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ORIGINAL PAPER

Accumulation fatty acids of in *Chlorella vulgaris* under heterotrophic conditions in relation to activity of acetyl-CoA carboxylase, temperature, and co-immobilization with *Azospirillum brasilense*

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Abstract The relation between fatty acid accumulation, activity of acetyl-CoA carboxylase (ACC), and consequently lipid accumulation was studied in the microalgae *Chlorella vulgaris* co-immobilized with the plant growth-promoting bacterium *Azospirillum brasilense* under dark heterotrophic conditions with Na acetate as a carbon source. In *C. vulgaris* immobilized alone, cultivation experiments for 6 days showed that ACC activity is directly related to fatty acid accumulation, especially in the last 3 days. In co-immobilization experiments, *A. brasilense* exerted a significant positive effect over ACC activity, increased the quantity in all nine main fatty acids, increased total lipid accumulation in *C. vulgaris*, and mitigated negative effects of nonoptimal temperature for growth. No correlation between ACC activity and lipid accumulation in the cells was established for three different temperatures. This study demonstrated that the interaction between *A. brasilense* and *C. vulgaris* has a significant effect on fatty acid and lipid accumulation in the microalgae.

Dedication: 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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Keywords Acetyl-CoA carboxylase · *Azospirillum* · *Chlorella* · Immobilization · Lipids

Introduction

Green microalgae of the genus *Chlorella* (Chlorophyceae) are unicellular, simple, nonmotile cells living in aquatic environments and were the first microalgae isolated by Martinus Beijerinck in 1890. Species of this microalgae provide a source for production of high-value, low-volume compounds, including those in the "healthy foods" market (Lebeau and Robert 2006), serve as wastewater treatment agents (de-Bashan and Bashan 2010), and as a basic model to study photosynthesis and respiration (Ilangovan et al. 1998). In recent years, because of several technological and economic advantages that microalgae have over oleaginous plants, microalgae, including *Chlorella* spp., were tested to produce biofuels, so far only experimentally (Chisti 2007; Brennan and Owende 2010; Mata et al. 2010; Rodolfi et al. 2009). The best raw materials for biofuels from microalgae are polyunsaturated fatty acids (PUFA), specifically triacylglycerols (TAG). PUFA can also be used in human food, especially for brain development and function (Bigogno et al. 2002; Khozin et al. 1997; Ratledge 2004).

Species of the genus *Chlorella* can grow under autotrophic, heterotrophic, and mixotrophic conditions. For heterotrophic growth, the most common carbon sources are glucose (Wan et al. 2012; Wu and Shi 2007; Xiong et al. 2008), Na acetate (Choix et al. 2012b; Perez-Garcia et al. 2011a), and glycerol (Isleten-Hosoglu et al. 2012; O'Grady and Morgan 2011). Under heterotrophic conditions, which are more economical for production of high-volume biomass (Brennan and Owende 2010; Perez-Garcia et al. 2011b; Wu and Shi 2007),

two microalgae cultures have been used to produce PUFA at a commercial scale and five *Chlorella* species were used to produce pigments, lipids, antioxidants, L-ascorbic acid, and carbohydrates at an experimental scale (Choix et al. 2012b, 2014; de-Bashan et al. 2002; Bumbak et al. 2011).

In plants and algae, acetyl-CoA carboxylase (ACC) is found in plastids, where primary fatty acid biosynthesis occurs, and in the cytosol, where synthesis of very long-chain fatty acids and flavonoids occur (Liu et al. 2007; Sato and Moriyama 2007; Yu et al. 2007). ACC activity had been studied in maize leaves (Egli et al. 1993; Herbert et al. 1996), pea leafs (Alban et al. 1994), rice seedling (Hayashi and Satoh 2006), and microalgae (Khozin-Goldberg and Cohen 2011; Livne and Sukenik 1992; Sukenik and Livne 1991).

An association between ACC activity and accumulation of fatty acids was shown. Inhibition of ACC leads to reduced fatty acid synthesis in lipogenic tissues in mammals (Tong and Hardwood 2006). Increase of ACC activity in *Escherichia 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 ACC (Sasaki and Nagano 2004). ACC is the key enzyme in lipid biosynthesis in potato tubers (Klaus et al. 2004). Thus far, this did not imply a direct correlation of ACC activity and lipid accumulation in microalgae (Roessler and Ohlrogge 1993; Sheehan et al. 1998).

For many agricultural, biotechnological, and industrial applications, immobilization of numerous microorganisms in polymers is a common practice (Bashan 1986; Bashan et al. 2002; 2014; Prasad and Kadokawa 2009). Co-immobilization of the microalgae *Chlorella* spp. with the plant growth-promoting bacteria (PGPB) *Azospirillum brasilense* is a potential technology for wastewater treatment (Covarrubias et al. 2012; Cruz et al. 2013; de-Bashan et al. 2002, 2004) and a model to study metabolic interactions between eukaryotic and prokaryotic cells (de-Bashan and Bashan 2008; de-Bashan et al. 2011). No direct correlation between ACC activity and lipid accumulation under autotrophic and heterotrophic conditions was demonstrated in this co-immobilized system (Leyva et al. 2014).

