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Cyanobacterial biological nitrogen fixation as a sustainable nitrogen fertilizer for the production of microalgal oil

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ABSTRACT

Oleaginous microalgae have a great potential as a feedstock for biodiesel and other biofuels. However, the current cost of producing biofuels from microalgae biomass is still high to envision massive and profitable commercialization in the near future. One of the drawbacks of implementing large-scale cultivation of these organisms is the unsustainable requirement of N-fertilizers. It is presumed that co-production of higher value by-products in the frame of a biorefinery would increase the profitability of producing oil from microalgae.
The aim of this work is to provide proof-of-concept for the complete substitution of chemical N-fertilization by on-site biological N_2 fixation in a process of microalgal oil production. We show the efficient conversion of biomass of a N_2-fixing cyanobacterium into oil-rich microalgae biomass when the eukaryotic alga is fed with a cyanobacterium extract as a sole source of nutrients. Oil production yields in environmental photobioreactor simulations were in the range of current yields obtained at the expense of synthetic N-fertilizer and up to 20-fold higher than those reported when using plant feedstocks.

1. Introduction

Microalgae- and or cyanobacteria-based bioprocesses represent a very promising set of alternatives to traditional plant-based agriculture for biofuels, food, feed and bulk chemical feedstock [1]. These bioprocesses might alleviate pressure on agriculture for food supply and extensive land-use change. In the case of microalgae as feedstock for biodiesel, this potential is mostly claimed because many strains produce very high yields of biomass and oil and can be cultivated in non-arable land [1,2].

However, the production of algal biomass is currently too expensive and too energy intensive for low commercial-value products such as biodiesel [3]. A recent Farm-level Algae Risk Model (FARM) simulation of the probabilistic cost of microalgae crude oil suggested costs from $\$77 \cdot gal^{-1}$ to $\$ 109 \cdot gal^{-1}$ using current technology, which is still far from a target price of around $\$2 \cdot gal^{-1}$ [4,5].

One of the drawbacks of implementing massive cultivation of microalgae is the unsustainable requirement of fertilizers, especially N. Microalgae have an average composition of $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, with N accounting for 4–8% on a dry biomass basis, making these bioprocesses considerably more N-intensive than traditional agriculture. For example, calculations from different laboratories indicated that for the production of 1 kg of triacylglycerol (the biodiesel's feedstock) from microalgae biomass, 0.30–0.36 kg of N (0.64–0.77 kg urea) would be needed [6,7].

This situation may not only impact negatively on production costs, but it also represents a significant share of the world's energy balance since more than 1% of the energy consumed by humans is devoted to the synthesis of N-fertilizers by the Haber–Bosch process [8].

In many regions of the developing world, such as Sub-Saharan Africa, the situation is worsened by restricted access to fertilizers mostly due to undeveloped industry, dependence on importation and high international prices, and limited means of transportation into rural zones, among other factors [9,10]. Thus, sustainable on-site production of N-fertilizers at low cost will be broadly advantageous and might promote some relief towards food and energy security, especially in the less developed regions of the world.

Several alternatives, such as the use of wastewater [11] and/or N-recycling from biomass [12] have been proposed as partial or complete substitutes for synthetic N-fertilizers for massive cultivation of

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microalgae. While wastewater can be regionally and/or seasonally exploited, in addition to frequent toxicological problems, it is not always coincidentally available together with useful lands [13]. Direct N-recycling after low temperature catalytic gasification of microalgae biomass together with the production of methane rich biogas has been shown [12]. Ammonia capture after gasification of a N_2-fixing cyanobacterium biomass has also been proposed from a life cycle analysis perspective as an alternative to the Haber–Bosch industrial process for the production of N-fertilizer [14,15]. More recently, N-recycling from algal biomass residuals that remain after lipids are extracted and carbohydrates are fermented to ethanol was successfully used as a N-source for culturing *Scenedesmus acutus* after removing some inhibitors form the fermentation spent broth [16].

Similar to agriculture [9,10], there is an increasing interest in taking advantage of biological N_2 fixation (BNF) for microalgae biomass production [17]. BNF is the conversion of N_2 from the air into ammonia and is only carried out by some bacteria and archaea. BNF is catalyzed by O_2 sensitive nitrogenases in a high energy-demanding reaction requiring 8 electrons and at least 16 ATP equivalents to fix 1 N_2. Some cyanobacteria display an elegant array of adaptations to harmonize photosynthetic O_2 evolution and O_2 sensitive N_2-fixation in a process that is entirely powered by light [18].

Although the genetic engineering of N_2-fixation into oleaginous microalgae or hyper accumulation of oil into N_2-fixing cyanobacteria for low N-intensive production of oil has been envisioned [3,17], the complete genetic engineering of either pathway could not be accomplished up-till-now likely due to cellular, genetic and/or biochemical complexities [9,10,17]. In turn, it has been shown that CO_2- and N_2-fixing synthetic consortia comprising a genetically modified N_2-fixing and heterotrophic bacterium, *Azotobacter vinelandii*, and oleaginous microalgae produce some oil while apparently exchanging products of C and N fixed from the air [19–21].

