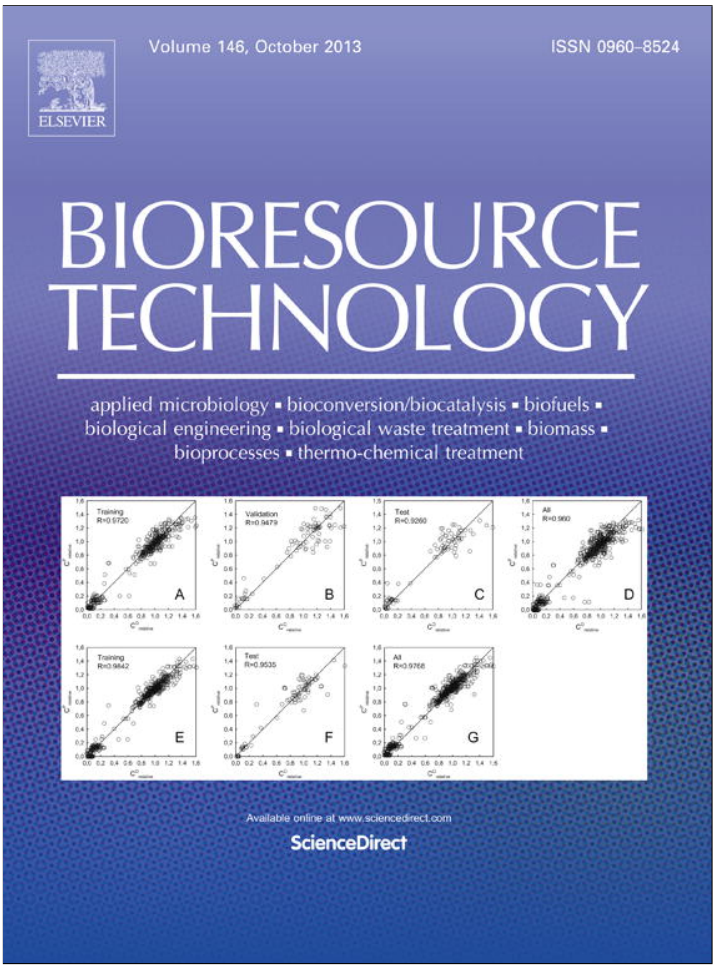

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High lipid productivity of an *Ankistrodesmus–Rhizobium* artificial consortium

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HIGHLIGHTS

- Bacterial strains were isolated from laboratory-acclimated microalgae cultures.
- *Rhizobium* strain 10II is likely a general microalgae-growth promoter.
- Growth promotion appears to be mediated by indol 3-acetic acid and B12.
- Microalgae inoculation with strain 10II resulted in up to 30% increment in biomass.
- Oil productivity of up to 0.1 g L^{-1} d^{-1} was obtained after optimization.

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ABSTRACT

Microalgae have great potential as alternative productive platforms for sustainable production of bioenergy, food, feed and other commodities. Process optimization to realize the claimed potential often comprises strains selection and improvement and also developing of more efficient cultivation, harvesting and downstream processing technology. In this work we show that inoculation with the bacterium *Rhizobium* strain 10II resulted in increments of up to 30% in chlorophyll, biomass and lipids accumulation of the oleaginous microalgae *Ankistrodesmus* sp. strain SP2-15. Inoculated cultures have reached a high lipid productivity of up to 112 mg L^{-1} d^{-1} after optimization. The resulting biomass presented significant levels of Ω3 fatty acids including stearidonic acid, suggesting potential as an alternative land-based source of essential fatty acids.

1. Introduction

Increasing demand of food and energy and concerns on environmental decay, including global climate change, represent some of the main current challenges for Humankind. However, what is even more challenging is how to harmonize these three often conflicting interests for sustainable development (Tilman et al., 2009). The production of biofuels, food, feed and biomaterials from microalgae or cyanobacteria biomass represent one promising alternative to bring into line all these concerns (Brennan and Owende, 2010; Chisti, 2007).

Similarly to plants, algae and cyanobacteria capture solar energy and store it as chemical energy of their biomass. Since this process is mostly accomplished by photosynthetic CO_2 fixation, carbon-containing biofuels have a varied tendency to be carbon neutral after combustion and hence might contribute to climate change mitigation. On the other hand, the possibility of large-scale culturing of photosynthetic microorganisms in non-agricultural lands might prevent competition with food supply and extensive land use changes (Brennan and Owende, 2010).

However, biofuels from microalgae are not commercial yet, and up to what extent the potential of biofuels from microalgae could be realized in the near future is still a matter of debate (Petkov et al., 2012). This is mostly because techno-economic modeling for biofuels from microalgae based on real productivities achieved at a range of scales of operation consistently supported the conclusion that the production of algae biomass is currently too expensive and too energy intensive (Borowitzka, 2013). Currently, microalgal biomass is produced commercially mostly for high value products such as human food supplements, animal feed, cosmetics and pharmaceuticals (Gong et al., 2011).

Microalgae strains selection has been identified as a key aspect to reduce production costs by increasing biomass and

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energy-carrier or target product content as well as other traits that would facilitate downstream processes. It has been proposed that the "ideal microalgae" should combine desirable traits as high biomass yield, high content of lipid or any other useful energy carriers, large cells with thin walls, flocculation properties, tolerance to high light intensity and oxygen concentration, resistance to contamination with other microorganisms or predators and high nutrient-recycling capacity and/or ability to utilize abundant and inexpensive alternative sources of N, P and other macro-elements (Ortiz-Marquez et al., 2013; Wijffels and Barbosa, 2010).

