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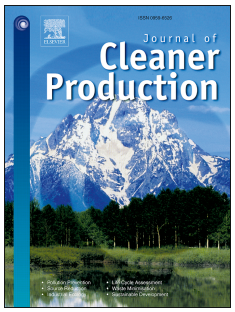

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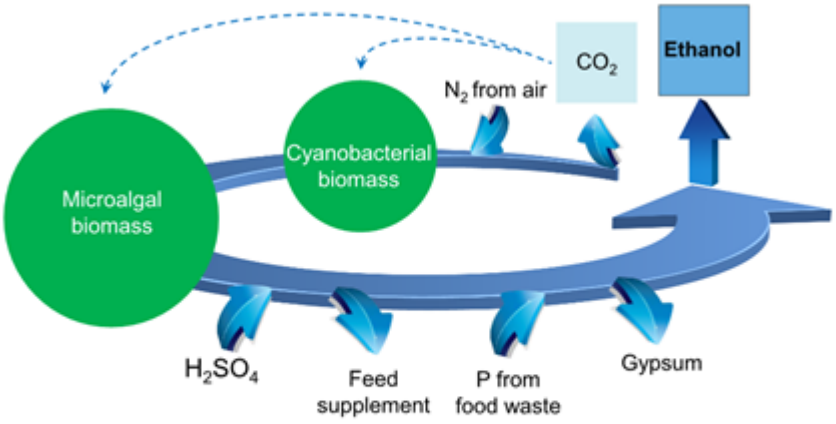

Production of feed and ethanol at the expense of N_2 from the air and P recovered from food waste by recycling H_2SO_4 used for biomass saccharification.

A semi-closed loop microalgal biomass production-platform for ethanol from renewable sources of nitrogen and phosphorous

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Abstract

Production of microalgal biomass for feed and fuels demands unsustainable large amounts of fertilizers. The most broadly considered alternative sources of nutrients/fertilizer for microalgae are wastewater and internal recycling in closed-loop production platforms. However, these strategies largely disable co-production of feed and fuel in biomass biorefineries for an increased economic and environmental feasibility.

In this study, we aimed at providing proof-of-concept for a semi-closed loop microalgal production-platform and biomass biorefinery for ethanol and feed from renewable resources of N and P. Atmospheric N_2 was assimilated into a N_2-fixing cyanobacterial biomass, which sustained growth of a microalga that accumulated high levels of carbohydrates (up to 60% (w/w)) as a sole source of fertilizer. The microalgal biomass was efficiently saccharified with H_2SO_4, which was recycled to release soluble PO_4^{3-} from bone meal as a renewable source of P. Fermenting these P-enriched preparations with yeasts quantitatively produced ethanol at theoretical yields, a concentration of up to 50 g ethanol . L^{-1} and a yield of 0.25 g ethanol . g biomass^{-1}. Calculations suggested a potential yield from 7,600 to 10,800 L ethanol . ha^{-1} . year^{-1}, under Buenos Aires environmental conditions, which would be higher than that currently obtained from maize feedstocks. The residual fermentation vinasse, supplemented with P and containing other downstream-process reagents, was recycled as a sole source of macronutrients for the cultivation of the N_2-fixing cyanobacterium to close the production cycle. Water recycling and co-production of residual biomass enriched in fat and protein as potential feed are also shown. This semi-closed loop biomass production-platform reconciles the concepts of microalgal biomass biorefineries for the co-production of feedstocks for biofuels and feed and nutrients recycling in closed-loop systems that largely minimizes production of waste.

Keywords: ethanol, feed, biological N_2-fixation, bone meal, sulfuric acid

1. Introduction

Global development has posed a growing dependence on both fossil fuels (for energy and materials) and industrialized agriculture (for food, feed and feedstocks for biofuels for renewable energy). This results in a serious challenge to the quality of the environment and the sustainability of the current production systems (Börjesson and Tufvesson, 2011). The most affected parameters are: *i*) the rate of biodiversity loss; *ii*) climate change, and *iii*) anthropogenic interference with the N cycle, mostly by production and use of synthetic N fertilizers in agriculture (Rockström et al., 2009). Demand and price of N and P fertilizers are increasing steadily up to an estimate of 120 Mt of elemental N and 47 Mt of P_2O_5 in 2018 (Heuer et al., 2017). While P fertilizers are produced from rocks or sediments, whose reserves are unevenly distributed and highly susceptible to depletion (Simons et al., 2013), N fertilizer is mostly obtained by the industrial Haber–Bosch process from atmospheric N_2 at the expense of large amounts of fossil fuel (Sutton et al., 2011). Whereas in some regions of the world the availability of fertilizers limits crops yields, an incorrect dose or timing of application results in up to 70% of the fertilizer lost in the environment in other regions. This not only represents an unnecessary waste of energy and non-renewable resources, but also produces a number of adverse conditions on climate change (Shcherbak et al., 2014), eutrophication (Lewis et al., 2011) and public health (Liu et al., 2013).

