Chapter 20
Interaction of *Azospirillum* spp. with Microalgae: A Basic Eukaryotic–Prokaryotic Model and Its Biotechnological Applications

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Abstract The interaction of the bacteria *Azospirillum* spp. with photosynthetic, single cell microalgae that are co-immobilized in alginate beads provides a significant shortcut for understanding the interaction of this plant growth-promoting bacteria (PGPB) with plants in general. This interaction is currently relevant for studying physiological, physical, biochemical, and molecular aspects. As an independent subfield of *Azospirillum* research, this interaction has some significant potential biotechnological applications, such as wastewater treatment, production of biofuel (ethanol and biodiesel), increased fertility of eroded soils combined with promoting growth of higher plants, production of pigments, and production of biomass. All of these applications have yet to be scaled up and evaluated for their true practical potential.

20.1 The Logic Behind Using This Interaction as a Model for Plant–Bacteria Interaction

A major obstacle in the study of interactions between *Azospirillum* spp. and plants is the complexity of the plant. Studies of basic plant–bacterium interactions of *Azospirillum* spp., done mainly with roots, are difficult because there are many

Dedication: This chapter is dedicated to the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1964–2011) of CSIC in Spain.

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tissue functions and numerous possible interactions with plant roots and plant metabolism, as well as interference with the soil matrix. Plants with relatively small genomes, such as *Arabidopsis thaliana* (125-Mb genome) and rice (389-Mb genome), were sequenced and used as models for *Azospirillum* spp. interaction. However, larger plant genomes, in which *Azospirillum* spp. commonly interacts, such as maize (2.5 Gb), oat (11.4 Gb), and wheat (16 Gb), even though some are undergoing sequencing procedures, are unlikely to be understood in detail for some time. Green microalgae, on the other hand, have the smallest plant genome (~40 Mb). *Chlorella* spp. (Chlorophyceae) are simple, nonmotile, unicellular, aquatic green microalgae that have been intensively studied regarding metabolic functions of the cell. The *Chlorella* genome is the smallest eukaryotic, photosynthetic microorganism characterized so far, which makes it an alternative to higher plants with large genomes interacting with *Azospirillum* spp., with a specific aim of studying plant metabolism and molecular mechanisms affected by *Azospirillum* spp. The reason for co-immobilization of both microorganisms in a polymer bead is to keep them together in very close proximity to ensure that each affects the other's metabolism. Consequently the three basic components of the experimental model are cells of the microalgae *Chlorella* spp. and cells of *Azospirillum* spp. that have been co-immobilized in small (3–4 mm in dia.) alginate beads.

20.2 Co-immobilization Techniques

Co-immobilization techniques are detailed in Fig. 20.1. Alginate beads containing the two microorganisms are presented in Fig. 20.2, where 20 mL of axenic cultures (*C. vulgaris* and *A. brasilense*) are mixed with a 2 % alginate solution. Beads are formed using automated equipment (de-Bashan and Bashan 2010; http://www.bashanfoundation.org/beads/macrobead.html accessed 10 July 2014) or by drops from a large syringe (less recommended). To immobilize the two microorganisms in the same bead, each culture is washed and then each is resuspended in 10 mL 0.85 % saline solution. The two mixes are then mixed with the alginate before the beads are formed. 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 is necessary in OAB medium (Bashan et al. 1993, see also chapter on formation of inoculants) or in a diluted, rich media BTB-1 or BTB-2 (Bashan et al. 2011 see also Chap. 26 on formation of inoculants).

20.3 Applications

20.3.1 Basic Studies of Prokaryotic–Eukaryotic Interaction

This conceptual experimental and simple quantitative model offers a convenient and basic approach to studies of complex interactions between plants and bacteria. These interactions are mainly physiological, biochemical, and molecular mechanisms