In this study, we provide proof-of-concept for an integrated bioprocess that would produce N-fertilizer on-site by a sustainable process entirely based on BNF. The N-rich cyanobacterial biomass can be efficiently used, without the need of additional nutrients, to produce oil-rich microalgae biomass as a suitable feedstock for biodiesel. On-demand access to high-quality N-fertilizer will relieve the need for N-recycling from microalgae biomass increasing the possibilities of biorefining the biomass for N-rich co-products.

2. Materials and methods

2.1. Algal strains and culture conditions

Nostoc sp. strain M2 was isolated from a freshwater body in southeastern Buenos Aires, Argentina (38°0′0″S 57°33′0″W). The strain was initially enriched in liquid $BG11_0$ medium and then single colonies were isolated from the same medium solidified with 1% agar–agar.

Stock and experimental cultures of *Nostoc* sp. strain M2 were routinely cultivated diazotrophically either in $BG11_0$ medium containing a negligible amount of an additional N-source [22]. *Nostoc* cell-free extracts were prepared by freezing, thawing, centrifuging at 6000 × *g* for 10 min and filtering through 0.22-μm sterile membranes. When indicated, *Nostoc* cell-free extracts were proteolyzed with partially purified pineapple bromelain at a substrate to enzyme ratio of 20:1 at 45 °C for 1 h. Hydrolysis was confirmed by SDS-PAGE [23].

The microalgae used were: *Chlorella sorokiniana* strain RP, *Scenedesmus obliquus* strain C1S, *Ankistrodesmus* sp. strain SP2-15 from our collection of native microalgae [22] and *Chlamydomonas reinhardtii* strain cc124 and *Nannochloropsis oceanica* (kindly provided by Christoph Benning, Michigan State University, U.S). All freshwater species except *C. reinhardtii* were cultivated in $BG11_0$ medium supplemented with 3 mM urea-N or 3 mM *Nostoc* cell-free extracts-N (0.26 $g \cdot l^{-1}$ total protein). Casein was assayed as an N-source at 3 mM protein-N according to an N-to-protein conversion factor of 6.25 [24]. *C. reinhardtii* was cultivated in a TAP medium supplemented with 3 mM NH_4^+ or *Nostoc* sp. cell-free extracts as a sole N-source. All freshwater strains were routinely maintained at 28 ± 1 °C and illuminated with constant white light at 50 μmol photons $m^{-2} \cdot s^{-1}$. The marine strain *N. oceanica* was cultivated in F/2 medium using 3 mM KNO_3 or with *Nostoc* sp. cell-free extracts as a sole N-source at 20 °C under white light at 35 μmol photons $m^{-2} \cdot s^{-1}$ and a photoperiod of 14 h light/10 h darkness.

Four different culture settings were used in this work: i) static cultures were carried out in a 25 ml medium inside 100-ml Erlenmeyer flasks shaken manually by 5–6 strokes twice a day; ii) air-bubbled cultures were run in 500-ml bottles containing a 200-ml medium sparged with 0.22-μm-filtered air from the bottom at 0.3–0.5 $l \cdot min^{-1}$; iii) 5-l air-lift photobioreactors (PBRs) containing 4.5 l of medium sparged with filter-sterilized air from the center of the riser tube at 6 $l \cdot min^{-1}$ (up flow circulation) and pure CO_2 from the bottom of the down flow circulation at 0.2 $l \cdot min^{-1}$, and illuminated with constant white light at 200 μmol photons $m^{-2} \cdot s^{-1}$; and iv) environmental photobioreactors (ePBRs), that are last generation laboratory-scale systems that provide a growth environment designed to replicate environmental conditions in algal production ponds and natural systems that are sufficiently representative of production conditions [25]. The ePBR conditions used in this study simulated the average spring weather conditions in November in Mar del Plata city (38°0′0″S 57°33′0″W) and were: light intensity at modeled noon of 1500 μmol photons $m^{-2} \cdot s^{-1}$ for a 14 h sinusoidal light cycle. Day^{-1} and maximum and minimum temperatures of 26 °C and 16 °C, respectively, also in a sinusoidal cycling mode, and bubbling with filtered air at 0.25 $l \cdot min^{-1}$.

2.2. Analytical methods

Molecular taxonomy determination of *Nostoc* sp. M2 was carried out as previously described [26,27]. Briefly, the 16S rDNA region was PCR amplified from genomic DNA by using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACG GYTACCTTGTMTACGACTT-3′) and sequenced by Macrogen Inc., Seoul, South Korea. The closest relatives were determined by using the Basic Local Alignment Searching Tool and multiple sequence analysis with selected sequences.

For growth curve analysis, cell number was determined under a Neubauer chamber or estimated by culture OD_{660}. Data were plotted using the GraphPad PRISM software (Intuitive Software for Science, San Diego, CA) and doubling times were obtained by fitting the experimental data to theoretical curves of exponential growth with $R^2 \geq 0.97$. Microalgae biomass dry weight was determined by centrifugation of the samples at 10,000 × *g* for 10 min before drying out the pellets in an oven at 70 °C until constant weight. For biomass total protein determination, samples were prepared by boiling resuspended cells at 100 °C for 10 min in the presence of 1 N NaOH. Sample aliquots were subjected to protein determination by the Lowry's method [28] using NaOH-treated bovine serum albumin as standard. Biomass total carbohydrates were determined from resuspended cells mixed with the Antrona's reagents [29] and compared with a standard curve prepared with fructose. Proteolytic activity was determined using azocasein as an artificial substrate [30] after concentrating *Nostoc* sp. cell-free extracts ten-fold in a lyophilizer.

