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ORIGINAL ARTICLE

Activity of acetyl-CoA carboxylase is not directly linked to accumulation of lipids when *Chlorella vulgaris* is co-immobilised with *Azospirillum brasilense* in alginate under autotrophic and heterotrophic conditions

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Abstract Activity of acetyl-CoA carboxylase (ACCase) and lipid accumulation were assayed in the microalga *Chlorella vulgaris* co-immobilised in alginate beads with *Azospirillum brasilense*, under autotrophic and heterotrophic growth conditions, with and without ammonium starvation. ACCase is a key enzyme in de novo fatty acid biosynthesis. Under the two growth conditions, co-immobilisation always enhanced the activity of ACCase and yielded a higher level of lipids when compared with immobilisation of the alga alone. The highest lipid content obtained under autotrophic conditions was with ammonium starvation. Cultivation under heterotrophic conditions without limitation of nitrogen, with or without the presence of bacteria, yielded a higher growth rate and accumulated more lipids than under autotrophic conditions. No correlation was found between total lipids and ACCase activity. Unusually, ammonium starvation significantly reduced lipid accumulation under heterotrophic conditions. Consequently, co-immobilisation, sufficient ammonium and heterotrophic growth conditions were the most significant parameters for lipid accumulation and ACCase activity in *C. vulgaris* where the two latter parameters are not directly linked.

Keywords Acetyl-CoA carboxylase · *Azospirillum* · *Chlorella* · Immobilisation · Lipids

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

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Introduction

The genus *Chlorella* is a commonly studied unicellular, non-motile, green microalga inhabiting aquatic environments. Many microalgae have diverse applications in high-value, low-volume compounds, such as pigments for the food industry, products for the health food market, human and animal foodstuffs, applications for wastewater treatments, and, potentially, as future biofuels (Lebeau and Robert 2006; de-Bashan and Bashan 2010; Mata et al. 2010). *Azospirillum* spp. are bacteria with known plant growth promoters that enhance the performance of many plant and algal species, including the unicellular *Chlorella* spp. (Bashan et al. 2004). This enhancement occurs via many simultaneously operating mechanisms, in tandem or cascading, a process recently termed "Multiple Mechanisms Theory" (Bashan and de-Bashan 2010). Among the recorded effects of the bacteria on *Chlorella* spp. during co-immobilisation are increase in population (Gonzalez and Bashan 2000), increase in nitrogen and phosphorus metabolism (de-Bashan et al. 2002b; 2004; Hernandez et al. 2006; de-Bashan et al. 2008b; Perez-Garcia et al. 2010; Covarrubias et al. 2012), lipid accumulation and effects on fatty acid and pigment production (de-Bashan et al. 2002a), enhanced carbohydrate and starch accumulation (Choix et al. 2012a, b), and mitigation of negative effects of pH (de-Bashan et al. 2005) or excess tryptophan (de-Bashan and Bashan 2008).

Chlorella vulgaris grows under both autotrophic and heterotrophic conditions. Under heterotrophic conditions, glycerol (O'Grady and Morgan 2011), Na-acetate (Perez-Garcia et al. 2011), and glucose (Wan et al. 2012) can be used as carbon sources. Heterotrophic cultures of two microalgae have been used to produce polyunsaturated fatty acids (PUFAs) on a commercial scale, and five species of *Chlorella* produced pigments, lipids, antioxidants, L-ascorbic acid, and polysaccharides on an experimental scale (de-Bashan et al. 2002a; Bumbak et al. 2011; Choix et al. 2012b).

Immobilisation of microorganisms, including microalgae and *Azospirillum* (each species separately), is a common biotechnological practice for many agricultural and industrial applications (Bashan 1986, 2002; Prasad and Kadokawa 2009). Co-immobilisation of *Chlorella* spp. with *A. brasilense* has been proposed as a potential technology for wastewater treatment (de-Bashan et al. 2002b; 2004; Cruz et al. 2013) and a model to study interaction between eukaryotic and prokaryotic cells (de-Bashan and Bashan 2008; de-Bashan et al. 2011).

Cells of microalgae contain lipids in the range of 4–65 % (Gouveia and Oliveira 2009) and those of *C. vulgaris* within the range of 5–58 % (Mata et al. 2010). Although almost all of this is usually located in the membranes, many microalgae accumulate neutral lipids over membrane lipids under certain growth conditions. In plants (Ohlrogge and Browse 1995; Francki et al. 2002; Klaus et al. 2004), as well as in microalgae (Radakovits et al. 2010), the most abundant lipids are derived from fatty acid and glycerolipid biosynthetic pathways.

In plants and algae, ACCase is found in plastids, where primary fatty acid biosynthesis occurs, and in the cytosol, where synthesis of very long-chain fatty acids and flavonoids occurs (Liu et al. 2007; Sato and Moriyama 2007; Yu et al. 2007). Several studies have shown an association between ACCase activity and accumulation of lipids. One study demonstrates that ACCase is the control point in lipid biosynthesis in potato tubers (Klaus et al. 2004). Inhibition of ACCase leads to reduced fatty acid synthesis in lipogenic tissues in mammals (Tong and Hardwood 2006). Increase of ACCase activity in *E. coli* leads to greater synthesis of fatty acids (James and Cronan 2004). The rate of synthesis of fatty acids in spinach changes greatly with light and dark regimes by activating or inactivating ACCase (Sasaki and Nagano 2004). In microalgae (diatoms), ACCase activity was detected, but it was not directly correlated to accumulation of lipids (Sheehan et al. 1998).