In present times, the most common biofuel is first generation bioethanol, which is produced from agricultural feedstocks such as corn or sugarcane in the US or Brazil, respectively. Despite the

great benefits associated with partial replacement of some fossil fuels, the fact that present and future global food security is still not fully warranted poses a serious concern on the use of these feedstocks for bioenergy purposes (Gray et al., 2006). A second generation of bioethanol from plant lignocellulosic feedstocks has been more recently envisioned. Compared with the previous generation, the second generation offers clear advantages, such as broad availability and low cost of the feedstock, and non-competition with food production. However, they face severe disadvantages due to the composition and structure of the lignocellulosic biomass, which requires quite intensive mechanical and physicochemical pretreatments, and due to expensive saccharifying enzymes for its conversion into ethanol (Kumar et al., 2016). Regardless of the nature of the feedstock, ethanol production from biomass generates large volumes of waste, called vinasse. The amount of vinasse generated after fermentation and distillation of ethanol can be up to 20-fold the production of ethanol. Safe disposal and recycling of vinasse for fertirrigation appears to be the best alternative, among others (Moran-Salazar et al., 2016). Sugarcane vinasse can satisfy the requirements of P and other minerals for most crops (Moran-Salazar et al., 2016). However, it is mostly N-deficient, and thus it tends to promote the accumulation of minerals in the soil up to levels that may become detrimental to the environment (Rodrigues Reis and Hu, 2017). Low pH, electric conductivity, and some chemical elements present in vinasse may also contribute, over long periods of time, to adverse effects on agricultural soils, rivers, lakes and biota (Christofoletti et al., 2013).

The motivation of the present research was to advance in the design of a microalgae-based alternative biomass production-platform for the generation of bioethanol and feed. This new approach takes advantage of inexpensive and renewable sources of N and P fertilizers, together with extensive recycling of vinasse and reagents used for biomass downstream processes.

Aquatic microalgae and cyanobacteria are increasingly considered a promising alternative to conventional crops as feedstocks for food and feed, biofuels, and other higher-value products (Yong et al., 2016). This is mainly because of a much higher photosynthetic productivity (a conservative potential of about 50-fold), a more favorable biochemical composition and structural properties than biomass of terrestrial crops as a feedstock for bioethanol, and independence of arable land for cultivation (Brennan and Owende, 2010). Despite their predominant aquatic lifestyle, microalgae have a more favorable water footprint than terrestrial crops as a comparable feedstock for biofuels (Rulli et al., 2016). Culturing in closed systems (e.g. photobioreactors), or partially closed systems (e.g. open ponds) (Brennan and Owende, 2010), microalgae cultivation allows a higher control of fertilizers and wastewater discharges into the environment, among other operational parameters.

According to a general formula of $C_{106}H_{181}O_{45}N_{16}P$ for microalgal biomass composition, nutrients are to be supplied at appropriate rates to attain maximum productivity, particularly CO_2, N and P. It has been calculated that the production of 1 L biodiesel from microalgal biomass requires 0.23 - 1.55 kg N and 29 - 145 g of P, depending of the cultivation conditions. The production of microalgal oil-based fuels for about 25% of the target established by the United States for 2022, would require 41–56% and 32–49% of the world N and P fertilizer surplus (Canter et al., 2015). Thus, massive cultivation of microalgae would result in a more intensive use of fertilizers than traditional agriculture, which represents a potential threat to food security due to competition for supplies (instead of land) (Rösch et al., 2012). This demand for nutrients/fertilizer can be expected to severely limit the extent to which the production of biofuels from microalgae can be sustainably expanded (Canter et al., 2015).

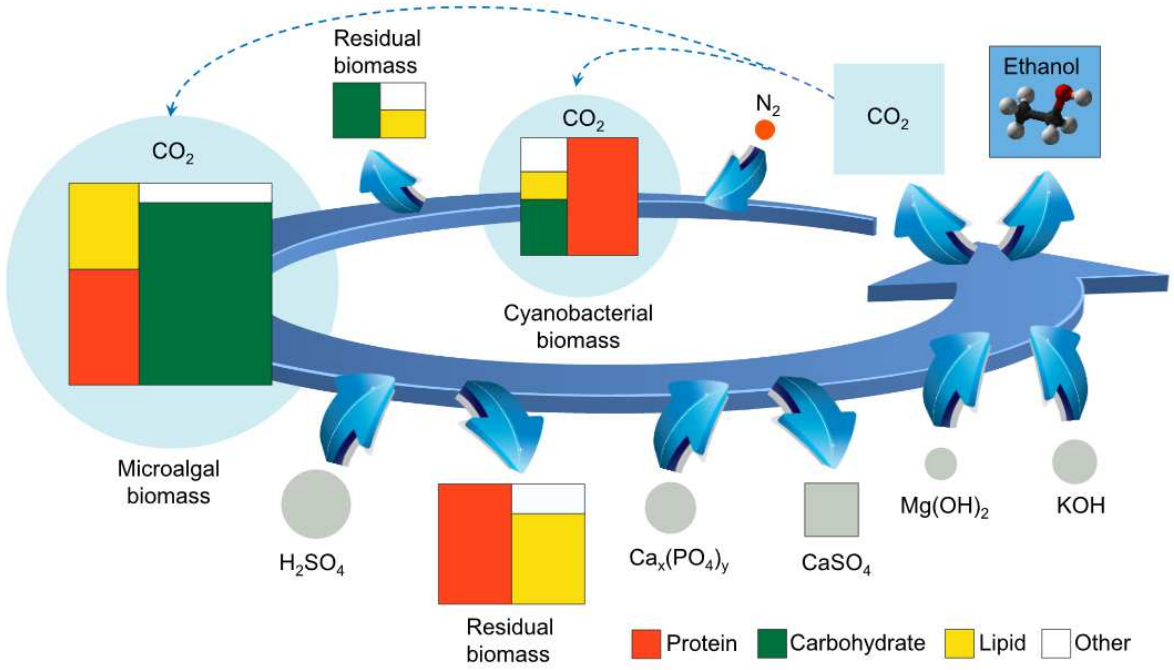
The most broadly considered alternative sources of nutrients/fertilizer for microalgae are wastewater and internal recycling in closed-loop production platforms (Canter et al., 2015). Wastewater composition is frequently variable, and nutrients are not always bioavailable.

