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Bioprospecting for native microalgae as an alternative source of sugars for the production of bioethanol

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ABSTRACT

While the production of biofuels holds potential to contribute to energy security, concerns on food prices, land use, and carbon emissions have arisen from increased production of first-generation bioethanol. While second-generation bioethanol from lignocellulosic agricultural waste faces difficult-to-overcome technological barriers, renewed promise is held in microalgae biomass as an alternative feedstock. In this work we show the results of bioprospecting for microalgae native of South America for accumulation of carbohydrates under conditions of nitrogen deficiency, and constant light and temperature. After a preliminary analysis of seventeen strains, we selected strain SP2-3, because its biomass could be enriched in carbohydrates over 70% (w/w) on a dry biomass basis, and *Desmodesmus* sp. strain FG for its fermentable sugars productivity. After optimization of microalgae culture conditions, biomass hydrolysis and fermentation with baker's yeast *Saccharomyces cerevisiae*, we demonstrated ethanol yields of up to 0.24 g ethanol·g of biomass^{-1}, and an ethanol concentration in the fermentation broth of 24 g ethanol·L of fermentation broth^{-1}, for up to 87.4% of the maximum theoretical value. These results contribute to support the potential of microalgae biomass as an alternative feedstock for bioethanol and the value of bioprospecting programs to identified candidate strains among natural biodiversity.

1. Introduction

Today, the most common renewable fuel is ethanol, derived mainly from sucrose or starch sources of agricultural stocks such as sugarcane and corn [1–3]. However, the human demand for food, especially for the years to come is still not fully warranted and poses a serious concern on the use of these feedstocks for bioenergy purposes.

Microalgae have been considered a promising feedstock for the production of biofuels [2–4]. since they present some potential advantages in comparison with conventional plant crops such as (*i*) higher productivity of biomass per unit of surface and time (for example a 4- to 5-fold higher oil productivity than the most productive crop plants currently used as biodiesel feedstock has been demonstrated [5,6]; *ii*) non competitiveness for land or food market with crops, since can be produced even on non-arable land [7]; (*iii*) better economy of water and nutrients through effective recycling [8]; (*iv*) possibility of using residues from industries as a source of inexpensive nutrients, especially CO_2, N and P, which might assist in municipal or industrial waste management, and help to mitigate climate change and reduce the demand of fertilizers [8].

Several microalgal strains accumulate carbohydrates in excess of 40% of the dry biomass [9–13] mainly as insoluble starch and cellulose [13–14]. Although microalgal biomass is not readily accessible to common fermenting microorganisms, for example for the production of bioethanol, it would be potentially easier to convert it into monosaccharides in comparison with plant lignocellulosic materials mostly because of the lack of lignin. It has been shown that microalgal biomass can be hydrolyzed by chemical (acid or alkaline)/physicochemical or enzymatic hydrolysis [15,16]. Acid hydrolysis under high temperatures and pressures is faster, easier and cheaper than other types of hydrolysis, but may lead to decomposition of sugars into inhibitors of the fermentation process [3,15,16]. Conversely, enzymatic hydrolysis can be completed under mild temperatures and pressure, but it is slower, more expensive, and still requires physical or chemical pretreatments [3,15,16]. Proof of principle for the successful conversion of saccharified microalgal biomass into ethanol by ethanologenic microorganisms has been provided in several studies during the last years [17, Table 3 and references therein].

Nevertheless, up to what extent the potential of biofuels from microalgae could be achieved in the near future is still a matter of debate. This is mostly because techno-economic modeling for biofuels from microalgae according to the available technologies and strains

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consistently support the conclusion that the production of algae biomass and its further processing into biofuels is currently too expensive and too energy intensive [18]. Meanwhile, microalgae strains selection has been identified as a key aspect to contribute to reduce production costs by increasing biomass and energy-carrier or target product content as well as other traits that would facilitate downstream processing of the biomass [19–23].

Thus, bioprospecting of indigenous microalgae is usually a starting point in the road map towards biofuels and other commodities from microalgal biomass. This is mostly because it is broadly assumed that robust native strains might be more suitable for outdoors cultivations since they might be naturally acclimated to the prevailing environmental conditions including successful displacement of potential competitors. Furthermore, mass cultivation of native strains might ameliorate the potential ecological risk associated with the introduction of exotic (or genetically engineered) strains and could also be favored by the public perception [20].