Nitrogen starvation commonly induces accumulation of lipids in several microalgae under autotrophic conditions (Xiong et al. 2010; Tang et al. 2011; Přibyl et al. 2012), and co-immobilisation of *Chlorella vulgaris* and *Azospirillum brasilense* affects several metabolic pathways in the microalga as described above. We hypothesised, therefore, that these factors will also enhance lipid accumulation and activity of ACCase under autotrophic and heterotrophic conditions and ACCase activity is linked to accumulation of lipids. Consequently, the objectives of this study were to: (1) measure the effect of immobilisation of *C. vulgaris* with *A. brasilense* on the activity of ACCase and accumulation of lipid, (2) measure the effect of ammonium starvation on this prokaryote–eukaryote interaction, (3) determine whether growth under autotrophic (illuminated) and heterotrophic (dark) conditions affects accumulation of lipids and ACCase activity during this interaction, and (4) find whether changes in activity of ACCase is directly linked to accumulation of lipids in *C. vulgaris*.

Materials and methods

Microorganisms and culture conditions

Chlorella vulgaris Beijerinck (UTEX 2714, University of Texas, Austin, TX, USA) and *Azospirillum brasilense* Cd (DSM 1843; Leibniz-Institut DMSZ, Braunschweig, Germany) were used in all experiments. *C. vulgaris* was cultured for 7 days in mineral growth media (C30; Gonzalez et al. 1997) and agitated at 140 rpm in an orbital shaker, 28±1 °C, and 60 μmoles photon m^{-2} s^{-1}. 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), and $NaMoO_4$ (0.021). *A. brasilense* was cultured for 18 h in nutrient broth (NB; #N7519 Fluka; Sigma-Aldrich, St. Louis, MO, USA) at 37±1 °C and agitated at 140 rpm.

Immobilisation of microalgae and bacteria in alginate beads

Microorganisms were immobilised using the method described by de-Bashan et al. (2004), where 40 mL *C. vulgaris* culture (6.0×10^6 cells mL^{-1}) was mixed with 160 mL of a sterile, 6,000 cP 2 % alginate solution (i.e., alginate mixed at 14,000 cP and 3,500 cP), and stirred for 15 min. Using an automatic bead maker, this mixture was dropped into a 2 % $CaCl_2$ solution under slow stirring (de-Bashan and Bashan 2010). The beads formed were stabilised for 1 h at 28±1 °C and washed in sterile saline solution. *Azospirillum brasilense* (approximately 1.0×10^9 CFU mL^{-1}) was immobilised similarly. The immobilisation normally reduces the number of organisms in the beads; therefore, a second incubation step was necessary (10 % NB overnight). For starvation conditions of *A. brasilense*, N-free OAB medium was used for the second incubation step (Bashan et al. 1993). To combine both species in the same beads, a similar procedure was performed, using 20 mL of each culture in a mixture (total of 40 mL). After the second incubation the beads were placed in 1-L Erlenmeyer flasks (40 g of beads per flask) containing 500 mL of synthetic growth medium (SGM; de-Bashan et al. (2011). SGM

medium contains (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), and NH_4Cl; (191). The flasks were placed in an orbital shaker for 6 days under the same conditions as described for culturing *Chlorella*. In the starvation experiments using the microalgae, the N-free SGM medium was used.

Autotrophic and heterotrophic experimental conditions

For autotrophic experiments, the flasks were incubated in an orbital shaker using the same light and temperature conditions described for culturing *C. vulgaris*. For heterotrophic conditions, the preculture was maintained in the dark without nitrogen for 24 h (Choix et al. 2012b), and during the experiments, the culture was maintained in total darkness at 28±1 °C, using 10 g L^{-1} of sodium acetate (#S7670; Sigma-Aldrich) as a carbon source (Choix et al. 2012a, b) and nitrogen (5 mM ammonium, 90 mg L^{-1}) and phosphorus (phosphate, 0.44 mM or 42 mg L^{-1}).

Samples

From each Erlenmeyer flask, 40 g of beads were taken (the beads swell when placed in the SGM) in 50-mL Corning tubes and frozen at −80 °C. These samples were used for all analyses listed below and in all experiments.

Counting microorganisms after treatment

After each experiment, beads containing microorganisms were dissolved in 4 % sodium bicarbonate solution at room temperature (~28 °C) for ~30 min., then microorganisms were counted. *C. vulgaris* was counted under a light microscope with a Neubauer hemocytometer (Gonzalez and Bashan 2000) connected to an image analyser (Image ProPlus 4.5; Media Cybernetics, Silver Spring, MD, USA). Growth rate (μ) of *C. vulgaris* was defined by: $\mu = (\ln Nt_1 - \ln Nt_0) / (t_1 - t_0)$, where Nt_1 is the number of cells at sampling time and Nt_0 is the number of cells at the beginning of the experiment, t_1 is the sampling time, and t_0 is the beginning of the experiment (Oh-Hama and Miyachi 1992). *A. brasilense* was counted after serial dilution by the plate count method on nutrient agar medium (#M7519; Sigma-Aldrich).