While many potential "production strains" have been identified worldwide, conversely to the development of modern plant crops there have been no breeding programs for microalgae strains (Georgianna and Mayfield, 2012). Although wild microalgae that exceed current yields may exist, it is presumed that achieving the efficiencies needed for economic production might required improvement of strains through breeding or recombinant DNA technologies (Georgianna and Mayfield, 2012; Rasala et al., 2013).

Additionally, a less explored concept that relies on either natural or synthetic multispecies microbial cell-factories comprising microalgae towards biomass, biofuels and biomaterials production has been proposed (Kazamia et al., 2012; Ortiz-Marquez et al., 2013; Subashchandrabose et al., 2011).

In nature microalgae live and have evolved in the context of multi-species microbial consortia. Either beneficial or antagonistic relationships take place in these associations. Beneficial interactions range from mutualistic to symbiotic and partners use to trade metabolites, mainly as cross-feeding, and tend to become specialized in specific functions that promote division of work (Wingreen and Levin, 2006). Accordingly, microbial consortia usually perform more complex tasks than monocultures and can carry out functions that are difficult or even impossible for individual strains or species (Brenner et al., 2008).

In most microalgae–bacteria consortia, microalgae provide photosynthetic oxygen and organic molecules. In natural systems, the algae release of dissolved organic carbon ranges from zero to 80% of photosynthates and it is around 6–16% in photobioreactors (Hulatt and Thomas, 2010). For bioenergy purposes, the latter can be seen as both a direct bioenergy loss and also a potential secondary loss due to the assembly of spontaneous and unattractive microorganism's consortia with low or null biotechnological value (Hulatt and Thomas, 2010; Lakaniemi et al., 2012). On the other hand bacteria could provide a broader array of substances from general respiration end-products as CO_2 and more specific compounds such as nutrients, vitamins, growth-promoting substances, etc. (Kazamia et al., 2012; Ortiz-Marquez et al., 2013).

Some microalgae–bacteria assemblies can persist even under conditions of laboratory cultivation of unialgal cultures which may resemble natural microalgal–bacterial consortia. In some cases, co-inoculation of bacterial strains isolated from long-term laboratory microalgal cultures had resulted in better growth than axenic microalgae (Subashchandrabose et al., 2011).

Different approaches have been used to identify microalgae-bacteria assemblies in laboratory cultures, uncovering a wide arrange of possible partners. However, it appears to exist a certain affinity of both macroalgae and microalgae to engage in associations with α-proteobacteria (Lakaniemi et al., 2012; Otsuka et al., 2008).

Nevertheless, although microalgae–bacteria associations have been extensively described, the effect of bacterial inoculation on microalgae biomass and energy carries yield and especially the basis of the bacterial algae-growth promotion are mostly speculative.

This work reports the isolation and identification of cultivable heterotrophic bacteria after 2–3 years enrichment by serial dilution of monoalgal cultures in mineral medium. An artificially assembled *Ankistrodesmus* sp.–*Rhizobium* sp. consortium was further characterized to show enhanced oleaginous biomass production rich in Ω3 fatty acids.

2. Methods

2.1. Algae strains and cultures

The strains used in this work were *Chlorella sorokiniana* strain RP, *Chlorella* sp. strain MI, *Scenedesmus obliquus* strain SP2-9, *Ankistrodesmus* sp. strain SP2-15, *Ankistrodesmus* sp. strain LP1 and *Pseudokirchneriella* sp. strain C1D and had been isolated by our group from southeastern Buenos Aires, Argentina (38°0′0″S 57°33′0″W) (Do Nascimento et al., 2012). All stock and experimental algal cultures were maintained in BG11 mineral medium at 29 ± 1 °C. Three different culture settings were used in this work. Static cultures were carried out in 25 mL medium containing 2 mM $NaNO_3$ as nitrogen source in 100 mL Erlenmeyer flasks shaken manually by 5–6 stokes twice a day. Air-bubbled cultures were run in 200 mL medium containing 2 mM $NaNO_3$ in 500 ml bottles sparged with 0.22 μm-filtered air from the bottom. For the induction of lipids accumulation cells were transferred to medium containing 0.1 mM $NaNO_3$ and further incubated for 6 days. Either static or air-bubbled cultures were exposed to constant light at 35 μmol photons m^{-2} s^{-1}. Air-lift photobioreactors contained 4.5 L medium with 3 mM $NaNO_3$ and were sparged with filter-sterilized air from the center of the riser tube to separate the liquid upflow and downflow circulations. CO_2 was sparged at the bottom of the downflow circulation external to the riser tube. The reactors were illuminated with constant white light at 180 μmol photons m^{-2} s^{-1}. Using this setting, lipids accumulation began as cells exhausted the nitrogen to completion in approximately 3 days.

When indicated, microalgae cultures were supplemented with thiamin, cyanocobalamin (B12), biotin, riboflavin, indol 3-acetic acid or tryptophan at the indicated concentrations.

