Algal Research 45 (2020) 101733

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Sucrose-to-ethanol microalgae-based platform using seawater

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ARTICLE INFO

Keywords:
Biofuels
Saccharification
Sugars
Bioethanol
Biorefinery
Water footprint

ABSTRACT

Microalgal biomass is increasingly considered a promising feedstock for the production of bioethanol because it has simpler biochemical composition and structural features than plant feedstocks. However, it still requires hazardous and/or expensive acid or enzymatic saccharification processes before its conversion into ethanol by fermentation. To bypass this limitation, we identified microalgal strains that accumulate up to 10% (*w*/w) of sucrose, a readily fermentable sugar. Conditions were optimized to produce sucrose in half-strength seawater, as well as efficient extraction by very mild procedures, and conversion into ethanol. We present a model based on cultivation in environmental photobioreactors that predicted a productivity of 4200 L ethanol·ha^{-1}·year^{-1} in raceway ponds in Brazil, approaching the average sucrose and ethanol productivity from sugarcane.

1. Introduction

Bioethanol represents an alternative to diminish rapid depletion of crude oil reserves and climate change [1]. It can be produced by fermentation of renewable agricultural feedstocks rich in sucrose or starch, such as sugarcane or corn, respectively. It is also the most used renewable fuel in the transportation sector [2]. However, sustainability concerns related to food security and environment conservation promoted the development of a second generation of bioethanol from lignocellulosic feedstocks. Yet, the complex chemical composition and structure of these materials demand harsh physicochemical pretreatments and costly enzymes for saccharification, posing a difficult-to-overcome barrier towards commercialization [3].

Microalgal biomass is increasingly considered as a promising feedstock for the production of third generation bioethanol because of several characteristics [4,5]. Firstly, microalgae have higher productivity of biomass and starch per unit of surface and time than the most productive crop plants. They have simpler biochemical and structural composition in comparison to plant feedstocks. They can be cultivated independently from arable land and freshwater, with the possibility of using sea or brackish water for cultivation. Also, there is the possibility of using residues from industries as a source of inexpensive nutrients, especially CO_2, N-sources and phosphates. This could additionally assist in municipal or industrial waste management, to reduce the demand for fertilizers, and to mitigate climate change [5–8].

However, the technology to transform algal biomass into fuels is not mature yet, and thus current capital and production costs are prohibitive for commercialization [9]. Among the aspects that need to be improved are: (i) high, robust, and consistent productivity under dynamic environmental conditions [10]; (ii) although currently superior to crop plants, the freshwater footprint and demand for fertilizers of microalgae cultivation must be further optimized [11]; (iii) biomass harvesting and downstream processes for conversion into biofuels also need improvement [12]. Furthermore, most techno-economic analyses suggest that co-production of biofuels and animal feed, or other high-value co-products, would be mandatory for cost-effectiveness [12].

Some microalgal strains accumulate large quantities of carbohydrates in their biomass, up to 70% (*w*/w) [4,6], mainly as insoluble starch and cellulose [6,13]. Although the lack of lignin and simpler structure of microalgal cell walls [14] largely simplifies biomass saccharification in comparison to plant feedstocks, algal biomass still requires chemical (acid or alkaline)/physicochemical or enzymatic hydrolysis to enable fermentation [4,5,7].

Similarly to plants, some microalgae and cyanobacteria accumulate sucrose [15], a soluble sugar that is readily fermentable by yeast and by most industrially relevant microorganisms [16,17]. Sucrose accumulates in cyanobacteria and microalgae as a response to salt, osmotic, desiccation, cold or heat stress [18,19]. Recently, several studies have shown increased sucrose accumulation by genetic engineering of unicellular or filamentous cyanobacteria [20–22]. Particularly, we showed the genetic modification of carbohydrates partitioning in a filamentous

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https://doi.org/10.1016/j.algal.2019.101733
Received 21 August 2019; Received in revised form 8 November 2019; Accepted 15 November 2019

cyanobacterium towards accumulation of sucrose up to 10% (*w*/w). After aqueous extraction from the transgenic biomass, sucrose-rich syrups were efficiently converted into ethanol without the need of exhaustive or expensive pretreatments and/or saccharification processes. Additionally, a large fraction of the cyanobacterial protein content could be separated after short pulses of heat, keeping the value of sugars, protein and lipids for different applications in the food, energy and/or other sectors of the market [23].

This study describes the identification of sucrose-accumulating native microalgae in response to NaCl stress. Conditions were optimized to induce sucrose accumulation up to 10% (*w*/w) using seawater. Sucrose was extracted by two alternative methods and directly fermented into ethanol at the maximum theoretical efficiency, leaving a large fraction of crude protein to be recovered for feed purposes. A semi-experimental model for bioethanol production using marine water in open raceway ponds is provided after cultivation simulations in environmental photobioreactors.

2. Materials and methods

2.1. Strains and culture conditions

The microalgal strains used in this work were obtained from previous bioprospecting efforts from Southeastern Buenos Aires, Argentina (38°0′0″ S 57°33′0″ W), in 2010 [24] or Jujuy, Argentina (24°11′08″ S 65°17′58″ W) in 2016 [25].

The reference culture condition was performed in 500 mL bottles containing 300 mL of $BG11_0$ medium (0.04 $g{\cdot}L^{-1}$ K_2HPO_4; 0.075 $g{\cdot}L^{-1}$ $MgSO_4{\cdot}7H_2O$; 0.036 $g{\cdot}L^{-1}$ $CaCl_2{\cdot}2H_2O$; 0.006 $g{\cdot}L^{-1}$ citric acid; 0.006 $g{\cdot}L^{-1}$ ferric ammonium citrate; 0.001 $g{\cdot}L^{-1}$ EDTA (disodium salt); 0.02 $g{\cdot}L^{-1}$ Na_2CO_3, and trace metal mix A5 (2.86 $mg{\cdot}L^{-1}$ H_3BO_3; 1.81 $mg{\cdot}L^{-1}$ $MnCl_2{\cdot}4H_2O$; 0.222 $mg{\cdot}L^{-1}$ $ZnSO_4{\cdot}7H_2O$; 0.39 $mg{\cdot}L^{-1}$ $NaMoO_4{\cdot}2H_2O$; 0.079 $mg{\cdot}L^{-1}$ $CuSO_4{\cdot}5H_2O$ and 0.049 $mg{\cdot}L^{-1}$ Co $(NO_3)_2{\cdot}6H_2O$)) supplemented with 0.84 $g \cdot L^{-1}$ $NaHCO_3$, and NaCl and $NaNO_3$ as indicated. Bottles were illuminated with constant white light at 540 $\mu mol{\cdot}photons{\cdot}m^{-2}{\cdot}s^{-1}$ (unless stated otherwise) on the surface of the bottle, sparged from the bottom with filtered 2% (*v*/v) CO_2-enriched air at 0.3–0.5 $L{\cdot}min^{-1}$ and maintained at 28 ± 1 °C. All cultures were started at an initial cell density (OD_{750nm}) of 0.2.