Quantification of lipids

Standard curve for lipids: The quantity of lipids was measured following the method described by Pande et al. (1963). Extraction of lipids followed the standard method described by Bligh and Dyer (1959) with modifications involving sonication to break down cell walls. Briefly, lipids were extracted by adding 4 mL methanol/chloroform solution (2:1, v/v) to dry beads. The beads were sonicated for 10 min (2 cycles of 5 min at 30 kHz) in an ice bath. The sonicated beads were incubated at 4 °C for 24 h in the dark and this procedure (only sonication) was repeated under the same conditions. The sample was then centrifuged (5,000 *g*, 20 min, 4 °C) and the supernatant was transferred to a clean tube. The rest of the analysis was done as originally described.

Quantification of lipids: Lipid assays, based on a potassium dichromate colour change reaction, were done according to Pande et al. (1963), using a calibration curve with tripalmitin (#T5888; Sigma-Aldrich), as a standard. The concentration of lipids was determined in a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm, recording the intensity of the green colour formed. Potassium dichromate has a yellow-reddish colour before the reaction and yellow-green after the reaction with lipids. The method quantified lipids in the range of 70 μg to 1.33 mg.

Enzymatic activity of ACCase

Extraction: Frozen bead aliquots were dissolved in two volumes of 4 % solution of $NaHCO_3$ for 40 min at room temperature. Each suspension was then centrifuged (5,000 *g*, 10 min, 4 °C), the supernatant was discarded and the pellet was washed twice in 0.85 % NaCl and centrifuged again. The pellet was frozen with liquid nitrogen and pulverised with pestle and mortar. Preliminary analyses using sonication (5× 1 min, 30 kHz with 1 min incubation on ice between sonications, 9 min total) yielded identical enzymatic activity results compared to the pestle and mortar technique that was generally used for convenience. For resuspension, 5 mL extraction buffer [100 mM Tris–HCl pH 8.2, 4 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF, #P7626; Sigma-Aldrich)] was added to the pellet. This was then centrifuged for 30 min at 10,500 *g* at 4 °C. The pellet was discarded and the supernatant was used as a crude extract for enzymatic reactions (de-Bashan et al. 2008a).

Quantification: The reaction buffer was composed of 50 mM Tris–HCl pH 7.5, 6 μM acetyl-CoA, 2 mM ATP, 7 mM $KHCO_3$, 8 mM $MgCl_2$, 1 mM DTT, and 1 mg mL^{-1} of bovine serum albumin (BSA; #B4287; Sigma-Aldrich). The crude extract was pre-incubated (30 min, 25 °C) with 10 mM potassium citrate and 2 mg mL^{-1} BSA. Then, 200 μL crude extract was added to 0.8 mL of reaction buffer and the enzymatic reaction was incubated for 1 h at 30 °C. The reaction was stopped with 0.5 mL 10 % perchloric acid (PCA, #244252; Sigma-Aldrich). The total reaction mix was filtered (0.22-μm membrane filter; EMD Millipore, Billerica, MA, USA). Then, 500 μL of this mixture was transferred to a 1.5-mL glass vial and injected into the HPLC according to the method described by Levert et al. (2002), using a Zorbax Eclipse Plus C-18 column (4.6 μm and 5×150 mm; Agilent Technologies, Santa Clara, CA, USA). The flow rate was

1 mL min^{-1} and the UV detector was adjusted to 262 nm. Solution A was 10 mM KH_2PO_4 at pH 6.7 and solution B was absolute methanol. The analysis was done in triplicate with two controls; in both controls, the PCA solution was added at the beginning of the reaction's development time. Using analytical software (ChemStation; Agilent Technologies), the peak areas were recorded and the quantity of acetyl-CoA was calculated using previously completed standard curves of acetyl-CoA and malonyl-CoA; hence, measuring either the disappearance of the substrate (acetyl-CoA) or the formation of the product (malonyl-CoA). Specific activity of ACCase could not be calculated because of the large quantity of unrelated proteins produced mainly by the bacterium during co-cultivation; a factor that masked all values. Therefore, activity was defined as nmoles of substrate transformed per min per one mL of sample (beads).

Determination of chlorophyll

To determine the quantity of chlorophyll *a*, extraction was done according to Sartory and Grobbelaar (1984) with small modifications. Quantification used the equation of Porra et al. (1989): Chl a=16.29 (A_{665}) – 8.54 (A_{652}). Briefly, 10 mL 100 % methanol was added to 5 mL of freshly thawed beads and heated for 10 min at 70 °C. After cooling, the samples were incubated in the dark for 24 h at 4 °C. Then, the samples were centrifuged for 10 min (4 °C; 6,000 *g*) and absorbance was recorded in the supernatants at 665 and 652 nm.

Experimental design and statistical analysis

Eight individual experiments were done using a factorial design. Three variants were used: (1) *Azospirillum* alone, (2) *Chlorella* alone, and (3) co-immobilisation of *Chlorella* and *Azospirillum*. Beads without microorganisms were not routinely used because preliminary determination showed that there was no effect on the measured parameters, total lipids, and ACCase activity. Two treatments were used: (1) ammonium starvation and (2) autotrophic or heterotrophic growth conditions. The treatments were tested with either a full supply of ammonium (90 mg NH_4^+ L^{-1} or 5 mM) or ammonium starvation (0 mg NH_4^+ L^{-1}), each under autotrophic (light) or heterotrophic (dark) conditions. In each treatment, three 1-L Erlenmeyer flasks containing 0.5 L SGM were used, where each flask served as a replicate. Each experiment was repeated twice and average data of both experiments were used for statistical analysis. In all eight experiments, three analyses were done: ACCase enzymatic activity, total lipids, and chlorophyll *a* content. Statistical analysis was done by Student's *t* test at $p<0.05$ (comparisons between autotrophic and heterotrophic conditions) or one-way ANOVA and LSD post-hoc analysis at $p< 0.05$ (comparisons among the three treatments), using Statistica 8.0 software (StatSoft, Tulsa, OK, USA).