2.2. Isolation of microalgae associated-bacteria

We used our *ad-hoc* collection of native microalgae (Do Nascimento et al., 2012) as a source of microalgae-related bacteria. Bacteria were isolated after 2–3 years of enrichment by serial dilution of monoalgal cultures in mineral medium lacking any source of organic carbon except that excreted by the algae. For the isolation of bacterial strains that could not be removed out of the microalgae cultures by exhaustive dilution we have developed an artificial medium (AEX medium) mimicking microalgae photosynthetic exudates (modified from Watanabe et al., 2006). The composition of AEX medium is: 34.2 mg/L sucrose, 7.2 mg/L casamino acids, 1.2 mg/L biotin, 170 mg/L $NaNO_3$ and trace metals mix (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2{\cdot}4H_2O$, 0.222 mg/L $ZnSO_4{\cdot}7H_2O$, 0.39 mg/L $NaMoO_4{\cdot}2H_2O$, 0.079 mg/L $CuSO_4{\cdot}5H_2O$ and 0.049 mg/L $Co(NO_3)_2{\cdot}6H_2O$) in half strength BG11 medium. Microalgae-associated bacteria were isolated onto AEX or AEX-supplement (ten-fold strength), solidified with 10 g/L water-washed agar–agar. Colonies were initially classified according to morphological characteristics such as colony size, shape, structure and color and microscopic inspection.

2.3. Identification of microalgae associated-bacteria

For the identification of strains, liquid cultures were set in ten-fold strength AEX medium and genomic DNA was extracted according to the method described by Cai and Wolk (1990). Molecular characterization was done by sequencing the 16S rDNA after amplicons production by PCR with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGT-

TACGACTT-3′). The 25 μL reactions contained 1 μL genomic DNA, 1X reaction buffer (Fermentas), 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.62 μM of each primer, and 2.5 U of Taq DNA polymerase (Fermentas). Cycling temperature program for PCR reaction was: 94 °C, 2 min and 30 cycles at 94 °C, 45 s, 45 °C, 45 s and 72 °C, 90 s and 72 °C, 10 min in an Eppendorf Mastercycler Gradient device (Eppendorf, Hamburg, Germany). PCR products were analyzed by electrophoresis on 1% agarose gels and send to the sequencing facility (Macrogen Inc., Seoul, South Korea) for the corresponding analysis. The closest relatives were determined using the Basic Local Alignment Searching Tool (BLAST) and by multiple sequence analysis using the Clustal W algorithm from The Molecular Evolutionary Genetics Analysis v5 (MEGA5) (Kumar et al., 2008). Phylogenetic trees were created using the Neighbor Joining algorithm from the MEGA5 software after 500 rounds of bootstrap resampling.

2.4. Effect of bacterial isolates on the growth of microalgae

To test the effect of the different bacterial isolates on microalgae growth, pair-wise combinations of most of the bacterial isolates and selected axenic microalgae from our collection were co-cultured. Inocula were prepared by cultivating either bacteria or microalgae in their specific medium (ten-fold AEX or BG11-NO_3, respectively). Cells were collected by centrifugation at 6000 rpm, for 10 min, washed twice in BG11 and resuspended in the same medium. Finally cells were inoculated at a bacterium to alga ratio of 10:1 (10^7 bacterial cells/mL–10^6 algal cell/mL) and incubated according to the different culture variants.

Microalgae growth was analyzed by direct cell counting using a Neubauer chamber, chlorophyll *a* and dry-biomass determination. Bacterial growth was analyzed by direct cell counting under the optical microscope and colony-forming-units (CFU) counting onto LB or AEX solid media (Ortiz-Marquez et al., 2012).

Cell-free bacteria spent medium was prepared from the supernatant of 2 days cultures (centrifuged for 15 min at 9000 rpm at 4 °C) and then filtered through 0.22 μm sterile filters. Algae spent medium were similarly prepared from 4 days cultures in BG11-$NaNO_3$ medium.

2.5. Lipids methods

Lipids extractions, gravimetric determinations, Nile Red staining, fatty acids methylation and gas chromatography analyses were performed essentially as previously described (Do Nascimento et al., 2012).

3. Results and discussion

3.1. Isolation and identification of bacterial strains associated to microalgae

In parallel to the isolation of oleaginous microalgae native to Buenos Aires, that resulted in more than thirty monoalgal and axenic cultures (Do Nascimento et al., 2012) we have continued subculturing monoalgal non-axenic cultures in mineral medium by 1:20 to 1:100 dilutions monthly during two to three years. In order not to bias the selection of bacteria, we recovered those strains able to develop onto a synthetic medium that mimics the composition of *C. sorokiniana* exudates (AEX medium). Most cultures resulted in the enrichment of a few bacterial strains discernible by colony size and shape onto AEX and LB medium and cell shape at the optical microscope (not shown).

For a preliminary screening, most isolated bacterial strains were mixed with axenic cultures of microalgae (Do Nascimento et al., 2012) at different dilutions and spotted onto AEX medium (not shown). Each bacterial strain that produced some noticeable effect on at least one of the assayed microalgae was subjected to ribotypification by sequence analysis of the 16S rRNA region. As shown in Fig. 1 most strains belong to proteobacteria, and about half of them were α-proteobacteria. A similar trend of this group of bacteria to form mixed assemblages together with a diversity of micro- and macroalgae has been appreciated in previous studies (Amin et al., 2012). Interestingly, most strains uncovered in this work belong to genera for which plant-growth-promoting strains are known and extensively studied such as *Rhizobium* spp. and *Herbaspirillum* spp. The latter has not been associated to microalgae before to the best of our knowledge. Similarly, strains closely associated to the known plant-pathogen *Xanthomonas campestris* have also become enriched by long term co-cultivation with microalgae. This apparent similarity of bacterial genera that have the ability to engage in stable associations with plants and algae has remained uncovered in previous work (Lakaniemi et al., 2012; Otsuka et al., 2008). Nevertheless, the analysis of a larger set of samples would be necessary for a better supported generalization.