Lipid extractions, gravimetric determinations, Nile Red staining, fatty acid methylation and gas chromatography analyses were performed essentially as described previously [22].

For total N or P determinations, samples were digested in H_2SO_4 or a mixture of HNO_3 and $HClO_4$, respectively, and elements were determined in a commercial facility (Fertilab — Laboratorio de Suelos S.A. Mar del Plata, Argentina).

LR-microcystin determination was performed according to the manufacturer's recommendation [Zeu-Immunotec, S.L. (Zaragoza, Spain)].

2.3. Protein purification

2.3.1. Partial purification of bromelain

Cubes of about 1 cm of a fresh pineapple were blended in 50 mM Tris/HCl, pH 7.5 buffer, filtered and clarified by centrifugation at 27,000 ×*g* for 30 min. Bromelain preparations consisted in proteins that precipitated between 30% and 70% ethanol at 4 °C and were then solubilized in 20 mM $NaPO_4$, pH 7.5 buffer containing 2.5 mM L-cysteine and 20% glycerol and stored at −20 °C [31].

2.3.2. Phychoerythrin purification

See Supplementary methods.

3. Results and discussion

3.1. Isolation and properties of Nostoc sp. strain M2

Aimed at isolating suitable microorganism for on-site production of N-fertilizers, we run a screening for native diazotrophic cyanobacteria from the surroundings of Mar del Plata, Buenos Aires, Argentina (38°0′0″S 57°33′0″W) during 2009. The isolate that grew faster was morphologically classified as belonging to the cyanobacterial order Nostocales, presumably to the genus *Nostoc* and was given the strain name M2. Sequence analysis of its 16S RNA (GenBank accession # KM272975) confirmed this taxonomic determination (Supplementary Fig. S1).

Nostoc sp. strain M2 presented a doubling time of its dry biomass of 1.9 ± 0.7 days ($\mu = 0.36 \cdot day^{-1}$) in $BG11_0$ medium (growing diazotrophically) with a final biomass yield of up to 2 g dry biomass$\cdot l^{-1}$ in 10–12 days. Similar yields (1.8 g dry biomass$\cdot l^{-1}$ in 15–20 days) were obtained in 3-l air-bubbled outdoor cultures in summer.

The biomass macromolecular composition of *Nostoc* sp. strain M2 was 46% protein, 35% carbohydrates and 10% lipids. Calculations using total N-to-protein conversion factors for cyanobacterial biomass [24], indicate that *Nostoc* sp. strain M2 would fix and assimilate within its biomass about 20 mg of $N_2 \cdot l^{-1} \cdot d^{-1}$ under laboratory controlled conditions or 13 mg$\cdot l^{-1} \cdot d^{-1}$ outdoors.

Interestingly, *Nostoc* sp. strain M2 produces trichomes that remain attached side-by-side to form macroscopic film-like aggregates that can be collected with a strainer and air-dried. This is certainly a very interesting characteristic of *Nostoc* sp. M2 biomass, since harvesting cyanobacteria or microalgae from diluted cultures is one of the main constraints for profitable production of biomass from these microorganisms mostly due to the small size of single cells [32].

Upon rehydration of the air-dried biomass, cells lyse and release part of their content. However, freezing and thawing *Nostoc* biomass resulted in a more efficient release of its cellular content that, in the case of total protein, was up to 90% (10% of the total protein remained in the cell paste). We took advantage of this property to prepare *Nostoc* sp. strain M2 cell-free extracts as N-biofertilizer. A typical preparation contained 0.9 g$\cdot l^{-1}$ N; 0.1 g$\cdot l^{-1}$ P; 5 g$\cdot l^{-1}$ protein; and 2.5 g$\cdot l^{-1}$ soluble carbohydrates.

Nostoc sp. strain M2 protein extracts are extremely rich in phycobiliproteins, especially phycoerythrin, that is a prime example of a high value commodity. Thus we show simple protocols for production of *Nostoc* phycoerythrin at different grades of purity that can contribute towards profitability of multispecies biorefineries (see Supplementary material, Figs. 2S and 3S).

3.2. Nostoc sp. strain M2 biomass used as a sole N-source for microalgae growth

As a first approach to evaluate *Nostoc* sp. strain M2 extracts as N-fertilizer for oleaginous microalgae, we analyzed the ability of *C. sorokiniana* strain RP to use *Nostoc* sp. strain M2 proteins hydrolyzed by bromelain treatment. Fig. 1A shows that *C. sorokiniana* used this N-source efficiently, with doubling times of 12.6 ± 4.3 h or 9.2 ± 0.6 h, for this N-source or urea, respectively. As controls we show that *C. sorokiniana* used a casein hydrolysate with a doubling time of 12.1 ± 0.8 h (Fig. 1A) but did not use non-hydrolyzed casein (Supplementary Fig. S4A).

