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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)].