Considering these findings, we hypothesized that ACC activity in *Chlorella vulgaris* is linked to accumulation of fatty acids when the microalgae is co-immobilized in alginate with *A. brasilense* under heterotrophic conditions. The objectives of this study were to (1) measure the quantity of the nine most abundant fatty acids in the microalgae in relation to enzymatic activity in *C. vulgaris* when immobilized alone, (2) determine if co-immobilization with a PGPB increased or decreased the quantity of each of the main fatty acids, (3) determine whether changing the optimum growth temperature to mildly stressful temperatures has an effect on this relationship, (4) determine whether lack of correlation between ACC activity and lipid accumulation, earlier described for specific temperature conditions, is true if the microalgae is cultivated over a range of temperatures, and (5) determine whether the PGPB provides a temperature-mitigating effect for the microalgae in accumulating fatty acids when cultured under nonoptimal temperatures.

Materials and methods

Microorganisms and culture conditions

C. vulgaris Beijerinck (UTEX 2714, University of Texas, Austin, TX) and *A. 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) at 140 rpm on an orbital shaker at 28±1 °C and 60 μmol photon m^{-2} s^{-1}. Medium C30 is composed of (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 (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 agar broth (NB; no. N7519, Sigma-Aldrich, St. Louis, MO) at 37±1 °C and agitated at 140 rpm.

Immobilization of microalgae and bacteria in alginate beads

Microorganisms were immobilized using the method described by de-Bashan et al. (2004), where 40 mL *C. vulgaris* culture (6.0×10^6 cells mL^{-1}) were mixed with 160 mL of a sterile, 6,000 cP 2 % alginate solution (alginate mixed at 14,000 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 conditions (de-Bashan and Bashan 2010). The beads were stabilized for 1 h at 28±1 °C and washed in sterile saline solution. *A. brasilense* (approximately 1.0×10^9 CFU mL^{-1}) was immobilized similarly. Immobilization normally reduces the number of *Azospirillum* spp. in the beads; therefore, a second incubation step was necessary by culturing in 10 % NB overnight. To combine both species in the same beads, a similar procedure was used, where 20 mL of each culture were mixed (total of 40 mL). After the second incubation, the beads were placed in 1 L Erlenmeyer flasks (40 g of beads/flask) that contained 700 mL of synthetic growth medium (SGM; de-Bashan et al. 2011). SGM medium contains (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 that were described for culturing *Chlorella*.

Experimental conditions

During the experiments, the flasks were incubated on an orbital shaker in total darkness at 24, 27, or 30±1 °C, using 10 g·L^{-1} of sodium acetate (no. S7670, Sigma-Aldrich) as the carbon source (Choix et al. 2012b) and nitrogen (5 mM ammonium, 90 mg L^{-1}) and phosphorus (phosphate, 0.44 mM or 42 mg L^{-1}). All experiments lasted 6 days with daily assays of samples.

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. Immediately before each analysis, the frozen samples were freeze-dried. 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., microorganisms were counted. *C. vulgaris* was counted under a light microscope with a Neubauer hemocytometer (Gonzalez and Bashan 2000) connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD). 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 sampling time and t_0 is the beginning of the experiment (Oh-Hama and Miyachi 1992). Data is presented as per-day units. *A. brasilense* was counted after serial dilution by the plate count method on nutrient agar medium (no. M7519, Sigma-Aldrich).

Fatty acid identification and quantification

Quantification and later identification of fatty acids were done according to the method described by Sato and Murata (1988), with small variations. The method is based on a direct transmethylation of fatty acids without previous extraction of total lipids. Freeze-dried bead samples (100–200 mg/sample) were placed in a screw-cap glass tube. Five milliliters of a mix of concentrated hydrochloric acid and absolute methanol (5:95: HCl/CH_3OH (*v*/*v*)) were added to each sample and the cap hermetically sealed with additional polytetrafluoroethylene (PTFE) film. The tubes were placed in a water bath at 90 °C for 2 h for transmethylation. These samples were cooled to room temperature (26–28 °C), and 2 mL pure hexane (HPLC grade, no. 650552, Sigma-Aldrich) and 0.5 mL MilliQ water (EMD Millipore, Billerica, MA) were added to each sample and gently mixed in vortex. After 10 min incubation at room temperature, when the layers were separated, the top hexane layer was transferred to a clean tube and the water layer was discarded. The hexane was evaporated under nitrogen gas, and the dry pellet was resuspended with a known volume of hexane (500 μL for *A. brasilense* and 1 mL for *C. vulgaris* alone or co-immobilized) and transferred to a crimp-top sealed vial (no. 5181-8801, Agilent Technologies, Santa Clara, CA) and injected into a gas chromatograph-mass spectrograph (HP-GDC1800B, Agilent Technologies) equipped with an Omegawax 250 column (30 m× 0.25 mm×0.25 μM; Supelco, Bellefonte, PA); the latter dimension is the size of the particles in the column. Running conditions were specified by the manufacturer: 1 μL of injected sample, high purity helium as the carrier gas, flow rate of 0.9 mL·min^{-1}, and injections of the sample was in the splitless mode. The temperatures of the injector and detector were 250 and 260 °C, respectively. Each run involved the following preprogrammed steps: initial temperature of 110 °C for 3 min, then an increase of 30 °C min^{-1} to 165 °C for 2 min. Then, the temperature was increased at the rate of 2.2 °C min^{-1} to 209 °C for 35 min. Identification of fatty acids was done by comparing the retention times of each methylated fatty acid with the corresponding fatty acid in the calibration curve of the gas chromatograph. Identification was confirmed by analyzing the mass spectrum of each fatty acid. The threshold of detection was set to 0.5 % of total fatty acids. The fatty acid analyses were based on 6 days of experiments, and the samples were taken at the end of each experiment.