Surprisingly, *C. sorokiniana* could grow at the expense of *Nostoc* sp. strain M2 cell-free extracts that had not been hydrolyzed with bromelain at the same rate as with proteolyzed preparations (Fig. 1B). *Nostoc* cell-free extracts presented proteolytic activity that would account for the release of short peptides supporting growth of the microalga (Supplementary Fig. S4B). Proteolysis was mostly due to *Nostoc* sp. strain M2 proteases (in contrast to possibly contaminating bacteria), since 0.22-μm-filtered cell-free extracts were still a good N-source for *C. sorokiniana* even in the presence of ampicillin to prevent further growth of bacteria (Supplementary Fig. S4A).

The use of *Nostoc* cell-free extracts as N-fertilizer for microalgae growth was tested in other distantly related strains such as *S. obliquus*, *C. reinhardtii*, *Ankistrodesmus* sp., and *N. oceanica*, which were able to use it as efficiently as *C. sorokiniana* (Fig. 2). In the case of *Ankistrodesmus* sp., *Nostoc* N-fertilizer clearly outperformed urea (Fig. 2C) or NO_3^- (not shown) as alternative N-sources.

Nostoc cell-free extracts, in addition to the N-source, appear to provide C-containing organic substrates to promote microalgal mixotrophic growth [33], and possibly other growth stimulating substances for some microalgae with special nutritional requirements [34]. Conversely to its closest phylogenetic relative, the microcystin-producing strain *Nostoc* sp. CENA88 (Supplementary Fig. S1), no evidence was found for the production of this cyanotoxin by *Nostoc* sp. strain M2 (Supplementary Tabl2 S1 and Supplementary Fig. S5). This was not surprising since microcystin production appears to be polyphyletic in cyanobacteria, since producing and non-producing strains normally cluster together in rRNA phylogenies [27].

Interestingly, *Nostoc* cell-free extracts seemed to provide all nutrients required for the production of *C. sorokiniana* biomass, since extracts prepared from extensively washed *Nostoc* cells supported growth of the microalga when diluted in deionized water without the supplementation of any additional nutrient (Fig. 1B). The final yield of microalgae cultures was proportional to the amount of *Nostoc* extract used as fertilizer, but growth showed identical initial slopes suggesting that, under these conditions, the release of bioavailable nutrients did not limit microalgae growth (Supplementary Fig. S6).

The depletion of *Nostoc* protein from the medium equaled the increase in *C. sorokiniana* proteins in six days of culture (Fig. 3), indicating that assimilation of N from *Nostoc* cell-free extracts into *C. sorokiniana* biomass occurred at an efficiency close to 100%.The overall incorporation of total N and total P from *Nostoc* cell-free extracts into *Chlorella* biomass in seven days was 80% and 84%, respectively (Table 1).

Thus, in addition to an efficient recycling of N from cyanobacteria into microalgae biomass, recycling up to 84% P is of prime interest especially considering that, contrary to N-fertilizer that can be produced by synthesis or biologically from atmospheric N_2, P-fertilizer is produced through mining from non-renewable resources that are expected to be depleted within 50–100 years [13].

During the course of the preparation of this manuscript an alternative approach for N-recycling has been shown. Gu et al.[16] applied quite successfully a biorefinery approach in which they pretreated *S. acutus* strain SCE 0401 biomass with 2% (w/w) H_2SO_4 at 150 °C to lyse cells and then separated lipids from the slurry and used the saccharified liquid as a source of sugars for the production of bioethanol by fermentation with yeasts. They further show that the fermentation spent medium substituted for N-fertilizer during culturing of *S. acutus* strain SCE 0401 providing proof-of-principle of a biorefinery producing oil and ethanol as the main outputs while minimizing the input of N-fertilizer by N-recycling in an internal loop. According to the authors this property might apparently be quite specific of *S. acutus* strain SCE 0401, but could potentially be transferred to other promising strains [16].

