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Enhanced activity of ADP glucose pyrophosphorylase and formation of starch induced by *Azospirillum brasilense* in *Chlorella vulgaris*

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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.

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ABSTRACT

ADP-glucose pyrophosphorylase (AGPase) regulates starch biosynthesis in higher plants and microalgae. This study measured the effect of the bacterium *Azospirillum brasilense* on AGPase activity in the freshwater microalga *Chlorella vulgaris* and formation of starch. This was done by immobilizing both microorganisms in alginate beads, either replete with or deprived of nitrogen or phosphorus and all under heterotrophic conditions, using D-glucose or Na-acetate as the carbon source. AGPase activity during the first 72 h of incubation was higher in *C. vulgaris* when immobilized with *A. brasilense*. This happened simultaneously with higher starch accumulation and higher carbon uptake by the microalgae. Either carbon source had similar effects on enzyme activity and starch accumulation. Starvation either by N or P had the same pattern on AGPase activity and starch accumulation. Under replete conditions, the population of *C. vulgaris* immobilized alone was higher than when immobilized together, but under starvation conditions *A. brasilense* induced a larger population of *C. vulgaris*. In summary, adding *A. brasilense* enhanced AGPase activity, starch formation, and mitigation of stress in *C. vulgaris*.

1. Introduction

The green unicellular microalgae *Chlorella vulgaris* can grow and accumulate starch heterotrophically (Choix et al., 2012b; Griffiths, 1965). Starch formation by microalgae has significant importance in several biotechnological applications, the most current are biofuels (Lebeau and Robert, 2006; Markou et al., 2012). For growth and formation of metabolites under heterotrophic conditions, *C. vulgaris* requires sources of energy and carbon. The most common carbon sources are glucose or sodium acetate (Perez-Garcia et al., 2011); although in some microalgae, including *C. vulgaris*, other carbon sources support accumulation of starch (Markou et al., 2012). Starch accumulation, under heterotrophic conditions, is primarily used in microalgae as a strategy for intracellular storage of carbon and energy to support cell growth (Markou et al., 2012).

In higher plants and microalgae, biosynthesis of starch involves three steps: glucose activation, chain elongation, and chain branching (Ballicora et al., 2004). The most important step in the biosynthesis is the activation of glucose, converting it into nucleoside-diphosphate-glucose (ADPGlc), which functions as a glucosyl donor for the elongation chain of starch. The formation of ADPGlc is catalyzed by the enzyme ADP glucose pyrophosphorylase (AGPase; E.C.2.7.7.27), which catalyze the synthesis of ATP and glucose-1-phosphate in ADPGlc. In biosynthesis of starch, formation of ADPGlc is a limiting step; ADPGlc directly affects formation of starch. If ADPGlc does not formed, starch will not accumulate. AGPase is subjected to high regulation.

The main factor that enhances accumulation of lipids and carbohydrates in *Chlorella* spp. under autotrophic conditions is nitrogen starvation (Bumbak et al., 2011; Markou et al., 2012; Přibyl et al., 2012; Tang et al., 2011; Xiong et al., 2010), which is species-dependent. Phosphorus starvation has the same effect in other microalgae (Khozin-Goldberg and Cohen, 2006). To increase starch content in microalgae, studies focused on enhancing AGPase activity (Slattery et al., 2000; Smith, 2008; Radakovits et al., 2010;

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Rismani-Yazdi et al., 2011) and cultivating microalgae under conditions of nitrogen and phosphorus starvation (Brányiková et al., 2011; Dragone et al., 2011).

The bacterium *Azospirillum brasilense* is a microalgae growth-promoting bacterium (MGPB; de-Bashan et al., 2004) because, under autotrophic conditions, it can increase growth of cells, the size of some microalgae populations (de-Bashan and Bashan, 2008; Gonzalez and Bashan, 2000), and the amount of photosynthetic pigments, lipids, and fatty acids (de-Bashan et al., 2002). Currently and practically, co-immobilizing *A. brasilense* and *C. vulgaris* in alginate beads is a new technology for tertiary wastewater treatment because *Chlorella* spp. efficiently remove nitrogen from wastewater (de-Bashan et al., 2002, 2004; Covarrubias et al., 2012; Cruz et al., 2013; Hernandez et al., 2006), where the leftover debris can be used to improve infertile soils and plant growth (Lopez et al., 2013; Trejo et al., 2012). In the wastewater treatment role, *A. brasilense* enhanced the activity of two enzymes, glutamate dehydrogenase and glutamine synthetase, involved in nitrogen metabolism of *Chlorella* spp. (de-Bashan et al., 2008b). This microalgae-bacteria relationship has potential biotechnological significance as food for humans and animals, feed for aquaculture, and production of pigments. Notably, *Chlorella* spp., co-immobilized with *A. brasilense*, can also increase total carbohydrates, mainly starch, under autotrophic and heterotrophic conditions (Choix et al., 2012a, b), useful for ethanol production for biofuels.

Considering these enhanced activities, our hypothesis was that, similar to higher enzymatic activity in nitrogen metabolism, *A. brasilense* would induce *C. vulgaris* to increase enzymatic activity that is involved in biosynthesis of starch, such as AGPase. The main objective of this study was to measure the effect of *A. brasilense* on AGPase activity when *C. vulgaris* is co-immobilized with *A. brasilense* in alginate beads. This was done under nitrogen and phosphorus starvation under heterotrophic conditions, when either D-glucose or sodium acetate were added as sources of carbon. Our main strategy for experimenting with two carbon sources and two starvation regimes were to determine whether the response of the microalga to the bacteria is centralized mainly on *C. vulgaris–Azospirillum* spp. interaction or is substrate related.

2. Materials and methods

2.1. Microorganisms and initial growth conditions

The unicellular microalga *Chlorella vulgaris* Beijerinck (UTEX 2714, University of Texas, Austin, TX) and the bacterium *Azospirillum brasilense* Cd (DSM 1843, Leibniz-Institut DMSZ, Braunschweig, Germany) were used. To produce inoculum of the microalgae, 10 mL of axenic culture from *C. vulgaris* was inoculated into 90 mL sterile 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 $\mu g\,L^{-1}$): 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 $27 \pm 2\,^{\circ}C$, and shaken at 140 rpm under light intensity 60 $\mu mol\ photon\ m^{-2}\ s^{-1}$ for 7 d (Gonzalez et al., 1997). The bacterium grew in BTB-2 medium (Bashan et al., 2011), 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), glycerol (8 mL), and yeast extract (5). The culture was adjusted to pH 7 with 1 M KOH and incubated at $32 \pm 2\,^{\circ}C$ and shaken at 120 rpm for 16 h.

2.2. Immobilization of microorganisms

Microorganisms were immobilized, using the procedure described in de-Bashan et al., 2004. Briefly, 20 mL of axenic cultures (*C. vulgaris* and *A. brasilense*) were mixed separately with 2% alginate solution. Beads (3–4 mm diameter) were formed using automated equipment (de-Bashan and Bashan, 2010). For co-immobilization of the two microorganisms in the same bead, after washing the cultures, each of them was re-suspended 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, to increase the numbers of *A. brasilense* to its original level, a second 24 h incubation of the beads was necessary in 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), NH_4Cl (1) and (in $\mu g\,L^{-1}$): 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) (Bashan et al., 1993). Under starvation conditions, nitrogen or phosphorus was removed from OAB medium, depending on the type of starvation performed later in each experiment.

2.3. Experimental culture conditions

After secondary incubation, the beads were washed three times with sterile saline solution (0.85% NaCl). For experiments, 12 g beads with microorganisms, either immobilized separately or co-immobilized, were inoculated in 150 mL synthetic 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), NH_4Cl; (191) (de-Bashan et al., 2011) with sufficient nitrogen or phosphorus. Starvation conditions were created by removing nitrogen or phosphorus from the SGM.

SGM was supplemented with $10\,g\,L^{-1}$ D-glucose or sodium acetate (G5767, S2889, Sigma, St. Louis, MO) as carbon sources. Both carbon sources were sterilized by filtration through a 0.2 μm a syringe filter (Acrodisc, Pall Corp., Port Washington, NY). *C. vulgaris* and *A. brasilense* can grow and use these substances under heterotrophic conditions (Chen and Chen, 2006; Perez-Garcia et al., 2011). All experiments were performed under heterotrophic conditions in complete darkness, shaken at 140 rpm at $27 \pm 2\,^{\circ}C$ for 72 h.

2.4. Counting C. vulgaris in beads

In each experiment, the microbial populations of three beads from each flask (replicate) of 250 mL were counted. Each bead was dissolved by immersion in 1 mL 4% $NaHCO_3$ solution for 30 min at ambient temperature of $25 \pm 4\,^{\circ}C$. *C. vulgaris* cells were counted under a light microscope, using a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD). Growth rate of *C. vulgaris* (μ) 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 (Oh-Hama and Miyachi, 1992).

2.5. Analytical methods

Samples of one gram (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 $80\,^{\circ}C$ for 12 h, and ground with a mortar and pestle, which yielded 10 mg samples.