3.2. Identification of microalgae-growth-promoting bacterial strains

For the identification of microalgae-growth-promoting strains, pair-wise co-cultivation runs were set between axenic microalgae of the genera *Scenedesmus*, *Chlorella*, *Ankistrodesmus* or *Pseudokirchneriella* and the bacterial isolates in erlenmeyer flasks shaken manually twice a day. Supplementary Fig. S1 shows representative growth curves of microalgae in the presence of selected bacterial strains. The most robust growth-promotion effect corresponded to the pair comprising the microalgae *Ankistrodesmus* sp. strains SP2-15 (Do Nascimento et al., 2012) and *Rhizobium* sp. strain 10II. Although to a lesser extent, this bacterium appeared to promote growth of a broad spectrum of microalgae. Quite surprisingly, this strain was less effective in promoting growth of the very closely-related microalgae *Ankistrodesmus* sp. strain LP1 (Do Nascimento et al., 2012). However, the most noticeable effect on growth promotion of this alga was produced by the closely related strain *Rhizobium* sp. HI (Supplementary Fig. S1). Our analyses resulted in uncertainties regarding the taxonomic assignment of strains between the genera *Rhizobium* or *Agrobacterium*, what was not surprising according to the fact that taxonomy of these genera is still under revision (Lindström and Young, 2011). Strain 10II grew diazotrophically under microaerobic conditions and gave negative results for the 3-ketolactose production assay (Moore et al., 2001) and was preliminary assigned to the *Rhizobium* genus.

3.3. Characterization of Ankistrodesmus–Rhizobium co-cultures

The microalgae growth-promotion effect exerted by *Rhizobium* sp. strain 10II was dependent on the initial bacterial inoculum size (Fig. 2A), required the activity of live bacteria and could be mimicked by amendments of the bacterial spent medium (Fig. 2B).

These results suggested that close contact between the algae and the bacteria might not be required for growth promotion and that the bacteria might produce a diffusible growth promoting substance.

Similar results were shown in a previous work using either a *Mezorrhizobium loti* strain isolated from a non-axenic culture of *Chlamydomonas nivalis* or the plant-nodulating rhizobia *M. loti* (strain MAFF 303099), *Rhizobium leguminosarum* (strain RL 3841) and *Sinorhizobium meliloti* (strain RM 1021) from culture collections on growth promotion of *Lobomonas rostrata*. It was shown that the mutualistic consortia exchange photosynthetic exudates from the algae for vitamin B12 produced by the rhizobia (Kazamia et al., 2012).