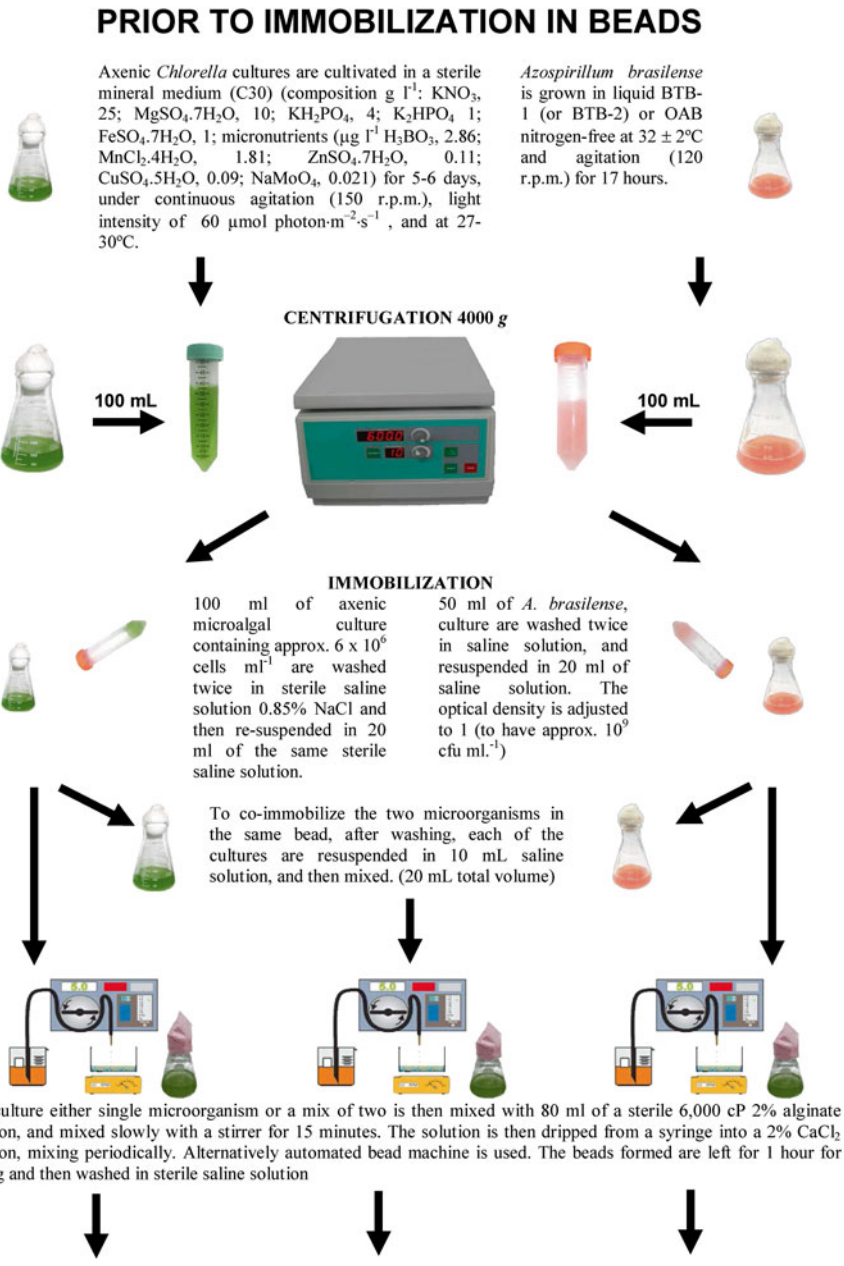

Fig. 20.1 Flow chart showing methods and techniques used to immobilize, co-immobilize, count, and cultivate microalgae and *A. brasilense* for various applications. Composition of media BTB-1, BTB-2, and OAB are given in this book (Bashan and de-Bashan, Inoculant preparation and formulations for *Azospirillum* spp.)

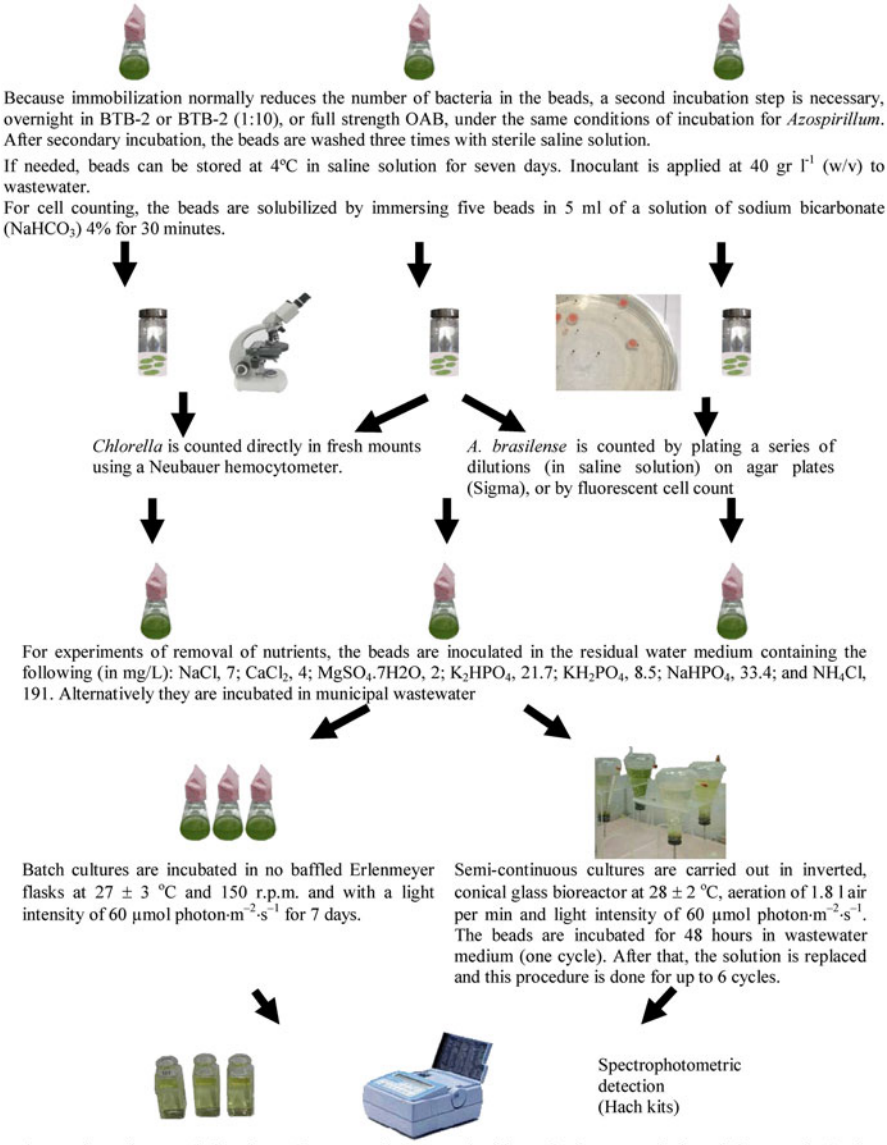

Fig. 20.1 (continued)

shared by higher plants and green microalgae (de-Bashan and Bashan 2008; de-Bashan et al. 2005). Many of the mechanisms proposed so far for *Azospirillum*–plant interactions (Bashan and de-Bashan 2010; Bashan et al. 2004) are relatively easy to study using this model. Apart from easy technical handling, the logic of choosing a microalga as the plant partner for *Azospirillum* spp. is straightforward.