This report shows the results of bioprospecting microalgae native from southeastern Buenos Aires for carbohydrates productivity under laboratory conditions of nutrients deprivation and the biomass quality as an alternative feedstock for bioethanol after fermentation with *S. cerevisiae*. These results contribute to support the potential of microalgal biomass as an alternative source of sugars worldwide and more specifically for potential future local developments in Argentina.

2. Materials and methods

2.1. Culture of microalgal strains

The seventeen microalgal strains analyzed in this study have been previously isolated from different brackish or freshwater ecological areas in the surroundings of Mar del Plata, Buenos Aires, Argentina (38°0′0″S 57°33′0″W) during the four seasons from 2009 to 2010. All the strains belong to the Division Chlorophyta and are routinely maintained in our laboratory collection.

The strains were routinely cultivated in BG11 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_2CO3, 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 $mg \cdot L^{-1}$ $Co(NO_3)_2 \cdot 6H_2O$)), containing 0–12 mM $NaNO_3$ as a nitrogen source. When indicated cultures contained no source of P or S.

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 $L \cdot min^{-1}$ and illuminated with constant white light at 100 μmol photons $m^{-2} \cdot s^{-1}$. Alternatively, selected strains were cultivated in 5-L airlift 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 $m^{-2} \cdot s^{-1}$. Under both culture systems temperature was maintained constant at 28 ± 1 °C.

2.2. Microalgae biomass hydrolysis and fermentation

For the reference diluted acid hydrolysis of biomass around 5% microalgal biomass (w/v) was incubated in 2% H_2SO_4 (v/v) for 30 min at 120 °C in an autoclave (Table 2). For biomass hydrolysis optimization concentrated microalgal slurry was hydrolyzed in diluted acid

Table 1
Biomass composition of native microalgal strains.

Strain	Carbohydrate (% dwt)	Protein (% dwt)	Lipid (% dwt)	Cell dwt ($g \cdot L^{-1}$)
Pseudokirchneriella sp. strain C1D ($n = 2$)	40.5 ± 0.7	31.8 ± 1.3	nd	0.8 ± 0.1
Ankistrodesmus sp. strain SP_2-15 ($n = 2$)	47.3 ± 9.5	31.6 ± 2.3	nd	0.5 ± 0.0
Chlorella sp. strain SP_2-1 ($n = 2$)	44.4 ± 2.2	30.8 ± 5.4	nd	0.7 ± 0.5
CH ($n = 2$)	51.2 ± 8.1	24.0 ± 3.2	nd	0.6 ± 0.1
Scenedesmus obliquus strain C1S ($n = 2$)	29.9 ± 8.3	15.6 ± 1.2	49.9 ± 3.0	1.5 ± 0.9
Scenedesmus sp. strain SP_2-9 ($n = 2$)	52.9 ± 4.1	13.9 ± 1.9	14.3 ± 0.5	0.9 ± 0.0
Chlorella sorokiniana strain RP ($n = 2$)	49.8 ± 2.4	14.8 ± 1.8	nd	0.7 ± 0.2
Scenedesmus sp. strain L_2 ($n = 2$)	38.1 ± 5.8	19.3 ± 2.4	nd	0.7 ± 0.0
C_1 ($n = 2$)	51.5 ± 4.5	17.5 ± 2.1	nd	1.4 ± 0.7
C_1C ($n = 4$)	52.9 ± 6.0	19.4 ± 3.4	nd	1.0 ± 0.4
Desmodesmus sp. strain FG ($n = 4$)	53.5 ± 14.2	16.3 ± 6.9	nd	0.7 ± 0.0
Scenedesmus sp. strain SP_1-20 ($n = 4$)	46.1 ± 6.4	25.0 ± 4.8	nd	0.7 ± 0.3
Ankistrodesmus sp. strain LP1 ($n = 4$)	51.3 ± 9.0	28.9 ± 3.6	nd	0.5 ± 0.1
Scenedesmus sp. strain RD ($n = 4$)	41.9 ± 13.2	11.9 ± 4.3	nd	0.7 ± 0.1
Scenedesmus sp. strain PL ($n = 4$)	45.7 ± 10.1	18.0 ± 7.5	nd	0.9 ± 0.2
SP_2-3 ($n = 4$)	70.4 ± 8.2	17.6 ± 8.6	10.3 ± 2.6	0.6 ± 0.2
Chlorella sp. strain MI ($n = 4$)	57.8 ± 16.6	17.5 ± 8.2	11.0 ± 2.2	0.7 ± 0.3