Starch was quantified by the method described by Brányiková et al., (2011), which is a modification of the method of McCready et al., (1950). It is based on total hydrolysis of starch by 30% perchloric acid and quantified by colorimetric means of the liberated glucose.

Uptake of D-glucose or sodium acetate from SGM by microorganisms was analyzed using the Megazyme D-glucose (glucose oxidase/peroxidase) assay kit (K-GLUC, gopod format, Megazyme

International, Bray, Ireland), and a kit to measure acetic acid (K-ACETAF 12/07, acetyl-coA synthetase format; Megazyme International). These tests were performed to the manufacturer's instruction.

To determine enzymatic activity of ADP-glucose pyrophosphorylase (AGPase), six grams of alginate beads from each treatment were taken 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 was washed three times with sterile saline solution (0.85% NaCl). Enzymatic activity of AGPase was measured by the method of Li et al., (2011). AGPase activity was present in buffer containing (in mM): HEPES at pH 7.4 (100), ADP-glucose (1.2), sodium pyrophosphate (3), $MgCl_2$ (5), dithiothreitol (4; D0632, Sigma), in a final volume of 500 μL. The 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. The supernatant (1000 μL) 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) and glucose-6-phosphate dehydrogenase (1 U). The enzymatic activity of AGPase is expressed as U mg^{-1} protein, where one unit is 1 nmol of ADP mg^{-1} protein min^{-1}. Proteins in the mixture were determined by the Bradford's assay (Bradford, 1976).

2.6. Localization of AGPase gene in Azospirillum spp.

Several full genome sequences of *Azospirillum* spp. are available: *Azospirillum lipoferum* 4B, *A.* sp. B510 (Kaneko et al., 2010), *A. brasilense* Sp245 (Wisniewsky-Dyé et al., 2011) and *A. brasilense* CBG497 (Wisniewsky-Dyé et al., 2012). The presence of the *glgC* gene, which encodes the enzyme AGPase in bacteria, has been identified in these strains by the whole genome random shotgun method (Southern, 1975).

2.7. Experimental design and statistical analysis

The setup of all experiments was in batch cultures. Each experiment was performed using three separate flasks per treatment. Each 250 mL Erlenmeyer flask, containing 150 mL suspension, served as a replicate. Each experiment was identically repeated two times. Three samples were taken from each flask for each analysis. Each setup contained four treatments: (1) beads without microorganisms, (2) beads containing *C. vulgaris*, (3) beads containing *A. brasilense* (all serving as controls), and (4) beads containing the two microorganisms (*C. vulgaris* and *A. brasilense*) ($n = 12$). The data from each treatment from the two repetitions ($n = 24$) 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 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 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 are grams of product formed in 24 h and N_t is the number of microalgae cells at this time (Rahn, 1930). Starch yield (quantity of starch produced per g of carbon source uptake of culture during 24 h) was calculated as: *Starch yield* (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 this time interval, S_1 is the substrate concentration (D-glucose or sodium acetate) after 24 h, and S_0 is the substrate at the beginning of this time interval. V is the volume of the medium (100 mL) (Choix et al., 2012b).

3. Results

3.1. Enzymatic activity of AGPase of C. vulgaris co-immobilized with A. brasilense when using D-glucose as the carbon source

All assays were conducted and results are presented, first for the entire culture and then calculated per individual cell, based on the actual number of cells that grew in each treatment and in each culture.

Growing in media replete with nitrogen, *A. brasilense* showed constant AGPase activity, with highest activity of 8.81 ± 3.44 U at 48 h. Activity declined at 96 h (Fig. 1a; capital letter analysis). *C. vulgaris* alone or co-immobilized with *A. brasilense* highest AGPase activity was at 48 h, 11.23 ± 2.69 U (immobilized alone) and 18.76 ± 3.04 U (co-immobilized with *A. brasilense*). Activity decreased until 96 h in both treatments (Fig. 1a; capital letter analysis). Significant differences between the activity of AGPase in *C. vulgaris* alone and with *A. brasilense* were not found at all incubation times (Fig. 1a; lower case analysis). Specific activity of AGPase in *C. vulgaris* immobilized with *A. brasilense* was significantly higher than when *C. vulgaris* was immobilized alone at all durations of incubation (Fig. 1a; lower case analysis).

Growing *A. brasilense* under nitrogen starvation, AGPase activity was 6.09 ± 2.06 U at 24 h, remaining constant at this level at all sampling times (Fig. 1b; capital letter analysis). *C. vulgaris* immobilized alone had the highest AGPase activity at 48 h and 72 h (10.23 ± 2.05 U), declining afterwards (Fig. 1b; capital letter analysis). There were no significant difference in AGPase activity of *C. vulgaris* immobilized alone and *A. brasilense* immobilized alone at 24, 48, and 96 h, but a significant difference at 72 h (Fig. 1b; lower case analysis). AGPase activity of *C. vulgaris* co-immobilized with *A. brasilense*, did not change significantly at any sampling time, which was 17.17 ± 3.58 U at 48 h (Fig. 1b; capital letter analysis). This was significantly greater than the two controls that were immobilized alone during almost all sampling times (Fig. 1b; lower case analysis).

When AGPase activity per cell was measured in *A. brasilense* growing with nitrogen replete ($4.3 \pm 1.95 \times 10^{-6}$ U $cell^{-1}$) or nitrogen starvation ($6.14 \pm 1.42 \times 10^{-6}$ U $cell^{-1}$), activity was very low and constant during the first 96 h of incubation (Fig. 1c, d; capital letter analysis). With sufficient nitrogen, *C. vulgaris* immobilized alone or with *A. brasilense* had the highest AGPase activity per cell at 24 h, $3.43 \pm 1.59 \times 10^{-3}$ U $cell^{-1}$ (immobilized alone) and $8.95 \pm 1.41 \times 10^{-3}$ U $cell^{-1}$ (co-immobilized with *A. brasilense*). At later sampling times, activity declined (Fig. 1c; capital letter analysis). This activity was significantly higher in *C. vulgaris* co-immobilized with *A. brasilense* than either microorganism immobilized alone at most sampling times (Fig. 1c; lower case analysis). Under nitrogen starvation, AGPase activity in *C. vulgaris* (immobilized alone) was constant during the first 72 h ($2.82 \pm 1.3 \times 10^{-4}$ U $cell^{-1}$) and then decreased (Fig. 1d; capital letter analysis). *C. vulgaris* co-immobilized with *A. brasilense* had maximum AGPase activity at 24 h ($1.14 \pm 1.08 \times 10^{-3}$ U $cell^{-1}$) and later declined (Fig. 1d; capital letter analysis). Only at 24 h was there a significant difference between *C. vulgaris* immobilized alone or co-immobilized with *A. brasilense* (Fig. 1d; lower case analysis).

With phosphorus replete for growth, AGPase activity in *A. brasilense* was 4.93 ± 1.54 U at 24 h and remained constant until 72 h, declined afterward (Fig. 2a; capital letter analysis). A similar pattern occurred in the AGPase activity of *C. vulgaris* (6.87 ± 1.6 U) when immobilized alone or co-immobilized with *A. brasilense* (9.7 ± 1.3 U). In all treatments, this enzymatic activity peaked at 24 h, remained unchanged until 72 h, and then declined at 96 h (Fig. 2a; capital letter analysis). There were no significant differences between activities for *C. vulgaris* or *A. brasilense* (Fig. 2a; lower case analysis). AGPase activity in *C. vulgaris* co-immobilized