Results

All experiments were performed when *C. vulgaris* was immobilised with *Azospirillum brasilense* in alginate beads under ammonium starvation and autotrophic and heterotrophic conditions. An optimum supply of ammonium was used in some experiments for comparison with ammonium starvation conditions. *Azospirillum* and *Chlorella* immobilised individually are also described.

Effects on total lipids

With an optimum supply of ammonium, cultures of *C. vulgaris* accumulated more lipids under heterotrophic than autotrophic conditions, whether cultured alone or with *A. brasilense* (Fig. 1a, lowercase analysis). Co-immobilisation under either type of growth cultivation produced more lipids than immobilisation of the microalgae alone (Fig. 1a, uppercase letter analysis). When cellular lipid content was calculated per cell of microalga, the cultivation conditions, autotrophic or heterotrophic, had no effect on the accumulation of lipids (Fig. 1b, lowercase analysis). Yet, each cell in the co-immobilised treatment had significantly more lipids than in other variants (Fig. 1b, uppercase letter analysis). Specific growth rates (μ; see Materials and Methods) under autotrophic conditions without starvation were 0.087±005 (microalgae immobilised alone) and 0.056±0.003 (co-immobilisation)for autotrophic with starvation 0.051±0.002 (immobilised alone) and −0.04±0.0 (co-immobilisation); for heterotrophic conditions without starvation 0.168±0.002 (immobilised alone) and 0.153±0.003 (co-immobilisation); for heterotrophic combined with starvation 0.023±0.004 (immobilised alone) and −0.048±0.004 (co-immobilisation). Growth rate of *Chlorella* was calculated per 6 days of growth when day 0 is immediately before the experiment started and day 6 after taking the sample. Values calculated are per day.

Under autotrophic conditions, ammonium starvation significantly enhanced lipid accumulation in all variants (microalgae and bacteria immobilised alone or co-immobilised) compared with cultivation with an optimal supply of ammonium (compare Fig. 1a and c). Contrary to conditions with an optimal supply of ammonium, cultures of *C. vulgaris* accumulated more lipids under autotrophic conditions (Fig. 1c, lowercase analysis), where *C. vulgaris* co-immobilised with *A. brasilense,* and cultured under autotrophic conditions combined with ammonium starvation, produced more lipids than any other variant (i.e., individually immobilised microalga or bacterium) (Fig. 1c, uppercase letter analysis). Under heterotrophic conditions, co-immobilisation did not enhance total lipid accumulation (Fig. 1c, uppercase letter analysis). This observation held true when lipid accumulation was calculated per cell (Fig. 1d, uppercase letter analysis and lowercase analysis, separately); however, it was