Wastewater sometimes can exert toxic effects on microalgal propagation and/or its resulting biomass, preventing other uses of the biomass as a fertilizer, and especially as feed/food (Markou et al., 2014). During the last years much attention was devoted to the possibility of recycling N, P, and other nutrients from oil-extracted biomass. The main investigated methods for nutrient recycling include anaerobic digestion (Zhu et al., 2016), catalytic hydrothermal gasification and hydrothermal liquefaction (Barbera et al., 2018). Most of these methods ensure efficient recycling of nutrients, which largely reduces fertilizer inputs for microalgal biomass production (Canter et al., 2015).

There is currently a generalized agreement that fuel-only pathways from microalgal biomass would be unviable from both an economic and an environmental standpoint (Zhu, 2015). The co-production of higher-value commodities from microalgal biomass in biorefinery facilities must be envisioned to ameliorate these drawbacks (Laurens et al., 2017). A recent study concluded that due to the general good properties of microalgal proteins for food/feed, its production alongside biofuels can increase the utilization of resources, lower the environmental impact, and thus pave the route to commercialization of commodities form microalgal biomass (Walsh et al., 2016).

In this study, we aimed at reconciling the concepts of microalgal biomass biorefineries for the co-production of feedstocks for biofuels and feed, and nutrients recycling in closed-loop microalgal biomass production platforms. We present a conceptual design (Fig. 1) and proof-of-concept for a semi-closed loop microalgal biomass production platform that is sustained by constant inputs of N and P from low-cost and renewable resources, such as air and bone meal, respectively. We show conditions for diluted sulfuric-acid saccharification of the microalgal biomass that retained most of the biomass oil and protein in an insoluble fraction as a potential animal feed supplement, and allowed ethanol production from the solubilized sugars at a ratio of 0.25 g ethanol . g biomass^{-1}. Optimized conditions for nutrients recycling from the fermentation

144 vinasse and saccharification reagents as a sole source of macronutrients for a new cycle of
145 biomass production are shown.



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148 **Figure 1**. Simplified schematic of the main matter transformations in a semi-closed loop
149 biomass biorefinery to produce feed and fuel. Circles represent the main inputs: CO_2, N_2,
150 H_2SO_4, $Ca_x(PO_4)_y$, $Mg(OH)_2$ and KOH. Squares represent the main outputs: ethanol, CO_2
151 (becomes a nutrient input), $CaSO_4$ and residual biomass as feed. The area of the shapes
152 represents the mass of each input or output in the platform.

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2. Materials and Methods

2.1. Reagents and chemicals

Reagents, chemicals supplier and chemical purity are shown in the Supplementary Table 1.

2.2. Culture of microalgae and cyanobacteria

Both the microalga *Desmodesmus* sp. strain FG (Do Nascimento et al., 2012) and the cyanobacterium *Nostoc* strain M2 (Do Nascimento et al., 2015) were routinely maintained and cultivated in $BG11_0$ medium (0.04 g . L^{-1} K_2HPO_4; 0.075 g . L^{-1} $MgSO_4$. $7H_2O$; 0.036 g . L^{-1} $CaCl_2$. $2H_2O$; 0.006 g . L^{-1} citric acid; 0.006 g . L^{-1} ferric ammonium citrate; 0.001 g . L^{-1} EDTA (disodium salt); 0.02 g . L^{-1} Na_2CO_3, and trace metal mix A5 (2.86 mg . L^{-1} H_3BO_3; 1.81 mg . L^{-1} $MnCl_2$. $4H_2O$; 0.222 mg . L^{-1} $ZnSO_4$. $7H_2O$; 0.39 mg . L^{-1} $NaMoO_4$. $2H_2O$; 0.079 mg . L^{-1} $CuSO_4$. $5H_2O$ and 0.049 mg . L^{-1} $Co(NO_3)_2$. $6H_2O$)), containing $NaNO_3$ or atmospheric N_2 as sole N source. Other growth media and experimental conditions are described in the main text.

Either for growth analysis or biomass characterization, microalgal strains were cultivated indoors in 500 mL bottles containing 250 mL medium sparged with filtered air from the bottom at 0.3 – 0.5 L . min^{-1} and illuminated with constant white light at 100 µmol photons m^{-2} . s^{-1}. For preparative purposes (biomass fermentation), both strains were cultivated in 5 L airlift photobioreactors containing 4.5 L of medium sparged with filter-sterilized air from the center of the riser tube at 6 L . min^{-1} up flow circulation and pure CO_2 from the bottom of the down flow circulation at 0.2 L . min^{-1}. Cultures were illuminated with constant white light at 200 µmol photons m^{-2} . s^{-1}. Under both culture systems temperature was maintained constant at 28 ± 1 °C.

2.3. Preparation of cyanobacterial extracts and mixotrophic culture of microalgae

Biomass pellets of *Nostoc* sp. strain M2 were allowed to dry out under a cold air stream at 11 ± 1 °C and milled with 15 % (w/w) sand in a mortar. Water soluble biomass-extracts were prepared by addition of 30 volumes of water (v/w) at room temperature (22 ± 2 °C) together with a few glass beads, vigorously agitated in a vortex and finally clarified by centrifugation at 6,000 x *g* for 10 min. A typical preparation contained 0.9 g . L^{-1} N; 0.1 g . L^{-1} P; 5 g . L^{-1} protein; and 2.5 g . L^{-1} soluble carbohydrates (Do Nascimento et al., 2015). For mixotrophic cultivation of microalgae, *Nostoc* extracts at stated dilutions substituted for BG11 medium containing $NaNO_3$.