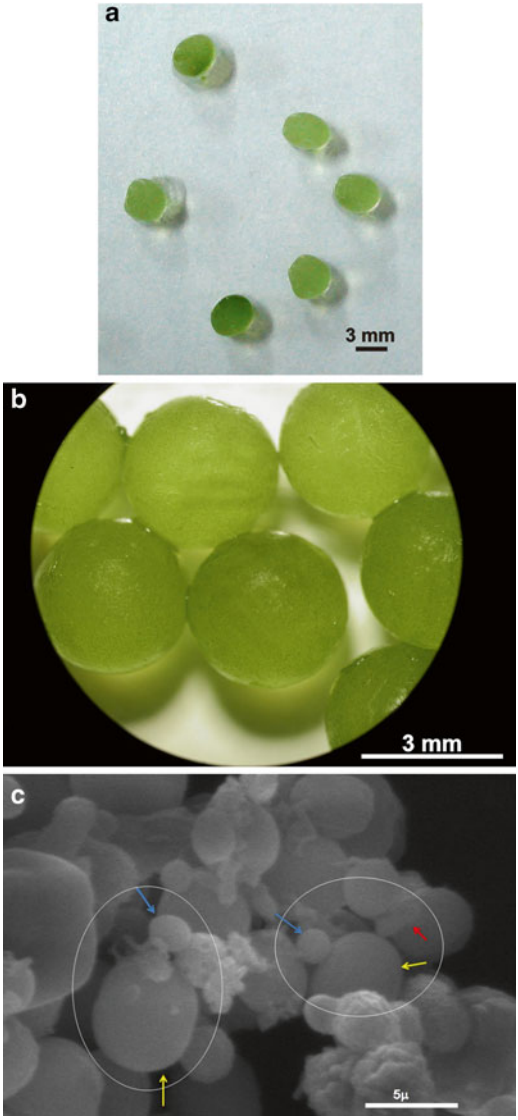

Fig. 20.2 (**a**) Beads containing *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* (**a**, **b**). (**c**) Scanning electron microscopy of the interaction between the two microorganisms inside the bead. *Circles* indicate interactions between *C. vulgaris* (*yellow arrows*) and cysts (*blue arrows*) and a vegetative cell (*red arrow*) of *A. brasilense*

The most basic definition of a green plant is that it contains chlorophylls *a* and *b*, starch as a storage material inside the chloroplast, and a cell wall made of cellulose. Higher plants and algae are part of the same group (Chlorobionta). There is 70–98 % genetic similarity between land plants and algae (Devereux et al. 1990). The size of the organism, the number of cells and differentiation into organs are not defining parameters of a plant. Consequently, single-cell microalgae are considered plants.

The following sequence of events occurs during the interaction between the two microorganisms within the polymeric bead. Initial immobilization is a random spread of particles inside a gel matrix (Gonzalez and Bashan 2000). Nutrients in the surrounding medium freely diffuse into the porous gel. Over time (6–48 h), depending on the pairing of microalgae and bacteria, both microorganisms are found in the same cavity within the bead, mainly just beneath the surface. Small parts of the internal structure of the bead matrix dissolve or split and separate as microcolonies develop and enlarge (Covarrubias et al. 2012; Lebsky et al. 2001; de-Bashan et al. 2011). The bacteria mainly excrete indole-3-acetic acid (IAA) and other undefined signal molecules that reach the nearby microalgal cells (de-Bashan et al. 2008a). At this stage, the activities of the microalgal enzymes (two were tested so far, glutamine synthetase and glutamate dehydrogenase) are not enhanced (de-Bashan et al. 2008c). At the next phase of interaction, beginning about 48 h after joint immobilization and continuing, glutamate synthetase and glutamate dehydrogenase activities are enhanced, photosynthetic pigment production is enhanced (de-Bashan et al. 2002a), nitrogen and phosphorus uptake into microalgal organelles is accelerated (de-Bashan et al. 2005), carbohydrates accumulation, especially starch, occurs (Choix et al. 2012a, b), as well as an increase in lipids and fatty acids (de-Bashan et al. 2002a; Leyva et al. 2015). At the same time, the co-immobilized system liberates oxygen produced by *Chlorella* spp. as a by-product of photosynthesis. The metabolic functions of this model, studied so far, are illustrated in Fig. 20.3. At the same time, the common phenotypic colonization of *Azospirillum* on roots, connection to the root surface by all sort of fibrillar material (Bashan et al. 1986; Levanony et al. 1989) are detected in the *Azospirillum–Chlorella* interactions (de-Bashan et al. 2011).

These favorable characteristics have biotechnological implications. The model is not restricted to *Chlorella vulgaris–Azospirillum brasilense* interactions that have comprised most of the studies done so far. Other PGPB, such as *Bacillus pumilus*, *A. lipoferum*, *Phyllobacterium myrsinacearum*, and other microalgae, such as *C. sorokiniana*, were successfully tested (de-Bashan et al. 2008b, c; Gonzalez-Bashan et al. 2000; Hernandez et al. 2009).

These options create opportunities for endless combinations of microalgae and PGPB and for many *Azospirillum* strains. Similarly, different alginates and derivatives from many macroalgae are commercially available (McHugh 2003) for entrapment and combination schemes, as needed. Because immobilization of microorganisms is also commonly used with other polymers (O'Reilly and Scott 1995), this model is not restricted to alginates; each polymer has its own advantages and disadvantages.

The practical and analytical aspects of this model are considerable. All ingredients are inexpensive, and the microorganisms are easy to cultivate and test in standard microbiology facilities. The results are available on a microbial time scale (days to a week). Reproducibility is very high, and replicates are merely Erlenmeyer flasks, allowing as many replicates as needed in a small space and in a soil-free system. Reviewing hundreds of published results using this system, it appears that the standard error is low and allows detection of minute effects between the interacting organisms. So far, we have not observed any disadvantages in experiments conducted over the past 15 years.

