* Sp6, peaked at 24 h and then declined, especially when supplied with synthetic IAA (Fig. 5b, capital letter analysis).

3.4.3. Starch accumulation in C. sorokiniana co-immobilized with A. brasilense

When nitrogen-starved, accumulation of starch was significantly reduced to about half in all treatments. However, the same pattern of accumulation of starch as under the nitrogen-replete condition, was conserved, but with small modifications. *C. sorokiniana*, when immobilized alone, produced the smallest amount of starch of all treatments, with peak production at 48 h, and then declined. When *Chlorella* received supplemental synthetic IAA, this temporarily enhanced starch accumulation and peaked at 48 h, followed by declining production; this was similar to the treatment receiving nitrogen (Fig. 4b, capital letter analysis). Peak accumulation of starch occurred in *C. sorokiniana* at 48 h when co-immobilized with *A. brasilense* Cd or *A. brasilense* Sp6, and then declined (Fig. 4b, capital letters analysis). This quantity of starch in *C. sorokiniana*, co-immobilized with either wild-type bacteria, was significantly higher than the content of starch in all other treatments and at all sampling times (Fig. 4b, lower case letter analysis of each cluster of columns). Accumulation of starch in *C. sorokiniana* was significantly higher at most sampling times, when co-immobilized with *A. brasilense* Sp6, compared with *A. brasilense* Cd (Fig. 4b, lower case letter analysis). When co-immobilized with attenuated *A. brasilense* SpM7918,

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accumulation of starch in *C. sorokiniana* was much lower (Fig. 4b, capital letter analysis). At 48 h, volumetric productivity (*Yp*) and starch yield (*Qs*) were significantly higher in *C. sorokiniana* when immobilized with the wild-type *A. brasilense* Sp6 than in the other treatments (Table 1).

3.4.4. Uptake of D-glucose in C. sorokiniana

Growing under either nitrogen condition, *C. sorokiniana* continuously consumed D-glucose in all treatments (Figs. 3c and 4c, capital letter analysis). Cultured alone, the lowest quantity of D-glucose was consumed. When *Chlorella* received supplemental IAA or was co-immobilized with the IAA-attenuated mutant *Azospirillum*, *Chlorella* consumed larger quantities of D-glucose. When cultured with the wild-type strains (*A. brasilense* Cd and Sp6), glucose consumption was significantly higher at most sampling times (Figs. 3c and 4c, lower case letter analysis); however, there was no significant difference between these two treatments (Figs. 3c and 4c, lower case letter analysis). The affinity of glucose (*Sa*), under either nitrogen condition, was also significantly higher in *Chlorella* when it was immobilized with either of the two wild-type strains than in the other treatments (Table 1).

3.4.5. Growth of both microorganisms when co-immobilized under heterotrophic conditions

In general, co-immobilization reduced the population of *Chlorella* in most treatments and at most sampling times (Figs. 3d and 4d). Supplementation with IAA enhanced population growth only under starvation conditions. This was true whether the cells were counted or the growth rate (μ) was calculated (Table S1, Supplementary data). Growth of the three *Azospirillum* strains under both nitrogen conditions was variable (Fig. S1, Supplementary data).

4. Discussion

Mutualistic interactions among different species are based on the exchange of resources and services [3,4,57]. The ability of *Chlorella* spp. to produce tryptophan, and of *Azospirillum* spp. to synthesize IAA from this tryptophan, can be considered as a major exchange of resources during synthetic mutualism of *C. sorokiniana* and *A. brasilense*. We chose to explore the exchange of tryptophan and IAA during mutualistic interaction on a metabolic pathway of *C. sorokiniana* that we had previously established – starch metabolism. Earlier, Choix et al. [38] demonstrated that *A. brasilense* increased the activity of the starch regulatory enzyme AGPase, and this increased the quantity of starch in *Chlorella* spp. under autotrophic and heterotrophic conditions [36,37]. The objectives of continuing this line of work were to assess whether IAA, produced by *A. brasilense* and induced by tryptophan production by the microalgae, is a mechanism affecting the main enzymes (AGPase and α-amylase) of starch metabolism in *C. sorokiniana*. Increasing accumulation of starch in microalgae, and high growth rates under heterotrophic conditions, are of major importance for several biotechnological applications, which are central to biofuel production.

We demonstrated that *C. sorokiniana* always exudes tryptophan. Others studies demonstrated that higher plants and other microalgae (not *Chlorella*) exude tryptophan [58,59]. Additionally, the *mma7* gene codifies tryptophan synthase, which participates in tryptophan biosynthesis, was isolated from the Chlorophyta *Chlamydomonas reinhardti* [60], the taxonomic group to which *Chlorella* spp. belongs. Possibly, this gene could be found in the *Chlorella* genome once it is fully sequenced.

Under both nitrogen conditions and without adding tryptophan to the medium, a high correlation was found between tryptophan production in *C. sorokiniana* and IAA production in wild-type *A. brasilense* strains (Cd and Sp6). This demonstrated that, during this mutualistic interaction, *C. sorokiniana* provides tryptophan to *A. brasilense* and these bacteria convert it to IAA. This hypothesis is supported by our finding that the IAA-attenuated mutant *A. brasilense* SpM7918 strain did not produce IAA in measurable quantities, although *C. sorokiniana* exuded tryptophan to the medium where it grew.