For the identification of sucrose-accumulating strains, microalgae were cultivated in BG11 medium containing 10 mM $NaNO_3$ for 2 days after which 150 mM NaCl was added and then left to grow for another 2 days. Light intensity was set at 325 $\mu mol{\cdot}photons{\cdot}m^{-2}{\cdot}s^{-1}$ on the surface of the bottles.

For algal cultivation in seawater, BG11 medium was prepared using synthetic seawater (24.54 $g{\cdot}L^{-1}$ NaCl; 0,74 $g{\cdot}L^{-1}$ KCl; 1.90 $g{\cdot}L^{-1}$ $MgCl_2$; 1.47 $g{\cdot}L^{-1}$ $CaCl_2{\cdot}2H_2O$, 6.16 $MgSO_4{\cdot}7H_2O$, 0.84 $g{\cdot}L^{-1}$ $NaHCO_3$; 0.04 $g{\cdot}L^{-1}$ K_2HPO_4; 0.036 $g{\cdot}L^{-1}$ $CaCl_2{\cdot}2H_2O$; 0.006 $g{\cdot}L^{-1}$ citric acid; 0.006 $g{\cdot}L^{-1}$ ferric ammonium citrate; 0.001 $g{\cdot}L^{-1}$ EDTA (disodium salt); 0.02 $g{\cdot}L^{-1}$ Na_2CO_3; and trace metal mix A5) and mixed at stated proportions with BG11 medium prepared in deionized water.

For simulation of outdoor cultivation, laboratory benchtop environmental photobioreactors (ePBRs Phenometrics™) were used [26]. To simulate 5-cm-depth ponds, ePBRs were filled with 90 mL of $BG11_0$ medium prepared in 25% (v/v) seawater containing 10 mM $NaNO_3$ and sparged during the daylight phase with 2% (w/v) CO_2 in air. ePBRs were programmed to simulate the mean summer season weather in Fortaleza, Brazil (3°46′53″ S 38°35′20″ O): maximum irradiance at noon of 1554 $\mu mol\ photons{\cdot}m^{-2}{\cdot}s^{-1}$; 13 h day duration; and 28 ± 3 °C. Data was obtained from the Power Data Access Viewer from NASA (https://power.larc.nasa.gov). Both light and temperature were adjusted to a sinusoidal pattern from dusk to dawn. ePBRs were inoculated at an initial cell density (OD_{750nm}) of 0.6. After 2 days, the simulated ponds were flooded with 45 mL or 90 mL seawater-BG11-medium to further induce sucrose accumulation for another 2 days. The resulting final pond depths were 7 cm or 10 cm, respectively.

2.2. Sucrose extraction and fermentation

Two alternative methods were used for preparative sucrose extraction, essentially as described before [23]. Briefly, for the method based on microwaves (MW), the cell paste was subjected to extraction by microwaves at 200 w of power, for 4 cycles of 2 min each, in a microwave oven (BGH Quick Chef® 15,140, Argentina). The soluble fraction was separated by centrifugation at 17,211 ×*g* for 15 min. The extraction was repeated twice, and the fractions were combined. For the method based on dry milling (D&M), the biomass was air-dried, milled with 15% sand (*w*/w) and rehydrated with water at a 1:3.5 ratio (*w*/*v*). After centrifugation at 11,952 ×*g* for 15 min, the soluble fraction was separated, and the aqueous extraction was repeated one more time. Both soluble fractions were combined, incubated at 100 °C for 5 min and then centrifuged 16,300 ×*g* for 5 min to remove proteins.

Fermentations were conducted as previously described [4] by inoculating the sucrose-rich syrups with *Saccharomyces cerevisiae* for 24 h at 28 °C and agitation at 120 rpm.

2.3. Analytical methods

Cell density was estimated by periodically measuring the OD at 750 nm with a UV-1800 spectrophotometer (Shimadzu, Japan). To estimate doubling time, data were plotted and adjusted to theoretical curves of exponential growth using GraphPad PRISM software (Intuitive Software for Science, US). Dry weight was determined from 25 mL of suspended cells in culture medium. Samples were first centrifuged at 3900 ×*g* for 10 min, transferred into 1.5 mL tubes and centrifuged again at 16,300 ×*g* for 5 min, at 4 °C. Pellets were dried in an oven at 90 °C until constant weight was reached (2–3 days).

For protein determination, samples were heated at 100 °C for 10 min in the presence of 1 N NaOH, followed by Lowry's method [27] using NaOH-treated bovine serum albumin as a standard. For total lipids determination, lipids were extracted and determined gravimetrically based on Bligh and Dyer [28] with previously described modifications [24]. Alternatively, lipids were determined by the sulfo-phospho-vanillin method (SFV) [29] using commercial canola oil as a lipid standard.

For total carbohydrates determination, the anthrone reagent was used [30]. Analytical sucrose extraction/determination was performed essentially as previously described [31]. Briefly, 14 to 45 mL of culture was centrifuged at 3600 ×*g* for 15 min, at 4 °C. Cell pellets were resuspended in 2 volumes of boiling alkaline water (pH 8) and incubated at 100 °C for 5 min, followed by centrifugation at 9600 ×*g* for 5 min, at 4 °C. These steps were repeated twice, and fractions were combined. Alternatively, extraction was done according to Waghmare and colleagues [32] with modifications. First, 1 to 3 mL of culture were centrifuged at 4800 ×*g* for 5 min, at 4 °C. Pellets were resuspended in 250 μL PBS buffer (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 140 mM NaCl, pH 7.4), and frozen and thawed 3 times. Then, one volume of anhydrous ethanol, previously warmed-up to 50 °C, was added. Samples were vortexed and centrifuged at 4800 ×*g* for 10 min, at 4 °C. Steps were repeated twice, fractions were combined and left to evaporate under vacuum in a Speed Vac Concentrator (HVL, Savant™). Samples were resuspended in 0.5 or 1 mL of deionized water. Sucrose determination was done by incubating the samples at 55 °C in the presence of 80 $\mu g{\cdot}mL^{-1}$ acid invertase (Sigma-Aldrich) and 10 mM NaOAc, pH 4.5 for 30 min. Conversion into glucose and fructose was determined by the Somogyi-Nelson's method with a standard curve using sucrose [31].

Ethanol was determined by an enzymatic assay as reported previously [4]. The standard ethanol assay contained 50 mM Tris-HCl, pH 8.4; 2.5 mM NAD^+ and 3 μg protein preparations enriched in alcohol dehydrogenase activity. Ethanol dependent reduction of NAD^+ was detected in a spectrophotometer at 340 nm and then compared with a standard curve made with 99% (*v*/v) analytical grade ethanol.

Table 1
Bioprospecting for sucrose-accumulating microalgae strains.