Enzymatic activity of ACC

Extraction Frozen bead aliquots were dissolved in two volumes of 4 % $NaHCO_3$ solution 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 pulverized 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 results of enzymatic activity, compared with the mortar and pestle technique that is generally used for convenience. For resuspension, 5 mL extraction buffer (100 mM Tris–HCl, pH 8.2, 4 mM ethylenediaminetetra acetic acid (EDTA), 10 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF; no. P7626, Sigma-Aldrich)) was added to the pellet. This was 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. 2008b).

Quantification The reaction buffer was composed of 50 mM Tris–HCl at 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; no. B4287, Sigma-Aldrich). The crude

extract was preincubated for 30 min at 25 °C with 10 mM potassium citrate and 2 mg mL^{-1} BSA. Then, 500 μL crude extract was added to 0.5 mL of reaction buffer, and the enzymatic reaction was incubated for 100 min at 30 °C. The reaction was stopped with 0.5 mL 10 % perchloric acid (PCA; no. 244252, Sigma-Aldrich). The total reaction mixture was filtered (0.22 μm membrane filter; EMD Millipore). 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 (5×150 mm and 4.6 μm; Agilent Technologies). 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 with 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). The specific activity was defined as nanomoles of substrate transformed per minute per 1 mg of protein.

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 small modifications to adapt it to microalgae, which involves 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 (two cycles of 5 min at 30 kHz) in an ice bath. Sonicated beads were then 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 color change reaction, were done according to Pande et al. (1963), using a calibration curve with tripalmitin (no. T5888, Sigma-Aldrich) as a standard. The concentration of lipids was determined in a microplate reader (Molecular Devices, Sunnyvale, CA) at 590 nm, recording the intensity of the green color formed. Potassium dichromate has a yellow-reddish color before reaction with lipids and yellow-green after the reaction with lipids. The method quantified lipids in the range of 70 μg to 1.33 mg.

Experimental design and statistical analysis and presentation of the results

Six individual experiments were done using a factorial design, all under heterotrophic conditions (dark, Na acetate as the C source), using three temperatures: 24, 27, or 30 °C. Three variants were tested: *C. vulgaris* immobilized alone, *A. brasilense* immobilized alone, and co-immobilization of the two microorganisms together. Beads without microorganisms were not routinely used because preliminary determination showed that there was no effect on the measured parameters. The effect of the bacteria on the microalgae (ACC activity, accumulation of fatty acids and lipids) is presented only for day 6 of culturing because the system needed stabilization. Data from earlier days of samplings showed higher variation in all parameters. This date is comparable to sampling date in earlier studies on this system (de-Bashan et al. 2002; Leyva et al. 2014). The treatments were tested with a full supply of ammonium (90 mg $NH_4^+L^{-1}$ or 5 mM). In each treatment, three 1 L Erlenmeyer flasks containing 0.7 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 six experiments, three analyses were done: ACC enzymatic activity, fatty acid profile, and total lipids. Statistical analysis was done with the Student's *t* test at $p<0.05$ (comparisons among two treatments) 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).

Results

Growth rate of ***C. vulgaris*** under six variants of immobilization and temperature

Growth rate of immobilized *C. vulgaris* was greatest when cultured at 27 °C (0.126±0.005 day^{-1}). This treatment was one of only two treatments that had a positive growth rate in the six experiments. The other treatment was *C. vulgaris* co-immobilized with *A. brasilense* at the same temperature. All other treatments had negative growth rates, indicating reduction in the populations over time, where the lowest growth was *A. brasilense* immobilized alone at 24 °C (−0.468± 0.223 day^{-1}; Table 1).

Relationship between ACC activity and fatty acid accumulation in ***C. vulgaris*** immobilized alone

An analysis over time of the relationship between ACC activity and accumulation of fatty acids was done using *C. vulgaris* immobilized alone under three temperature regimes, optimal

Table 1 Growth rate per day of *Chlorella vulgaris* and *Azospirillum brasilense*, based on data of days 1 and 6 of each experiment

Variant	24 °C	27 °C	30 °C
A. brasilense	−0.468±0.223 Ac (14×10^6–1.4×10^6)	−0.250±0.048 Ac (10.7×10^6–4×10^6)	−0.348±0.056 Ab (6.33×10^6–0.8×10^6)
C. vulgaris	−0.015±0.007 Ba (1.99×10^6–1.82×10^6)	0.126±0.005 Aa (1.24×10^6–2.64×10^6)	−0.297±0.040 Cab (13.8×10^6–2.33×10^6)
C. vulgaris+*A. brasilense*	−0.061±0.010 Bb (2.27×10^6–1.58×10^6)	0.059±0.006 Ab (1.36×10^6–1.93×10^6)	−0.182±0.076 Ca (2.53×10^6–0.85×10^6)

Values in rows (separately) denoted by different capital letters indicate significant differences at different temperatures. Values in columns (separately) denoted by different lowercase letters indicate significant differences among the different treatments at the same temperature. Statistical analyses were done by one-way ANOVA and LSD post hoc analysis at $p<0.05$. The *values below each growth rate* are the number of cells (days 1 and 6) used to calculate the growth

(27 °C), and mildly stressful (24 and 30 °C). Figure 1 shows this relationship; at 24 and 27 °C, there is a relation between the two variables. The best fit is from days 3 to 6 (Fig. 1a, b, d, e). This was evident as either per culture (Fig. 1 a, b) or when ACC and fatty acids were calculated for a single cell (Fig. 1d, e). At 30 °C, no direct relationship was found. However, at this temperature, the highest production of fatty acids was found at day 2 (over 6 μg mg^{-1} per culture analysis and over 3 μg mg^{-1} $cell^{-1}\times10^{-6}$ per cell analysis). Content of ACC was in the range of ~1–8×10^{-3} mU when the analysis was made per culture and 1–5×10^{-9} when the analysis was made per cell. The highest enzymatic activity in all experiments was found at day 2 at 27 °C either when the analysis was made per culture or per number of cells (almost 8×10^{-3} mU per culture and almost 4×10^{-9} mU per cell).