Biosynthesis of IAA by *Azospirillum* spp. from tryptophan is well-known [22]. These bacteria have several proven tryptophan-dependent pathways and a proposed tryptophan-independent pathway [21,51,61]. It was also demonstrated that IAA enhanced exudation of amino acids, including tryptophan, from root cells in *Sorghum bicolor* [62]. Enhancement of tryptophan exudation also occurred in our experiments. This may happen because: (1) we found a highly significant correlation between tryptophan and IAA production by *C. sorokiniana* and wild-type *Azospirillum* strains, but not with the IAA-attenuated mutant; and (2) *C. sorokiniana* supplemented with synthetic IAA induced the highest tryptophan synthesis, although this increase was transient. The mechanism for the latter decline in enhancement is still under investigation.

AGPase activity in higher plants and in the microalga *Pseudochlorococcum* sp. [53] is usually directly correlated with starch accumulation [25,63]. Under all our experimental conditions, the highest AGPase activity occurred when the microalgae were interacting with wild-type strains of *Azospirillum*. In contrast, when interacting with the IAA-attenuated mutant strain, the activity of this enzyme remained low. Hence, peak starch yield (*Qs*) and volumetric productivity (*Yp*) were obtained when *C. sorokiniana* was co-immobilized with the wild-type strains of *A. brasilense* after 24 h. Collectively, these facts indicate that IAA produced by *A. brasilense*, in response to tryptophan exudation by *C. sorokiniana*, can be involved in increasing starch metabolism in *C. sorokiniana*, which is supported by findings that IAA usually alters the metabolism of *Chlorella* spp. [20,64]. Earlier, two plausible ways to increase AGPase activity by IAA production by *A. brasilense* Cd (employed as the positive control in this study) were hypothesized [38]. Briefly, IAA directly affects or regulates the activity of AGPase, or IAA increases absorption of glucose that may later translate, via AGPase, into higher starch accumulation by the following mechanism. *Chlorella* cells possess an inducible, active, hexose/H^+ symport membrane protein system. The symport system is responsible for uptake of glucose from the medium under heterotrophic conditions [65]. If IAA affects the

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symport of *C. sorokiniana*, in a yet-to-be-discovered way, this may cause greater absorption of glucose. This may also load each cell with more energy and carbon. Synthesis of reserve polysaccharide in bacteria and plants is greatest when cellular carbon and energy are in excess [63]. Thus, this state of carbon/energy excess enhances AGPase activity, which directly leads to accumulation of starch. This study provides evidence for these arguments as a plausible cellular metabolism operating in this synthetic mutualism. It shows that when synthetic IAA was added to *C. sorokiniana* cultures, AGPase activity increased only after 24 h and decreased later. It did not reach the same level of activity as when *C. sorokiniana* interacted with the wild-type *A. brasilense* strains (Cd and Sp6), since both continuously supplied IAA in abundance. The continuous effect on the carbohydrate metabolism of *C. sorokiniana* is further explained by the fact that *Azospirillum* spp. possess several potential mechanisms that can alter the metabolism of microalgae, not only IAA production. Taken together, a stronger effect on carbohydrate metabolism was constantly recorded. Further, involvement of IAA in *C. vulgaris* with other cellular mechanisms occurs when IAA acts as an enhancer toward other cellular mechanisms and other hormones [66].

Under nitrogen-replete conditions, activity of the starch-degrading enzyme α-amylase was lower than when cultured under nitrogen-starved conditions. It is well known that limited or absent nitrogen affects several metabolic pathways in microorganisms, and not only nitrogen metabolism. For example, limited nitrogen upregulates genes related to carbohydrate metabolism, such as α-amylase in the rice blast pathogen *Magnaporthe grisea* [67]. In rice, barley and *Arabidopsis thaliana*, deprivation of a single nutrient such as nitrogen or phosphate cross-activates expression of α-amylase and other hydrolases responsible for coordinated mobilization of the full complement of different nutrients [68,69]. Under nitrogen starvation, the Chlorophyceae *Pseudochlorococcum* sp. and *C. vulgaris* used starch as the main carbon source to produce neutral lipids as secondary storage products [54,70]. During normal nitrogen conditions for starch accumulation, α-amylase activity was undetectable in *C. vulgaris* cells, but when nitrogen was not available, α-amylase activity increased [71]. In our study, when *C. sorokiniana* was co-immobilized with the wild type strains of *A. brasilense* under nitrogen starvation, α-amylase activity declined concomitantly with increasing production of IAA by *A. brasilense*. It is known that IAA reduces α-amylase activity in plants and fruit [72]. Inhibition of α-amylase activity by IAA can be explained by the fact that IAA has the ability to form strong hydrogen bonds with the active site of the enzyme [73]. This deactivates the enzyme's active site and results in greater starch accumulation in higher plants [74,75]. This is likely to happen in *C. sorokiniana*, which has a similar starch metabolism.

Under both nitrogen conditions, glucose uptake was also higher in *C. sorokiniana* interacting with the wild-type *Azospirillum* spp. and not with the IAA-attenuated mutant. This finding is supported by glucose affinity (*Sa*), that shows that the highest affinity occurs during the interaction with the wild type strains of *A. brasilense*. This may be explained by an effect on the cell membrane. Previous studies demonstrated that *A. brasilense* Cd alters the activity of cell membranes of plants [76], and this increases glucose uptake in *C. vulgaris* [36]. The ability of IAA to alter the metabolism of higher plants and microalgae is tied to an electrical and structural combination, which can be identified by membrane transporters in *Chlorella* [66]. *Chlorella* spp. has orthologous genes that codify for membrane transporters of auxins, mainly IAA [64,77].

In summary, this study demonstrated that, during synthetic mutualistic interaction of *C. sorokiniana* with *A. brasilense*, mutual support (feedback) of tryptophan and IAA by the two microorganisms is a mechanism that generates several changes in starch metabolism in *C. sorokiniana*, leading to increased starch content.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.02.005.

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