Strain	Dry weight ($g \cdot L^{-1}$)	Sucrose (% DW)	Sucrose productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	Strain origin
Ankistrodesmus sp. A14	1.39 ± 0.06	5.42 ± 0.69	18.80 ± 3.36	[25]
Ankistrodesmus sp. LP-1	1.01 ± 0.37	3.64 ± 0.51	9.66 ± 4.68	[24]
Ankistrodesmus sp. SP2–15	1.34 ± 0.12	5.20 ± 0.26	17.34 ± 0.72	[24]
Chlorella sorokiniana sp. *RP*	1.36 ± 0.29	4.57 ± 0.39	15.79 ± 4.60	[24]
Chlorella sp. CH	1.34 ± 0.12	5.34 ± 0.73	18.16 ± 4.04	[24]
Chlorella sp. MI	1.05 ± 0.28	4.13 ± 0.51	11.26 ± 4.29	[24]
Chlorella sp. Prm	1.06 ± 0.20	7.26 ± 2.34	20.38 ± 9.86	[25]
Chlorella sp. Rys	1.37 ± 0.03	3.98 ± 0.89	13.54 ± 2.74	[25]
Chlorella sp. SP2–1	1.24 ± 0.20	5.34 ± 0.22	16.63 ± 3.35	[24]
Chlorophyta sp. C1	1.51 ± 0.16	7.19 ± 1.71	26.04 ± 3.21	[24]
Chlorophyta sp. L-20	1.30 ± 0.35	5.58 ± 1.18	19.22 ± 8.76	[24]
Chlorophyta sp. SP1-20	1.69 ± 0.22	4.79 ± 0.84	19.79 ± 0.93	[24]
Chlorophyta sp. MH	1.08 ± 0.31	6.81 ± 0.44	17.99 ± 4.04	[24]
Coelastrella sp. P2	1.09 ± 0.13	8.84 ± 0.83	23.75 ± 0.75	[25]
Desmodesmus sp. A1	0.44 ± 0.01	10.49 ± 4.32	11.89 ± 5.17	[25]
Desmodesmus sp. FG	1.40 ± 0.23	4.76 ± 1.01	17.29 ± 6.28	[24]
Desmodesmus sp. P1	1.40 ± 0.001	5.98 ± 0.55	20.93 ± 1.94	[25]
Desmodesmus sp. P13	1.43 ± 0.13	6.43 ± 0.35	23.04 ± 3.34	[25]
Desmodesmus sp. P5	1.54 ± 0.40	10.94 ± 1.11	40.67 ± 6.23	[25]
Desmodesmus sp. P7	1.59 ± 0.15	7.34 ± 0.04	29.09 ± 3.01	[25]
Haematococcus pluvialis HP	1.67 ± 0.18	6.10 ± 0.21	25.56 ± 3.59	[24]
Haematococcus pluvialis	0.32 ± 0.06	3.86 ± 1.15	2.88 ± 0.32	UTEX 2505
Pseudokirchneriella sp. C1D	1.33 ± 0.28	7.68 ± 0.44	25.27 ± 3.91	[24]
Pseudokirchneriella sp. F21	1.73 ± 0.07	5.10 ± 0.23	21.99 ± 0.14	[25]
Pseudokirchneriella sp. P23	1.70 ± 0.10	5.49 ± 0.51	23.43 ± 3.51	[25]
Scenedesmus obliquss sp. C1S	1.14 ± 0.30	5.31 ± 1.41	16.23 ± 8.04	[24]
Scenedesmus sp. F15	2.11 ± 0.01	7.80 ± 0.30	41.09 ± 1.41	[25]
Scenedesmus sp. P31	1.45 ± 0.28	4.81 ± 1.79	16.21 ± 3.13	[25]
Scenedesmus sp. RD	1.51 ± 0.19	2.81 ± 0.85	11.01 ± 4.53	[24]
Selenastraceae sp. A3	1.49 ± 0.08	6.35 ± 1.65	23.38 ± 4.86	[25]

2.4. Statistical analysis

To analyze the statistical significance of the difference in sucrose productivity in the experiment of salt stress priming, a *t*-test was performed using Sigma-Plot (Systat Software, Inc., US).

3. Results and discussion

3.1. Bioprospecting for sucrose-accumulating microalgal strains

Based on previous analyses of the properties of microalgal strains native to the Central [24] or Northern [25] regions of Argentina, we selected 29 freshwater strains, representing most genera of Chlorophyta, to identify sucrose-accumulating strains. Initially, all strains were challenged with a moderate salt stress at 150 mM NaCl for 2 days. Strains accumulated sucrose in the range of 2.8% to 11% (*w*/w) of their dry biomass (Table 1). While most of the strains accumulated sucrose at about 5% (w/w), only a few accumulated it over 10% (w/w). However, high sucrose accumulation does not necessarily translate into high sucrose productivity since it also depends on biomass productivity, which is affected by the strain's tolerance to salt stress. Under the standardized sucrose accumulation conditions used in this study (4 days, two under salt stress), the most productive strains were *Desmodesmus* sp. P5 and *Scenedesmus* sp. F15, which accumulated 41 mg $sucrose \cdot L^{-1} \cdot day^{-1}$ and a final sucrose accumulation level in the biomass of 11 ± 1% (*w*/w) and 7.8 ± 0.3% (w/w), respectively (Table 1).

Strains were cultivated in the presence of 10 mM $NaNO_3$ for 48 h and induced with 150 mM NaCl for another 48 h. Data represent the mean and range of two independent experiments.

Sucrose accumulation and sucrose metabolizing enzymes were described decades ago in some Chlorophytes [33]. However, to the best of our knowledge, the present study represents the first systematic effort to investigate the accumulation of sucrose in green algae. A literature survey indicated *Neochloris oleoabundans* as one of the most thoroughly studied strains with respect to sucrose accumulation as a response to salt stress [34]. This strain accumulates up to 4.8% (*w*/w) sucrose in its biomass [35], an average sucrose accumulator when compared to the strains analyzed in this study.

None of the genomes of the algal strains used in this study have been sequenced yet to allow a direct comparison of genes for sucrose metabolism. However, we have confirmed by BLAST analysis [36] the presence of sequences homologous to plant and cyanobacterial sucrose-phosphate synthase and sucrose synthase genes [15] in the available draft genomes comprising most Chlorophytes (https://greenhouse.lanl.gov/greenhouse/organisms/). These results tend to confirm that sucrose metabolism in green algae would be as ubiquitous as it is in plants and cyanobacteria [15]. Furthermore, recent transcriptomic analyses showed up-regulation of sucrose metabolism genes in *Chamydomonas reinhardtii* [37] and *N. oleoabundas* [34] as a response to NaCl stress.

We previously showed that genetic engineering of a model cyanobacterium was necessary to increase sucrose accumulation up to 10% (*w*/w) [23]. In the present study we identified promising native strains which accumulated about the same content of sucrose relative to transgenic cyanobacteria. As a sucrose-producing platform, native strains would represent an alternative likely to face fewer regulatory hurdles when compared to genetically engineered strains regarding potential environmental impacts [38].