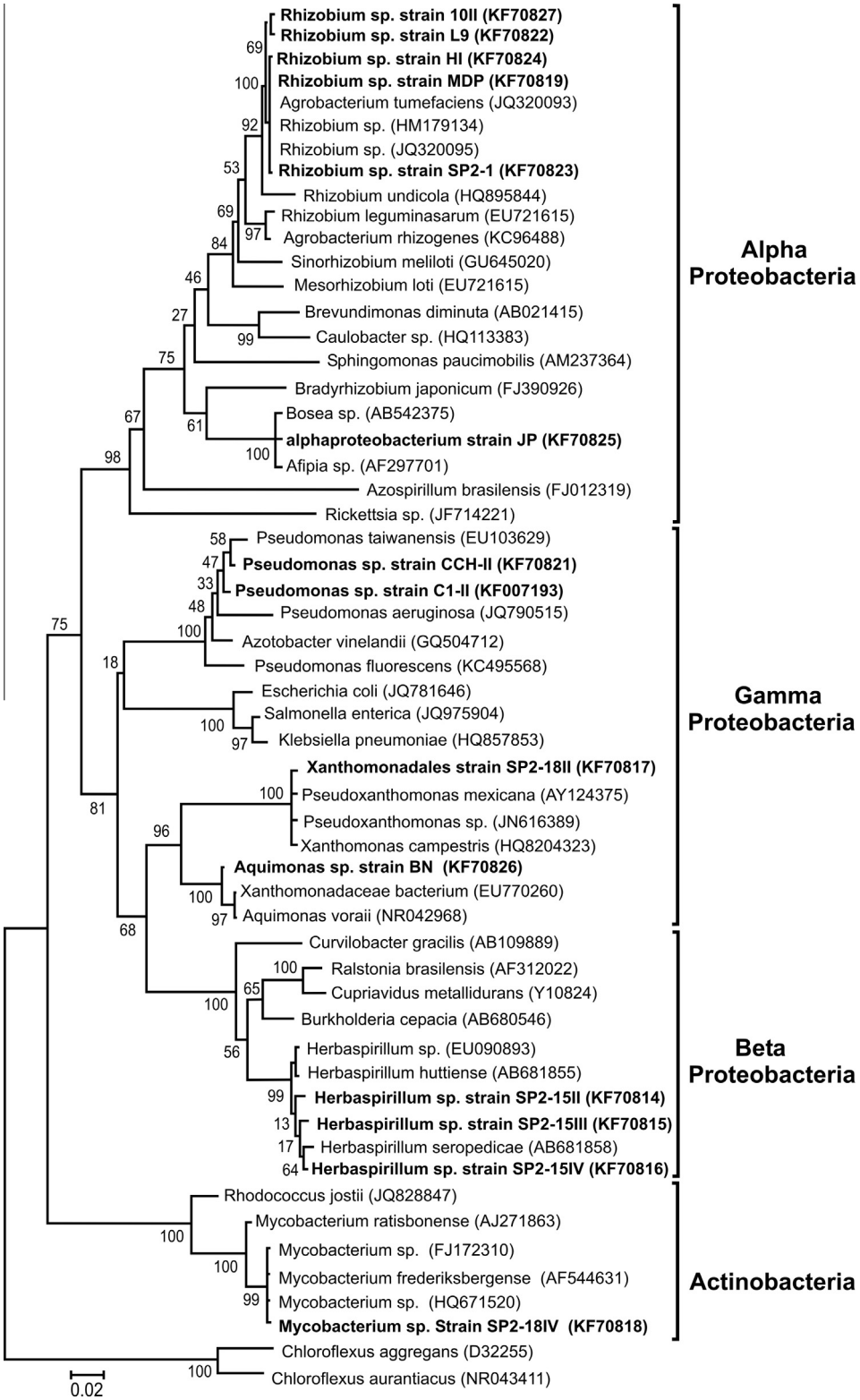

Fig. 1. Phylogenetic analysis of the 16S rRNA region of the microalgae associated bacteria. Sequences accession numbers were indicated at the right of each strain name. The sequences corresponding to the strains isolated in this work were indicated in bold case and the corresponding GeneBank accession numbers were indicated. The multiple sequence alignments were generated using the Clustal W algorithm from The Molecular Evolutionary Genetics Analysis v5 (MEGA5) software. Phylogenetic trees were created using the Neighbor Joining algorithm from the MEGA5 software after 500 rounds of bootstrap resampling.

In contrast to *C. nivalis* and *L. rostrata*, the microalgae strain used in this study, *Ankistrodesmus* sp. strain SP2-15, is not an obligate auxotroph for B12 and can be cultivated without supplementation of the vitamin. Nevertheless, to get insights about the basis of the algae growth-promotion, we analyzed the effect of different amendments into the growth medium to see which of them could mimic the algae-growth stimulation by *Rhizobium* sp. 10II. The bacterium did not support the alga growth in the absence of

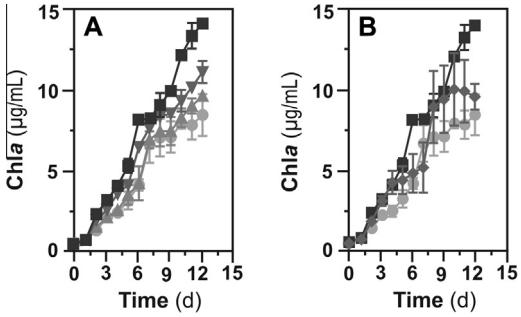

Fig. 2. Effect of different bacteria to algae ratios (A) or bacerial spent medium (B) on algal growth in static culture. (A) *Ankistrodesmus* sp. strain SP2-15 was inoculated with *Rhizobium* sp. strain 10II at relative cell ratios of (●) 1:0; (▲) 1:0.1; (▼) 1:1; or (■) 1:10. (B) Effect of supplementing axenic *Ankistrodesmus* sp. strain SP2-15 culture (●) with 50% *Rhizobium* sp. strain 10II spent medium (♦). The data represent the mean and standard deviation of two independent experiments.

Table 1
Production of indole-3-acetic acid by the bacterial strains isolated in this study.

Indole-3-acetic acid production (µg/mL)		
Strain	–	Tryptophan (100 µg/mL)
15I	3.16 ± 0.26	4.30 ± 0.96
15II	3.68 ± 0.30	7.24 ± 2.42
15III	4.71 ± 1.15	21.51 ± 1.99
18II	4.58 ± 1.34	3.90 ± 0.69
18IV	3.63 ± 1.15	5.26 ± 0.38
C1II	3.79 ± 0.84	6.34 ± 1.92
10I	6.34 ± 0.38	17.78 ± 0.34
10II	9.60 ± 0.38	43.60 ± 2.03
SP1-20 II	6.89 ± 1.15	22.86 ± 0.07
Mot	1.10 ± 0.34	3.11 ± 0.49
CR	1.02 ± 0.99	5.23 ± 1.19
CCH IV	3.52 ± 0.53	6.70 ± 2.42
MdP	7.16 ± 0.03	23.03 ± 0.23
C2C	6.34 ± 0.38	22.86 ± 0.23
H1	7.43 ± 0.38	48.25 ± 1.92
L9	7.70 ± 0.76	42.70 ± 0.53

supplemented nitrogen, indicating that nitrogen fixation products from the bacteria (if any) are unlikely to contribute to the algae growth-stimulation. Conversely, the growth of axenic algae was moderately stimulated by a mixture of vitamins B12, thiamine and biotin and also presented a dose-dependent response to indole-3-acetic acid (IAA), from promotion to complete inhibition (Fig. 3).