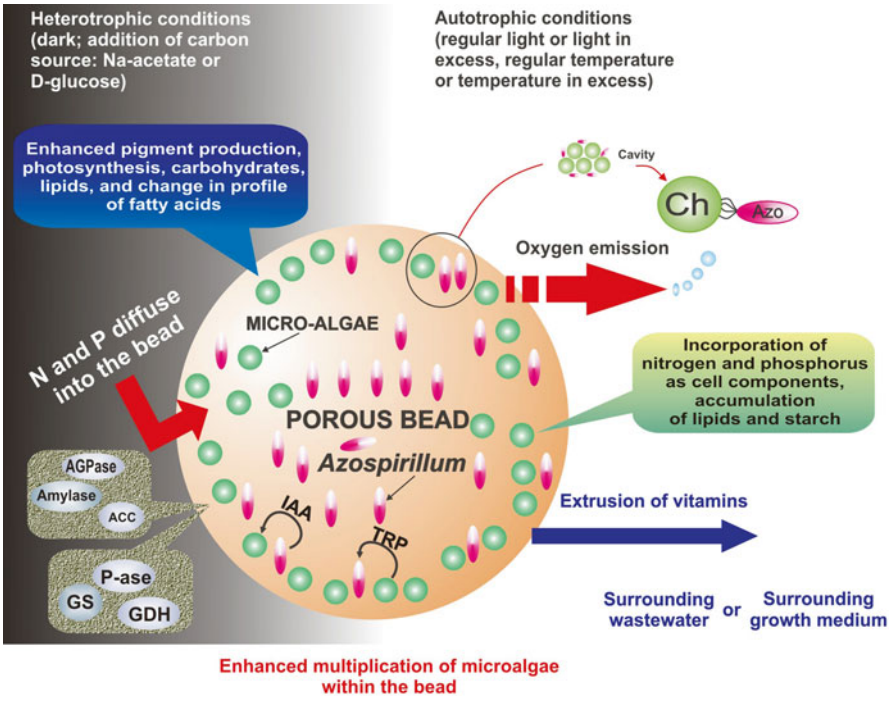

Fig. 20.3 A conceptual model of *Azospirillum* spp. co-immobilized with microalgae in alginate beads to study prokaryotic–eukaryotic interaction under autotrophic and heterotrophic conditions. *Azo Azospirillum* spp., *Ch Chlorella* spp., *GS* glutamine synthetase, *GDH* glutamate dehydrogenase, *IAA* indole-3-acetic acid, *P-ase* phosphatase, *Amylase* α amylase, *ACC* acetyl-CoA carboxylase, *AGPase* ADP glucose pyrophosphorylase. This is an updated version of a model previously published in de-Bashan et al. 2012. Applied Soil Ecology 61: 171–189

20.3.1.1 Methods

To observe the physical interaction and fibril formation between the two microorganisms during their association, the following techniques are used. These are common techniques that employ few small modifications to this bacterial species and are described in detail in the following references. However, when there are many small modifications, or when these small modifications have a significant importance for obtaining the expected results using this association, a detailed description of the method needs to be supplied.

- Scanning electron microscopy (SEM). There are standard techniques for SEM for plants, with modifications to adapt to the interaction with microalgae (Bashan et al. 1986; Covarrubias et al. 2012).
- Transmission electron microscopy (TEM) by conventional techniques (Lebsky et al. 2001).

- Fluorescent in situ hybridization (FISH), using one of the conventional techniques for FISH but adapted by many minor details specifically for this interaction. The images are observed by confocal laser microscopy (de-Bashan et al. 2011) or under fluorescent microscopy.
  - *Fixation and preparation of samples*. There are two ways to analyze the interaction inside the beads: dissolve the beads to free the microorganisms to measure the strength of attachment between the two partners in the model or slice the beads with a scalpel, in which case the physical distribution of the microorganisms inside the beads can be observed.
  - To dissolve beads (DB), at least ten beads are dissolved in 1 mL 4 % sodium bicarbonate for 30 min. One mL DB is centrifuged (14,000 × *g*); the pellet is washed twice in 1X PBS (15 % v/v 200 mM sodium phosphate buffer/130 mM NaCl at pH 7.4); it is then fixed with 4 % paraformaldehyde for 1 h at 4 °C. After fixation, the pellet is washed twice with 1X PBS and stored in a mix of 1X PBS/96 % ethanol (1:1 v/v) at −20 °C until used. Previous to hybridization, 10 μL of the sample is added to gelatin (0.1 % w/v, 0.01 % w/v chromium potassium sulfate)-coated microscope slide, air-dried, and dehydrated by successive 50, 80, and 96 % ethanol washes (3 min each). Samples are air-dried again (Daims et al. 2005).
  - For sliced beads (SB), each slice is mounted on gelatin (0.1 % w/v, 0.01 % w/v chromium potassium sulfate)-coated microscope slides, attached to the slide by adding 1 drop of warm, low-melt, agarose solution (0.25 % w/v), and dried at 37 °C for 45 min. The samples are then fixed with 50 μL 4 % paraformaldehyde and incubated at 4 °C for 1 h. Then the paraformaldehyde is removed by pipetting. The samples are washed with 0.85 % saline solution, dehydrated by successive 50, 80, and 96 % ethanol washes (3 min each), air-dried, and stored at 4 °C until hybridization.
  - In situ *hybridization*. This assay is based on the technique described by Assmus et al. (1995), with numerous small modifications. Hybridization is performed at 35 % formamide stringency at 46 °C for 2 h. Samples are washed at 48 °C for 5 min with 50 mL pre-warmed washing buffer. The slides are then rinsed for a few seconds with ice-cold, deionized water, and then air-dried. Slides can be stored at −20 °C in the dark until visualization. An equimolar mixture of probes is used: EUB-338 I (Amann et al. 1990), II, and III (Daims et al. 1999). These three probes, when combined, detected almost all bacteria. For *A. brasilense*, the specific probe Abras 1420 (Stoffels et al. 2001) is used. The EUB-338 I, II, and III probes are labeled with the fluorochrome FITC and the *Abras* 1420 probe is labeled with the fluorochrome Cy3. The final concentration of the probes is 30 ng·μL^{-1} for probes labeled with Cy3 and 50 ng·μL^{-1} for probes labeled with FITC. Before visualization, the slides are mounted in AF1 anti-fading reagent (Citifluor).

  - *Visualization*. With confocal laser scanning microscopy (CLSM), a LSM 510 META system with an Axiovert 100 M inverse microscope (Carl Zeiss, Oberkochen, Germany), or equivalent, can be used (Schmid et al. 2009). A helium neon laser provides the excitation wavelength of 543 nm (Cy3) and an argon ion laser provides the excitation wavelength of 488 nm (FITC). To distinguish between the fluorescence from Cy3 and FITC-labeled oligonucleotide probes, the specific signals are depicted in red and green, respectively. The third color channel (helium laser, 633 nm singular wavelengths) is used to visualize autofluorescence of the microalgae and is assigned a blue color. The three signals are combined and depicted as a red-green-blue (RGB) image. An Apochromat 63 X/1.2 water immersion lens is used for all analyses and acquisition of images. Analyses of images use LSM 510 4.2 software (Carl Zeiss).