3.2. Effect of salt stress on microalgal growth and biochemical composition of the biomass

Both *Desmodesmus* sp. P5 and *Scenedesmus* sp. F15, displaying the highest sucrose productivity at 41 mg $sucrose \cdot L^{-1} \cdot day^{-1}$, and *Pseudokirchneriella* sp. C1D, as an example of a moderate accumulator (25 mg $sucrose \cdot L^{-1} \cdot day^{-1}$) were selected for a more detailed analysis.

As expected for freshwater strains, higher salt concentrations negatively affected growth as ascertained by a decrease in OD_{750} (Fig. 1), an increase in doubling time, and a reduction of dry biomass production after 8 days (Table 2). Fig. 2 and Supplementary Fig. S1 show a time course of the effect of NaCl on the biochemical composition of the

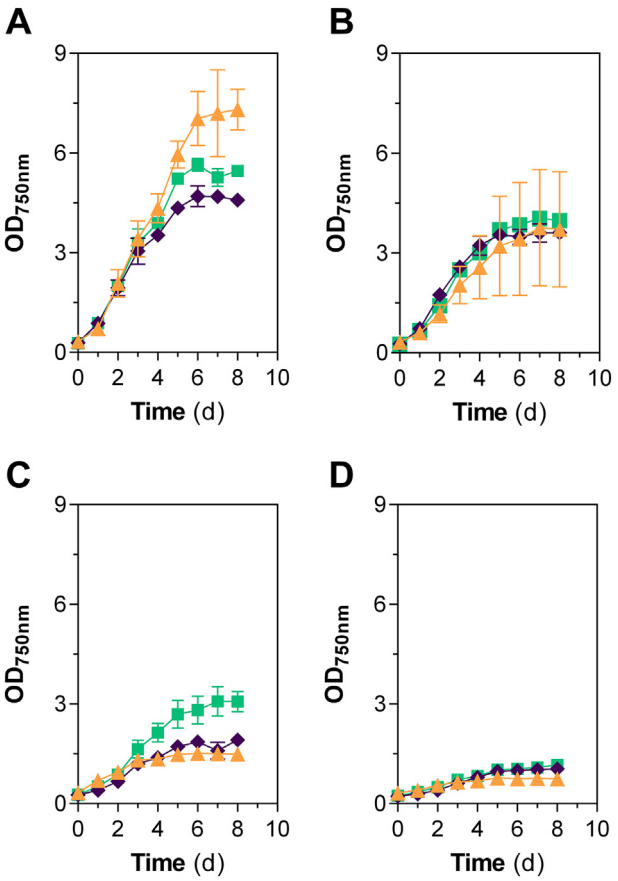

Fig. 1. Growth curves of (▲) *Pseudokirchneriella* sp. C1D, (■) *Desmodesmus* sp. P5 and (♦) *Scenedesmus* sp. F15 after 8 days of culture in BG11 medium containing 10 mM $NaNO_3$ and (A) 0 mM, (B) 100 mM, (C) 200 mM, or (D) 400 mM NaCl. Data represent the mean and range of two independent experiments.

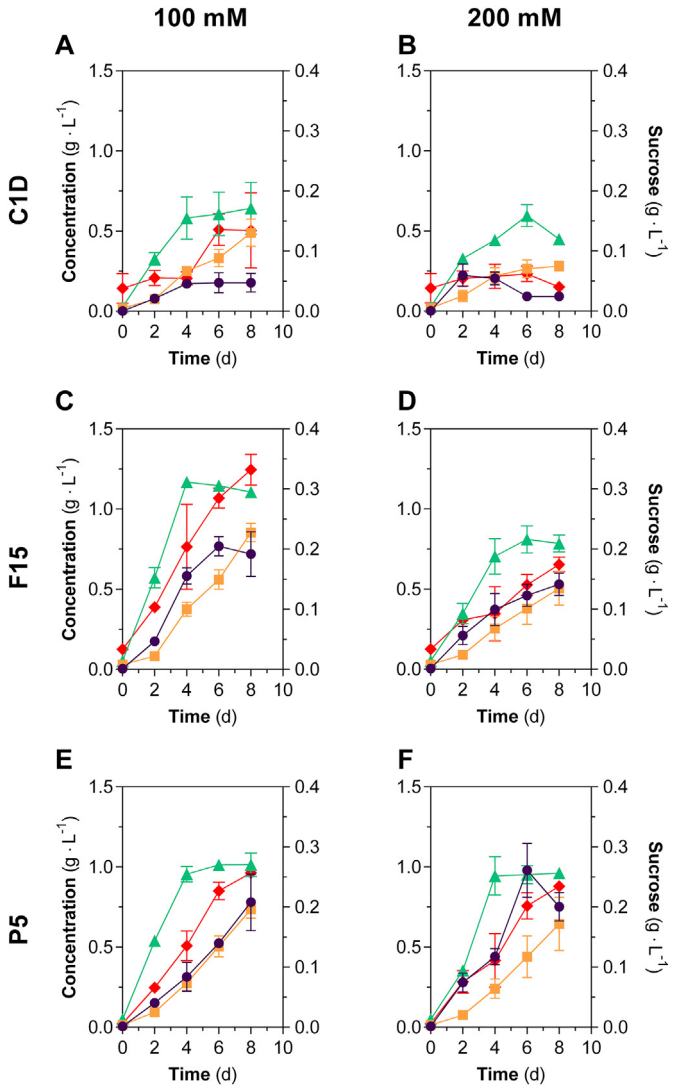

Fig. 2. Time course of (▲) proteins, (♦) total carbohydrates, (●) sucrose and (■) lipids accumulation in (A, B) *Pseudokirchneriella* sp. C1D; (C, D) *Scenedesmus* sp. F15; or (E, F) *Desmodesmus* sp. P5, in the presence of (A, C, E) 100 mM, or (B, D, F) 200 mM NaCl. Data represent the mean and range of two independent experiments.

biomass of the selected microalgal strains. Generally, under non-stressing conditions all strains produced mainly crude protein and carbohydrates (Supplementary Fig. S1 A, C, E). To estimate biomass enrichment in each of the macromolecular fractions, total protein or carbohydrates productivities were normalized by culture density (OD_{750}). For all strains, these fractions remained mostly constant, independently of the salt concentration (Supplementary Fig. S2). Thus, volumetric productivity of these macromolecules was mostly constrained by the effect of NaCl on microalgal growth and overall biomass

Table 2
Characterization of growth and final biomass composition of selected strains.