2.4. Microalgal biomass hydrolysis and fermentation

Biomass pellets of *Desmodesmus* sp. strain FG were dry out under a cold air stream at 11 ±1 °C and milled with 15 % (w/w) sand in a mortar. For diluted acid hydrolysis, biomass at 20 % (w/v) load was incubated in the presence of 2% H_2SO_4 (v/v) for 30 min at 120 ºC in an autoclave and further clarified by centrifugation at 6,000 x *g* for 10 min. Both analytical or preparative preparations (1 or 20 mL) were brought to pH 4.5 with $Mg(OH)_2$ crystals and inoculated with the yeast *Saccharomyces cerevisiae* (Levex®, Argentina) for fermentation at an initial OD_{600} of 0.25 in 3 or 25 mL vials, respectively (Sanchez Rizza et al., 2017). Each hydrolysate fermentation was routinely accompanied by parallel fermentations of YPD medium at a dextrose concentration in the range of the sugar content of the samples.

2.5. Phosphorous supplementation to saccharified biomass

For vinasse-like preparations from pure reagents, 60 mM P from $Ca_3(PO_4)_2$, $Ca_5(PO_4)_3(OH)$, or bone meal were reacted sub-stoichiometrically with 360 mM H_2SO_4 (corresponding to 2% H_2SO_4 (v/v) as optimized for microalgal biomass saccharification) according to the following reaction:

$Ca_3(PO_4)_2 + 6\ H_2SO_4 \rightarrow 2\ H_3PO_4 + 3\ CaSO_4 + 3\ H_2SO_4$

Gypsum was separated by centrifugation and filtration. The pH of the preparations was brought from pH 0.5 to 4.5 with $Mg(OH)_2$ and KOH as follows:

$2\ H_3PO_4 + 3\ H_2SO_4 + 3\ Mg(OH)_2 + 2\ KOH \rightarrow 2\ KH_2PO_4 + 3\ MgSO_4$

(pKa_{H2SO4} = -10; 2; and pKa_{H3PO4} = 2.2; 7.2; 12.3)

Thus, according to this stoichiometry, soluble salts of S, P, Mg and K remained at similar relative ratios as those in the reference culture medium $BG11_0$ (Rippka et al., 1979), representing the whole complement of macronutrients for diazotrophic cyanobacteria.

For P, Mg and K supplementation to saccharified biomass, essentially the same procedure was followed. After fermentation with baker's yeast, cells were separated by centrifugation at 6,000 x *g* for 10 min. Next, ethanol was determined and evaporated at 80 °C for 1 h for removing 90 – 95 % of its content, mimicking distillation for recovery. These preparations were used at an appropriate dilution as a complete source of macronutrients for diazotrophic cultivation of the cyanobacterium *Nostoc* sp. strain M2.

2.6. Analytical methods

Cell density for growth analysis was estimated by recording OD at 750 nm using a spectrophotometer.

For microalgal biomass dry weight determination, samples (50 mL of culture) were centrifuged at 14,000 x *g* for 10 min and pellets were dried out in an oven at 60 - 70 °C until constant weight (2 - 3 days).

Total protein determinations were obtained after boiling resuspended cells at 100 °C for 10 min in the presence of 1 N NaOH. Aliquots were subjected to protein determination by the Lowry's method (Lowry et al., 1951) using NaOH-treated bovine serum albumin as a standard.

For biomass total carbohydrates determination, resuspended cells were directly reacted with the anthrone method reagents (Dreywood, 1946). Carbohydrates content was calculated from a standard curve using glucose.

Analytical determinations of organic matter, ash, crude protein, crude fat and water soluble carbohydrates were performed at a commercial facility (https://inta.gob.ar/servicios/). For organic matter and ash, microalgal biomass was calcined in a muffle furnace at 600 º C for 2 h for ash content determination. Organic matter was calculated as the difference between dry matter and ash content. Crude protein was calculated after the combustion of the samples in an atmosphere of ultrapure O_2 and helium at 850 ºC, determination of total N in a LECO FP 528 system using EDTA as calibration standard, and applying the standard N-to-protein conversion factor 6.25. For crude fat determinations, dry and milled samples were extracted with petroleum ether in an Ankom XT10 equipment. Water soluble carbohydrates were extracted in a boiling aqueous solution, filtered, and determined by the anthrone reagent as described above.

Ethanol was determined from the *S. cerevisiae* fermentation spent-medium by an enzymatic assay as reported previously (Sanchez Rizza et al., 2017). Briefly, the standard ethanol assays contained 50 mM Tris-HCl, pH 8.4; 2.5 mM NAD^+ and 3 µg protein preparations enriched in alcohol dehydrogenase activity. Samples were mixed in a total volume of 100 µl and incubated at room temperature for 25 min. Ethanol in samples was determined as the ethanol dependent reduction of NAD^+ in a spectrophotometer at 340 nm and comparison with a standard curve made with 99% (v/v) analytical grade ethanol.

3. Results and Discussion

3.1. Conceptual design of a semi-closed loop microalgal biomass production platform

The aim of this study was to design a biomass production platform using renewable resources as fertilizer inputs. This system produces fermentable sugars as a feedstock for biofuels and protein for feed as the main outputs, while minimizing the amount of waste. Figure 2 shows a conceptual design based on a multispecies microbial cell factory approach that relies in the technological coupling of the activity of different microorganisms that excel at single tasks. This platform would take N directly from the air (substituting for the synthetic N-fertilizer) by the activity of a N_2-fixing cyanobacterium that accumulates high levels of protein. The N-rich cyanobacterial biomass would be used as an organic fertilizer to produce biomass of eukaryotic microalga that accumulates high levels of fermentable carbohydrates. Biomass treatment with H_2SO_4 would render a saccharified liquid stream for producing ethanol by a fermenting microorganism, and a solid fraction as animal feed. Fermentation vinasse could be recycled as a source of nutrients for the cultivation of the N_2-fixing cyanobacterium to close one production cycle. Conversion of the spent H_2SO_4 into H_3PO_4 by reaction with calcium phosphates from different sources would transform a hazardous waste into a very useful P-fertilizer. Recovery of proteinaceous pigments from *Nostoc* biomass has been shown before (Do Nascimento et al., 2015).