Cultures were inoculated at OD_{660} of 0.2–0.3 in $BG11_0$ medium supplemented with 1 mM $NaNO_3$ and allowed to proliferate for 10 days for the analyses of biomass composition.

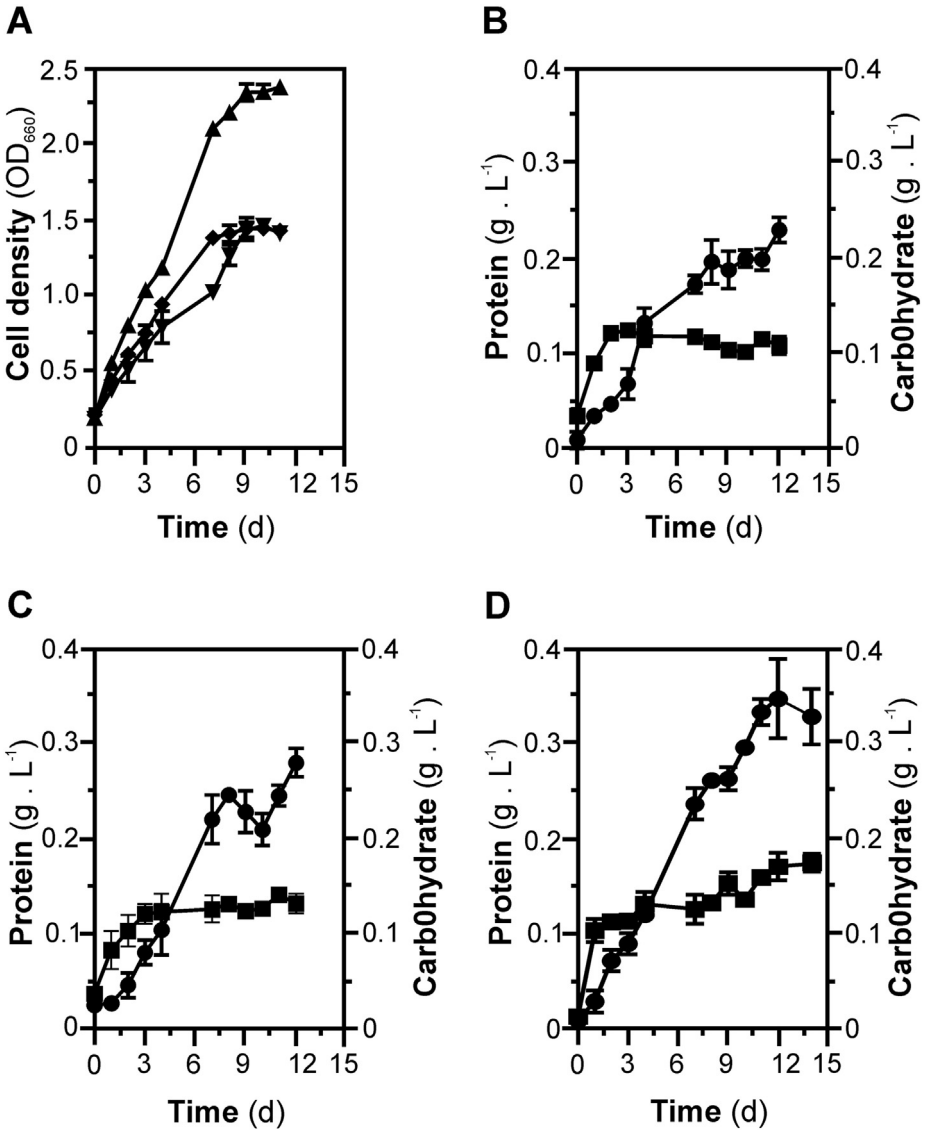

Fig. 1. Growth curve, protein and sugars content of different microalgae. (A) Microalgae growth. OD_{660}, optical density at 660 nm; (▲) *Desmodesmus* sp. strain FG; (▼) strain SP2-3; (♦) *Chlorella* sp. strain MI. (B–D) Microalgae biochemical composition; (B) *Chlorella* sp. strain MI; (C) strain SP2-3; (D) *Desmodesmus* sp. strain FG. (■) Protein; (●) carbohydrate. Strains were cultivated in BG11 medium supplemented with 1 mM $NaNO_3$. The data represent the mean and standard deviation of two independent experiments.