Strain	NaCl (mM)	Doubling time (d)	Dry weight ($g \cdot L^{-1}$)	Protein (% DW)	Lipid[a] (% DW)	Lipid[b] (% DW)	Carbohydrate (% DW)	Sucrose (% DW)
Pseudokirchneriella sp. C1D	0	0.8 ± 0.2	2.38 ± 0.04	46.3 ± 7.0	30.8 ± 3.5	31.3 ± 0.9	47 ± 2	2.6 ± 0.3
	100	1.3 ± 0.2	1.4 ± 0.4	45.1 ± 0.6	35.2 ± 3.4	35 ± 2	33 ± 8	3.2 ± 0.2
	200	1.6 ± 0.1	0.85 ± 0.05	53.1 ± 4.9	33.1 ± 1.4	42 ± 2	18 ± 3	2.9 ± 0.6
	400	2.8 ± 0.9	0.4 ± 0.1	40.39[c]	21.8 ± 1.2	N/D	16 ± 10	6 ± 1
Desmodesmus sp. P5	0	0.9 ± 0.2	2.62 ± 0.02	42.9 ± 1.0	31.0 ± 2.6	24 ± 3	40 ± 4	4.2 ± 0.2
	100	1.06 ± 0.03	2.53 ± 0.02	40.0 ± 2.5	29.0 ± 2.3	28 ± 2	38.1 ± 0.4	8 ± 2
	200	1.2 ± 0.1	2.5 ± 0.2	38.8 ± 3.0	25.4 ± 4.0	29 ± 1	35 ± 3	8.0 ± 0.2
	400	2.3 ± 0.1	1.63 ± 0.02	33.3 ± 0.5	26.3 ± 3.6	29 ± 2	37 ± 3	9.1 ± 0.2
Scenedesmus sp. F15	0	0.81 ± 0.05	2.66 ± 0.07	28.5 ± 2.5	26.5 ± 4.4	26.6 ± 0.2	51 ± 4	2.50 ± 0.06
	100	0.77 ± 0.08	2.82 ± 0.04	39.2 ± 0.4	30.3 ± 2.4	28.62 ± 0.03	44 ± 3	7 ± 1
	200	1.3 ± 0.1	1.8 ± 0.1	42.6 ± 0.3	27.1 ± 2.3	28 ± 3	35.5 ± 0.3	7.6 ± 0.6
	400	2.1 ± 0.1	1.43 ± 0.03	37.0 ± 2.5	27.2 ± 2.7	33 ± 1	34 ± 1	5 ± 3

Samples were collected after 8 days of culture.
Data represent the mean and range of two independent experiments.
[a] Determined with the SPV method.
[b] Determined gravimetrically.

productivity (Fig. 1). Conversely, during salt stress, algal cells shifted towards sucrose and lipid accumulation (Fig. 2 and Supplementary Figs. S1 and S2). Although sucrose enrichment in the biomass was maximal at 400 mM NaCl in all strains (Table 2 and Supplementary Fig. S2J-L), sucrose productivity tended to decline at higher salt concentrations and longer times (Supplementary Fig. S3). Maximum sucrose productivities were 72 mg sucrose·L^{-1}·day^{-1} for *Desmodesmus* sp. P5 at 200 mM from day 4 to 6; 54 mg sucrose·L^{-1}·day^{-1} for *Scenedesmus* sp. F15 at 100 mM NaCl from day 2 to 4; and 29 mg sucrose·L^{-1}·day^{-1} *Pseudokirchneriella* sp. C1D at 200 mM NaCl from day 0 to 2 (Supplementary Fig. S3). As a reference, sucrose productivity of *N. oleoabundans* was 7.1 mg sucrose·L^{-1}·day^{-1} when induced with a NaCl increase of 400 mM from day 0 to 1, as recalculated from Band and co-workers (1992) [35]. However, the difference in culture conditions between both studies prevents a more direct comparison.

3.3. Combined effect of NaCl and N on co-production of sucrose and lipids

A previous work by our group showed that *Desmodesmus* sp. P5 is an oleaginous strain [25]. Here, we showed that this strain can also accumulate sucrose under moderate-to-high NaCl stress (Tables 1 and 2, Figs. 2 and Supplementary Fig. S1). Hence, we chose it to analyze the possibility of co-producing sucrose and lipids.

Since it is known that lipids accumulation in microalgae is normally triggered by N deficiency [39], we analyzed the combined effect of NaCl and $NaNO_3$ on sucrose and total lipids accumulation by this strain. While the effect of N-deficiency on lipids accumulation was more noticeable under non salt-stressing conditions (Fig. 3 A, C, E, G), accumulation levels tended to be almost identical when cells grew in the presence of 200 mM NaCl, regardless of the availability of N (Fig. 3 B, D, F, H). Additionally, sucrose productivity was attenuated under N-deficiency (Fig. 3 A-D) due to a decrease in both biomass productivity (Table 3 and Supplementary Fig. S4 A-B) and accumulation of the sugar in the biomass (Table 3 and Supplementary Fig. S5 A-D). These results indicated that, under salt-stressing conditions, N-sufficiency ensures a reasonably high productivity of both lipids and sucrose (Table 3 and Fig. 3 F, H).

3.4. Effect of acclimation to NaCl on sucrose and lipids productivity

It is generally acknowledged that priming by prior exposure to an eliciting factor makes organisms more tolerant to future stress exposure [40]. Thus, we analyzed this aspect by culturing *Desmodesmus* sp. P5 first in the presence of 100 mM NaCl (acclimation) and then increasing NaCl concentration to 400 mM (salt-stress). A three-day acclimation period was chosen since maximum growth rate is maintained for that period, at 100 mM NaCl (Fig. 1B). By doing this, we sought to maximize sucrose productivity per volume and days of culture. After 8 days of culture in the presence of 3 mM $NaNO_3$, sucrose productivity of non-acclimated microalgae was 70 mg·L^{-1} in the presence of 100 mM (Fig. 4A) or 200 mM (Fig. 3D), or 100 mg·L^{-1} at 400 mM NaCl (Fig. 4B). Acclimated and stressed cells produced 130 ± 8 mg sucrose·L^{-1} (Fig. 4C) or 279 ± 29 mg sucrose·L^{-1} (Fig. 4D) when supplied with 3 or 10 mM $NaNO_3$, respectively; showing a an statistically statistically significant difference between N-deficiency and sufficiency (*t*-test, $p < .001$). Thus, N sufficiency enabled a 2-fold higher sucrose productivity, and acclimation to NaCl an additional 30% increase. Standardizing these data to the culture's cell density confirm sucrose accumulation under these conditions (Supplementary Fig. S6). Acclimation to NaCl decreased lipid productivity by 23% or 38% in the presence of 3 mM (Fig. 4C) or 10 mM (Fig. 4D) $NaNO_3$, respectively, Supplementary Fig. S6.

Induction of sucrose accumulation in microalgae has been reported to take place soon after the onset of salt stress [34,35]. Rapid accumulation of sucrose in *Chlorella emevsonii* exposed to salt stress appeared to be independent of photosynthesis [41]. However, priming by a previous exposure to a low-stressing NaCl concentration appeared to result in an increased sucrose productivity by enabling a higher overall biomass productivity. Short-term acclimation of *C. reinhardtii* cells to 200 mM NaCl for 2 days resulted in upregulation of genes involved in the stress response, glycerophospholipid signaling, and genes for the transcription and translation machinery [37].