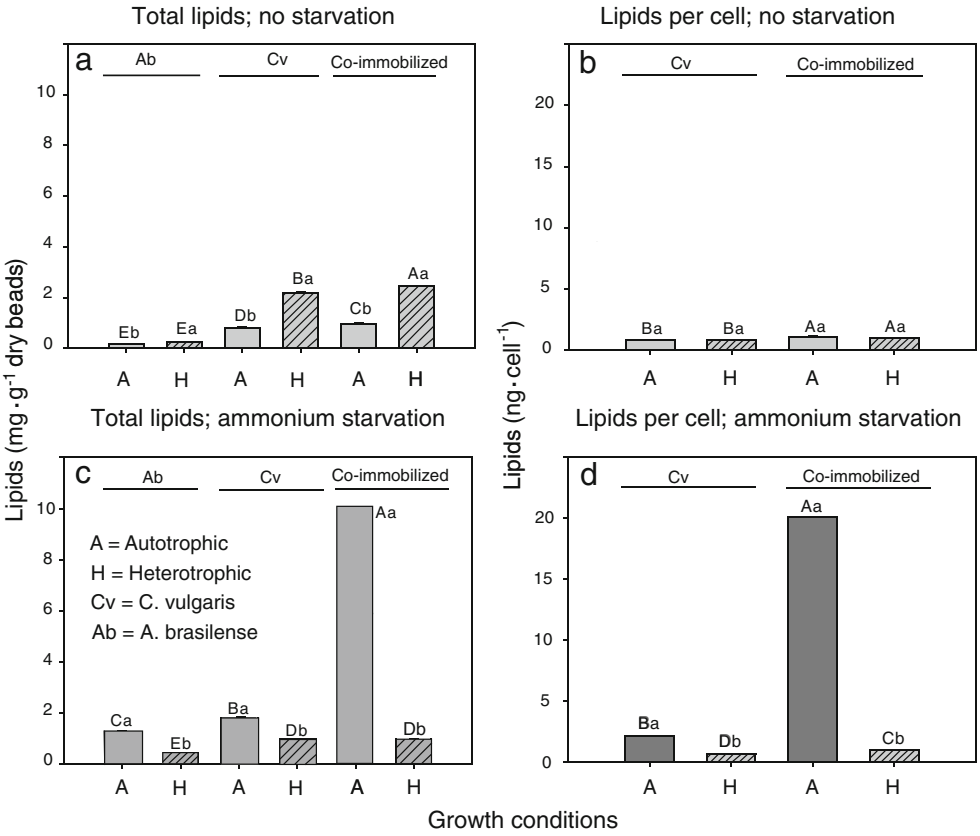

Fig. 1 Total lipid contents in *Chlorella vulgaris* (UTEX 2714) immobilised alone and combined with *Azospirillum brasilense* Cd (DSM 1843) under autotrophic and heterotrophic conditions and after incubation for 6 days and initial supply of 90 mg NH_4^+ L^{-1}. **a** Total lipids per culture (mg g dw beads^{-1}); **b** total lipids per cell. **c** and **d** are the same situations under ammonium starvation. Statistics was done separately for each subfigure. *Columns denoted by a different lowercase letter* for autotrophic versus heterotrophic conditions for immobilisation alone and co-immobilisation, which differ significantly at $p < 0.05$ using Student's *t* test. *Columns denoted by a different uppercase letter* differ significantly by one-way ANOVA and LSD post hoc analysis at $p < 0.05$. *Bars* SE. A missing bar (in subfigures **c** and **d**) signifies negligible SE. *A* Autotrophic conditions; *H* heterotrophic conditions

observed that, under heterotrophic conditions, co-immobilisation produced slightly more lipids per cell than when the microalgae were immobilised alone (Fig. 1d, uppercase letter analysis).

The effect of starvation under autotrophic and heterotrophic conditions (by a *t* test) on accumulation of lipids per culture and per cell is presented in Table S1, employing the raw data that was used as the source for analyses presented in Fig. 1. Under autotrophic conditions, regardless of the variant (immobilisation of microalgae and bacteria alone or combined), starvation led to more total lipids in *C. vulgaris*. Under heterotrophic conditions, starvation reduced the amount of accumulated lipids, with the sole exception of analysis of co-immobilisation at the cell level, where no decrease was observed.

Effect on ACCase activity

Records of activities of ACCase were different, depending on the way activity was calculated, i.e., per culture or per single cell. In cultures with an optimal supply of ammonium, immobilised cultures of *C. vulgaris* alone cultivated heterotrophically had higher ACCase activity than under autotrophic conditions. In co-immobilised cultures, the opposite phenomenon was recorded (Fig. 2a, lowercase analysis). While similar trends were observed when activity was calculated per cell for co-immobilisation, the effect on ACCase activity in single immobilisation disappeared (Fig. 2b, lowercase analysis). Activity of ACCase was highest under autotrophic co-immobilisation (Fig. 2a, b, uppercase letter analyses).

Under conditions of ammonium starvation, the general level of ACCase activity was lower than in non-starved cultures at the culture level (compare Fig. 2a and c), but activity was mostly higher at the cell level (compare Fig. 2b and d). The effects of ammonium starvation on ACCase activity were the same regardless of whether autotrophic or heterotrophic culture conditions were used (Fig. 2c, d, lowercase analyses). ACCase activity was however, always higher in co-immobilisation, compared to single immobilisation (Fig. 2c, d, uppercase letter analyses).

The effect of starvation under autotrophic and heterotrophic conditions (by a *t*-test) on ACCase activity per culture and per cell is presented in Table S2, employing the raw data that was used as the source for analyses presented in Fig. 2. Under autotrophic conditions, ammonium starvation significantly decreased ACCase activity per culture and per cell. Under heterotrophic conditions at the culture level, no effect