according to the following variables: 0%, 0.5%, 1%, 2% or 4% H_2SO_4 (v/v); biomass load of 2.5%, 5% or 10% (w/v); temperatures of 60 °C, 90 °C or 120 °C in an autoclave and incubation times of 0, 15, 30 or 60 min. Both solubilized sugars and the sugars remaining in the biomass were determined as indicated in Section 2.3. When indicated microalgal biomass hydrolysates were concentrated by lyophilization using a freeze dryer (Thermo, model ModulyoD).

Hydrolyzed preparations were brought to pH 5.5–6.0 with $Mg(OH)_2$ crystals and used directly or after concentration by freeze-drying for *S. cerevisiae* fermentation. Micro-fermentations (1 mL) were conducted by inoculating the saccharified preparations with *Saccharomyces cerevisiae* cells (Levex®, Argentina) at an initial OD_{600} of 0.25 and incubation in 3 mL vials sealed with foil (Parafilm®) finely perforated five times with a 25 G needle. For the standard fermentations (Table 2) ethanol was assayed from the spent medium at 30 h of incubation at 28 °C with shaking at 120 rpm in an orbital shaker. Each hydrolysate fermentation was routinely accompanied by a fermentation of YPD medium at a dextrose concentration in the range of sugar content of the samples.

Table 2
Ethanol production from microalgal biomass by *S. cerevisiae*.

Strain	Sugar utilization ($g \cdot L^{-1}$)[a]		Ethanol yield[b]		Sugar to ethanol (% of theoretical maximum)[a]
	$t = 0$ h	$t = 30$ h	($mg \cdot L$ culture^{-1})	($mg \cdot g$ biomass^{-1})	
Desmodesmus sp. FG	1.9 ± 0.2	0.1 ± 0.0	98 ± 30	134 ± 47	75 ± 15
SP2-3	1.1 ± 0.2	0.1 ± 0.0	37 ± 20	64 ± 28	84 ± 38
Chlorella sp. MI	2.2 ± 0.5	0.1 ± 0.0	61 ± 37	91 ± 55	60 ± 47

Data shown represent the mean and standard deviation of 3 ([a]) or 7 ([b]) independent experiments.