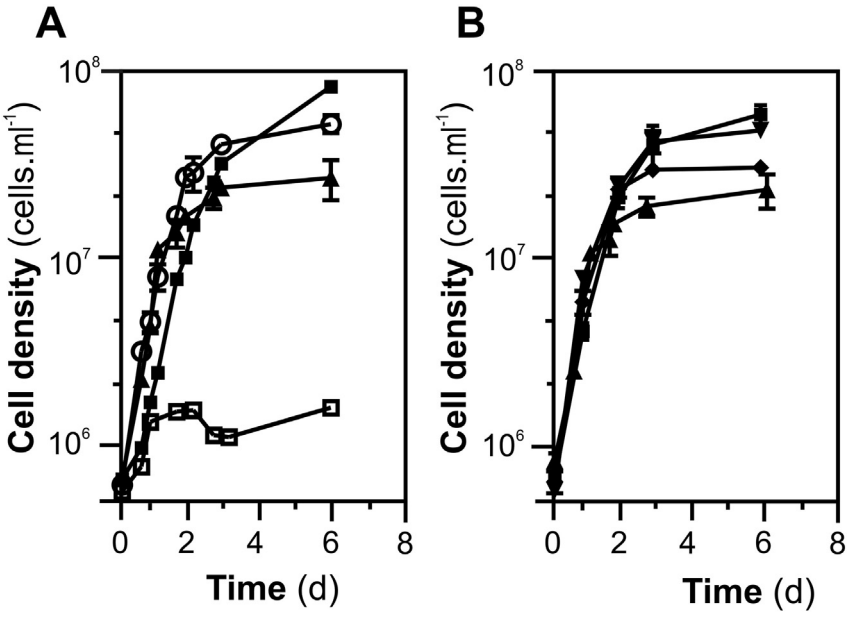

Fig. 1. Growth curves of *C. sorokiniana* using *Nostoc* extracts as a N-sources. (A) (□) $BG11_0$ medium (only traces of bioavailable N for microalgae); (■) $BG11_0$ supplemented with 3 mM urea-N; (○) $BG11_0$ supplemented with 3 mM protein-N from casein peptone; (▲) $BG11_0$ supplemented with 3 mM protein-N from proteolyzed (treated with bromelain) *Nostoc* sp. strain M2 cell-free extracts. (B) Effect of proteolysis of *Nostoc* sp. strain M2 cell-free extracts on growth of *C. sorokiniana*. (■) $BG11_0$ supplemented with 3 mM urea-N; (▲) $BG11_0$ supplemented with 3 mM protein-N from proteolyzed (treated with bromelain) *Nostoc* sp. strain M2 cell-free extracts; (▼) $BG11_0$ supplemented with 3 mM protein-N from non-treated *Nostoc* sp. strain M2 cell-free extracts; (♦) 3 mM protein-N from non-treated *Nostoc* sp. strain M2 cell-free extracts in deionized water. Cultures were conducted in 500-ml bottles bubbled with filtered air. The data represent the mean and standard deviation of two independent experiments.

The alternative approach we propose in this work differentiates in at least four key aspects: 1) it uses BNF as a constant input of N, thus it has a great potential to extend biorefineries to further co-produce protein as an additional output for feed, food and other applications; 2) no pretreatment and chemicals are needed since algae readily take *Nostoc* extract, and even as a complete source of nutrients; 3) it is not exclusive of a single microalgae strain, since at least five out of five biotechnologically relevant microalgae grew at the expense of *Nostoc* extracts; and 4) a multispecies biorefinery might increase the possibilities for production of additional commodities, as shown here for cyanobacterial pigments.

3.3. Microalgae oil production at the expense of Nostoc sp. strain M2 fertilizer

C. sorokiniana cell cultures accumulated neutral lipids when subjected to N deprivation, as expected [22] (Fig. 4A and B). The overall level of lipid accumulation in the culture was offset by the extremely low biomass production [22]. Conversely, supplementation of a growth medium with either 3.0 mM urea-N (to $BG11_0$ medium) or 2.2 mM protein-N from *Nostoc* cell-free extracts (as a complete source of nutrients) increased overall lipid accumulation by 10- to 15-fold, respectively, although with a delay of 3 to 4 days. Neutral lipid accumulation was faster in cultures at the expense of *Nostoc* cell-free extracts, with the maximum difference at the 5th day of culture. At this time, solvent extraction and gravimetric determination indicated total lipid content of 32.3 ± 1.0% (w/w) on a dry weight basis for *C. sorokiniana* cells cultivated in *Nostoc* extracts as a complete source of nutrients. Afterwards, cells continued accumulating lipids, although at a slightly slower rate, up to 33.4 ± 0.7% (w/w) on the 8th day of culture. On the other hand, cells from cultures supplemented with urea accumulated total lipids up to 12.1 ± 1.1% (w/w). Interestingly, *Nostoc* cell-free extracts triggered a higher level of accumulation of neutral lipids than the complete

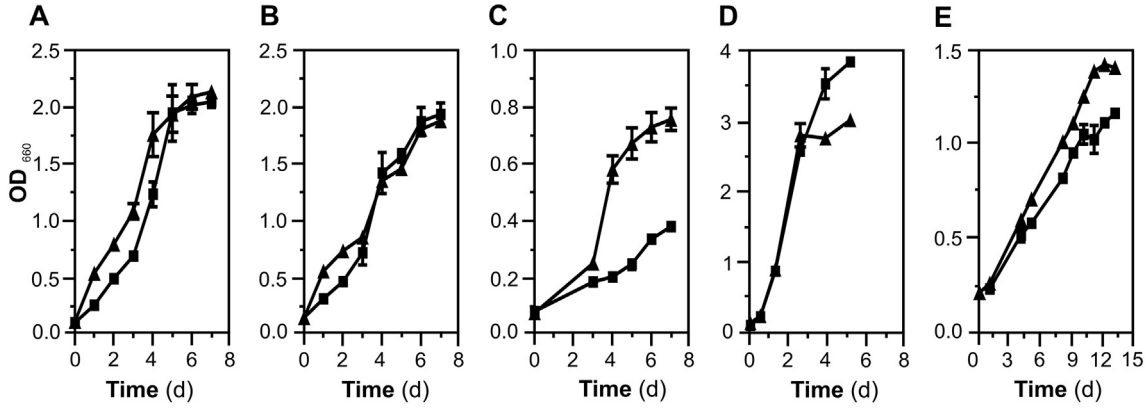

Fig. 2. Growth curves of different microalgae using *Nostoc* sp. strain M2 cell-free extracts as sole N-source. (A) *C. sorokiniana*; (B) *S. obliquus*; (C) *Ankistrodesmus* sp.; (D) *C. reinhardtii*; (E) *N. oceanica*. (■) $BG11_0$ supplemented with 3 mM urea-N; (▲) $BG11_0$ supplemented with 3 mM protein-N from non-preproteolyzed *Nostoc* sp. strain M2 cell-free extracts. Cultures were conducted in static flasks shaken manually twice a day (see Materials and methods section). The data represent the mean and standard deviation of two independent experiments.