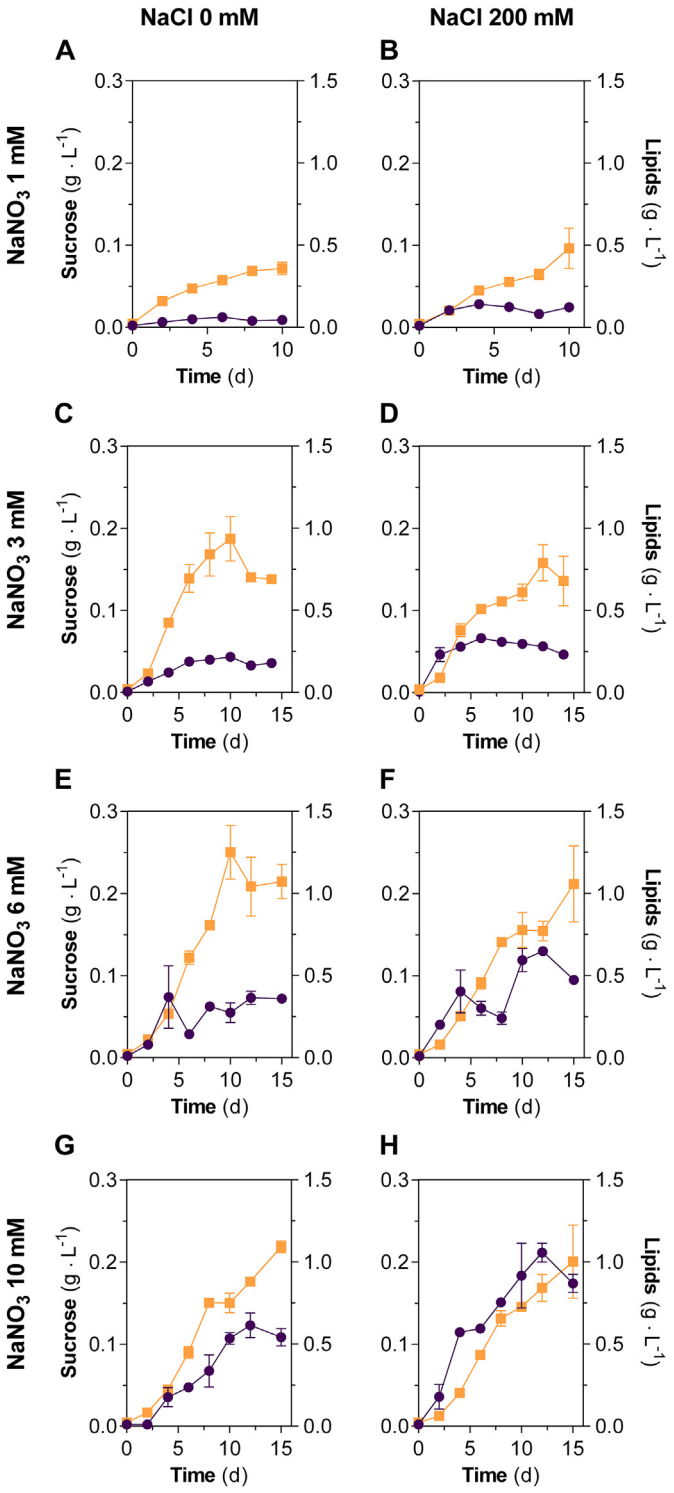

Fig. 3. Time course of (●) sucrose and (■) lipids accumulation of *Desmodesmus* sp. P5 cultivated in the presence of (A, C, E, G) 0 mM or (B, D, F, H) 200 mM NaCl and at the expense of (A, B) 1 mM; (C, D) 3 mM; (E,F) 6 mM; or (G, H) 10 mM $NaNO_3$. Data represent the mean and range of two independent experiments.

Table 3
Effect of $NaNO_3$ and NaCl concentration on biomass composition of *Desmodesmus* sp. P5.

$NaNO_3$ (mM)	NaCl (mM)	Dry weight ($g \cdot L^{-1}$)	Sucrose (% DW)	Lipids[c] (% DW)	Lipids[d] (% DW)
1[a]	0	0.88 ± 0.04	1.3[e]	40 ± 2	27 ± 5
	200	0.92 ± 0.02	2.64 ± 0.01	37.88[e]	22 ± 8
3[b]	0	2.01 ± 0.04	1.8 ± 0.11	35.62 ± 0.05	ND
	200	1.90 ± 0.04	2.6 ± 0.1	38 ± 10	ND
6[b]	0	2.87 ± 0.01	2.52[e]	37 ± 4	25 ± 2
	200	2.92 ± 0.03	3.25 ± 0.06	36 ± 7	35.9 ± 0.5
10[b]	0	4 ± 1	2.7 ± 0.4	28 ± 8	17 ± 5
	200	3.18 ± 0.08	5.5 ± 0.5	31 ± 6	31.53

ND - not determined. Data represent the mean and range of two independent experiments.
[a] 10 days of culture.
[b] 15 days of culture.
[c] Determined with the SPV method.
[d] determined gravimetrically.
[e] Single determination.

3.5. Effect of acclimation and induction of sucrose and lipids production by seawater

Algal and cyanobacterial acclimation to NaCl have attracted considerable attention as a model system to better understand survival of organisms in saline environments and to gather insights towards the development of biotechnological alternatives to increase crops productivity in saline soils [42]. However, the real challenge for algal sucrose production would be to study the induction of its accumulation in freshwater microalgae with seawater [43,44]. In addition to NaCl, seawater contains essential elements such as calcium, magnesium and potassium salts of sulfate and carbonate in a complex buffer system which normally supports microalgal growth [43].

Desmodesmus sp. P5 cells were acclimated in BG11 medium containing 100 mM NaCl and 10 mM $NaNO_3$ for 3 days and then supplemented with one or three volumes of the same culture medium but prepared in artificial seawater. The final salt concentration was around 350 mM or 475 mM, respectively. After 9 days, these cultures produced dry biomass at 3.9 ± 0.3 $g \cdot L^{-1}$ or 3.0 ± 0.2 $g \cdot L^{-1}$; and sucrose at 6.3 ± 0.7% (*w*/w) or 10.3 ± 0.4 (w/w), respectively. However, regardless of the almost two-fold increase of sucrose in the biomass of cultures containing a higher fraction of seawater, the overall sucrose productivity was fully compensated due to the higher biomass productivity of cultures induced with one volume of seawater (Fig. 5A and B).