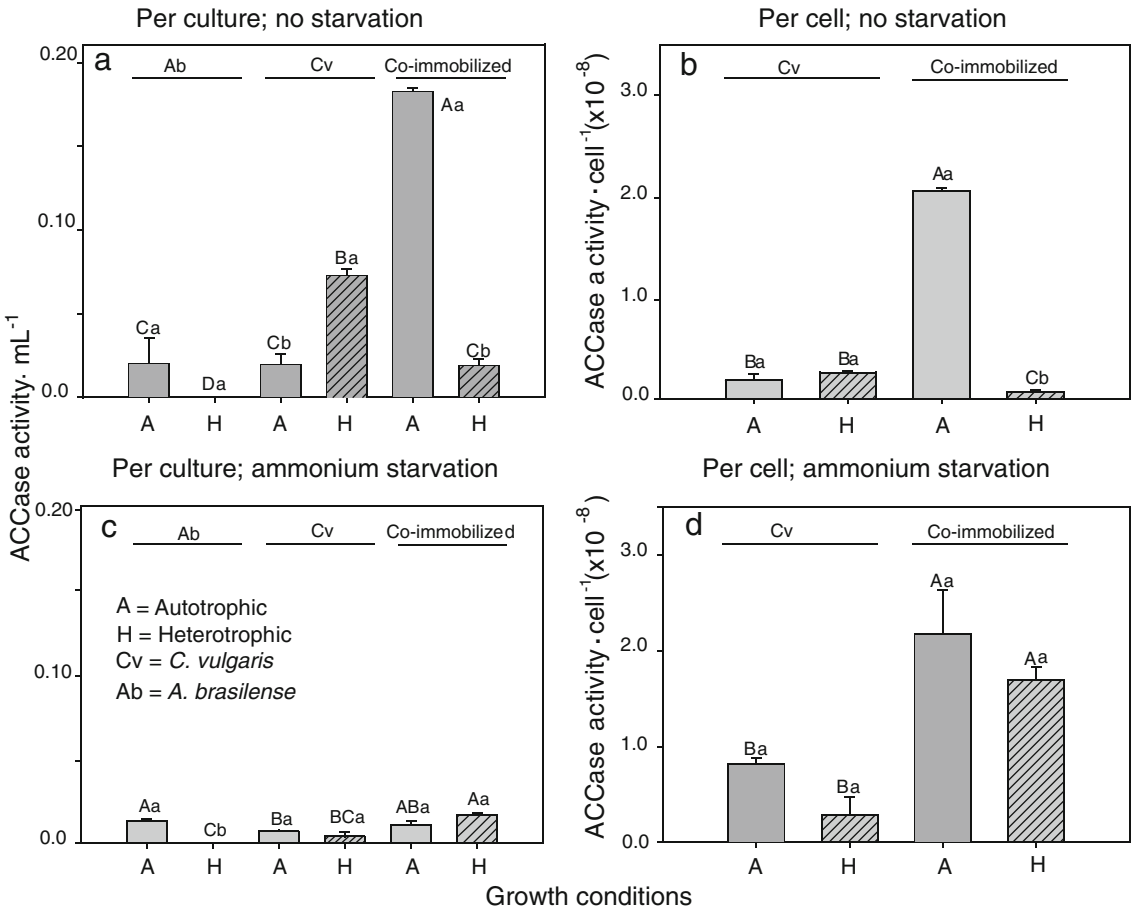

Fig. 2 ACCase activity in *Chlorella vulgaris* (UTEX 2714) immobilised alone and when immobilised with *Azospirillum brasilense* Cd (DSM 1843) under autotrophic and heterotrophic conditions and after incubation for 6 days and initial supply of 90 mg NH_4^+ L^{-1}. **a** ACCase activity per 1 mL culture; **b** total lipids per cell. **c** and **d** are the same under ammonium starvation. Statistics for each subfigure applied separately. *Columns denoted by a different lowercase letter* for autotrophic versus heterotrophic conditions for immobilised and co-immobilisation, which differ significantly at $p<0.05$ using Student's *t* test. *Columns denoted by a different uppercase letter* differ significantly by one-way ANOVA and LSD post hoc analysis at $p<0.05$. *Bars* SE. A missing bar signifies negligible SE. *A* Autotrophic conditions; *H* heterotrophic conditions

on ACCase activity was observed. But per cell ACCase activity is higher for co-immobilised *Chlorella.*

Effect on chlorophyll a content

Regardless of the growth conditions or starvation regime, the content of chlorophyll *a* in cells of *C. vulgaris* was always higher in co-immobilisation (Fig. 3). The highest content was recorded for non-starved autotrophic cultures (Fig. 3a). Comparable lower levels of chlorophyll *a* were recorded in starved cultures (autotrophic and heterotrophic) or non-starved heterotrophic cultures.

Discussion

Among the many changes in cell components and metabolism of *C. vulgaris* induced by immobilisation with the microalgae growth-promoting bacterium *A. brasilense* (under non-limited nitrogen and autotrophic growth conditions) is an increase in total lipids (de-Bashan et al. 2002a). However, the precise mechanisms for accumulating lipids in the microalgae in the combined and singly immobilised systems are unknown. This work is built upon this initial observation and measures the effects of ammonium starvation, combined with either autotrophic or heterotrophic growth conditions, on lipid accumulation and ACCase activity and the possible link between the two. ACCase is a key enzyme in de novo synthesis and in regulating fatty acid synthesis rate (Hu et al. 2008).

From all the tested parameters, co-immobilisation and heterotrophic growth conditions were the main factors affecting accumulation of lipids in *C. vulgaris*. A general analysis of the data obtained from the eight experiments is presented in Table 1. Under autotrophic and heterotrophic conditions, co-immobilisation always yielded higher amounts of lipids. The main factor that enhances accumulation of lipids in *Chlorella* spp. under autotrophic conditions is nitrogen starvation (Xiong et al. 2010; Tang et al. 2011; Přibyl et al. 2012). Our