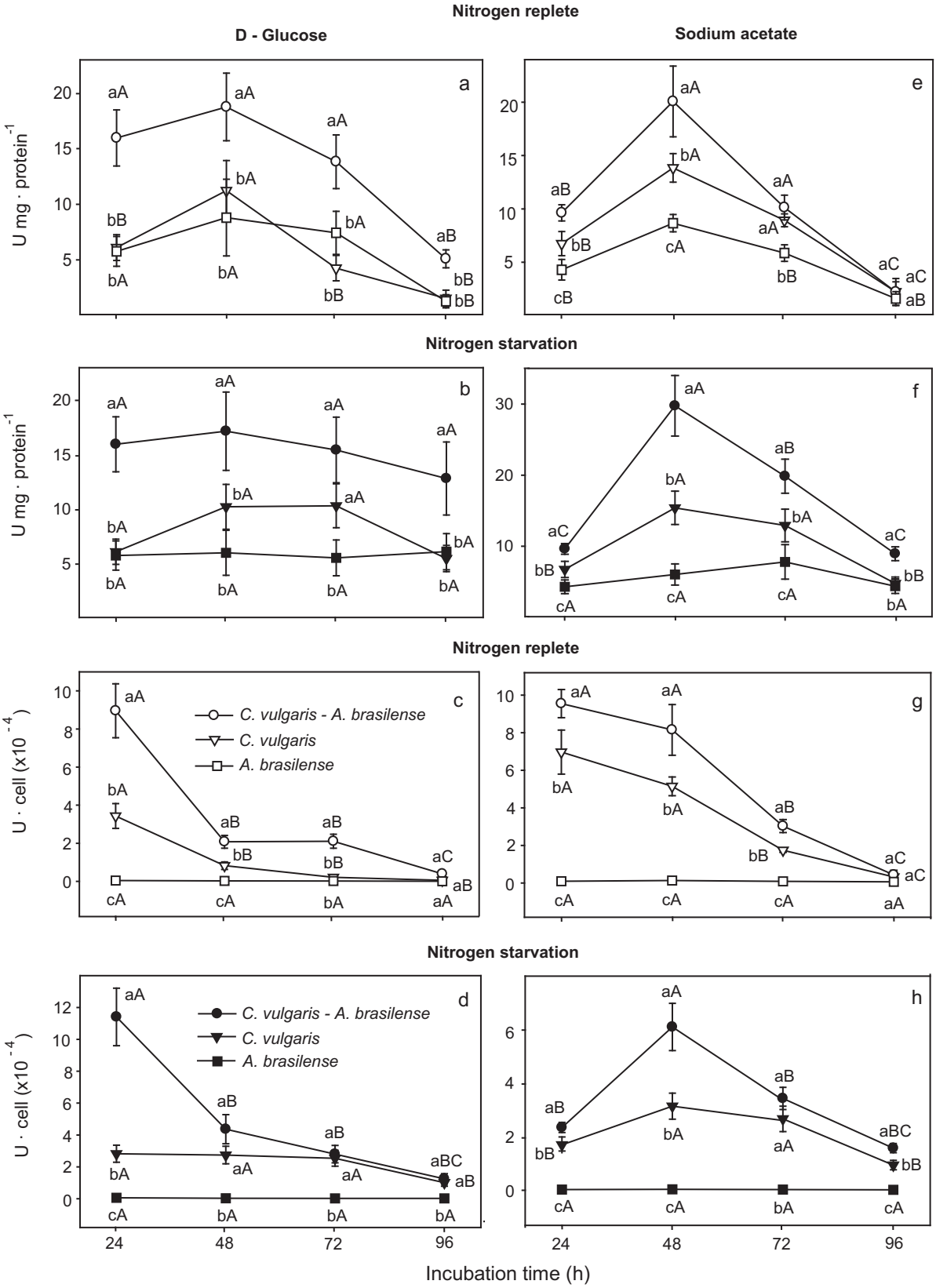

Fig. 1. Activity of AGPase with time in *Chlorella vulgaris*, *Azospirillum brasilense* immobilized alone and in *C. vulgaris* co-immobilized with *A. brasilense* in alginate beads under nitrogen starvation and nitrogen replete, using D-glucose or sodium acetate as carbon sources. Specific activity of AGPase (a, b, e, f), activity of AGPase measured per single cell (c, d, g, h). Values on curves denoted by different capital letters differ significantly where values at each time interval denoted by different lowercase letters differ significantly, both using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$.

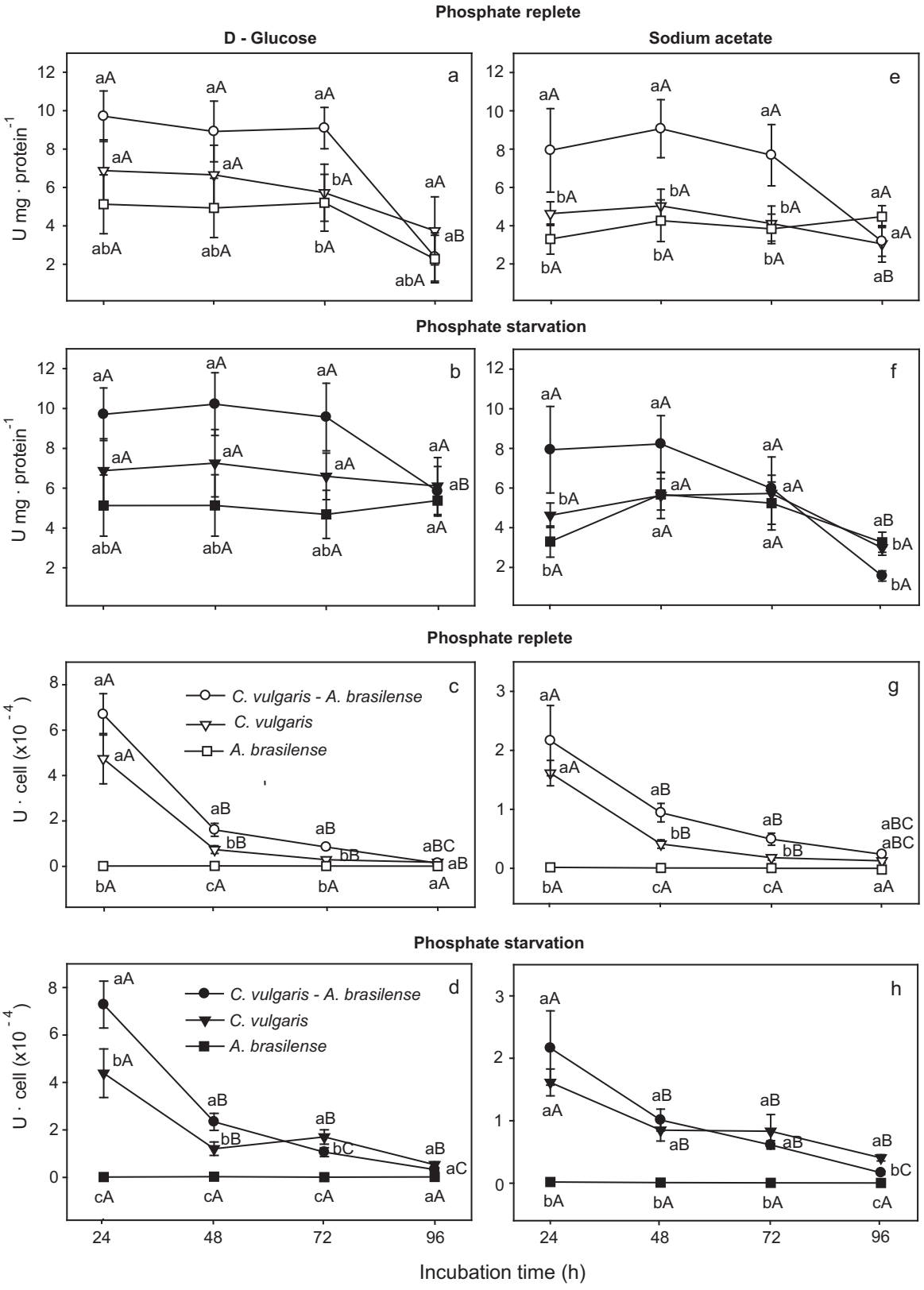

Fig. 2. Activity of AGPase with time in *Chlorella. vulgaris*, *Azospirillum brasilense* immobilized alone and in *C. vulgaris* co-immobilized with *A. brasilense* in alginate beads under phosphorus starvation and phosphorus replete, using D-glucose or sodium acetate as carbon sources. Specific activity of AGPase (a, b, e, f), activity of AGPase measured per single cell (c, d, g, h). Values on curves denoted by different capital letters differ significantly where values at each time interval denoted by different lowercase letters differ significantly, both using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$.

with *A. brasilense* was higher than when either microorganism was immobilized alone, there were no statistically significant differences between activity levels at any sampling time (Fig. 2a; lower case analysis).

Under phosphorus starvation, AGPase activity in *A. brasilense* was the same for the first 96 h (5.12 ± 1.53 U) (Fig. 2b; capital letter analysis). In *C. vulgaris*, when immobilized alone, AGPase activity (7.25 ± 1.69 U) was the same at all sampling times (Fig. 2b; capital letter analysis). This activity in *C. vulgaris*, immobilized alone, was higher than in *A. brasilense*, immobilized alone, but was not significantly different at most sampling times (Fig. 2b; lower case analysis). In *C. vulgaris* co-immobilized with *A. brasilense*, AGPase activity remained around 10.21 ± 1.57 U until 72 h and then declined (Fig. 2b; capital letter analysis). Regardless of higher activity, there was not significant difference in the activity of AGPase in microorganisms immobilized together or separately (Fig. 2b; lower case analysis).

A different pattern was detected when AGPase activity was calculated per cell. Activity of AGPase in *A. brasilense* with phosphorus ($1.31 \pm 1.23 \times 10^{-6}$ U cell^{-1}) or without phosphorus ($1.52 \pm 1.11 \times 10^{-6}$ U cell^{-1}) remained constant and very low at all incubation times (Fig. 2c; d capital letter analysis). With phosphorus, separately or co-immobilized, *C. vulgaris* had its highest AGPase activity at 24 h and declined thereafter (Fig. 2c; capital letter analysis) in both treatments where AGPase activity was $4.74 \pm 1.1 \times 10^{-4}$ (immobilized) and $6.69 \pm 2.22 \times 10^{-4}$ U cell^{-1} (co-immobilized). Significant differences between immobilized and co-immobilized *C. vulgaris* were found at 48 and 72 h of incubation (Fig. 2c; lower case analysis). A similar pattern occurred under phosphorus starvation. Highest activity of AGPase occurred at 24 h in immobilized and co-immobilized *C. vulgaris*. Activity decreased thereafter (Fig. 2d; capital letter analysis). At 24 h, AGPase activity per cell was $4.39 \pm 1.02 \times 10^{-4}$ (immobilized alone) and $7.28 \pm 2.42 \times 10^{-4}$ U cell^{-1} (when co-immobilized). Significant differences occurred at several time intervals (Fig. 2d; lower case analysis). No effect was recorded in beads without microorganisms (data not shown).