Fatty acid profile of immobilized and co-immobilized *C. vulgaris*

When *C. vulgaris* was immobilized alone or co-immobilized with *A. brasilense*, the profile and quantity of each fatty acid revealed nine main fatty acids. Eight were identified and quantified and one remained unidentified (Table 2). The quantified fatty acids were: palmitic (16:0), palmitoleic (16:1 ω7), an unidentified unsaturated fatty acid (probably a palmitic acid derivative according to its time of retention and mass spectrum), hexadecatetraenoic acid (16:4 ω3), stearic (18:0), oleic (18:1 ω9), linoleic (18:2 ω6), linolenic (18:3 ω3), and arachidic (20:0). Fatty acid 18:4 ω3 (common in green microalgae) was not found. The ratio of unsaturated/saturated fatty acids is approximately 1.5. In *A. brasilense*, the analysis detected only oleic acid as a main fatty acid (contributing with ~50, 55, and 63 % of total fatty acids content for 30, 27, and 24 °C, respectively). For all fatty acids at the three temperatures, co-immobilization of the microalga with the bacterium showed a higher quantity of the fatty acid than found in *C. vulgaris* immobilized alone. Arachidic acid was found only in the co-immobilized treatment at 30 °C in measurable amounts, and its quantities were only slightly above detection level at the other temperatures. An important observation of the entire profile shows that every fatty acid increased under co-immobilization where the lowest percentage of increase was 20 % (linoleic at 24 °C). The increase rose with temperature for every fatty acid. Palmitoleic, heptadecenoic, oleic, and linolenic acids have an increase of >350 % at 30 °C. Another four fatty acids (linoleic, stearic, unidentified, and palmitic) have an increase of ~150 % at the same temperature; at 27 °C, the increases range from 37 % for the unidentified unsaturated fatty acid to 309 % for palmitoleic acid. At 24 °C, the increases were more moderate, in the range of 20–100 %, yet, statistically significant (Table 2).

ACC activity, fatty acids, and lipids in co-immobilization experiments

Co-immobilization experiments positively affected the three analyzed variables: ACC activity, fatty acids, and total lipids (Fig. 2). The highest ACC activity occurred where microorganisms were co-immobilized at 27 °C (~6×10^{-3} mU per culture and ~3×10^{-9} mU per number of cells). At the culture level, statistical analysis shows that enzymatic activity in co-immobilized microorganisms is significantly different from *C. vulgaris* and *A. brasilense* immobilized alone (Fig. 2a, capital letter analysis). The co-immobilized treatment at 27 °C is statistically higher that the other two treatments (Fig. 2a, lowercase analysis). No differences occurred with the three treatments at the higher or lower temperatures when either temperature was analyzed separately (Fig. 2a, lowercase analysis). At the cell level, enzymatic activity in the co-immobilized treatments at 24 and 27 °C is higher than *C. vulgaris* alone (Fig. 2b, capital letter analysis). At 30 °C, no differences between co-immobilized and separately immobilized treatments were found. The lowest value for per cell ACC activity was in *C. vulgaris* immobilized alone. However, statistically it is not different from *C. vulgaris* immobilized alone at 24 and 30 °C and co-immobilized at 30 °C (Fig. 2b, lowercase analysis by one-way ANOVA).

At the culture level, accumulation of fatty acids was highest when the two microorganisms were co-immobilized at 30 °C, significantly different from co-immobilized cultures at 24 and 27 °C (Fig. 2c, lowercase analysis). When the analysis was separately made for each temperature (Fig. 2c, capital letter