The following sections provide proof-of-concept for every single step of the platform along with a discussion of specific aspects and further possibilities.

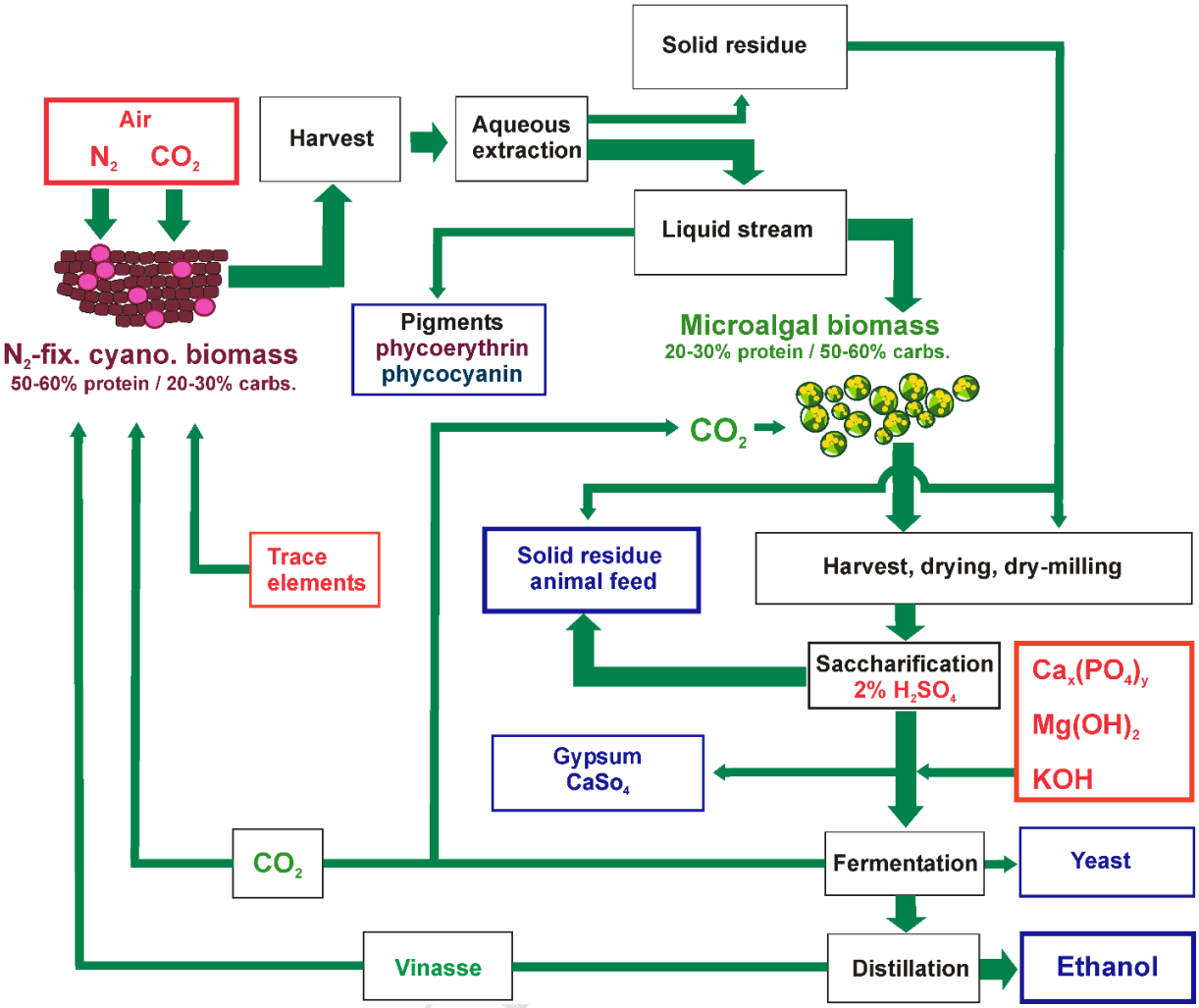

Figure 2. Simplified process design of a biorefinery for the production of ethanol and feed from CO_2 and N_2 from the air. The main stream towards ethanol and feed is indicated by wider arrows. Main inputs are marked in red boxes, main outputs in blue boxes and operations or streams in black boxes. Narrow arrows indicate recycling of reagents into nutrients or production of secondary products.

3.2. Production of fermentable sugars at the expense of C and N from the air

One of the aims of our approach was to gain access to N_2 from the air as a renewable and continuous source of N-fertilizer for the production of eukaryotic microalgal biomass. We used a filamentous N_2-fixing cyanobacterium (*Nostoc* sp. strain M2) that had been selected previously because of its high productivity, biomass composition (up to 60% w/w protein content), and ease of biomass collection and downstream processing into cell-free protein rich-extracts (Do Nascimento et al., 2015). Here we optimized conditions for low energy-intensive biomass processing into an organic fertilizer. Dry biomass powder was extracted with water at room temperature to recover up to 40% (w/w) of its protein content. This protein recovery yield was lower than the one previously obtained by freezing-thawing the biomass for a few cycles (up to 90 % w/w protein recovery) (Do Nascimento et al., 2015). These methods can be considered two alternatives that differ in their energy intensity at the expense of a yield reduction.