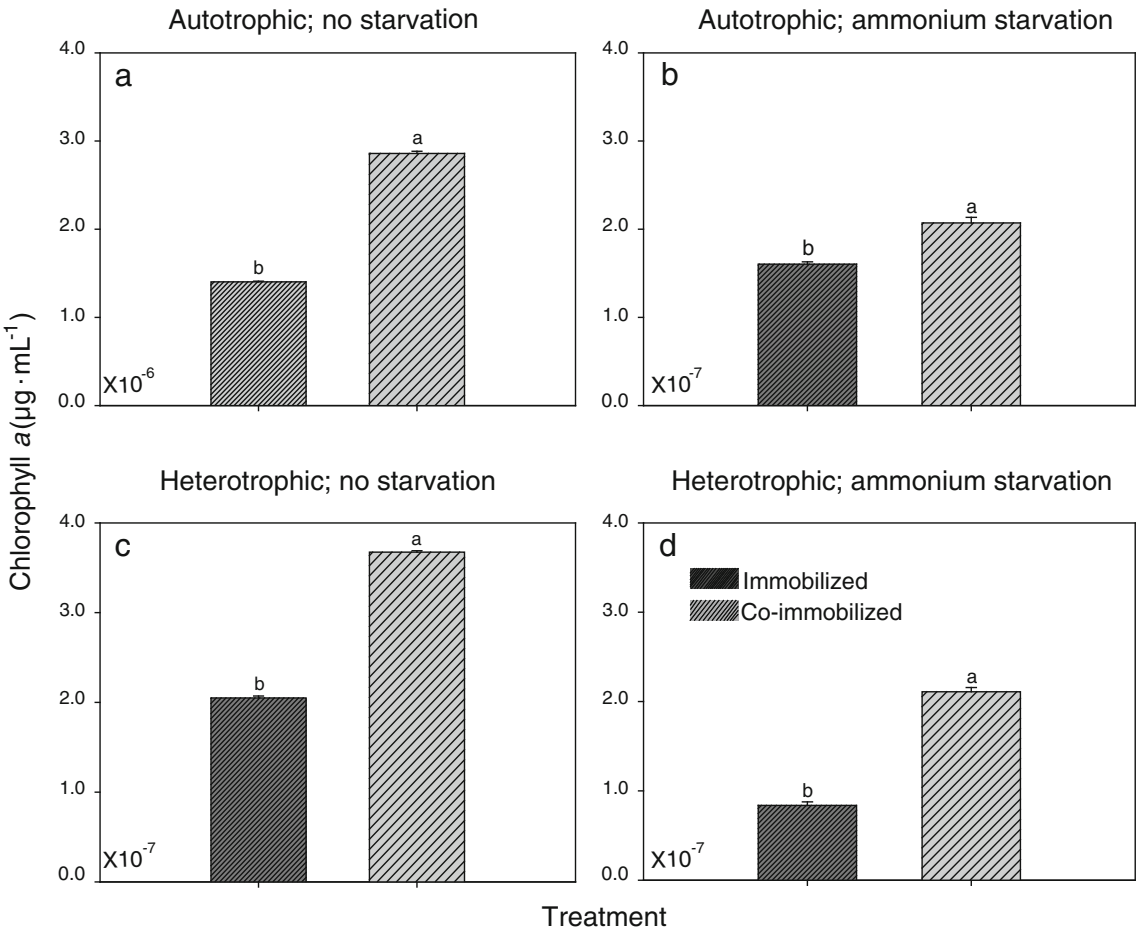

Fig. 3 Chlorophyll *a* content of *Chlorella vulgaris* (UTEX 2714) immobilised alone and combined with *Azospirillum brasilense* Cd (DSM 1843) under autotrophic and heterotrophic conditions and after incubation for 6 days and initial supply of 90 mg NH_4^+. **a**, **c** Without ammonium starvation; **b**, **d** under starvation conditions. Statistics for each subfigure were applied separately. *Columns denoted by a different lowercase letter* for autotrophic versus heterotrophic conditions for immobilised and co-immobilisation, which differ significantly at $p < 0.05$ using Student's *t* test. *Bars* SE. A missing bar signifies negligible SE

findings regarding autotrophic growth and nitrogen starvation are in accordance with this paradigm; the highest lipid content in this study under autotrophic conditions was obtained with ammonium starvation, but only when the microalga was co-immobilised with the bacterium. An unusual event that was detected was that under heterotrophic conditions ammonium starvation reduced lipid accumulation. Cultivation under heterotrophic conditions without limitation of nitrogen, however, and with or without the presence of bacteria, accumulated more lipids than under autotrophic conditions. A similar result was reported for single species cultivation where an increase of up to 900 % in lipid content in heterotrophic cultures of *Chlorella zofingiensis* fed 30 g L^{-1} glucose was found (Liu et al. 2011). In our study, therefore, it is unlikely that accumulation of lipids in *C. vulgaris* primarily results from limited N. While nitrogen starvation is a universal pre-requisite for lipid over-expression in most microalgae under autotrophic conditions, this study showed that *A. brasilense* clearly and strongly contributes to this effect.

Under co-immobilisation conditions with *A. brasilense,* accumulation of lipids differs between the quantity of lipids each cell accumulates in a specific culture and the total lipid accumulation capacity of a culture. This depends mainly on the size of developing microalgal population in each culture. This was demonstrated earlier for nitrogen uptake (de-Bashan et al. 2005) and carbohydrate and starch accumulation (Choix et al. 2012a, b) with our system of co-immobilisation. This phenomenon can be explained by the growth conditions of this co-culture. de-Bashan et al. (2005) and Choix et al. (2012a, b) demonstrate that culturing conditions have a significant effect on the metabolism of *Chlorella* spp. Small populations can uptake large quantities of nitrogen or accumulate more carbohydrate and starch, whereas larger populations were less efficient. In this study we demonstrated that this also occurred for lipid accumulation.

As a key enzyme in fatty acid synthesis, ACCase has been studied in several plants, including maize leaves (Egli et al. 1993; Herbert et al. 1996), pea leaves (Alban et al. 1994), rice

Table 1 General analysis of data obtained from the eight experiments of immobilised *Chlorella vulgaris* and *Azospirillum brasilense* alone and combined, with and without ammonium starvation, under autotrophic and heterotrophic conditions