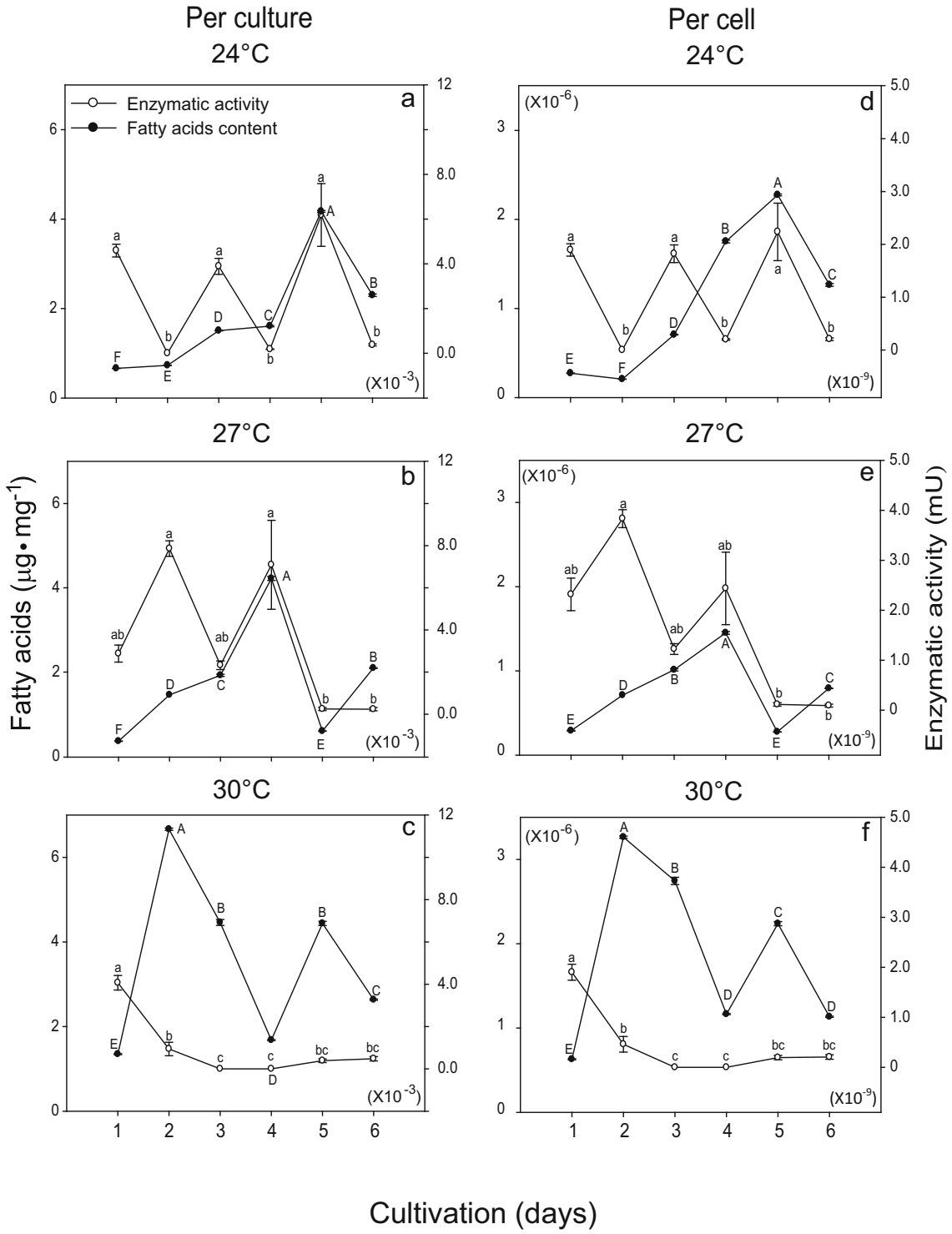

Fig. 1 Relationship between acetyl-CoA carboxylase (ACC) activity and fatty acid accumulation over time in *Chlorella vulgaris* immobilized in alginate beads under heterotrophic conditions. The analysis was done separately "per culture" and "per cell" at 24, 27, or 30 °C. In each subfigure, *different capital letters along the curve of fatty acids* denote statistically significant differences. Values for ACC activity are denoted with *different lowercase letters* to signify statistically significant differences. Both analyses were done by one-way ANOVA and LSD post hoc analysis at $p<0.05$. No comparative analysis among ACC activity and fatty acid accumulation was done because the variables are not statistically comparable

analysis), the co-immobilized treatment was always higher than when *C. vulgaris* was immobilized alone. The amount of fatty acids in *C. vulgaris* immobilized alone was always higher than those for *A. brasilense* immobilized alone. At the

Table 2 Fatty acid profile and quantity of immobilized and co-immobilized *Chlorella vulgaris* in alginate beads

Treatment	24 °C	27 °C	30 °C	Fatty acid
All values are $\times 10^{-7}$ (μg mg^{-1})				
C. vulgaris	5.025 Ba	3.007 Bc	4.81 Bb	Palmitic (16:0)
C. vulgaris+A. brasilense	6.873 Ab	4.604 Ab	13.1 Aa	
% increase	36	53	172	
C. vulgaris	ND	0.29 Bb	0.345 Ba	Palmitoleic (16:1)
C. vulgaris+A. brasilense	0.505 Ac	1.188 Ab	1.88 Aa	
% increase	100	309	443	
C. vulgaris	0.337 Ba	0.255 Bc	0.280 Bb	Unidentified unsaturated
C. vulgaris+A. brasilense	0.472 Ab	0.349 Ab	0.719 Aa	
% increase	40	37	156	
C. vulgaris	0.309 Ba	0.256 Bb	0.207 Bc	Hexadecatetraenoic acid (16:4 ω3)
C. vulgaris+A. brasilense	0.427 Ab	0.438 Ab	0.946 Aa	
% increase	38	71	356	
C. vulgaris	0.520 Bb	ND	0.801 Ba	Stearic (18:0)
C. vulgaris+A. brasilense	0.688 Ab	0.319 Ab	1.96 Aa	
% increase	29	100	145	
C. vulgaris	2.713 Bb	1.865 Bc	2.88 Ba	Oleic (18:1 ω9)
C. vulgaris+A. brasilense	4.28 Ab	3.84 Ab	10.59 Aa	
% increase	59	106	268	
C. vulgaris	3.105 Ba	1.8 Bc	2.48 Bb	Linoleic (18:2 ω6)
C. vulgaris+A. brasilense	3.735 Ab	2.842 Ab	5.72 Aa	
% increase	20	58	130	
C. vulgaris	0.641 Ba	0.454 Bc	0.497 Bb	Linolenic (18:3 ω3)
C. vulgaris+A. brasilense	0.794 Ab	0.737 Ab	2.49 Aa	
% increase	24	62	402	
C. vulgaris	ND	ND	ND	Arachidic (20:0)
C. vulgaris+A. brasilense	DNQ	DNQ	0.556 Aa	
% increase			100	