3.2. Starch accumulation, using glucose as the carbon source

When nitrogen or phosphorus were supplied, immobilized and co-immobilized *C. vulgaris* showed the same pattern of starch accumulation, mostly significantly higher in co-immobilized than in immobilized treatments (Fig. 3a; b lower case analysis). With nitrogen, starch content in *C. vulgaris* immobilized alone was 51.97 ± 6.31 mg g^{-1}; when co-immobilized with *A. brasilense*, starch content was 64.43 ± 3.10 mg g^{-1}, which peaked after 48 h (Fig. 3a; capital letter analysis). With phosphorus, starch accumulation peaked at 48 h when immobilized alone (51.09 ± 3.12 mg g^{-1}) and, when co-immobilized with *A. brasilense*, reached 60.03 ± 3.71 mg g^{-1} (Fig. 3b; capital letter analysis).

Under starvation of nitrogen or phosphorus, *C. vulgaris*, alone or co-immobilized with *A. brasilense*, reached peak starch content after 72 h (Fig. 3c, d; capital letter analysis). Under nitrogen starvation, *C. vulgaris*, immobilized alone, starch content peaked at 65.14 ± 2.88 mg g^{-1}; when co-immobilized, starch content peaked at 79.16 ± 3.22 mg g^{-1}. Under phosphorus starvation, starch content was 55.34 ± 4.49 mg g^{-1} (immobilized alone) and 66.25 ± 4.76 mg g^{-1} (co-immobilized). Significant differences occurred at 48 h and 72 h between immobilized alone and co-immobilized *C. vulgaris* (Fig. 3d; lower case analysis). Under all conditions, starch content (*Qs*) and volumetric productivity (*Yp*) were highest at 48 h, although there were no significant differences between immobilized and co-immobilized *C. vulgaris*, except when nitrogen was present; then *Yp* was higher for co-immobilized *C. vulgaris* (Table 1). *Qs* and *Yp* decreased after 48 h (data not shown). No effect was recorded in beads without microorganisms (data not shown).

3.3. Uptake of D-glucose

When nitrogen or phosphorus was supplied, whether immobilized alone and co-immobilized, *C. vulgaris* consumed significant amounts of glucose at 48 h (Fig. 4a for N and Fig. 4b for P; capital letter analysis). At this time, the same level of consumed glucose was reached in both treatments (Fig. 4a, b; lower case analysis). After 48 h, glucose uptake remained constant in immobilized *C. vulgaris*, up to 96 h; while significantly more uptake of glucose occurred when co-immobilized with *A. brasilense* (Fig. 4a, b; capital letter analysis and lower case analysis, separately). A very similar pattern occurred under starvation of either nitrogen or phosphorus (Fig. 4c, d; capital letter analysis and lower case analysis, separately). Independent of the experimental condition, affinity for glucose was highest at 48 h, with significant differences between *C. vulgaris* immobilized alone or co-immobilized with *A. brasilense* (Table 1). Afterwards, affinity declined in both treatments (data not shown). No effect was recorded in beads without microorganisms (data not shown).

3.4. Growth of C. vulgaris

Grown with nitrogen or phosphorus, cell density of *C. vulgaris* immobilized alone was significantly higher than cell density when co-immobilized (Fig. 5a, b; lower case analysis). With nitrogen, population peaked at 96 h in immobilized *C. vulgaris* (5.37 ± 0.22 Log cell density^{-1}) and when co-immobilized (5.10 ± 0.10 Log cell density^{-1}) (Fig. 5a; capital letter analysis). At this time, the growth rate was 0.87 ± 0.26 d^{-1} when immobilized along and 0.66 ± 0.06 d^{-1} when co-immobilized. With phosphorus, the population peaked at 96 h. The highest cell density was 5.30 ± 0.13 Log cell density^{-1} (immobilized alone) and 5.20 ± 0.12 Log cell density^{-1} (co-immobilized) (Fig. 5b, capital letter analysis), reaching a growth rate of 0.88 ± 0.15 d^{-1} (immobilized alone) and 0.81 ± 0.11 d^{-1} (co-immobilized).

An opposite pattern was found under nitrogen or phosphorus starvation. Under nitrogen starvation, peak cell density was reached at 96 h, when *C. vulgaris* was immobilized alone or co-immobilized (Fig. 5c; capital letter analysis). However, *C. vulgaris* cell density was significantly higher when co-immobilized (4.98 ± 0.16 Log cell density^{-1}) than when immobilized alone (4.66 ± 0.26 Log cell density^{-1}) (Fig. 5c; lower case analysis); reaching growth rates of 0.25 ± 0.18 d^{-1} (immobilized alone) and 0.67 ± 0.14 d^{-1} (co-immobilized). Under phosphorus starvation, cell density was 5.01 ± 0.21 Log cell density^{-1} (immobilized alone) and 5.22 ± 0.13 Log cell density^{-1} (co-immobilized) (Fig. 5d; capital letter analysis). Under this condition, growth rates were 0.63 ± 0.20 d^{-1} (immobilized alone) and 0.85 ± 0.09 d^{-1} (co-immobilized).

3.5. Enzymatic activity of AGPase of co-immobilized C. vulgaris with A. brasilense, using sodium acetate as the carbon source

Using sodium acetate as the carbon source and cultivating all combinations of *C. vulgaris* and *A. brasilense* described above, a similar patterns and levels of AGPase activity and starch accumulation as occurred with glucose as the carbon source (compare results when ammonium was available or absent): Fig. 1a vs. e, b vs. f, c vs. g, and d vs. h and when phosphate was available or absent: Fig. 2a vs. e, b vs. f, c vs. g, and d vs. h. The specific activity of AGPase and AGPase activity per cell were significantly higher when *C. vulgaris* was immobilized with *A. brasilense* than when either microorganism was immobilized alone. This happened whether nitrogen was

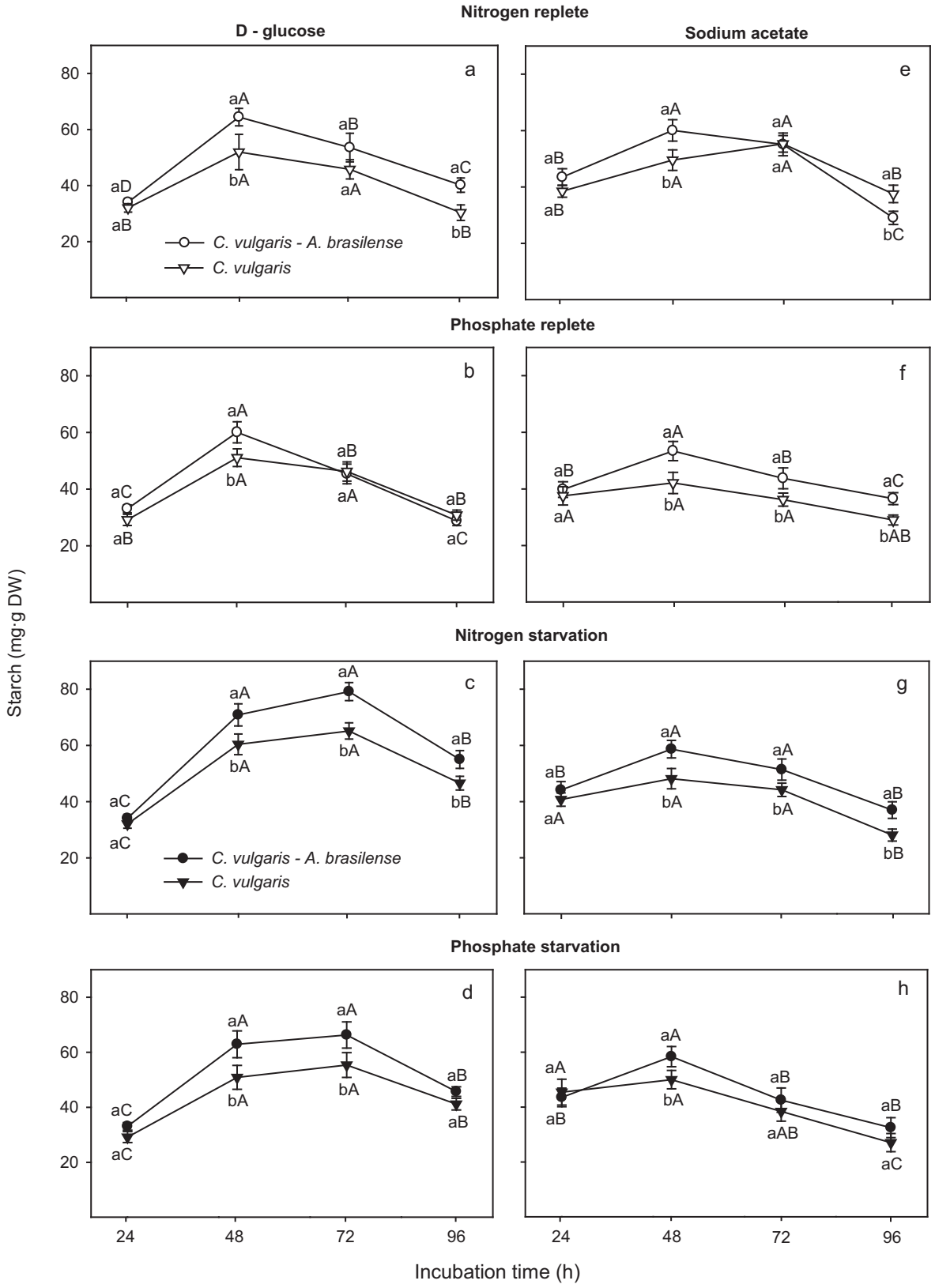

Fig. 3. Starch accumulation, with time, in *Chlorella vulgaris* immobilized alone and in *C. vulgaris* co-immobilized with *Azospirillum brasilense* in alginate beads under nitrogen (a, e) and phosphorus replete (b, f), or nitrogen (c, g) and phosphorus starvation (d, h), using D-glucose or sodium acetate as carbon sources. Values on curves denoted by different capital letters differ significantly where values at each time interval denoted by different lowercase letters differ significantly, both using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$.