3.6. Sugar extraction from biomass and production of ethanol by fermentation

We have previously optimized two methods for sugar extraction: one consisted in drying and milling (D&M) the biomass, followed by an aqueous extraction at room temperature (about 22 °C) and the other one was based on a microwaves (MW) treatment of the wet biomass [23]. Both methods allowed complete sucrose recoveries from the biomass of *Desmodesmus* sp. P5 at efficiencies of 145 ± 5% and 154 ± 10%, respectively. These results indicate that the applied methods (i.e. D&M or MW) are more efficient in extracting sucrose than the one used to determine the sucrose content in whole biomass. Higher concentrations of sucrose in the syrup could be obtained using the D&M method (up to 3% (*w*/*v*)), compared to the 1.5% (w/v) obtained by the MW method (Fig. 5C). An alternative strategy that has been improved over the last years involved over- and/or down-regulation of appropriate sucrose-metabolism genes and a sucrose exporter in cyanobacteria, for a final concentration of sucrose in the spent medium of 0.6% (w/v) [45].

Sugar syrups prepared by either D&M or MW methods, were quantitatively converted into ethanol by fermentation with *S. cerevisiae* at 15 ± 1 $g \cdot L^{-1}$ or 7.8 ± 0.3 $g \cdot L^{-1}$, respectively (Fig. 5C). The efficiency of sucrose conversion into ethanol in 24 h was of 99% or 110% of the maximum theoretical value of 0.51 g ethanol · g glucose^{-1}, respectively. This is likely due to the presence of other fermentable sugars in the syrups.

High concentration of fermentable sugars in the syrup, minimally in the range of 8% (w/v) is needed for the production of 40 g ethanol · L^{-1}, which can be recovered by distillation at a competitive cost [46]. Thus, the present study represents a step forward towards the production of fermentation feedstocks from algal biomass without the need of biomass hydrolysis with acids and/or enzymes, promoting environmental safety and economic viability. This study focuses on its use for the production of ethanol; however, it can be used for the production of most fermentation products.

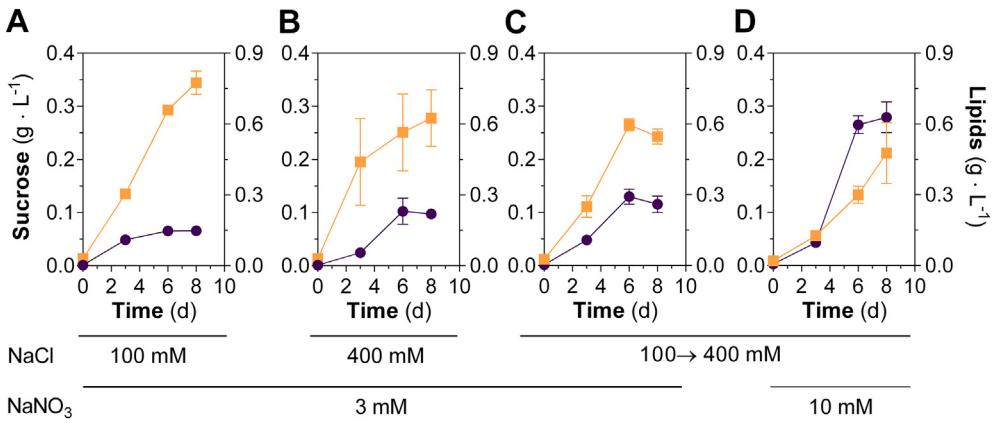

Fig. 4. Time course of (●) sucrose and (■) lipids accumulation of *Desmodesmus* sp. P5 grown at the expense of (A–C) 3 mM or (D) 10 mM $NaNO_3$, and in the presence of (A) 100 mM NaCl, (B) 400 mM NaCl, or (C, D) acclimated to 100 mM NaCl and then induced with 400 mM NaCl. Data represent the mean and standard deviation of two or three independent experiments.

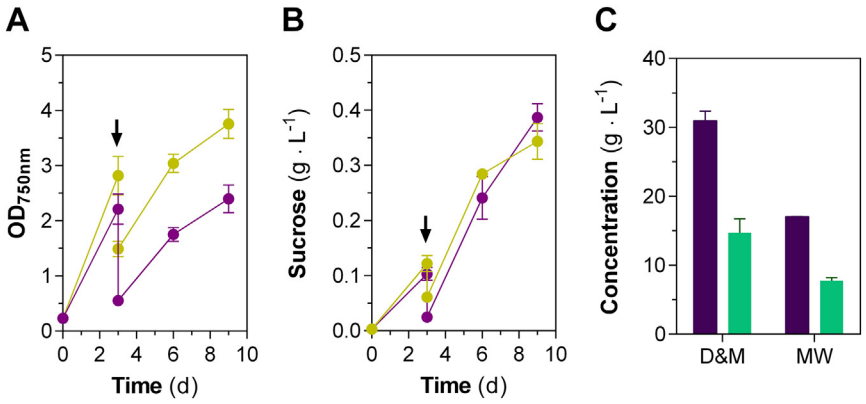

Fig. 5. (A) Growth curves of *Desmodesmus* sp. P5; and (B) time course of sucrose accumulation when cultures were supplemented with (●) 1 or (●) 3 volumes of BG11 medium prepared in seawater (ASWO). Arrow indicates supplementation time point. Data point at day 2 after dilution in panel B was calculated, and not determined. Data represent the mean and standard deviation of three or four independent experiments. (C) Fermentation of sucrose extracts obtained by the drying and milling (D&M) or the microwaves-based (MW) methods: (■) Initial sucrose concentration in the extracts and (■) ethanol produced after 24 h of fermentation by *S. cerevisiae*. Data represent the mean and range of two independent experiments.

3.7. Algal biomass biorefinery for the recovery of other cellular fractions

The microalgal strains used in this study, especially *Desmodesmus* sp. P5 accumulated lipids at about 30% (*w*/w) as a result of mild induction by NaCl (Tables 2 and 3 Supplementary Fig. S5), even in the presence of otherwise repressing concentrations of $NaNO_3$. This aspect is very interesting since N-deficiency largely increases lipids content in the biomass but offsets lipids productivity due to the decreasing algal proliferation and biomass production (Supplementary Fig. S4) [24,39,47].

It is broadly claimed that separation of multiple co-products in biomass biorefineries would largely improve third generation biofuels profitability [12]. The non-destructive methods used for separation of soluble sugars and crude protein would leave insoluble polysaccharides and lipids in the leftover biomass for further isolation by conventional methods for the extraction of feedstocks for biofuels and/or other applications. As recently proposed [23], separation of crude protein from the extracted aqueous phase by short pulses of heat could find applications as a feed supplement.