Percentage increase means the difference in quantity of a single fatty acid between *C. vulgaris* immobilized alone and *C. vulgaris* co-immobilized with *Azospirillum brasilense*. Each single pair of data (*C. vulgaris* and *C. vulgaris+A. brasilense*) was analyzed by Student's *t* test at $p<0.05$. Different lowercase letters mean significant difference. Values in rows denoted by different capital letters indicate statistical difference by one-way ANOVA and LSD post hoc analysis at $p<0.05$. All values are in the range of $\times 10^{-7}$

DNQ detected but not quantified, negligible quantities, slightly above detection level (level=0.5 %)

cell level, similar accumulations of fatty acids were detected; at the three temperatures, co-immobilized cultures had more fatty acids than cultures of *C. vulgaris* immobilized alone (Fig. 2d, capital letter analysis). Comparing all the treatments, the highest concentration was in co-immobilized cultures at 30 °C and the lowest was for *C. vulgaris* immobilized alone at 27 °C (Fig. 2d, lowercase analysis).

At the culture level, the total number of lipids was highest for *C. vulgaris* immobilized alone at 30 °C and lowest for *A. brasilense* immobilized alone at 27 and 30 °C (Fig. 2e, lowercase analysis). Co-immobilization only showed higher quantities of lipids than *C. vulgaris* immobilized alone at 27 °C (Fig. 2e, capital letter analysis). At 24 and 30 °C, *C. vulgaris* immobilized alone contained higher quantities of lipids than the co-immobilized treatment. At the cell level, accumulation of lipids changed slightly; only at 24 °C did *C. vulgaris* immobilized alone have more lipids than the co-immobilized treatment. At 27 °C and 30 °C, co-immobilized treatments produced more lipids than *C. vulgaris* immobilized alone (Fig. 2f, capital letter analysis).

Discussion

The key enzyme in the de novo biosynthesis of fatty acids, ACC (Hu et al. 2008), accumulation of fatty acids, and consequently, formation of lipids, are the central elements of the potential attractiveness of microalgae for biofuel production for over two decades. Any positive increase in the formation of fatty acids or lipids is advantageous, although not all fatty acids are well suited to this biotechnological application.