It was further observed that the isolated bacteria presented a varied capacity to excrete IAA into the growth medium, while *Rhizobium* strains, especially 10II, was the top producer among the analyzed strains. As expected (Ahmad et al., 2005), supplementation with tryptophan resulted in enhanced IAA production by most of the strains (Table 1). Attempts to further increase microalgae growth-promotion in co-culture with rhizobia by supplementation with tryptophan were unsuccessful because even in the absence of bacteria the aminoacid was detrimental for the microalgae growth (Supplementary Fig. S2). A similar effect was observed in plants while studying the effect of inoculation with IAA producing *Azotobacter* spp. or *Pseudomonas* spp. (Ahmad et al., 2005).

Although algae response to plant-like hormones has been established, comprehensive knowledge of algal regulatory systems controlled by phytohormone-like substances is fragmentary and mostly poorly understood (De Smet et al., 2011; Stirk et al., 2013).

It has been shown that co-immobilization with the plant growth-promoting bacteria *Azospirillum brasilense* resulted in increased pigment and lipid content, lipid variety, carbohydrates, and cell and population size of the microalgae *Chlorella* spp. (Choix et al., 2012). It was further shown that while four wild type IAA-producing *A. brasilense* strains promoted growth and ammonium

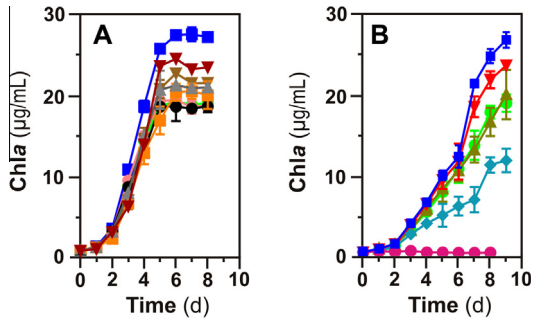

Fig. 4. Effect supplementations on *Ankistrodesmus* sp. strain SP2-15 growth of cultured in air bubbled bottles. (A–B) (●) Axenic *Ankistrodesmus* sp. strain SP2-15; (■) co-culture *Ankistrodesmus* sp. strain SP2-15–*Rhizobium* sp. strain 10II. (A) Axenic *Ankistrodesmus* sp. strain SP2-15 supplemented with (■) thiamin (0.1 µg/mL); (▼) B12 (0.01 µg/mL); (■) riboflavin (10 µg/mL); (●) biotin (0.012 µg/mL); or (▲) a mixture of the four vitamins at the same concentrations. (B) Axenic *Ankistrodesmus* sp. strain SP2-15 supplemented with indol 3-acetic acid at (▲) 0.1 µg/mL; (▼) 1 µg/mL; (♦) 10 µg/mL AIA); or (●) 30 µg/mL. The data represent the mean and standard deviation of two (A) and four (B) independent experiments.

removal capacity of *C. vulgaris*, the corresponding mutant strains deficient in IAA production remained unable to do so. These studies have supported the proposal that IAA might be implicated not only in plant-bacteria interactions, but also in microalgae-bacteria interaction (de-Bashan et al., 2008). Thus the dose-dependent response of *Ankistrodesmus* sp. strain SP2-15 to IAA and its interac-

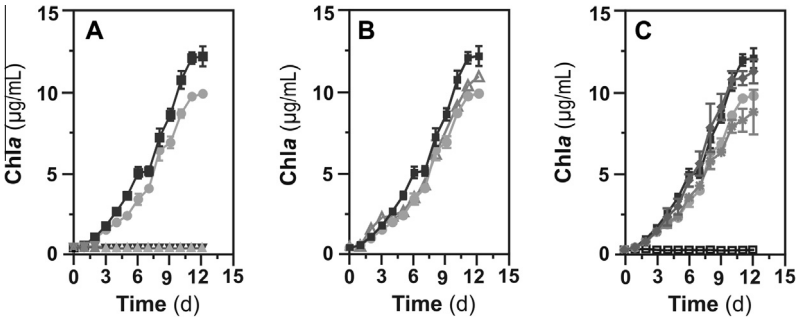

Fig. 3. Effect of different amendments or limitations on growth of *Ankistrodesmus* sp. strain SP2-15 in static culture. (A–C) (●) Axenic *Ankistrodesmus* sp. strain SP2-15; (■) co-culture *Ankistrodesmus* sp. strain SP2-15 and *Rhizobium* sp. strain 10II. (A) (▼) N-deficient axenic *Ankistrodesmus* sp. strain SP2-15 (0.1 mM $NaNO_3$); (▲) N-deficient co-culture *Ankistrodesmus* sp. strain SP2-15 and *Rhizobium* sp. strain 10II (0.1 mM $NaNO_3$). (B) (Δ) Axenic *Ankistrodesmus* sp. strain SP2-15 supplemented with vitamins mix B12 (0.01 µg/mL), biotin (0.012 µg/mL µg/mL) and thiamin (0.1 µg/mL). (C) Axenic *Ankistrodesmus* sp. strain SP2-15 supplemented with indol 3-acetic acid at (♦) 0.1 µg/mL; (∗) 1 µg/mL; or (□) 10 µg/mL. The data represent the mean and standard deviation of four independent experiments.

tion with *Rhizobium* sp. strain 10II may represent an alternative experimental model to complement current efforts to address this subject.

For simplicity the previous experiments were done in static culture. Next we challenged the microalgae-bacteria co-cultures in air bubbled bottles to set the air saturation of the cultures constant and to improved algae growth. Under these conditions, the growth promotion effect of the bacteria, supplementation with IAA and/or vitamins became even more robust than that observed for static cultures. It was also shown that most of the effect of vitamins supplementation could be attributed to B12 and no synergistic effect of IAA and B12 could be observed (Fig. 4).