The N_2-fixing cyanobacterium *Nostoc* sp. strain accumulates up to 60 % (w/w) proteins in its biomass together with low levels of carbohydrates (less than 30%) while producing a very low yield of ethanol after diluted acid saccharification/fermentation (not shown). We have conducted some bioprospecting studies to identify microalgae suitable as a feedstock for bioethanol. These studies resulted in the identification of *Desmodesmus* sp. strain FG which accumulates up to 60% carbohydrates that could be almost fully fermented into ethanol by the baker's yeast *S. cerevisiae* (Sanchez Rizza et al., 2017). Figure 2 shows mixotrophic cultivation of *Desmodesmus* strain FG at the expense of *Nostoc*-based organic fertilizer as a sole source of nutrients at a very high biomass concentration of 8 – 10 g . L^{-1} (dry w/v). Results show a biomass productivity of 0.6 g dry biomass . L^{-1} . day^{-1} and a maximum carbohydrates accumulation up to 6 g . L^{-1} of culture medium. The cultures were operated in a semi continuous mode with 75 % of water recycling and cell-harvesting at days 10 and 20. This system allowed efficient channeling of N_2 from the air into microalgal biomass by means of the natural process

of biological N_2-fixation and the complete recycling of other nutrients already assimilated in the cyanobacterial biomass. This is of prime importance considering that there is no known microalga o eukaryote able to fix N_2-from the air. At the time of harvesting, the microalgae contained up to 60 % carbohydrates but as low as 10 – 20 % (w/w) protein. Since this organic fertilizer allowed quantitative recycling of cyanobacterial protein into microalgal protein (Do Nascimento et al., 2015), this mixotrophic mode of cultivation allowed a 2- to 3-fold increase in biomass production with respect to the spent cyanobacterial biomass. Most of this increase corresponded to the accumulation of carbohydrates by the microalga under the used culture conditions (Fig. 3). Water recycling up to 75 % (v/v) per cultivation cycle under a semi-continuous cultivation regime proved to sustain an equivalent biomass productivity and an even slightly higher carbohydrate's yield.

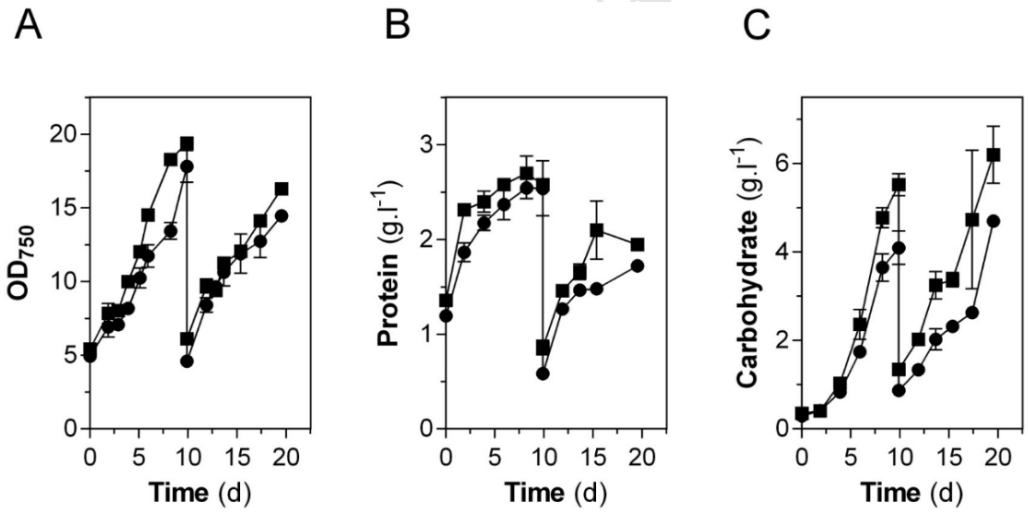

Figure 3. Mixotrophic growth of microalgae at the expense of a cyanobacterial extract. A-C) Time course of OD_{750} (A), protein (B) or carbohydrates (C) accumulation are represented. •) $BG11_0$ medium containing 8 mM NO_3^--N (positive control); or ■) *Nostoc* water-soluble extracts at 8 mM protein-N as a sole source of nutrients. Each data point represents the mean and range of two independent experiments.

3.3. Saccharification of microalgal biomass and ethanol production

Similar to biomass from other sources, microalgal biomass can be saccharified by different means, including chemical and/or enzymatic methods, among others. It appears that diluted H_2SO_4 treatment is currently the most cost-effective alternative for industrial applications (Li et al., 2014). Here, microalgal biomass was saccharified at a high biomass load of 20% (dry w/v) solids in the presence of 2% H_2SO_4 (v/v) at 120 °C for 30 min. The saccharified liquid stream was brought to pH 4.5 with hydroxides and contained up to 98.3 +/- 1.2 g sugars . L^{-1}. After fermentation with the yeast *S. cerevisiae*, it yielded up to 49.1 +/- 0.6 g ethanol . L^{-1}. The observed total carbohydrates to ethanol conversion was very close to the theoretical maximum conversion yield of 0.51 g ethanol per g of glucose and a biomass to ethanol conversion efficiency of 0.25 g ethanol per g biomass. The corresponding amount of CO_2 release and the production of low amounts of yeast biomass were confirmed, as reported before (Sanchez Rizza et al., 2017). We showed a large improvement of ethanol yields from *Desmodesmus* sp. strain FG biomass compared with previous work in microalgal biomass transformation into ethanol (Sanchez Rizza et al., 2017). Here, we further improved sugars and ethanol concentration by about 2-fold by increasing the biomass load during diluted acid saccharification from 10 to 20 % (w/v), with no signs of inhibition of fermentation yet. This is noteworthy since an economically-competitive production of ethanol requires a minimum of 40 g ethanol . L^{-1} of fermentation broth to reduce distillation costs (Möllers et al., 2014).