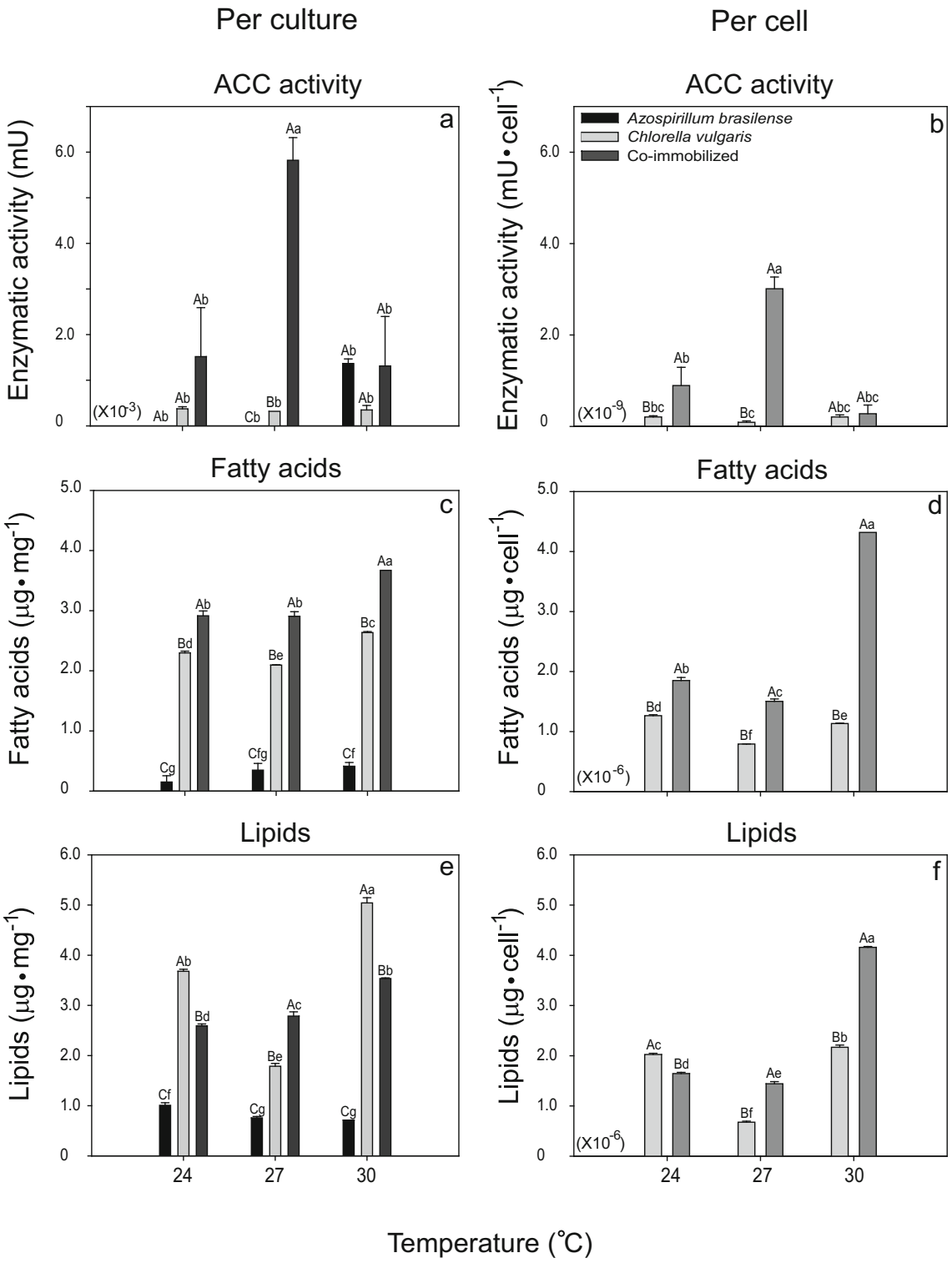

Fig. 2 Acetyl-CoA carboxylase (*ACC*) activity, fatty acid, and total lipid accumulation in *Chlorella vulgaris* immobilized alone and co-immobilized with *Azospirillum brasilense* in alginate beads under heterotrophic conditions. The data are shown "per culture" and "per cell". In the "per culture" subfigure, *columns denoted by different capital letters* indicate statistically significant differences among the three treatments at the same temperature. Columns denoted by *different lowercase letters* indicate statistical significant differences among the nine variants at the three different temperatures. Both analyses were done by one-way ANOVA and LSD post hoc analysis at $p<0.05$. In the "per cell" subfigure, *pairs of columns denoted by different capital letters* indicate statistically significant differences between the two variants at the same temperature by Student's *t* test at $p<0.05$. In each subfigure of this type, *different lowercase letters* indicate statistically significant differences among the six treatments at the three different temperatures done by one-way ANOVA and LSD post hoc analysis at $p<0.05$

Thus far, the main demonstrated factor responsible for increasing lipids accumulation in *Chlorella* spp. under autotrophic conditions is ammonium starvation (Přibyl et al. 2012; Xiong et al. 2010). Under heterotrophic conditions, using sodium acetate as the carbon source, *C. vulgaris* (Leyva et al. 2014) and *Chlorella zofingiensis* (Liu et al. 2011) produced more lipids. A direct correlation was found between ACC activity and formation of fatty acids in plants (Klaus et al. 2004; Sasaki and Nagano 2004; Tong and Hardwood 2006). Despite the significant culturing and molecular efforts to increase lipids in photosynthetic microorganisms for biofuel production (Radakovits et al. 2010; Rawat et al. 2013), no direct correlation was established between ACC activity and formation of lipids in different species of microalgae despite several attempts to increase ACC activity (Roessler and Ohlrogge 1993; Dunahay et al. 1996; Sheehan et al. 1998; Leyva et al. 2014). Consequently, this study explored the possible relation between accumulation of fatty acids in *C. vulgaris* and ACC activity under heterotrophic conditions and under the influence of an external PGPB.

Previous studies, using the same system of immobilization of microorganisms in alginate beads, demonstrated that accumulation of metabolites in the microalgae depends mainly on the population size developed in each culture. This was shown for nitrogen uptake (de-Bashan et al. 2005) and accumulation of carbohydrates and starch (Choix et al. 2012a, 2012b, 2014). These studies demonstrate that culturing conditions have significant effects on metabolism of *Chlorella* spp. and on the number of cells that are developed. Low populations can uptake large quantities of nitrogen or accumulate more carbohydrates and starch, but large populations are some times less efficient. This is also true for accumulation of lipids, as shown in this study, where most growth rate values in all treatments, apart from cultures growing at an optimal temperature, were negative.

This study demonstrated the relationship between ACC activity and accumulation of fatty acids in *C. vulgaris* immobilized in alginate beads under heterotrophic conditions. The 6-day experiments performed at three temperatures showed that the system requires about 3 days for stabilization. From day 4 onward of incubation, the relationship between variables matches, at least at the two lower temperatures of culturing. At the higher temperature, no relation between the two variables was found. Perhaps, this occurred because 30 °C is a less suitable temperature for growth of this microalgae species. These results could not be compared with similar studies because no such studies are available. Yet, this finding supports a previous cell–cell attachment study using this interaction to show that the association of these microorganisms becomes stronger at days 4–5 of co-incubation (de-Bashan et al. 2011). This suggests that a longer period of incubation regarding lipid metabolism of *C. vulgaris* is required, likely a gradual accumulation process that is manifested at later phases of incubation (de-Bashan et al. 2002).

Naturally, *A. brasilense* has ACC activity and possess active fatty acid metabolism. However, in our study, at temperatures that offer optimal growth, or just slightly below optimal temperature (24 and 27 °C), these variables were relatively very small, compared with ACC activity and fatty acid accumulation of the microalgae growing alone or in the co-immobilized variant. Only at 30 °C, temperature better for *A. brasilense* growth and less favorable for *C. vulgaris* growth, the bacterial ACC activity was comparable to those of the microalgae. However, this did not translate to accumulation of fatty acids. Perhaps this happens because the common, and favorable, energy storage molecule of *A. brasilense* Cd is poly-β-hydroxybutyrate (Tal and Okon 1985) and not lipids.