3.8. Simulation of biomass and sucrose productivity in environmental photobioreactors

To get a reasonably reliable model of ethanol productivity from microalgal biomass cultured in diluted seawater, we run *Desmodesmus* sp. P5 cultures in Phenometrics environmental Photobioreactors™ (ePBRs). ePBRs control LED-light intensity and temperature from a script of the corresponding weather variables for simulation of microalgal growth in open raceway ponds. These devises allow a low-risk and cost-effective way of modeling microalgal productivity at any geographical location [26]. Thus, we modeled biomass and sucrose productivity of *Desmodesmus* sp. strain P5 in Fortaleza (Brazil) in the summer season, which has been predicted as a potentially high productivity region by a mathematical model [48]. Similar to the continuous light experiments, cells were allowed to acclimate and grow in simulated 5 cm-depth ponds containing 25% seawater for 2 days, at which an OD_{750} of 2.3 was achieved. Preliminary experiments indicated that growth rate slowed down soon after and negatively affect final sucrose productivity (data not shown). Then, the simulated ponds were flooded with half or one volume of seawater supplemented with BG11 nutrients, including 10 mM $NaNO_3$ for further induction of sucrose accumulation. These conditions corresponded to final proportions of seawater of 50% (*v*/v) (300 mM salt) or 63% (v/v) (375 mM salt), respectively. As expected, a higher proportion of seawater resulted in slower growth (Fig. 6A) and a lower final biomass concentration, also due to an increased dilution. Final dry biomass concentration after 4 days of culture was 1.69 ± 0.08 $g{\cdot}L^{-1}$ or 1.14 ± 0.06 $g{\cdot}L^{-1}$ for cultures at 50% (v/v) or 63% (v/v) seawater, respectively. Sucrose production was largely enhanced by dilution with seawater (Fig. 6B) for a final concentration in the dry biomass of 10.1 ± 0.7% (*w*/w) or 8.3 ± 0.4% (w/w) for cultures induced with 50% (v/v) or 63% (v/v) seawater, respectively. This short-cycle cultivation program, comprising a first step for biomass production and acclimation to salt and a second step for enhanced induction of sucrose accumulation resulted in the highest productivity of sugar of the platform. The calculated biomass and sucrose productivities for the 50% (v/v) seawater model were 18.0 ± 0.8 $g \cdot m^{-2}{\cdot}day^{-1}$, and 1.79 ± 0.06 $g{\cdot}m^{-2}{\cdot}day^{-1}$, respectively, which can be extrapolated to 66 $MT{\cdot}ha^{-1}{\cdot}year^{-1}$ or 6.5 $MT{\cdot}ha^{-1}{\cdot}year^{-1}$ (Fig. 6C), assuming a very low seasonal variation in productivity in the modeled location, according to public weather historical records (https://power.larc.nasa.gov). Additionally, the 50% (v/v) seawater model would allow lower biomass collection costs due the higher biomass concentrations achieved when compared to the 63% (v/v) seawater model [49].

As demonstrated in this study, sucrose can be extracted from *Desmodesmus* sp. strain P5 biomass by mild methods, bypassing the need of hazardous chemicals and/or expensive enzymes for biomass saccharification, and converted into ethanol by common fermentation technology at a 100% efficiency. This would render an alcohol productivity of about 4200 L $ethanol{\cdot}ha^{-1}{\cdot}year^{-1}$, which compares very well with productivity from sugarcane or sugar beet at 6800 L $ethanol{\cdot}ha^{-1}{\cdot}year^{-1}$ or 5100 L $ethanol{\cdot}ha^{-1}{\cdot}year^{-1}$, respectively [50].

Prospects of this alternative would be remarkable for different reasons. *i*) This platform relies mostly on the use of seawater for a considerable improvement of the water footprint of agricultural production; *ii*) it avoids the use of chemicals and/or enzymes for saccharification; and *iii*) alcohol production from about 10% of the biomass, leaves the rest of the cellular fractions (crude protein, polysaccharides and lipids) available as feedstocks for biofuels, feed, or other applications. Recently, we presented results that suggested that fermentation of carbohydrate-rich microalgal biomass, saccharified with diluted H_2SO_4 at 120 °C produces at least 7600 L $ethanol{\cdot}ha^{-1}{\cdot}year^{-1}$. This represents a promising alternative to corn kernel bioethanol production at typical productivities of about 4000 L $ethanol{\cdot}ha^{-1}{\cdot}year^{-1}$ [8]. That same report showed full recycling of the ethanol fermentation vinasse as nutrients for the cultivation of microalgae, enabling savings in fertilizers and lowering the environmental impact of the proposed production platform [8].

Nevertheless, and beyond the likely high potential productivity of the proposed platform and the advantages of circumventing biomass saccharification, more general cost associated with production of algal biomass would still need to be improved for economic feasibility compared to sugarcane or corn ethanol [12].

4. Conclusion

This study identifies useful microalgal strains and provides optimized conditions for sucrose production using seawater. Independence from saccharification and efficient conversion into ethanol by mild methods at a modeled productivity of 4200 L $ethanol{\cdot}ha^{-1}{\cdot}year^{-1}$ would place this platform as an alternative to ethanol production from

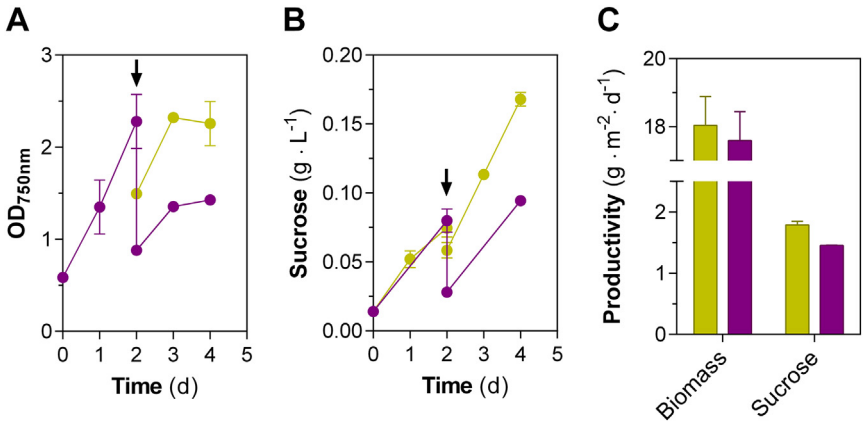

Fig. 6. (A) Growth curves of *Desmodesmus* sp. P5, (B) time course of sucrose accumulation, and (C) areal productivity when cultures were carried out in BG11 medium prepared in (●) 50% or (●) 63% seawater (ASWO). Arrow indicates supplementation time point. Data represent the mean and range of two independent experiments.

conventional crops and agricultural systems. Both the mild conditions used for sugar and protein extraction, and increased lipids productivity in seawater under N-sufficiency conditions would largely favor the development of algal biomass biorefineries towards economic feasibility.

Declaration of competing interest

None.

Acknowledgements

The authors are very grateful to M Do Nascimento for helpful discussions and N Almada for technical assistance. This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica, Argentina [grants numbers PICT2012-2589 and PICT2015-3559] to LC.

Contributions

MESS and CDC performed all experiments, analyzed results, prepared figures and collaborated in writing the manuscript. GLS contributed with funding. LC conceived and designed the experiments, supervised data analysis and interpretation, wrote the manuscript and contributed with funding.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101733.

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