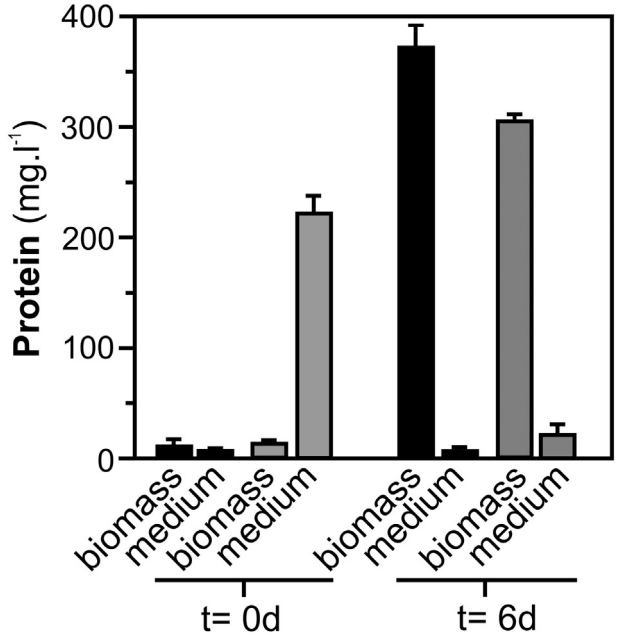

Fig. 3. Partitioning of total protein in cultures of *C. sorokiniana* at the expense of *Nostoc* cell-free extracts. Total protein levels were determined both in culture media and in *C. sorokiniana* biomass at times 0 and 6 days of culture. Culture medium was either $BG11_0$ medium supplemented with 3 mM urea-N (black boxes) or 3 mM protein-N from non-preproteolyzed *Nostoc* sp. strain M2 cell-free extracts diluted in deionized water (gray boxes). Note: while at 0 day biomass refers to proteins present in the *C. sorokiniana* inoculum, at 6 days it refers to proteins in the *C. sorokiniana* biomass accumulated in the culture. The data represent the mean and standard deviation of two independent experiments.

withdraw of the N-source, suggesting that other components in the *Nostoc* cell-free extracts contribute to enhance the accumulation of lipids in addition to N-deficiency.

The potential maximum production of microalgae oil was also tested in scaled-up microalgal cultures run in 5-l air-lift photobioreactors (PBRs). *Nostoc* cell-free extracts (3 mM total N) was used as complete source of nutrients, and conditions for CO_2 supplementation, and constant light and temperature were optimized (see Materials and methods section). The lipid productivity at the 5th day of culture was 89 $mg \cdot l^{-1} \cdot d^{-1}$ at a lipid content of 37% (w/w). At the 8th day biomass turned 42% lipids (w/w), although productivity tended to decrease in the absence of microalgae growth. This productivity approached the 100 $mg \cdot l^{-1} \cdot d^{-1}$ threshold for very high lipid productivity according to an extensive literature survey [35].

The fatty acid composition of oil extracted from *C. sorokiniana* cells cultivated for eight days in *Nostoc* extracts as a complete source of nutrients was more similar to that of cells resuspended in $BG11_0$ medium (traces of a useful N-source) than cells using urea as the N-source (Fig. 4C). Enrichment in oleic acid (18:1), as observed in microalgae depleted for N or cultivated in *Nostoc* extracts, is characteristic of the accumulation of triacylglycerols [22]. Thus, fertilization of microalgal cultures with *Nostoc* extracts increases process yield without changing the fatty acid composition of the microalgal oil that might have implications in the resulting biodiesel such as oxidative stability, and low temperature fluidity[22].

Table 1
Assimilation of N and P from *Nostoc* cell-free extract into *C. sorokiniana* biomass.

	N ($mg \cdot l^{-1}$)	P ($mg \cdot l^{-1}$)
$BG\text{-}11_0$ medium[a]	0.15	4.50
$BG\text{-}11_0$ + *Nostoc* extract[a]	32.07	9.31
C. sorokiniana biomass[b]	24.07 ± 1.56	7.36 ± 0.46
Spent medium[b]	7.58 ± 1.57	ND[c]

[a] Single determinations.
[b] Data represent the mean and standard deviation of two independent culture runs at the 7th day of culture.
[c] Not detected.