Lipids; per culture		
Growth condition	With 90 mg NH_4^+	Without NH_4^+ (ammonium starvation)
Autotrophic	Comb > Chlo > Azo	Comb > Chlo > Azo
Heterotrophic	Comb > Chlo > Azo	Comb = Chlo > Azo
Best regime	Hetero > Auto	Auto > Hetero
The best treatment is co-immobilisation under autotrophic conditions and ammonium starvation		
Lipids; per cell		
Growth condition	With 90 mg NH_4^+	Without NH_4^+ (ammonium starvation)
Autotrophic	Comb > Chlo	Comb > Chlo
Heterotrophic	Comb > Chlo	Comb > Chlo
Best regime	Auto = Hetero	Auto > Hetero
The best treatment is co-immobilisation under autotrophic conditions and ammonium starvation		
ACCase activity; per culture		
Growth condition	With 90 mg NH_4^+	Without NH_4^+ (ammonium starvation)
Autotrophic	Comb > Chlo = Azo	Comb = Azo > Chlo
Heterotrophic	Comb < Chlo > Azo	Comb > Chlo > Azo
Best regime	Auto > Hetero	Auto = Hetero
The best treatment is co-immobilisation under autotrophic conditions with 90 mg NH_4^+		
ACCase activity; per cell		
Growth condition	With 90 mg NH_4^+	Without NH_4^+ (ammonium starvation)
Autotrophic	Comb > Chlo	Comb > Chlo
Heterotrophic	Comb = Chlo	Comb > Chlo
Best regime	Auto > Hetero	Auto = Hetero
The best treatment is co-immobilised under autotrophic conditions with 90 mg NH_4^+		

Comparisons were done separately per culture or per cell for each pair of variables, using Student's *t* test at $p<0.05$. *Azo Azospirillum brasilense*, *Chlo Chlorella vulgaris*, *Comb* immobilisation of *C. vulgaris* with *A. brasilense*, *Auto* autotrophic growth conditions, *Hetero* heterotrophic growth conditions

seedlings (Hayashi and Satoh 2006) , and in microalgae (Sukenik and Livne 1991; Livne and Sukenik 1992; Khozin-Goldberg and Cohen 2011). In our study, ACCase activity at the culture level was variable; however, high activity was obtained with non-starved autotrophic cultures or with co-immobilisation, sufficient ammonium, and autotrophic growth conditions. At the cellular level, the highest activity was obtained when there was co-immobilisation (3 out of 4 analyses) and the best regime, either in cultures or in cells, was autotrophic (Table 1).

Although many major efforts have been made to improve production of lipids in photosynthetic microorganisms, mainly for biofuels production (Radakovits et al. 2010; Rawat et al. 2013), nonetheless, ACCase activity has not been directly correlated to lipid production in diatoms (Roessler and Ohlrogge 1993; Dunahay et al. 1996; Sheehan et al. 1998). Yet, the association between fatty acid accumulation and ACCase activity has been demonstrated (James and Cronan 2004; Klaus et al. 2004; Sasaki and Nagano 2004; Tong and Hardwood 2006). One of the most significant findings of our study is that we did not find a correlation between total lipid content and ACCase activity regardless of the variables tested. It is worth emphasising, however, that co-immobilisation under autotrophic and heterotrophic conditions (the former with and the latter without starvation) generally increased ACCase activity. We propose three plausible explanations of why there is no direct correlation between total lipid content and ACCase activity. The first explanation might be connected to the common method we used to quantify total lipids. This method quantifies other cellular components, such as chlorophyll, carotenoid, sterol, and some vitamins whose structures are lipid in nature (Pande et al. 1963), and whose synthesis is not directly controlled by ACCase, in contrast to the case for common fatty acids. Some of these compounds are enhanced in *C. vulgaris* when co-immobilised with *A. brasilense* (de-Bashan et al. 2002a; this study). This may have the strongest effect on our results in cultures with co-immobilisation, where we showed that chlorophyll *a* contents were always higher compared with individual immobilisation. These side effects may distort the values of lipids attributed to the production of total lipids from

fatty acids by ACCase. The second explanation, strongly supporting our results with *C. vulgaris*, is based on intensive studies to increase production of lipids in diatoms (Sheehan et al. 1998). These studies concluded that the plausible reason for there being no increase in lipid production, despite performing successful ACCase over-expression, is because the lipid biosynthesis pathway may be subjected to feedback inhibition. Therefore, the increased activity of ACCase is compensated for by other pathways within the cell. The third explanation might be that, although ACCase is the unique enzyme in the pathway of formation of fatty acids which leads to production of lipids, lipids in the plant cell can also be produced by other pathways such as the isoprenoid pathway (Ohlrogge and Browse 1995). Thus, an increase or decrease in ACCase activity does not necessarily translate into an increase or decrease in lipid production.

At this stage of our investigations, one needs to add a reservation regarding the separation of the activities of microalgal ACCase and bacterial ACCase, which were both present in the co-immobilisation experiments. The analyses cannot separate the contribution to enzymatic activity of the prokaryotic partner, *A. brasilense*, to the mix. While this contribution is small regarding accumulation of lipids, the bacterium has about the same level of enzymatic activity as microalga immobilised alone under autotrophic conditions, but far lower than in the co-immobilisation treatment.

This study confirmed two observations regarding cultivation of *C. vulgaris* and *A. brasilense* when immobilised together. First, under some conditions, the growth rate of *C. vulgaris*, when co-immobilised with the bacterium, is lower than when the microalga is immobilised alone (Choix et al. 2012b). This demonstrated that the effect of *Azospirillum* is not necessarily on cell multiplication, but rather on metabolic activity, as reported previously (de-Bashan et al. 2005; 2008a). Secondly, sodium acetate is an effective carbon source for heterotrophic studies of this interaction.

In summary, when the eukaryotic–prokaryotic model (microalga–bacterium) is used, the effect exerted by *A. brasilense* on *C. vulgaris* can be quantified as an increase in total lipids and increased ACCase activity. Heterotrophic growth conditions were favourable for production of lipids, ACCase activity, and population growth of *C. vulgaris,* but its enhanced activity of ACCase is not linked to enhanced accumulation of lipids.

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