- For epifluorescence microscopy, an Axioplan 2 (Carl Zeiss), equipped with a mercury lamp (HXP120, Osram) and Carl Zeiss filter sets for FITC/GFP (Emitter BP 525/50, Beamsplitter FT 495, Exciter BP 470/40), Cy3 (Emitter BP 605/70, Beam splitter FT 570, Exciter BP 545/25), and Cy5 (Emitter BP 690/50, Beam splitter FT 660, Exciter BP 640/30) excitation is used. An Apochromat 63 X/1.2 water immersion lens (Carl Zeiss) is used for all observations. Images are recorded with the CCD camera AxioCam MRm controlled by AxioVision Rel. 4.6 software (Carl Zeiss) and processed with Adobe Photoshop 8.0 software (Adobe Systems).
- A major technical difficulty observing microalgae–bacteria interactions by FISH is that autofluorescence of the microalgae is far stronger than the relatively faint FISH labeling of the bacteria. Consequently, it is impossible to obtain microalgae and bacteria in one sharp image. However, this does not affect the actual observation, since the laser's intensity can be manipulated. For precise observations, a technique used for solar photography is adapted, where the ultrabrilliant microalgae are obscured by a black circle, allowing observation of the nearby less-fluorescent bacteria. *A. brasilense* does not have autofluorescence. Consequently, after performing FISH with the probes described above, *A. brasilense* cells should exhibit fluorescence only in the green and red channels. Additionally, to enhance clarity of the images, exposure time is increased or decreased for each of the three channels, depending on the intensity of the observed autofluorescence and specific FISH signals. As a result, positive fluorescence signals from *A. brasilense* vary in their fluorescence color from yellow-green to orange, arising from different intensities of the separately recorded red and green channels. Similarly, microalgae show slightly different tones, ranging from magenta to light cyan. The major difference, however, is the presence of the blue color fraction, which is absent in *A. brasilense* signals.

  - *Quantification*. Cell counting and measuring populations and cluster size of the microalgae and bacteria in FISH images obtained from the confocal laser scanning and epifluorescence microscopies can be quantified using image analyzing software (Image Pro-Plus 4.1, Media Cybernetics).

20.3.2 Wastewater Treatment

A combination of microalgae *Chlorella vulgaris* or *C. sorokiniana* with *A. brasilense* strain Cd, co-immobilized in small alginate beads, was developed to remove phosphorus and nitrogen nutrients from municipal wastewater. Co-immobilization of the two microorganisms was superior to removal efforts by the microalgae alone, reaching up to 100 % ammonium, 15 % nitrate, and 36 % phosphorus within 6 days (varied with the source of the wastewater), compared to 75 % ammonium, 6 % nitrate, and 19 % phosphorus by the microalgae alone (de-Bashan and Bashan 2010; Covarrubias et al. 2012; Cruz et al. 2013). This happens in synthetic residual wastewater (de-Bashan et al. 2002b) or domestic wastewater (de-Bashan et al. 2004) at ambient temperature (~25 °C) or extreme temperature (>40 °C) and irradiation (up to 2,500 μmol m^2 s^{-1}), using microalgal strains that are resistant to these conditions (de-Bashan et al. 2008b) and under autotrophic and heterotrophic conditions (Perez-Garcia et al. 2010, 2011). Artificial, sterile (by autoclaving) wastewater used in some of these studies is prepared using the following (mg/L): NaCl, 7; $CaCl_2$, 4; $MgSO_4 \cdot 7H_2O$, 2; K_2HPO_4, 21.7; KH_2PO_4, 8.5; Na_2HPO_4, 33.4; and NH_4Cl, 191. For continuous and semi-continuous cultures, KH_2PO_4, at levels in the range of 12–15 mg/L, was used as the sole source of phosphorus.

Biological removal of phosphorus is a harder task than removing nitrogen. In domestic wastewater, phosphorus removed by *C. sorokiniana* was significantly enhanced after a starvation period of 3–5 days in saline solution, combined with co-immobilization with *A. brasilense* Cd. The best phosphorus removal treatment of a batch of synthetic or domestic wastewater was with tandem treatments of wastewater treatment first with pre-starved, co-immobilized microalgae and replacement of this culture after one cycle of removing phosphorus with a new, similarly starved culture. This sequential treatment with two cultures was capable of removing up to 72 % of the phosphorus from the wastewater (Hernandez et al. 2006). It appears that starvation periods, combined with co-immobilization with *A. brasilense* have synergistic effects on absorption of phosphorus from wastewater by microalgae.

The advantage of this technology is that microalgae that is co-immobilized with bacteria are always more effective at removing nitrogen and phosphorus than microalgae without bacteria. As the two microorganisms are immobilized in alginate beads that are easily and rapidly removed from wastewater by sedimentation, this technology could be a cost-effective alternative to chemical precipitation, which is the standard treatment of wastewater. It solves two problems in standard microalgal technology: increasing the population of microalgae to a level sufficient to clean the wastewater and using the waste biomass in soil remediation when the cleaning process is completed.

20.3.2.1 Methods

- Bioreactors of various sizes (Cruz et al. 2013).
- Water analytical methods (Eaton et al. 2005) for the following parameters: NH_4^+ (μM), NO_3^- (μM), NO_2^- (μM), PO_4^{3+} (μM), pH, conductivity (mS m^{-1}), salinity (‰), silicates (μM), total hardness (mg L^{-1}, $CaCO_3$), Cl (mg L^{-1}), SO_4^{2-} (mg L^{-1}),

acidity (mg L^{-1}), total suspended solids (mg L^{-1}), dissolved solids, and sediments (mg L^{-1}).
- SEM and FISH coupled with specialized image-analysis quantification software (Covarrubias et al. 2012; also see above)

20.3.3 Increased Fertility of Eroded Soil

A potential application of this model of interaction is that the biological residues from a biological wastewater treatment (described above) is a resource for improving quality of degraded soils and improved plant growth. After tertiary wastewater treatment (removal of nutrients), debris composed of alginate beads containing the co-immobilized microorganisms can be used as an amendment for eroded and infertile soils with low levels of organic matter, where the microalgae serves as organic matter and *A. brasilense* as a PGPB. *A. brasilense* survived in these used, dried alginate beads for at least 1 year. Three consecutive applications of the dry debris increased organic matter, organic carbon, and microbial carbon in the soil. Growth of sorghum in the amended soil was greater than sorghum grown in soil with low organic matter, untreated soil, or soil amended with beads containing other combinations of alginate, microalgae, or bacteria. The surface of plant roots growing in the amended soil was heavily colonized by *A. brasilense*, with no endophytic colonization; root tips were the preferred sites of colonization (Trejo et al. 2012). Application of this residue significantly changed the bacterial rhizosphere population of plants growing in these soils (Lopez et al. 2013).