3.4. Potential performance of outdoor *C. sorokiniana* cultures growing at the expense of *Nostoc* extracts

ePBR simulations were run to estimate the potential outdoors performance of *C. sorokiniana* cultivation in 20-cm-deep raceway ponds at the expense of *Nostoc* extracts as a sole source of nutrients (no CO_2 supplementation). Fig. 5 shows a representative run simulating average spring weather conditions in Mar del Plata city (38°0′0″S 57°33′0″W). Under these conditions, *Chlorella* cells exhausted the supplied *Nostoc* protein in 2 days. In 6 days, the protein recovery in *C. sorokiniana* biomass equaled that of the initial amount of protein supplied in the culture medium. Following a slight decrease in the protein content of their cells, the microalga started to accumulate lipids, which by the 9th day, became 33% of the dry weight for a final biomass yield of 0.6 $g \cdot l^{-1}$ (13.3 $g \cdot m^{-2} \cdot day^{-1}$) and an oil productivity of 20 $mg \cdot l^{-1} \cdot d^{-1}$. Outdoor productivity was one fourth of its apparent maximum potential, as estimated by culturing in air-lift PBRs (see above), and it was probably due to slower growth.

Nevertheless, assuming a biomass average year-round productivity of 75% of the spring estimation, as has been observed for year-round outdoor cultures of *Nannochloropsis oculata* in this city [36], 13,216-l microalgal oil $\cdot ha^{-1} \cdot year^{-1}$ could be obtained by this means.

The surface required to produce *Nostoc* biomass for coupled on-site production of N-fertilizer and oil production by oleaginous microalgae would reduce oil yield on an aerial basis according to the culturing system for the bacterium. In this study we could not make ePBR estimations of the potential productivity of the cyanobacterium in raceway ponds, because the long filaments have a tendency to stick around the sensors of the system. Nevertheless, assuming a productivity in raceway ponds as that observed in previous work for a related cyanobacterium (*Anabaena* sp. ATCC 33047) of 56.6 t dry biomass $\cdot ha^{-1} \cdot year^{-1}$[14], about 33% of the land area should be devoted for on-site production of the N-fertilizer. This calculation is based on the observed efficiency of conversion of *Nostoc* to *C. sorokiniana* biomass and the biochemical composition of each microorganism. This would reduce oil productivity of the coupled system to 9937-l microalgal oil $\cdot ha^{-1} \cdot year^{-1}$. Alternatively, in case that the *Nostoc* biomass productivity we observed for outdoor culturing in 3-l cylinders could be achieved in heavy-gauge polyethylene tubing enclosed in cylindrical cages of about 30 cm of diameter and 200 cm height, or any other system similar to those commonly used in aquaculture [37], less than 5% of the land area would be enough for on-site production of the N-fertilizer for the microalgae for an oil productivity of 12,770-l microalgal oil $\cdot ha^{-1} \cdot year^{-1}$.

This estimated yield of 10.000–13.000-l microalgae oil $\cdot ha^{-1} \cdot year^{-1}$ is about 20-fold higher than that reported for soybean as the prevailing feedstocks for biodiesel and remains within the range for microalgae oil production of operating raceway ponds at the expense of synthetic N-fertilizer and CO_2 supplementation [3,35].

In addition to oil, this process would roughly produce from 2 kg of *Nostoc* biomass about 1 kg of *Nostoc* residues (mostly carbohydrates) and 1.0 kg of carbohydrates and 1 kg protein from *C. sorokiniana* biomass (the estimation is based on cyanobacterium to microalga biomass conversions and biomass macromolecular composition). This represents a very high amount of photosynthetic biomass for claiming carbon credits [38] and an opportunity for further developing a biorefinery for the co-production of N-rich food and feed, among other alternatives, according to available technology and markets [39,40] that would largely increase the profitability of the process. Access to a fully renewable source of N would significantly increase the sustainability of the proposal while diminishing its environmental impact.

A recent life cycle analysis suggested that gasification of *Anabaena* sp. ATCC 33047 biomass (a strain related to *Nostoc* sp. M2), stripping