Accumulation of lipids in cells of microalgae, followed by nutrient starvation, mainly nitrogen, is common knowledge (Ratledge 2004). Nonetheless, more than once (for an example, Rodolfi et al. 2009) under nutrient limitations, the population of microalgae in a culture significantly decreased. Consequently, high lipid productivity of the individual cells is not sufficient to compensate for the smaller population and for maintaining high productivity of lipids in culture. This is the main reason why nutrient starvation is not routinely used in lipid production processes of microalgae. We demonstrated earlier (Leyva et al. 2014) that the synthetic growth medium we used contains sufficient nutrients to maintain the growth of the culture for 6 days without starvation. Therefore, our current study shows the effect of the PGPB on ACC activity and accumulation of fatty acids in *C. vulgaris*.

Immobilization of *C. vulgaris* with the PGPB *A. brasilense* had a profound effect on fatty acid and lipid metabolism in *C. vulgaris*: four of six analyses show statistically significant higher content of both features in *A. brasilense* and *C. vulgaris* than when either is immobilized alone. Quantities of the main fatty acids significantly increased, some dramatically. At the same time, the PGPB did not multiply, yet, it exerted effects for the first 6 days of each experiment. Similarly, in some treatments, the population of the microalgae decreased over time. Longer cultivation periods were not studied.

By analyzing the fatty acid profile where co-immobilization occurred, we found nine major fatty acids. This fatty acid profile is similar to the general profile reported for *C. vulgaris* (Ötles and Pire 2001; Petkov and Garcia 2007; Tang et al. 2011a). The ratio of unsaturated to saturated fatty acids that we found is also similar to Tang et al. (2011b). They showed that the mass balance for *Chlorella* spp. is that 95–98 % of fatty acids are compounds with chains of 16 and 18 carbons (Tang et al. 2011b). Our findings support this. This profile is valid at the three temperatures we tested and for immobilized and co-immobilized treatments.

de-Bashan et al. (2002) report that, when *C. vulgaris* is co-immobilized with *A. brasilense*, there are de novo fatty acids detected in the interaction as well as an increase in the

accumulation of total lipids. These new fatty acids may have been present in the cells, but their quantity was below the detection threshold of the equipment and was revealed only during the interaction with the PGPB. It is important to mention that the nine fatty acids we detected increased in quantity in the presence of the PGPB.

In our study, all the fatty acids reported by de-Bashan et al. (2002) were found except lauric acid and a "de novo" fatty acid, arachidic acid, only in co-immobilized treatment at 30 °C. At lower temperatures this fatty acid was detected but not quantified because it was produced in negligible quantities and slightly above detection level. The chemical nature of fatty acids in *C. vulgaris* is under debate. Petkov and Garcia (2007) state that fatty acids with more than 18 carbons in a chain might be a laboratory contamination because *Chlorella* do not produce that fatty acids. However, Ötles and Pire (2001) report even longer fatty acids in *C. vulgaris*. Tang et al. (2011a) did not find longer-chain fatty acids (>18 carbons) in *C. pyrenoidosa*. Yet, they found such a long-chain fatty acid in *Scenedesmus obliquus*, another freshwater chlorophyte. Our study supports that 18–20 carbon atom chains are a significant part of the fatty acids in the strain we work with. Perhaps the difference among analyses is based on inherited differences among strains, as is well known for many microbial parameters. Analysis of more strains may resolve this issue.

This study analyzed only ACC. Future analyses could quantify activity of downstream enzymes, such as fatty acid synthase and diacylglycerol acyltransferase, which synthetizes triglycerides (Hu et al. 2008) to understand fatty acid synthesis and profiles under different culturing conditions. This idea is important because, although some reports indicate successful overexpression of ACC in some microalgae, accumulation of lipids in transformed microalgae did not reflect overexpression (Dunahay et al. 1996; Roessler and Ohlrogge 1993). This absence of correlation between ACC activity and lipid formation was also detected in this immobilized system (Leyva et al. 2014) and expanded in our present study. The explanation for this may tied to feedback regulation of ACC activity (Sheehan et al. 1998).

The PGPB *Azospirillum* spp. is well known for mitigating several types of stress in plants and microalgae when conditions become restricting or stressing for plant growth (Bashan and de-Bashan 2010). Temperatures of 24 and 30 °C present a mild stress for the microalgae and result in less than optimal growth of this strain of *Azospirillum.* Growth rate data showed negative growth of the microorganisms at these temperatures. Yet, the association of *C. vulgaris* with the PGPB improved fatty acid accumulation to the same level found at the optimal temperature (27 °C) or even higher accumulation of fatty acids in co-immobilized treatments at 30 °C. This finding demonstrates additional mitigating effect of *Azospirillum* associated with *C. vulgaris*.

The prokaryote–eukaryote model of *C. vulgaris–A. brasilense* interaction (de-Bashan and Bashan 2008) proposes that this simple cell-to-cell interaction can be a useful replacement for studying the effects of PGPB on some metabolic pathways in higher and more complex plants. Thus far, this model demonstrated that nitrogen (de-Bashan et al. 2008b) and phosphorus metabolism (Hernandez et al. 2006), hormonal effects (de-Bashan et al. 2008a), carbohydrate, especially starch metabolism (Choix et al. 2012a; b, 2014), and carbon assimilation metabolism (Perez-Garcia et al. 2010, 2011b) can be studied. This study and its predecessor (Leyva et al. 2014) extend the scope of this model to fatty acid and lipid metabolism.

In summary, this study shows the relation between ACC activity and accumulation of fatty acids in *C. vulgaris* when it is immobilized in alginate beads. No correlation was found between ACC and accumulation of lipids at three temperatures. Co-immobilization with *A. brasilense* exerted a positive effect over ACC activity, quantity of fatty acids and variety, and on total lipids.

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