20.3.3.1 Methods

- Extraction of DNA from degraded soil is a modification of the method described by de-Bashan et al. (2010a, b), using a kit (Fast DNA SPIN for soils, MP Biomedicals) and applied according to the manufacturer's instructions. To remove humic acids, the binding matrix–DNA complex can be rinsed with saturated 5.5 M guanidine thiocyanate (Fluka-Sigma-Aldrich). Each DNA extraction is performed with a 0.6 g soil sample.
- Polymerase chain reaction (PCR). A modification of PCR procedure described by de-Bashan et al. (2010a, b) is used. The V9 variable region of the 16S rRNA gene is amplified with the bacteria primers 1070F (5′-ATG GCT GTC GTC AGC T-3′) and 1406R (5′-ACG GGC GGT GTG TAC-3′) with a 40 bp GC clamp (Ferris et al. 1996). A modification of PCR for DGGE by Colores et al. (2000) is used. These modifications include: Each PCR mixture (25 μL) contains 1 × PCR buffer with 15 mM $MgCl_2$ (Qiagen Sciences), 200 μM of each deoxyribonucleoside triphosphate (Sigma), 0.2 μM each primer, 5 % dimethyl sulfoxide (Sigma), 0.4 μg L^{-1} bovine serum albumin (Sigma), 0.6 units μL^{-1} HotStarTaq DNA polymerase (Qiagen Sciences), and ~100 ng template DNA. PCR is run in a thermocycler (Eppendorf) at 95 °C for 15 min for 30 cycles (94 °C for 45 s,

55 °C for 45 s, 72 °C for 30 s, and an extension at 72 °C for 7 min). PCR products are viewed after electrophoresis by running a 2 % agarose gel (Sigma) with a gel stain (SYBR Safe, Molecular Probes). PCR products are quantified in a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific).

- Denaturing gradient gel electrophoresis (PCR-DGGE) analysis. A modification of DGGE of the 16S rRNA gene products by de-Bashan et al. (2010a, b) is performed using a D-code universal mutation detection system (Bio-Rad Laboratories). Acrylamide gels (6 %) are prepared with a 40–60 % urea-formamide denaturing gradient, according to the manufacturer's protocol. Lanes are loaded with 15 μL PCR product. The external reference ladder may consist of different known species of bacteria. Electrophoresis is run at 40 V for 10 min at 60 °C and subsequently at a constant 60 V for 16.5 h at 60 °C. Gels are stained with nucleic acid gel stain (SYBR Green I, Molecular Probes) and gel images are recorded with a gel documentation imaging system (Gel Doc XR, Bio-Rad Laboratories).
- Identification of *A. brasilense* in PCR-DGGE profiles. Presumptive bands of *A. brasilense* Cd are excised from DGGE gels (45–60 % gradient) using sterile razor blades under UV illumination. The excised bands are eluted in 300 μL ultrapure water and incubated at 37 °C for 1 h. Aliquots are diluted 1:10 in ultrapure water; 2 μL of this dilution is used as a template to re-amplify the replicon by using the same PCR conditions and DGGE primers described earlier. The size of the PCR product is confirmed on 2 % agarose gel after each round of amplification. Successive PCR-DGGE gels were run to verify the identity and purity of the excised bands by comparing the re-amplified PCR products to the profile of the external reference ladder containing *A. brasilense*. PCR products that exhibited the highest identity to the *Azospirillum* band in the DGGE gel are purified using the QIAquick PCR purification kit protocol (Qiagen Sciences), and then submitted for commercial sequencing using primer 1070F (Genewiz). The original *A. brasilense* inoculum and its corresponding band in the external reference ladder are also sequenced at the same time as the experimental samples.
- Statistical analysis of DGGE gels. Analysis of gels is incomplete without detailed statistics of the bands. The band profiles obtained from DGGE gels are analyzed for similarity using the Dice coefficient. A dendrogram is built either from the Weighted Pair Group Matching Average (WPGMA) or the Unweighted Pair Group Matching Average (UPGMA). Similarity varies from 0 to 1, where 1 indicates 100 % similarity. Additionally, the observed similarities between profiles of DGGE are analyzed by multivariate statistical analysis, such as Kruskal's non-metric multidimensional scaling (NMDS; Venables and Ripley 2002) using computing software (Statistica 8.0, StatSoft). The Kruskal stress coefficient was used to reflect goodness-of-fit of the model. Values of Kruskal stress <0.1 are considered a good fit. Canonical analysis is also used for that purpose (de-Bashan et al. 2010b).

  Bacterial richness considered each band as an individual Operative Taxonomic Unit (OTU) (Kisand and Wikner 2003). This is obtained from the Band Type Report of the Quantity One 4.6.7 imaging software (Bio-Rad Laboratories) that provides the number of bands detected in DGGE profiles. Bacterial diversity is

calculated by analyzing the relative intensity of each peak (corresponding to a defined band) in the densitometric profile with Shannon's Diversity Index (Iwamoto et al. 2000), calculated by the formula: $H = -\Sigma Pi \log_{10} Pi$, where Pi is the importance probability of the bands in a gel lane and is calculated as $Pi = ni/N$, where ni is the intensity of a peak and N is the sum of all peak intensities of bands (Iwamoto et al. 2000). Data is then analyzed by one-way ANOVA and then by Tukey's post hoc analysis (or any other post hoc analysis) at $P < 0.05$, using statistical software.

- Root colonization by FISH. The technical details are presented above. Colonization by *Azospirillum* spp. is counted from images of FISH with imaging software (Image Pro Plus 6.3.1.542, Media Cybernetics) (modification of Treiser et al. 2007). Using the software RGB color code definitions, the specific magenta color (or any other color that the bacterium was labeled for) of *Azospirillum* detects qualitatively by FISH in these images is composed R-255, G-000, and B-255. The software measures the number of pixels that harbor this specific fluorescence and ignores other colors. The coverage (in %) of this fluorescence per area of root (in μm^2) is measured; this reflects the presence and level of colonization of each of the ten segments measured for each root part. These ten segments cover the entire root tip.
- Microbial biomass (expressed as microbial carbon) of soil is determined with a combination of the fumigation-extraction-oxidation of dichromate techniques described elsewhere (Joergensen and Brookes 2005).