Table 1
General analysis of starch in *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* in alginate beads growing in nutrient replete or starvation and under heterotrophic conditions, using glucose as the carbon source.

Time		Affinity (mg day^{-1})		Volumetric productivity (*Yp*) (mg 100 mL^{-1} day^{-1})		Starch yield (*Qs*) (mg 100 mL^{-1} day^{-1})	
		Co-immobilized	Immobilized alone	Co-immobilized	Immobilized alone	Co-immobilized	Immobilized alone
24–48 h	**−N**	0.87 ± 0.05a	0.73 ± 0.05a	36.81 ± 3.34a	28.34 ± 3.70a	19.16 ± 1.74a	19.52 ± 2.54a
	+N	0.25 ± 0.01a	0.13 ± 0.01b	30.37 ± 4.28a	19.92 ± 5.77b	16.12 ± 2.27a	12.34 ± 3.57a
	−P	0.72 ± 0.04a	0.37 ± 0.02b	29.82 ± 5.35a	21.70 ± 5.80a	13.58 ± 2.43a	12.92 ± 3.45a
	+P	0.48 ± 0.04a	0.23 ± 0.01b	26.95 ± 4.32a	21.93 ± 4.49a	13.42 ± 2.15a	12.39 ± 2.54a

Different lower case letters for each pair of values separately indicate significant differences between *Chlorella vulgaris* immobilized alone and *C. vulgaris* co-immobilized with *Azospirillum brasilense* analyzed by Student's *t*-test $P < 0.05$. (±) Represents SE.

available or absent (Fig. 1e, f, g, h) or whether phosphorus was available or absent (Fig. 2e, f, g. h).

Similar to using glucose as the carbon source, all treatment combinations using sodium acetate followed a similar patterns when nitrogen or phosphorus was available or absent regarding starch content (Fig. 3a, b, c, d [glucose] vs. Fig. 3e, f, g, h [sodium acetate]), uptake of substrate (Fig. 4a, b, c, d [glucose] vs. Fig. 4e, f, g, h [sodium acetate]), and cell density (Fig. 5a, b, c, d [glucose] vs. Fig. 5e, f, g, h [sodium acetate]). At most time intervals for all parameters, content of starch for *C. vulgaris*, when co-immobilized with *A. brasilense*, were significantly higher than when *C. vulgaris* was co-immobilized alone (Fig. 3; lower case analyses, Table 2).

4. Discussion

ADP-glucose pyrophosphorylase (AGPase) is the regulatory enzyme of starch biosynthesis in microalgae and higher plants (Ballicora et al., 2004). For example, starch content is directly correlated to AGPase activity in potato tubers (Slattery et al., 2000), in wheat endosperm and the microalgae *Chlamydomonas reinhardtii* (Ballicora et al., 2004). Earlier studies show that co-immobilization in alginate beads of *A. brasilense* with *Chlorella* spp. induced significant benefits for the microalgae (de-Bashan and Bashan, 2008; Gonzalez and Bashan, 2000). Recently, Choix et al., 2012a, b demonstrated that *A. brasilense* enhanced accumulation of starch in *C. vulgaris* under autotrophic and heterotrophic conditions. In this study, our hypothesis was that the increase in starch content in *Chlorella* spp. induced by *A. brasilense* is linked to increased AGPase activity in the microalgae; AGPase is a key enzyme in starch formation. Consequently, we evaluated AGPase activity in *C. vulgaris* co-immobilized with *A. brasilense* and cultivated when nitrogen and phosphorus were freely available or under starvation of these elements under heterotrophic conditions. Nutrient starvation is well documented to enhance accumulation of storage compounds in various microalgae, including *Chlorella* spp. (Khozin-Goldberg and Cohen, 2006; Přibyl et al., 2012; Tang et al., 2011; Xiong et al., 2010).

This study has shown that, under heterotrophic conditions and nitrogen or phosphorus starvation, *C. vulgaris* co-immobilized with *A. brasilense* exhibits higher enzymatic specific activity of AGPase than when cultivated alone. This held true when enzymatic activity was calculated as common enzymatic units or activity per single cell. Enhancement can be attributed to activity of *A. brasilense* on the microalgae because it usually alters the metabolism of *Chlorella* spp., possibly by phytohormone production (de-Bashan et al., 2008a). Most species of *Azospirillum* produce various auxins, cytokinins, gibberellins, or combinations of these (Bashan and de-Bashan, 2010), where the main effects on metabolism of *Chlorella* spp. by *A. brasilense*, demonstrated thus far, is probably hormonal, mainly from indole-3-acetic acid (IAA) (de-Bashan et al., 2008a). There are at least two plausible explanations how this may happen. Neither has been investigated so far for *Chlorella vulgaris* or other microalgae. (1) IAA directly affects or regulates the activity of AGPase. This was earlier demonstrated in rice (Fu et al., 2013). Application of IAA induced accumulation of starch in sorghum grains (Bhatia and Singh, 2002). (2) IAA increases absorption of glucose that may later translate, via AGPase, into higher starch content by the following mechanism. *Chlorella* cells possess an inducible, active hexose/H^+ symport system. These symporters are responsible for uptake of glucose from the medium under heterotrophic conditions (Tanner, 2000). If IAA affects any of the symporters, in a yet to discover way, this may cause greater absorption of glucose, so that each cell is loaded with more energy and carbon. Synthesis of reserve polysaccharide in bacteria and plants is maximal when cellular carbon and energy are in excess (Ballicora et al., 2004). Thus, this state of carbon/energy excess enhances AGPase activity that leads to accumulation of starch. Parts of this hypothetical scenario were demonstrated in this study: higher consumption of glucose, higher AGPase activity, and greater accumulation of starch.

In bacteria, the gene codifying for AGPase is *glgC*. We identified this gene in publically-available, full sequences of several *A. brasilense* strains (Kaneko et al., 2010; Wisniewsky-Dyé et al., 2011, 2012). Yet, in this bacterium, this enzyme regulates glycogen biosynthesis (Caiola et al., 2004; Lerner et al., 2009). Glycogen in *Azospirillum* spp. improves survival under stress. The *glgC* gene is upregulated under hyperosmotic and desiccating conditions in *Sinorhizobium meliloti* and *Yersinia pestis*, high temperature in *Salmonella typhimurium* and *Saccharomyces cerevisiae*, and nutrient starvation in *Escherichia coli* (Lerner et al., 2009).

Under heterotrophic conditions, glucose assimilated by *C. vulgaris* and *C. sorokiniana* is rapidly incorporated as starch, while sodium acetate induces far lower levels of starch accumulation (Choix et al., 2012b). Yet, using either carbon source, starch content was consistently higher in *C. vulgaris* immobilized with *A. brasilense*. This may theoretically happen, but has not yet been demonstrated, because IAA produced by *A. brasilense* induces a massive cascade of significant metabolic changes in the microalgae, including effects on carbohydrate metabolism, probably regardless of the specific carbon source used (Bashan and de-Bashan, 2010; Choix et al., 2012a,b; Tsavkelova et al., 2006). The resulting higher content of starch coincides with higher AGPase activity in *C. vulgaris* that is co-immobilized with *A. brasilense* whether nitrogen or phosphorus is amply available or unavailable. Commonly, greater AGPase activity and starch was found in potato tubers (Slattery et al., 2000), but rarely in freshwater microalgae. Recently, Li et al., (2011) demonstrated that the maximum level of starch in the terrestrial microalga *Pseudochlorococcum* sp. (Chlorophyceae) coincides with the increase in AGPase activity.