A recent work shows that co-cultivation of *Bradyrhizobium japonicum* with *C. reinhardtii* strain cc849, increased the microalga's growth by 26%. It was concluded that the increase in respiration rate and fast O_2 consumption in the co-cultures was the major reason for the improvement (Wu et al., 2012). Although not experimentally tested in this work, the observation that bacterial growth promotion improves in air bubbled bottles suggested that increase O_2 consumption might not be the major mechanism by which *Rhizobium* sp. strain 10II stimulates growth of *Ankistrodesmus* sp. strain SP2-15.

As expected *Rhizobium* strain 10II did not grow axenically in microalgae mineral medium, unless microalgae cells were co-inoculated. The bacteria grew comparatively well in axenic microalgae spent-medium. In the presence of 10 mM sucrose a ten-fold increment in viable *Rhizobium* strain 10II counts was observed suggesting that bacterial growth was carbon limited while in co-culture with microalgae (Supplementary Fig. S3).

3.4. Productivity of Ankistrodesmus–Rhizobium co-cultures

Inoculation with *Rhizobium* sp. strain 10II resulted in a 39% increase in chlorophyll content and a 29% increase in biomass production of *Ankistrodesmus* sp. strain SP2-15. The final lipid content on a dry biomass basis remained unaltered at 38%, indicating that lipid productivity mirrors that of biomass (Table 2). The effect of inoculating strain 10II on microalgae growth was further studied in 5 L tubular air-lift photobioreactors fed with CO_2 (Fig. 5). The doubling time of *Ankistrodesmus* sp. strain SP2-15 was 71, 43 or 11 h in static cultures, air bubbled bottles or tubular air-lift photobioreactors, respectively (Table 2). Microalgae growth stimulation by *Rhizobium* strain 10II was also verified in photobioreactors. In this case increases of 20% and 28% were registered for biomass yield (1.7 and 2.0 g/L) and lipid productivities (87 and 112 mg L^{-1} d^{-1}), respectively at day 6 of culture. At this time, non-inoculated or inoculated microalgae biomass had a lipid content of 33% and 35% (dw basis), respectively. Although at day 8 the cells attained lipid contents of 44% and 46% (dw basis), respectively, lipid productivity tended to decrease and continued decreasing in subsequent days of culture (not shown).

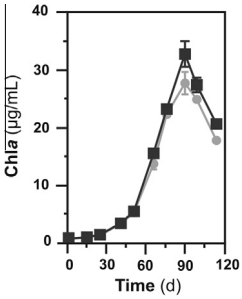

Fig. 5. Growth curves of *Ankistrodesmus–Rhizobium* co-cultures in photobioreactors. Non-inoculated (●) or inoculated with *Rhizobium* sp. strains 10II (■) *Ankistrodesmus* sp. strain SP2-15. Each data point represents the mean and standard deviation of two independent experiments.

In a review that compared growth rates, lipid content and lipid productivities of 55 species in different laboratories of the world it was found that the overall average lipid productivity for all species from the literature was 50–52 mg L^{-1} d^{-1}. A few species with lipid productivities above 100 mg L^{-1} d^{-1} were regarded as very high lipid producers and *Ankistrodesmus falcatus* was among them with a calculated productivity of 109 mg L^{-1} d^{-1}. The study compared lipid productivities under conditions of nutrients sufficiency, since although is known that nutrients deficiency increases lipid accumulation in most microalgae, data of lipid productivity under those conditions were less ready available in the literature (Griffiths and Harrison, 2009). Very often, enhanced lipid content under nutrient limitation is offset by a severe growth retardation that impacts negatively on lipid productivity (Do Nascimento et al., 2012). In the optimized conditions reported in the present study we showed that *Ankistrodesmus* sp. strains SP2-15 grew very fast at nearly 2.5 doublings a day until nitrogen (initial dose of 3 mM $NaNO_3$) was exhausted from the medium and the cells started to accumulate lipids for productivities higher than 100 mg L^{-1} d^{-1} as soon as day 6.

Conversely to the static or air-bubbled bottles, cultures in the air-lift photobioreactors could not be run axenically (or gnotobiotically when inoculated with *Rhizobium* strain 10II). Thus, the fact that *Rhizobium* strain 10II exerted its algae growth-stimulation effect even in complex assemblages of microalgae and bacteria suggests that it must be a good competitor under these circumstances. Additionally, bacterial stimulation of *Ankistrodesmus* sp. strain SP2-15 appears to be somehow specific for *Rhizobium* sp. strain 10II. Although not verified in this work, it could be speculated that inoculation with *Rhizobium* strain 10II could be useful to discourage other detrimental microbes to contaminate microalgae cultures when controlling contaminants by more classical means is not practical.

A recent study showed that inoculation with B12-producing rhizobia improved the growth performance of *C. reinhardtii* during

Table 2
Biomass and lipid productivity of axenic or *Rhizobium* sp. strain 10II inoculated *Ankistrodesmus* sp. strain SP2-15 for different culture systems.