We had simulated before the productivity of a microalga at the expense of *Nostoc*-based organic fertilizer in environmental photobioreactors mimicking open-pond conditions (Do Nascimento et al., 2015). According to that productivity and the biomass-to-ethanol conversion efficiency demonstrated in this work (about 0.25 g ethanol per g biomass), this platform might produce, under Buenos Aires environmental conditions, from 7,600 to 10,800 L ethanol . ha^{-1} . $year^{-1}$, depending on whether *Nostoc* is cultivated in raceway ponds or in tubular

photobioreactors, respectively (Do Nascimento et al., 2015). These preliminary calculations would suggest that this kind of production platforms might represent an interesting alternative to corn kernel or stover feedstocks for 1G or 2G bioethanol production at typical productivities of 3,680 or 1,594 L ethanol . ha^{-1} . $year^{-1}$, respectively (Karlen et al., 2011; Pimentel and Patzek, 2005).

Since the mixotrophic nature of the proposed production platform at the expense of a rich organic medium would make it prone to contamination, closed photobioreactors as those used in this study would be more suitable for escalation trails. In this case, the expected productivities should be significantly higher, in the range of 3-fold (Jorquera et al., 2010), but at the expense of a proportional increase in capital and operational costs (Richardson et al., 2012).

In addition to a significant production potential and possibilities of culturing in non-arable lands, this strategy completely substitutes air N_2 for synthetic N-fertilizer by means of a cyanobacterial biological N_2-fixation that, as photosynthetic C-fixation, is powered by light.

3.4. Biochemical composition of the residual biomass

The fraction that remained insoluble after the microalgal biomass saccharification retained a considerable amount of crude protein, became especially enriched in crude fat and, as expected, was largely depleted of carbohydrates (Table 1). This composition would make this fraction very attractive as animal feed. However, true nutritional value, digestibility, palatability and potential toxicity should be experimentally determined (Gong et al., 2018). Although obtained in a quantitative smaller amount, yeast biomass would indeed represent a wanted animal feed ingredient (Øverland and Skrede, 2017).

372 **Table 1.** Basic chemical composition of the solid fraction after saccharification of *Desmodesmus*
373 biomass with H_2SO_4

	Solid fraction after saccharification[a]	Whole biomass[b]
Organic matter (% w/w)	86.4 ± 3.1	81.4
Ash (% w/w)	15.6 ± 0.6	18.6
Crude protein (% w/w)	10.4 ± 2.1	10.8
Crude fat (% w/w)[c]	43.4 ± 3.4	4.5
Carbohydrates (% w/w)[d]	0.4 ± 0.4	11.3

374
375 [a]Mean and error of two independent preparations. [b]Single determinations. [c]Ether extract.
376 [d]Water soluble carbohydrates
377

378

379 *3.5. Sulfuric acid management and vinasse upgrading and recycling as nutrients*

380 A few alternatives have been proposed to recycle and to upgrade vinasse. It has been shown
381 that some microalgae can be cultivated at the expense of nutrients in vinasse (Santana et al.,
382 2017).

383 To investigate the microalgal fertilizing properties of microalgal biomass fermentation vinasse,
384 we evaporated most of the ethanol (about 5% v/v) after fermentation of saccharified microalgal
385 biomass at 80 °C (simulating distillation for ethanol recovery). Preliminary experiments indicated
386 that the resulting vinasse, at a dilution of 0.4% (v/v) contained all the nutrients required for
387 cultivation of N_2-fixing *Nostoc* up to similar levels than the reference mineral medium BG11.
388 Supplementation of N was required for cultivation of the microalga *Desmodesmus* sp. strain FG,
389 indicating deficiency of this nutrient in the microalgal biomass vinasse (not shown). In both
390 cases, supplementation with P produced a higher biomass yield.

With the multi-purpose of managing the spent H_2SO_4 and upgrading both the vinasse and alternative sources of P-fertilizer, we reacted the H_2SO_4 of the liquid stream of the saccharified microalgal biomass either with *(i)* the insoluble forms of P $Ca_3(PO_4)_2$, $Ca_5(PO_4)_3(OH)$, or *(ii)* bone meal to produce highly soluble H_3PO_4, and insoluble $CaSO_4$ (gypsum), which could be easily recovered by sedimentation/centrifugation. Before fermentation, the pH was brought to 4.5 with KOH and $Mg(OH)_2$. The sources of S, P, K and Mg were added in such a proportion to match the relative amounts of soluble forms of S, P, K and Mg in $BG11_0$, a reference culture medium for diazotrophic cyanobacteria. Both a simulated vinasse-like preparation from pure reagents (Supplementary Fig. S1) and true vinasse after biomass saccharification and supplementation, represented an improved growth medium for the cyanobacterium in comparison to the reference medium $BG11_0$ (Fig. 4).