20.3.4 Increased Bulk for Animal and Human Feed

Co-immobilization of *C. vulgaris* and *A. brasilense* under autotrophic condition yield, under a variety of environmental conditions, a significantly increased growth of the microalga. Dry and fresh weight, total number of cells, size of the microalgal clusters (colonies) within the bead, number of microalgal cells per cluster, and cell size significantly increased (de-Bashan et al. 2002a, 2005; Gonzalez and Bashan 2000). An even higher cell yield can be induced under heterotrophic conditions with D-glucose or Na-acetate as carbon sources (Perez-Garcia et al. 2010). When the microalgae is growing under less than optimal conditions, co-immobilization with *A. brasilense* mitigates the effect of these adverse condition on growth and metabolism of the microalgae (de-Bashan and Bashan 2008; de-Bashan et al. 2008c; Choix et al. 2014). This system has not been scaled up for biomass production.

20.3.4.1 Methods

- *Microbial counts*. Beads are solubilized for cell counts by immersing five beads (one bead per milliliter) in a solution of 4 % $NaHCO_3$ for 30 min at ambient temperature of 25 ± 4 °C. *A. brasilense* is counted by plating a series of dilutions

(in PBS) on BTB agar plates (Bashan et al. 2011). Alternatively, *A. brasilense* cells are first stained with fluorescein diacetate (Sigma) (Chrzanowski et al. 1984) and then directly counted under a fluorescent microscope. *C. vulgaris* is counted using a Neubauer hemocytometer connected to image analyzer or manually under light microscopy (Gonzalez and Bashan 2000). Growth rate of *C. vulgaris* (μ) is 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).

- *Determining biomass*. Ten grams of beads containing co-immobilized microalgae and bacteria are dissolved in 100 mL, as described above. The suspension is then filtered through a 3 mm (pore size) plankton net, leaving a pellet of microalgae on the net. This pellet is suspended in 100 mL PBS. Aliquots (10 mL) are centrifuged for 3 min at $1{,}400 \times g$ in tubes containing filter paper at the bottom. The supernatant containing the bacteria is discarded. The dry weight of the microalgae is measured after extracting and drying the filter paper at 105 °C for 1 h that contains the microalgal pellet.

20.3.5 Increased Photosynthetic Pigments

Green microalgae are commonly used for production of pigments for food and cosmetics (Lebeau and Robert 2006). Pigment production of the four major microalgal pigments; chlorophyll *a* and *b*, lutein, and violaxanthin of *C. vulgaris* and *C. sorokiniana*, co-immobilized with *A. brasilense*, significantly increased (de-Bashan et al. 2002a). This is very similar to the increase of these pigments in wheat plants inoculated with *A. brasilense* (Bashan et al. 2006). This system has not been as yet scaled up for pigment production.

20.3.5.1 Methods

- Pigments other than chlorophylls are detected, analyzed, and quantified by a HPLC method used mainly for pigments in plants (Bashan et al. 2006).
- Determination of chlorophyll. To determine the quantity of chlorophyll *a*, (the major component of this molecule in the microalgae) extraction is 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 is added to 5 mL of freshly thawed beads and heated for 10 min at 70 °C. After cooling, the samples are incubated in the dark for 24 h at 4 °C. Then, the samples are centrifuged for 10 min (4 °C; $6{,}000 \times g$) and absorbance is recorded in the supernatant at 665 and 652 nm.

20.3.6 Increase Carbohydrate Production

The interaction of *Azospirillum* spp. with microalgae enhances accumulation of total carbohydrate and starch in microalgae, either under autotrophic conditions or in the dark under heterotrophic conditions when D-glucose or Na-acetate is supplemented as a carbon source. Cells of *Chlorella* accumulated the highest amounts of carbohydrate after incubation for 24 h. After incubation for 72 h, mainly under co-immobilization treatments of both microorganisms, the cultures reached their highest total carbohydrate content (mainly as starch). This coincides with enhanced activity of ADP-glucose pyrophosphorylase (AGPase) that regulates starch biosynthesis in higher plants and microalgae. This demonstrates the potential of *A. brasilense* to affect carbohydrates and starch accumulation in *Chlorella* spp. when both microorganisms are co-cultured. This can be an important tool for future applications of microalgae, as in biofuel production (Choix et al. 2012a, b, 2014).

20.3.6.1 Methods

- Extraction and determination of carbohydrates. One gram of alginate beads is washed in distilled water, dried at 80 °C for 12 h, and ground with a mortar and pestle to yield a 10 mg sample. This sample is resuspended in 5 mL 1 M H_2SO_4 and sonicated for 4 min at 22.5 kHz with an ultrasonic cell disruptor. Carbohydrates are extracted by acid hydrolysis of the slurry after 60 min at 100 °C. Total carbohydrates are quantified by the phenol–sulfuric method (Dubois et al. 1956), adapted to microplates, using glucose as the standard.
- Starch is quantified by the method described by Brányiková et al. (2011), which 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 Na-acetate from the growth medium by microorganisms is analyzed using the Megazyme D-glucose (glucoseoxidase/peroxidase) assay kit (K-GLUC, gopod format, Megazyme International) and a kit to measure acetic acid (K-ACETAF 12/07, acetyl-coA synthetase format; Megazyme International).
- Enzymatic activity of ADP-glucose pyrophosphorylase (AGPase):
  - *Extraction*: To determine enzymatic activity, 6 g alginate beads are dissolved in 30 mL 4 % $NaHCO_3$ solution and centrifuged at 2,000 × *g* for 6 min. The supernatant is discarded and the pellet is washed three times with sterile saline solution (0.85 % NaCl). Enzyme extraction is done in 3 mL 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 20 mM β-mercaptoethanol, 12.5 % (v/v) glycerol, and 5 % (w/v) insoluble polyvinylpolypyrrolidone-40 at 4 °C (Nakamura et al. 1989).
  - *Quantification*: Enzymatic activity of AGPase is measured by the method of Li et al. (2011), with modifications as follows: the reaction buffer contains (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.