Under all of our experimental conditions, uptake of the carbon source and affinity to D-glucose or sodium acetate were always relatively higher in *C. vulgaris* co-immobilized with *A. brasilense*. The highest uptake of the carbon source usually occurred after 48 h. This could be attributed to the treatment procedure, where the cultures were first subjected to nitrogen or phosphorus starvation for 24 h prior to culturing with either carbon source. Vonshak and

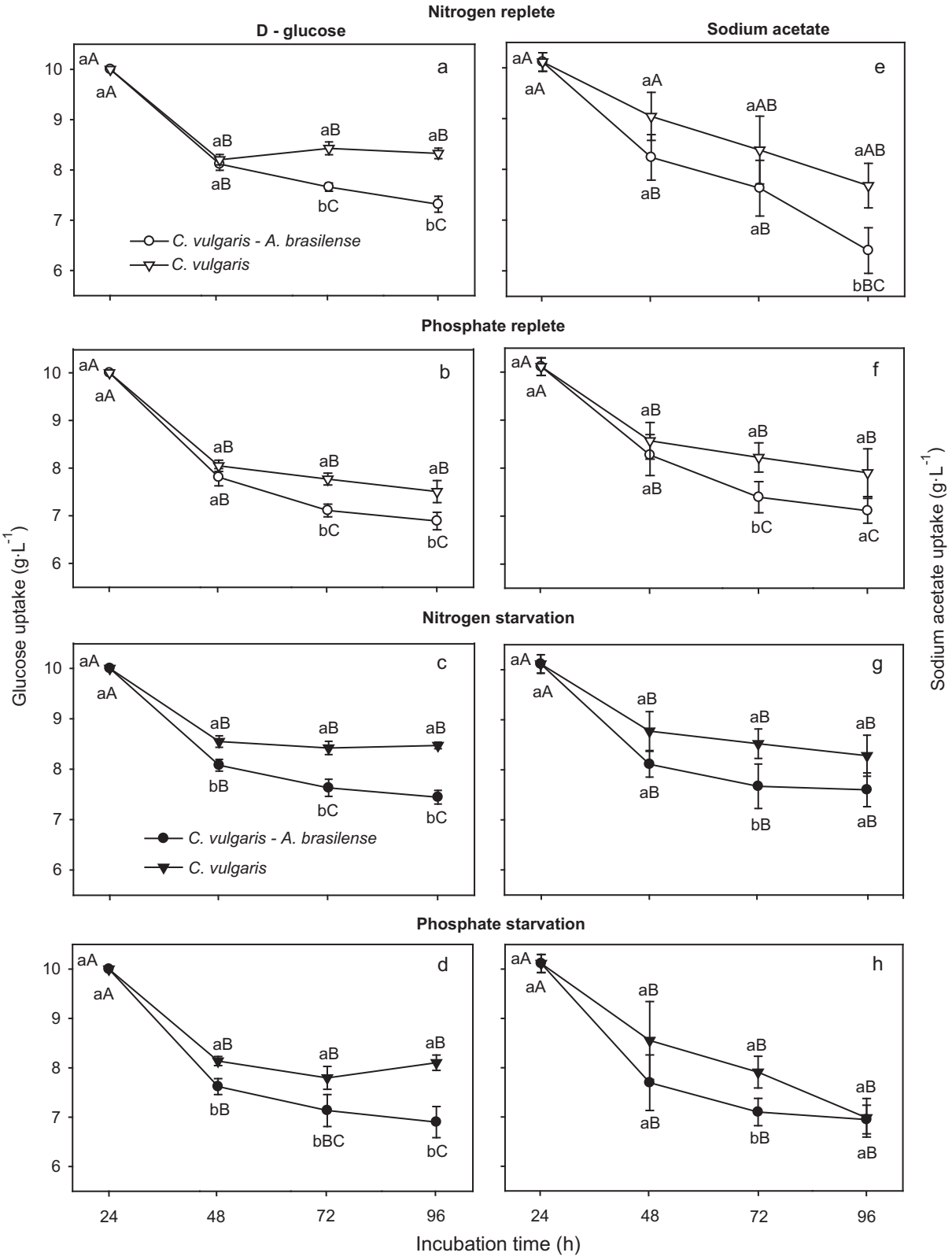

Fig. 4. Glucose uptake, with time, in *Chlorella vulgaris* immobilized alone and in *C. vulgaris* co-immobilized with *Azospirillum brasilense* in alginate beads under nitrogen (a, e) and phosphorus replete (b, f), or nitrogen (c, g) and phosphorus starvation (d, h), using D-glucose or sodium acetate as carbon sources. Values on curves denoted by different capital letters differ significantly where values at each time interval denoted by different lowercase letters differ significantly, both using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$.

Torzillo, (2004) postulated that under nutrient starvation, microalgae require biochemical and metabolic adjustments to balance the absorption of energy (as ATP) and the production of reducing power (as NADPH) for cell maintenance. High and rapid nutrient uptake under starvation by *Scenedesmus bicellularis* was reported (Kaya and Picard, 1995). In our study, the high affinity for a carbon source after 48 h of incubation coincides with peak starch yield and volumetric productivity. This demonstrates that higher consumption

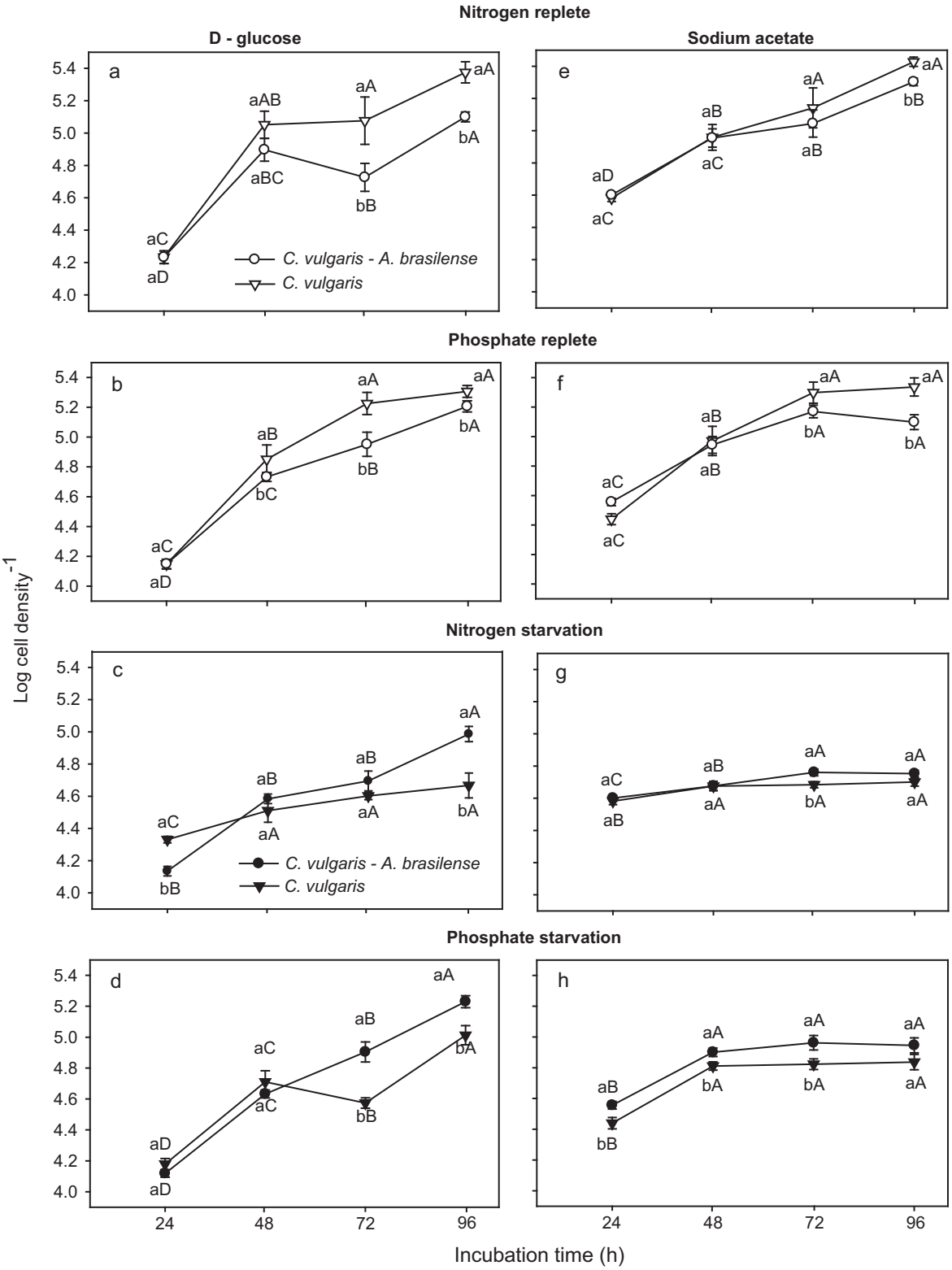

Fig. 5. Cell density, with time, of *Chlorella vulgaris* immobilized alone and in *C. vulgaris* co-immobilized with *Azospirillum brasilense* in alginate beads under nitrogen (a, e) and phosphorus replete (b, f), or nitrogen (c, g) and phosphorus starvation (d, h), using D-glucose or sodium acetate as carbon sources. Values on curves denoted by different capital letters differ significantly where values at each time interval denoted by different lowercase letters differ significantly, both using one-way ANOVA and LSD ad-hoc analysis at $P < 0.05$.

of the carbon source and AGPase activity can be related to the higher starch accumulation in *C. vulgaris* co-immobilized with *A. brasilense*. Similarly Choix et al., (2012b) demonstrated that under heterotrophic conditions *A. brasilense* promoted the conversion of glucose or sodium acetate in *C. vulgaris* and *C. sorokiniana* to starch.