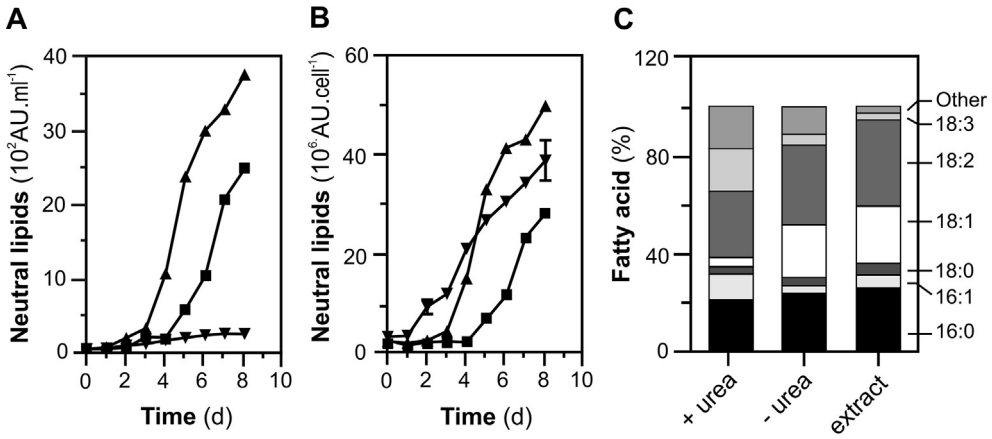

Fig. 4. Accumulation of neutral lipids in *C. sorokiniana* cultures growing with the following N-sources: (▼) $BG11_0$ medium; (■) $BG11_0$ supplemented with 3 mM urea-N; and (▲) 3 mM protein-N from non-preproteolyzed *Nostoc* sp. strain M2 cell-free extracts. (A) volumetric accumulation of neutral lipids analyzed by Nile Red fluorescence; (B) accumulation of neutral lipids on a cellular basis; (C) fatty acid profiles of oil extracted from *C. sorokiniana* cells cultured in $BG11_0$ supplemented with 3 mM urea-N; $BG11_0$; or 3 mM protein-N *Nostoc* extracts, as indicated, at the 8th day of culture. Cultures were conducted in 500-ml bottles bubbled with filtered air. The data represent the mean and standard deviation of two independent experiments. In (C) standard deviation was ≤5%.

and converting the ammonia from the biogas residue to ammonium sulfate, drying the ammonium sulfate solution to ammonium sulfate crystals, transporting the ammonium sulfate and converting it to liquid ammonia and concentrated sulfuric acid, would save about $1 \cdot 10^5$ MJ of non-renewable energy and 3100 kg CO_2 equivalent of global warming potential per 1 t of liquid ammonia, as compared with the Haber–Bosch process to obtain the same amount of N-fertilizer [14].

The alternative proposed herein for on-site production of N-fertilizer for microalgae culture is somehow more straightforward thanks to the special properties of *Nostoc* sp. M2 biomass for releasing copious amounts of protein that autoproteolyze into shorter peptides that can be readily taken by a variety of biotechnologically relevant microalgae. Future work is needed to confirm if this technology is at least as benign to the environment as the gasification and capture of ammonium sulfate crystals.

4. Conclusion

This work provides proof-of-principle for on-site production of biologically reactive N as a substitute for synthetic N-fertilizer used in microalgae culture. We show the simultaneous production of oil as fuel feedstock and protein-rich biomass with potential uses as food, feed and fine chemicals in a system driven by light energy and CO_2 and N_2 from the air.

Thus, the strategy proposed in this work would stand as an alternative to the environmentally disputed industrial process for synthetic N-fertilizer worldwide towards a sustainable green economy. It is expected that advancement in biorefineries, especially those that maximize sustainability by exploiting freely-available renewable resources (for example atmospheric N_2 and CO_2, as shown in this work) will gradually increase interest worldwide.

On the other hand, although there is an incentive to produce alternative crops for biofuels and other purposes in non-developed regions of the world, the task is hard to accomplish in part because agrochemical production, purchase and/or transportation are mostly prohibitively expensive for small farmers from these countries [38,39]. It is possible that in some of these regions where weather, land and labor availability are favorable, multipurpose biorefineries based on simplified technology and freely-available renewable resources, might contribute to produce an economic and social impact on small farm holders towards fulfilling basic needs for food and energy and as one of the starting points to create value by producing high value commodities for foreign markets.

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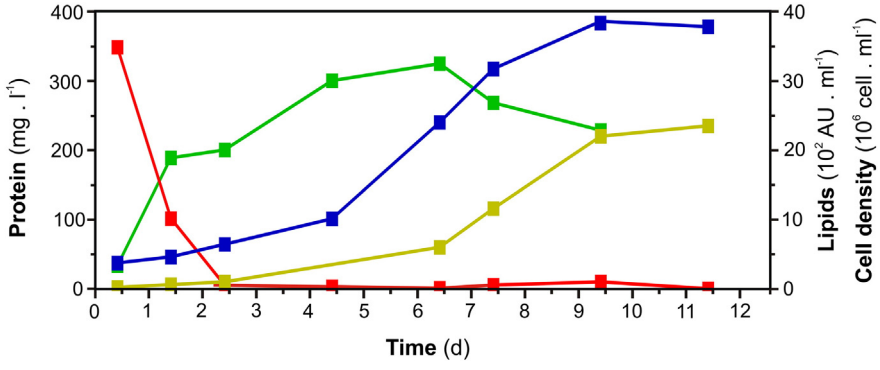

Fig. 5. Environmental photobioreactor simulation of *C. sorokiniana* outdoors oil productivity at the expense of *Nostoc* extracts as a sole source of nutrients. The ePBR conditions used in this study simulated the average spring weather conditions in November in Mar. del Plata city. (Red) Protein in the medium/spent medium; (green) protein in *C. sorokiniana* biomass; (blue) *C. sorokiniana* cells; and (yellow) neutral lipids (Nile Red-reactive in fluorescence arbitrary units). As a reference, the light period was shaded in the background of the plot and the temperature cycling was indicated by the dash trace. Amplitudes were indicated in Materials and methods section. The plot shows a representative run.

career researcher at the CONICET, Argentina. This work was supported by Grants from the Agencia Nacional de Promoción Científica y Tecnológica, PICT-2589; and CONICETPIP-1032 to L. C.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2015.08.017.

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