500 μL of the extracted enzyme is added to the reaction buffer. This reaction mixture is incubated at room temperature (26 ± 2 °C) for 20 min. The reaction is stopped by heating in boiling water for 2 min. Then, 600 μL distilled water is added, and the mixture is centrifuged at 13,000 × *g* for 10 min. The supernatant (1,000 μL) is mixed with 0.3 mg $NADP^+$. The activity is 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 are determined by the Bradford assay (Bradford 1976).

20.3.7 Increase Fatty Acids and Lipid Production

The interaction yielded more fatty acids and more lipids, mainly in the microalgae (de-Bashan et al. 2002a). Under autotrophic and heterotrophic growth conditions, co-immobilization always enhanced the activity of acetyl-CoA carboxylase (ACC), a key enzyme in de novo fatty acid biosynthesis, and yielded more lipids, when compared with immobilization of the microalga by itself. The highest lipid content under autotrophic conditions was obtained by also using an ammonium starvation period. Cultivation under heterotrophic conditions, without limitation of nitrogen, yielded a higher growth rate and accumulated more lipids than under autotrophic conditions (Leyva et al. 2014). Considering the major efforts to produce biodiesel from microalgae (Brennan and Owende 2010), this interaction has a significant, yet unexplored, biotechnological potential.

20.3.7.1 Methods

- Quantification and subsequent identification of fatty acids are done according to the method described by Sato and Murata (1988), with several small, but important, 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 per sample) are placed in a screw-cap glass tube. Five mL of a mix of concentrated hydrochloric acid and absolute methanol (5:95: HCl:CH_3OH v/v) are added to each sample and the cap hermetically sealed with additional polytetrafluoroethylene (PTFE) film. The tubes are placed in a water bath at 90 °C for 2 h for transmethylation. These samples are cooled to room temperature (26–28 °C) and 2 mL pure hexane (HPLC grade, #650552, Sigma-Aldrich) and 0.5 mL MilliQ water (EMD Millipore) are added to each sample and gently mixed in a vortex. After 10 min incubation at room temperature, when the layers are separated, the top hexane layer is transferred to a clean tube and the water layer is discarded. The hexane is 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 (#5181-8801, Agilent Technologies) and injected into a gas chromatograph-mass spectrograph (HP-GDC1800B, Agilent Technologies) equipped with a 30 m × 0.25 mm × 0.25 μM column (Omegawax 250, Supelco). The latter dimension is the size of the particles in the column. Running conditions are 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 in the splitless mode. The temperatures of the injector and detector are 250 °C and 260 °C, respectively. Each run involved the following pre-programmed 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 is increased at the rate of 2.2 °C·min^{-1} to 209 °C for 35 min. Identification of fatty acids is 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 is 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 are 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 are dissolved in two volumes of 4 % $NaHCO_3$ solution for 40 min at room temperature. Each suspension is then centrifuged (5,000 × *g*, 10 min, 4 °C); the supernatant is discarded, and the pellet is washed twice in 0.85 % NaCl and centrifuged again. The pellet is frozen with liquid nitrogen and pulverized with pestle and mortar. 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; #P7626, Sigma-Aldrich)] is added to the pellet. This is centrifuged for 30 min at 10,500 × *g* at 4 °C. The pellet is discarded and the supernatant is used as a crude extract for enzymatic reactions. The last steps are according to de-Bashan et al. (2008b).
  - *Quantification*: The reaction buffer is composed of 50 mM Tris–HCl pH 7.5, 6 μM acetyl-CoA, 2 mM ATP, 7 mM KHCO3, 8 mM MgCl2, 1 mM DTT, and 1 mg·mL^{-1} of bovine serum albumin (BSA; #B4287, Sigma-Aldrich). The crude extract is pre-incubated for 30 min at 25 °C with 10 mM potassium citrate and 2 mg·mL^{-1} BSA. Then, 500 μL crude extract is added to 0.5 mL of reaction buffer and the enzymatic reaction is incubated for 100 min at 30 °C. The reaction is stopped with 0.5 mL 10 % perchloric acid (PCA; #244252, Sigma-Aldrich). The total reaction mixture is filtered (0.22 μm membrane filter; EMD Millipore). Then 500 μL of this mixture are 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 5 m × 150 mm × 4.6 μm column (Zorbax Eclipse Plus C-18, Agilent Technologies). The flow rate is 1 mL·min^{-1} and the UV detector is adjusted to 262 nm. Solution A is 10 mM KH_2PO_4 at pH 6.7 and solution B is absolute methanol. Using analytical software (ChemStation, Agilent Technologies), the peak areas are recorded and the quantity of acetyl-CoA is 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 is defined as nmoles of substrate transformed per minute per 1 mg of protein.

- Lipids.
  - *Standard curve for lipids*: The quantity of lipids is measured following the method described by Pande et al. (1963). Extraction of lipids follows the standard method described by Bligh and Dyer (1959), but with small, yet very important, modifications to adapt it to microalgae, which involves sonication to break down cell walls. Briefly, lipids are extracted by adding 4 mL methanol/chloroform solution (2:1, v/v) to dry beads. The beads are sonicated for 10 min (2 cycles of 5 min at 30 kHz) in an ice bath. Sonicated beads are then incubated at 4 °C for 24 h in the dark and this procedure (only sonication) is repeated under the same conditions. The sample is then centrifuged (5,000 × *g*, 20 min, 4 °C), and the supernatant is transferred to a clean tube. The rest of the analysis is done as originally described.
  - *Quantification of lipids*: Lipid assays, based on a potassium dichromate color change reaction, are done according to Pande et al. (1963), using a calibration curve with tripalmitin (#T5888, Sigma-Aldrich), as a standard. The concentration of lipids is determined in a microplate reader (Molecular Devices) at 590 nm, recording the intensity of the green color that is formed. Potassium dichromate has a yellow-reddish color before reaction with lipids and a yellow-green color after the reaction with lipids. The method quantifies lipids in the range of 70 μg to 1.33 mg.

20.4 Conclusions

The interaction of *Azospirillum* spp. with photosynthetic, single-celled microalgae provides an important shortcut for understanding the interaction of this PGPB with plants, in general. This interaction is relevant for studying physiological, biochemical, and molecular aspects of the interaction. As an independent subfield of *Azospirillum* research, this interaction has some important biotechnological applications; most are yet to be tried in larger scale production and evaluation of their potential.

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