The highest AGPase activity was measured after 24 h in *C. vulgaris* co-immobilized with *A. brasilense*. This allowed sufficient

Table 2
General analysis of starch in *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* in alginate beads growing in nutrient replete or starvation and under heterotrophic conditions, using sodium acetate as the carbon source. Different lower case letters, for each pair of values separately, indicate significant differences between *Chlorella vulgaris* immobilized alone and *C. vulgaris* co-immobilized with *Azospirillum brasilense* analyzed by Student's *t*-test $P < 0.05$. (±) Represents SE.

Time		Affinity (mg day^{-1})		Volumetric productivity (*Yp*) (mg 100 mL^{-1} day^{-1})		Starch yield (*Qs*) (mg 100 mL^{-1} day^{-1})	
		Co-immobilized	Immobilized alone	Co-immobilized	Immobilized alone	Co-immobilized	Immobilized alone
24–48 h	**−N**	2.40 ± 0.45a	1.36 ± 0.42b	14.53 ± 4.39a	7.43 ± 4.02a	7.25 ± 2.19a	5.54 ± 3.00a
	+N	0.48 ± 0.04a	0.23 ± 0.01b	16.55 ± 4.07a	11.01 ± 4.30a	8.81 ± 2.38a	10.29 ± 4.02a
	−P	0.54 ± 0.13a	0.41 ± 0.18a	14.83 ± 5.32a	4.53 ± 5.27a	8.15 ± 2.92a	2.90 ± 3.37a
	+P	0.30 ± 0.06a	0.16 ± 0.04b	13.59 ± 4.09a	4.56 ± 4.78a	7.38 ± 2.22a	2.95 ± 3.09a

interaction time for *Azospirillum* to stimulate higher activity of the enzyme in *C. vulgaris*, and accumulation of starch in the cultures within the first 72 h of incubation under all of our experimental conditions. This suggests that, under nutrient starvation of nitrogen or phosphorus, microalgae rapidly change their metabolism from synthesis of proteins for growth to synthesis of starch for survival (Dragone et al., 2011; Ho et al., 2012; Markou et al., 2012). The starch accumulated in the cells provides energy for later growth when conditions for growth are resumed (Markou et al., 2012). Siaut et al., (2011) found that, under nutrient starvation, *Chlamydomonas reinhardtii* uses starch as the main energy storage material and lipids are used under conditions of prolonged stress. This same pattern was recently found in *C. vulgaris* (Ho et al., 2013). Nonetheless, in our study, when *C. vulgaris* was supported by amply available nutrients, starch content was similar to what occurred under starvation conditions. This may happen because, once nutrients are available to *C. vulgaris*, the previously starved population cannot recover to their normal cell density.

Heterotrophic growth of *Chlorella* spp. under unlimited and uninterrupted nutrient supply generally generates high growth rates and high cell densities (Perez-Garcia et al., 2011). However, we found that *C. vulgaris* immobilized alone or co-immobilized with *A. brasilense*, when nutrients were available or absent, cell density was relatively low. MacIntyre and Cullen, (2005) stated that when nutrients are absent, metabolism of microalgae cultivated alone decreased and growth ceased because nutrients are essential to generate DNA, RNA, ATP, and proteins. When one of these nutrients is removed, reduced, or completely absent, low growth rates and consequently low biomass followed (Ballin et al., 1988; Bumbak et al., 2011; Markou et al., 2012; Zachleder et al., 1988). The expected recovery of cell density when the nutrient supply was resumed did not happen. This could have happened because the phase of secondary growth of *C. vulgaris* was done under nitrogen or phosphorus starvation. As discussed above, starvation generates an unbalanced growth that may cause the microalgae to die. Furthermore, the physiological response of different species of microalgae to starvation differs and varies from minutes to days (Vonshak and Torzillo, 2004). Hernandez et al., (2006) demonstrate that *C. vulgaris*, growing under nutrient starvation could not recover its initial cell density even when the nutrient supply was resumed. In our study, in cultures of *C. vulgaris* immobilized alone or co-immobilized with *A. brasilense*, it is plausible that cell density could not recover. The decreased metabolism and the low cell population induced by starvation could explain the similarity of starch accumulation in *C. vulgaris* under a resupply of nutrients after a short period (24 h) of starvation.

Contrary to the above, we found that, under conditions of nitrogen or phosphorus starvation, *A. brasilense* promoted a relatively higher cell density of *C. vulgaris*, compared to when the microalgae was immobilized alone. This could happen because *A. brasilense* is known to reduce many environmental stressors in plants (Bashan et al., 2004; Bashan and de-Bashan, 2010), as well as in *Chlorella* spp. (de-Bashan et al., 2005; de-Bashan and Bashan, 2008), by numerous small-scale mechanisms, none of which have been studied in microalgae. Yet, when *C. vulgaris* was growing with nitrogen or phosphorus replete, it reached higher populations when immobilized alone than when co-immobilized with *A. brasilense*.

Our main strategy of evaluating two carbon sources and two regimes of nutrient starvation were to assess whether the response of the microalgae to the bacteria is a general response to the intimate presence of the prokaryote or is a response to a compound or to environmental conditions. The very similar response of the microalgae in the presence of the bacteria, with either carbon source or either nutrient starvation (nitrogen or phosphorus), indicates that the main cause for the observed effect on the AGPase activity and accumulation of starch is the association of the bacteria with the microalgae.

5. Conclusions

These results, taken together with earlier studies on starch accumulation (Choix et al., 2012a,b) show that:

- *A. brasilense* has the ability to increase the enzymatic activity of AGPase in *C. vulgaris* under heterotrophic conditions, yielding a higher quantity of starch in this microalgae–bacteria interaction.
- When grown under replete or starvation conditions of either nitrogen or phosphorus and using either D-glucose or sodium acetate as a carbon source, *C. vulgaris* had the same positive pattern of responses to *A. brasilense*.
- Under condition of starvation of nitrogen or phosphorus, *A. brasilense* reduces nutrient stress for *C. vulgaris* and increases its cell density.

Acknowledgments

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References

Ballicora, M.A., Iglesias, A.A., Preiss, J., 2004. ADP-glucose pyrophosphorylase: a regulatory enzyme for plant starch synthesis. Photosynth. Res. 79, 1–24.

Ballin, G., Doucha, J., Zachleder, V., Šetlík, I., 1988. Macromolecular syntheses and the course of cell cycle events in the chlorococcal alga *Scenedesmus quadricauda* under nutrient starvation: effect of nitrogen starvation. Biol. Plantarum 30, 81–91.

Bashan, Y., de-Bashan, L.E., 2010. How the plant growth promoting bacterium *Azospirillum* promotes plant growth – a critical assessment. Adv. Agron. 108, 77–136.