Culture system	Culture condition	Growth parameter			
		DT (h)	Chl*a* (µg/mL)	DW (mg/L) max	TL (%)
Static	Axenic microalgae	71.31 ± 6.74	9.05 ± 0.88	ND	ND
	Co-culture	60.80 ± 6.15	11.85 ± 0.70	ND	ND
Air-bubbled bottle	Axenic microalgae	43.53 ± 3.06	20.35 ± 1.62	699,96 ± 31.34	37.42 ± 4.81
	Co-culture	37.73 ± 2.36	28.44 ± 1.80	901,11 ± 18.59	37.62 ± 3.73
Air-lift photobioreactor	Axenic microalgae[a]	10.86 ± 0.58	27.69 ± 3.44	1671.95 ± 164.65	33.36 ± 1.13
	Co-culture	10.29 ± 0.91	30.23 ± 5.98	1996.35 ± 139.96	35.11 ± 0.90

DT, doubling time; Chl*a*, chlorophyll *a* content; DW, maximal dry weight; TL, total lipids as a percentage of dried biomass.
[a] Photobioreactors could not be maintained axenic.

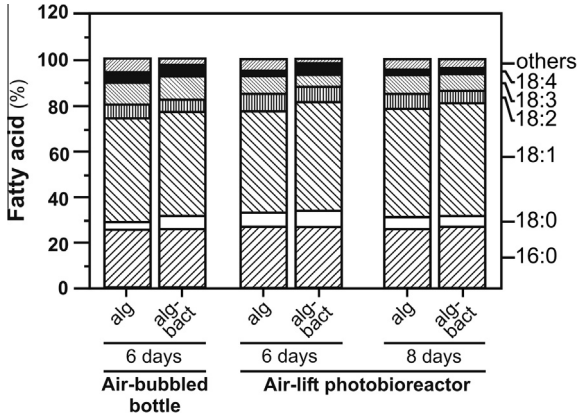

Fig. 6. Fatty profiles of *Ankistrodesmus-Rhizobium* co-cultures. Results are the mean and standard deviation (⩽5%) of two independent experiments.

high temperature stress (Xie et al., 2013). Thus crop-algae protection during suboptimal or stressing conditions could be an additional benefit of using rhizobia to inoculate outdoor ponds under some situations.

3.5. Ankistrodesmus sp. strain SP2-15 could be an alternative source of Ω3 fatty acids

Either the fatty acids found in *Ankistrodesmus* sp. strains SP2-15 or their relative amounts (Do Nascimento et al., 2012) remained unchanged regardless the cultivation system or the inoculation with *Rhizobium* strain 10II and/or the uncontrolled contamination with other bacteria as that observed in photobioreactors (Fig. 6). This indicates that even in the intentionally inoculated cultures the resulting oil might contain only very low amounts of bacterial lipids and that the bacteria did not induced specific metabolic changes in the microalga's fatty acids metabolism.

Ankistrodesmus sp. strain SP2-15 accumulates up to 16% and 8% of its fatty acids as the Ω3 fatty acids linolenic acid (c18:3ω3) and stearidonic acid (c18:4ω3) (Do Nascimento et al., 2012). In this work, we confirmed an average content of stearidonic acid of 5% for *Ankistrodesmus* sp. strain SP2-15 fatty acids despite the production system, indicating that the productivity of this fatty acid might mirror that of biomass. The use of land-based source of stearidonic acid (SDA), among other Ω3 fatty acids (alternative to fish or fish oil) has been proposed for a sustainable long-term approach for human nutrition, cardiovascular diseases prevention and general health improvement (Walker et al., 2013). In addition to fish and seafood oils, seeds from members of the Boraginaceae family of plants, have been identified as potential alternative sources of SDA. While echium is currently available as a commercial plant source of SDA, genetically modified soybean with a SDA content of 18–28% of total fatty acids was proposed as a more cost–effective source of SDA than echium (Walker et al., 2013).

Although to a less prominent level than in this study, accumulation of SDA in other *Ankistrodesmus* strains have been appreciated before. This was someway related with potential applications of *Ankistrodesmus* biomass as feed in aquaculture, still connected to human nutrition and health (Renaud et al., 1994). Benefits of SDA supplementation of farmed-fish feed in comparison to other common Ω3 fatty acids has also been demonstrated (Bharadwaj et al., 2010). According to projected comparative productivities of plants and microalgae (Chisti, 2007) and the results presented in this work, the possibility of developing a microalgae-based platform for the alternative production of SDA appears promising.

This work leads us to propose several non-mutually-exclusive alternative applications of *Ankistrodesmus* sp. strain SP2-15 from bulk production of oil as a feedstock for biodiesel to feed or food supplement. Further work is needed to ascertain how much of the potential of the productivities demonstrated in this work can be achieved in an outdoors setting. However, the fact that contrary to the expectation, an extensive literature survey showed that productivity in outdoor photobioreactors was on average five times higher than in the laboratory (Griffiths and Harrison, 2009), suggests that a productive platform comprising some of the elements of this work might be feasible.

4. Conclusions

We have isolated bacterial strains that engage in long term assemblages with microalgae in culture. We further identified *Rhizobium* strain 10II as a likely general microalgae-growth promoter. Inoculation with this strain resulted in increments of up to 30% in chlorophyll, biomass and lipids accumulation of the oleaginous microalgae *Ankistrodesmus* sp. strain SP2-15. The stimulation effect is apparently related to indol 3-acetic acid and/or vitamin B12 produced by the bacterium. An important lipid productivity of up to 112 mg L^{-1} d^{-1} was demonstrated. Interestingly *Ankistrodesmus* sp. strain SP2-15 oil could be an alternative source of Ω3 fatty acids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.07.085.

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