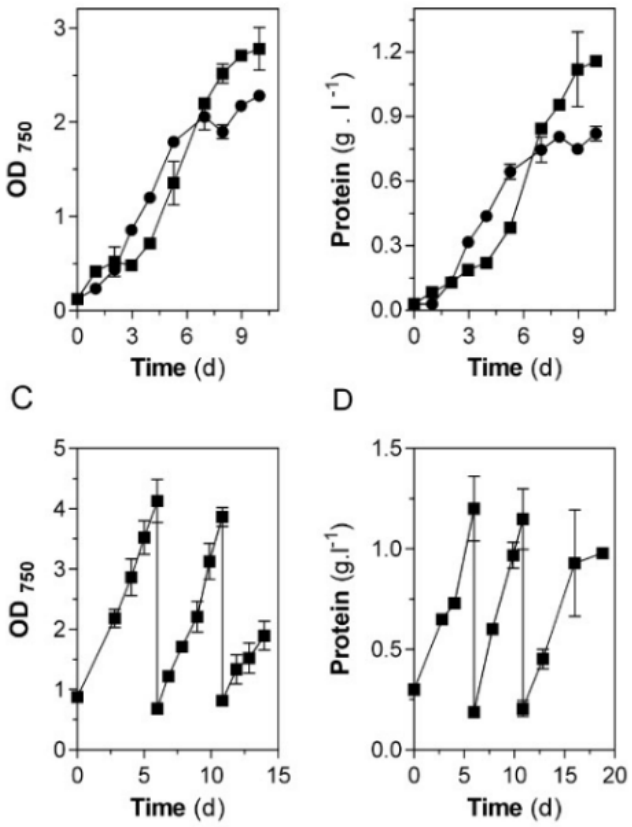

Figure 4. Fermentation vinasse recycling and up-grading. A and B) Growth curves of *Nostoc* sp. M2 at the expense of the fermentation vinasse of a microalga biomass saccharified with H_2SO_4 and supplemented/reacted with bone meal. •) BG11 medium (positive control); or ■) fermentation vinasse as a unique source of P, S, K and Mg. C and D). Semi continuous cultures of *Nostoc* sp. M2 at high density at the expense of P-supplemented vinasse. Each data point represents the mean and range of two independent experiments.

As expected, non-reacted $Ca_3(PO_4)_2$ was not a useful source of P for the cyanobacterium, neither *Nostoc* could be cultivated in the absence of added P or S (Supplementary Fig. S1).

Using this medium, under a semi-continuous mode of culture with 75 % (v/v) water recycling per cycle, cyanobacterial biomass up to 2 g(dw) . L^{-1} and a productivity of 0.3 g(dw) . L^{-1} . day^{-1} using atmospheric N_2 as the sole source of N were obtained for up to 3 cycles (Fig. 4 C and D). The collected biomass became the feedstock for the next production cycle of microalgal biomass rich in fermentable carbohydrates (Figs. 1 and 2).

Figure 1 depicts a simplified schematic of the main matter transformations demonstrated here for this semi-closed loop platform. The area of the shapes represents the relative amounts of each input (CO_2, N_2, H_2SO_4, $Ca_x(PO_4)_y$, $Mg(OH)_2$ and KOH) or output ethanol (for fuel); CO_2 (which becomes a nutrient input); residual biomass (as feed) and $CaSO_4$ (as a building material, cement additive, soil conditioner, etc.). Circles around biomass squares represent the assumed amount of CO_2 fixed to produce biomass at an estimated ratio of 1.8 kg CO_2 . kg^{-1} biomass.

H_2SO_4 plays a central role in this platform and represents its main input from “non-renewable” resources. H_2SO_4 is currently the most widely used reagent in the chemical/petrochemical industry (Nleya et al., 2016) and is mainly produced at petroleum refineries, natural-gas-processing plants, and coking plants in a process mostly intended to reduce the S levels of combustion gases. Over the last two decades, environmental considerations have placed increasing pressure towards reduction of S in the fuels. Sulfur emissions promote acid rain, which causes severe deleterious results on human health, biodiversity, as well as the integrity of buildings and machinery materials (Burns et al., 2012). It is anticipated that, driven by energy and environmental security, exploitation of lower quality fossil fuel reserves with higher content of S will sustain production of H_2SO_4 at a low cost. Notably, no H_2SO_4 waste is produced in the proposed platform since it is all converted into gypsum and fertilizer/biomass.

On the other hand, on-site production of P-fertilizer from natural resources containing $Ca_3(PO_4)_2$ or $Ca_5(PO_4)_3(OH)$ as phosphate rock or bone meal, would be economically advantageous. Using phosphate rock would be feasible in some regions and/or specific contexts. However, since phosphate rock is a finite natural resource unevenly distributed across geographical regions, the recovery of P from bone meal as a renewable byproduct (or waste) of food industry would be even more attractive from a circular economy and sustainability points of view. The production of P will accompany food demand worldwide (Mirabella et al., 2014). For example, Ethiopia produces approximately 192,000 to 330,000 tonnes of bone waste annually which would have yielded around 28 to 58% of the annual P fertilizer of the country and savings of US$ 50 to 104 million from importing an equivalent amount of P fertilizer. However, this strategy has been insufficiently explored (Simons et al., 2013).

4. Conclusions

This study shows the design and proof-of-concept of a semi-closed loop microalgal production platform for ethanol and feed from CO_2 and N_2 from the air, and P from food waste. This approach reconciles co-production of fuel and feed and internal recycling of macronutrients other than N and P.

We demonstrated a clear improvement in the state-of-the-art fermentation of microalgal biomass by producing saccharified liquid streams containing up to 100 g sugars . L^{-1} which yielded, after fermentation, up to 50 g ethanol . L^{-1}. The modeled potential yield in the field would be higher than those currently obtained from maize feedstocks.

Some unique features of the platform are: *i*) a multispecies approach comprising three different microorganisms that excel at single operations (N_2 fixation, carbohydrates accumulation, and fermentation); *ii*) H_2SO_4 for integrating biomass saccharification and recovery of soluble P from bone meal; and *iii*) intensive internal recycling of water and nutrients in fermentation vinasse. No H_2SO_4 waste is produced in the platform since it is all converted into gypsum and fertilizer/biomass for additional applications.

Each of these concepts has been poorly addressed in the past and, to the best of our knowledge, never integrated into a single production platform that contributes alternatives from circular economy into microalgal biotechnology for cleaner production of commodities.

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A semi-closed loop microalgal biomass production-platform for ethanol from renewable sources of nitrogen and phosphorous

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Highlights

- A multitrophic semi-closed loop biomass production platform is proposed.
- N and P fertilizers were produced on site from air and bone meal, respectively.
- Ethanol was produced at 0.25 g . g microlgal biomass^{-1} along with animal feed.
- Sulfuric acid integrated biomass saccharification and efficient P recovery.
- Nutrients in vinasse and water were recycled to close the production cycle.