Bashan, Y., Holguin, G., de-Bashan, L.E., 2004. Azospirillum-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can. J. Microbiol. 50, 521–577.
Bashan, Y., Holguin, G., Lifshitz, R., 1993. Isolation and characterization of plant growth-promoting rhizobacteria. In: Glick, B.R., Thompson, J.E. (Eds.), Methods in Plant Molecular Biology and Biotechnology. CRC Press, Boca Raton, FL, pp. 331–345.
Bashan, Y., Trejo, A., de-Bashan, L.E., 2011. Development of two culture media for mass cultivation of *Azospirillum* spp. and for production of inoculants to enhance plant growth. Biol. Fert. Soils 47, 963–969.
Bhatia, S., Singh, R., 2002. Phytohormone-mediated transformation of sugars to starch in relation to the activities of amylases, sucrose-metabolising enzymes in sorghum grain. Plant Growth Regul. 36, 97–104.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011. Microalgae–novel highly efficient starch producers. Biotechnol. Bioeng. 108, 766–776.
Bumbak, F., Cook, S., Zachleder, V., Hauser, S., Kovar, K., 2011. Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. Appl. Microbiol. Biot. 91, 31–46.
Caiola, M.G., Canini, A., Botta, A.L., Del Gallo, M., 2004. Localization of *Azospirillum brasilense* Cd in inoculated tomato (*Lycopersicon esculentum* Mill.) roots. Ann. Microbiol. 54, 365–380.
Chen, G.-Q., Chen, F., 2006. Growing phototrophic cells without light. Biotechnol. Lett. 28, 607–616.
Choix, F.J., de-Bashan, L.E., Bashan, Y., 2012a. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: I. Autotrophic conditions. Enzyme Microb. Technol. 51, 294–299.
Choix, F.J., de-Bashan, L.E., Bashan, Y., 2012b. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: II. Heterotrophic conditions. Enzyme Microb. Technol. 51, 300–309.
Covarrubias, S.A., de-Bashan, L.E., Moreno, M., Bashan, Y., 2012. Alginate beads provide a beneficial physical barrier against native microorganisms in wastewater treated with immobilized bacteria and microalgae. Appl. Microbiol. Biotechnol. 93, 2669–2680.
Cruz, I., Bashan, Y., Hernández-Carmona, G., de-Bashan, L.E., 2013. Biological deterioration of alginate beads containing immobilized microalgae and bacteria during tertiary wastewater treatment. Appl. Microbiol. Biotechnol. 97, 9847–9858.
de-Bashan, L.E., Antoun, H., Bashan, Y., 2005. Cultivation factors and population size control the uptake of nitrogen by the microalgae *Chlorella vulgaris* when interacting with the microalgae growth-promoting bacterium *Azospirillum brasilense*. FEMS Microbiol. Ecol. 54, 197–203.
de-Bashan, L.E., Antoun, H., Bashan, Y., 2008a. Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. J. Phycol. 44, 938–947.
de-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillo, J.J., 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Can. J. Microbiol. 48, 514–521.
de-Bashan, L.E., Hernandez, J.-P., Morey, T., Bashan, Y., 2004. Microalgae growth-promoting bacteria as "helpers" for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res. 38, 466–474.
de-Bashan, L.E., Magallon, P., Antoun, H., Bashan, Y., 2008b. Role of glutamate dehydrogenase and glutamine synthetase in *Chlorella vulgaris* during assimilation of ammonium when jointly immobilized with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. J. Phycol. 44, 1188–1196.
de-Bashan, L.E., Bashan, Y., 2008. Joint immobilization of plant growth-promoting bacteria and green microalgae in alginate beads as an experimental model for studying plant-bacterium interactions. Appl. Environ. Microbiol. 74, 6797–6802.
de-Bashan, L.E., Schmid, M., Rothballer, M., Hartmann, A., Bashan, Y., 2011. Cell–cell interaction in the eukaryote-prokaryote model of the microalgae *Chlorella vulgaris* and the bacterium *Azospirillum brasilense* immobilized in polymer beads. J. Phycol. 47, 1350–1359.
de-Bashan, L.E., Bashan, Y., 2010. Immobilized microalgae for removing pollutants: review of practical aspects. Bioresour. Technol. 101, 1611–1627.
Dragone, G., Fernandes, B.D., Abreu, A.P., Vicente, A.A., Teixeira, J.A., 2011. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl. Energy 88, 3331–3335.
Fu, J., Xu, Y.-J., Chen, L., Yuan, L.-M., Wang, Z.-Q., Yang, J.-C., 2013. Changes in enzyme activities involved in starch synthesis and hormone concentrations in superior and inferior spikelets and their association with grain filling of super rice. Ric. Sci. 20, 120–128.
Gonzalez, L.E., Bashan, Y., 2000. Increased growth of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol. 66, 1527–1531.
Gonzalez, L.E., Cañizares, R.O., Baena, S., 1997. Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. Bioresour. Technol. 60, 259–262.
Griffiths, D.J., 1965. The accumulation of carbohydrate in *Chlorella vulgaris* under heterotrophic conditions. Ann. Bot. 29, 347–357.
Hernandez, J.-P., de-Bashan, L.E., Bashan, Y., 2006. Starvation enhances phosphorus removal from wastewater by the microalga *Chlorella* spp. co-immobilized with *Azospirillum brasilense*. Enzyme Microb. Technol. 38, 190–198.
Ho, S.-H., Chen, C.-Y., Chang, J.-S., 2012. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour. Technol. 113, 244–252.
Ho, S.-H., Huang, S.-W., Chen, C.-Y., Hasunuma, T., Kondo, A., Chang, J.-S., 2013. Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E. Bioresour. Technol. 135, 157–165.
Kaneko, K., et al., 2010. Complete genomic structure of the cultivated rice endophyte *Azospirillum* sp. B510. DNA Res. 17, 37–50.
Kaya, V.M., Picard, G., 1995. The viability of *Scenedesmus bicellularis* cells immobilized on alginate screens following nutrient starvation in air at 100% relative humidity. Biotechnol. Bioeng. 46, 459–464.
Khozin-Goldberg, I., Cohen, Z., 2006. The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. Phytochemistry 67, 696–701.
Lebeau, T., Robert, J.M., 2006. Biotechnology of immobilized microalgae: a culture technique for the future? In: Rao, S. (Ed.), Algal Cultures, Analogues of Blooms and Applications, vol. 2. Science Publishers, Enfield, NH, USA, pp. 801–837.
Lerner, A., Castro-Sowinski, S., Lerner, H., Okon, Y., Burdman, S., 2009. Glycogen phosphorylase is involved in stress endurance and biofilm formation in *Azospirillum brasilense* Sp7. FEMS Microbiol. Lett. 300, 75–82.
Li, Y., Han, D., Sommerfeld, M., Hu, Q., 2011. Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under nitrogen-limited conditions. Bioresour. Technol. 102, 123–129.
Lopez, B.R., Bashan, Y., Trejo, A., de-Bashan, L.E., 2013. Amendment of degraded desert soil with wastewater debris containing immobilized *Chlorella sorokiniana* and *Azospirillum brasilense* significantly modifies soil bacterial community structure, diversity, and richness. Biol. Fert. Soils 49, 1053–1063.
Markou, G., Angelidaki, I., Georgakakis, D., 2012. Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Appl. Microbiol. Biotechnol. 96, 631–645.
MacIntyre, H.L., Cullen, J.J., 2005. Using cultures to investigate the physiological ecology of microalgae. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington, MA, pp. 287–326.
McCready, R.M., Guggolz, J., Silviera, V., Owens, H.S., 1950. Determination of starch and amylose in vegetables. Anal. Chem. 22, 1156–1158.
Oh-Hama, T., Miyachi, S., 1992. Chlorella. In: Borowitzka, M.A., Borowitzka, L.J. (Eds.), Microalgae Biotechnology. Cambridge University Press, Cambridge, UK, pp. 3–26.
Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. Water Res. 45, 11–36.
Přibyl, P., Cepák, V., Zachleder, V., 2012. Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. Appl. Microbiol. Biotechnol. 94, 549–561.
Radakovits, R., Jinkerson, R.E., Darzins, A., Posewitz, M.C., 2010. Genetic engineering of algae for enhanced biofuel production. Eukaryot. Cell 9, 486–501.
Rahn, O., 1930. The formula for the fermenting capacity of a single cell. J. Bacteriol. 19, 383–387.
Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K., Peccia, J., 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: pathway description and gene discovery for production of next-generation biofuels. BMC Genomics 12, 148.
Siaut, M., Cuiné, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylides, C., Li-Beisson, Y., Peltier, G., 2011. Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnol. 11, 7.
Slattery, C.J., Kavakli, I.H., Okita, T.W., 2000. Engineering starch for increased quantity and quality. Trends Plant Sci. 5, 291–298.
Smith, A.M., 2008. Prospects for increasing starch and sucrose yields for bioethanol production. Plant J. 54, 546–558.
Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.
Tang, H., Chen, M., Garcia, M.E.D., Abunasser, N., Simon, N.K.Y., Salley, S.O., 2011. Culture of microalgae *Chlorella minutissima* for biodiesel feedstock production. Biotechnol. Bioeng. 108, 2280–2287.
Tanner, W., 2000. The *Chlorella* hexose/H^+-symporters. Int. Rev. Cytol. 200, 101–141.
Trejo, A., de-Bashan, L.E., Hartmann, A., Hernandez, J.-P., Rothballer, M., Schmid, M., Bashan, Y., 2012. Recycling waste debris of immobilized microalgae and plant growth-promoting bacteria from wastewater treatment as a resource to improve fertility of eroded desert soil. Environ. Exp. Bot. 75, 65–73.
Tsavkelova, E.A., Klimova, S.Y., Cherdyntseva, T.A., Netrusov, A.I., 2006. Microbial producers of plant growth stimulators and their practical use: a review. Appl. Biochem. Micro. 42, 117–126.
Vonshak, A., Torzillo, G., 2004. Environmental stress physiology. In: Richmond, A. (Ed.), Handbook of Microalgae Culture: Biotechnology and Applied Phycology. Blackwell Publishing, Oxford, UK, pp. 57–82.

Wisniewsky-Dyé, F., Borziak, K., Khalsa-Moyers, G., Alexandre, G., Sukharnikov, L.O., et al., 2011. *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. Plos Genet. 7 (12), e1002430.

Wisniewsky-Dyé, F., Lozano, L., Acosta-Cruz, E., Borland, S., Drogue, B., Prigent-Combaret, C., Rouy, Z., Barbe, V., Herrera, A.-M., González, V., Mavingui, P., 2012. Genome sequence of Azospirillum brasilense CBG497 and comparative analyses of Azospirillum core and accessory genomes provide insight into niche adaptation. Genes 3, 576–602.

Xiong, W., Gao, C., Yan, D., Wu, C., Wu, Q., 2010. Double CO_2 fixation in photosynthesis–fermentation model enhances algal lipid synthesis for biodiesel production. Bioresour. Technol. 101, 2287–2293.

Zachleder, V., Ballin, G., Doucha, J., Šetlík, I., 1988. Macromolecular syntheses and the course of cell cycle events in the chlorococcal alga *Scenedesmus quadricauda* under nutrient starvation: effect of phosphorus starvation. Biol. Plantarum 30, 92–99.