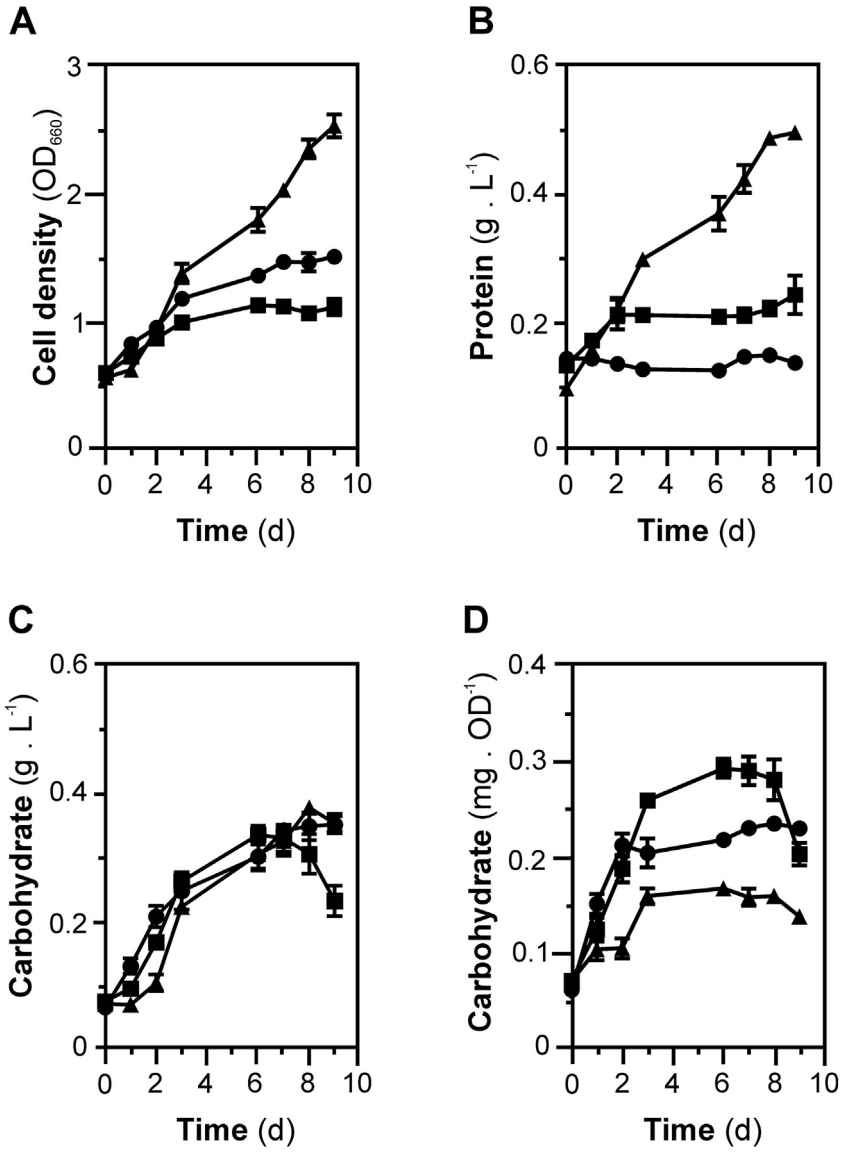

Fig. 2. Effect of N, S, or P deficiency on *Desmodesmus* sp. strain FG growth and accumulation of protein and carbohydrates. (A) Growth curve (OD_{660}); (B) accumulation of total protein; (C) accumulation of total carbohydrate; (D) mg carbohydrates·OD_{660}^{-1}. (●) N-deprivation; (▲) P-deprivation; or (■) S-deprivation. The data represent the mean and standard deviation of three independent experiments.

2.3. Analytical methods

For growth curves analysis, cell density was estimated by recording OD at 660 nm using a spectrophotometer. Microalgae biomass dry weight was determined from resuspended cells in the culture medium. Samples (50 mL of culture) were centrifuged at 14,000 ×*g* for 10 min and the pellets were dried out in an oven at 70 °C until constant weight (2–3 days). 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 [24]. Aliquots were subjected to protein determination by the Lowry's method [25] using NaOH-treated bovine serum albumin as a standard. For biomass total carbohydrates determination, resuspended cells were directly reacted with the Anthrone method reagents [26]. Carbohydrates content was calculated from a standard curve using glucose. Total lipids were determined gravimetrically after lipids extraction basically according to Bligh and Dyer [27] with modifications [20].

Ethanol was determined from the *S. cerevisiae* fermentation spent-medium by an enzymatic assay as reported by Bonnichsen and Theorell [28] with modifications. Partially purified *S. cerevisiae* alcohol dehydrogenase was obtained as reported before [29] with modifications. Preparations were obtained from cells cultivated in YDP medium overnight. Cells were disrupted by sonication in a buffer containing 20 mM Tris-HCl, pH 6.5; 5 mM $MgCl_2$; 0.4 mM ethylenediaminetetraacetic acid; 0.5 mM phenylmethylsulfonyl fluoride; 5 mM β-mercaptoethanol and extracts were clarified by centrifugation at 14,000 ×*g* for 15 min. Cell free extracts were incubated at 55 °C for 15 min and precipitated proteins were discarded after centrifugation at 14,000 ×*g* for 15 min. Ice cold acetone was slowly mixed with the supernatant at 0 °C and the partially purified alcohol dehydrogenase fraction corresponded to proteins precipitated from 40 to 50% (v/v) acetone. The standard ethanol assays contained 50 mM Tris-HCl, pH 8.4; 2.5 mM NAD^+ and 3 μg protein of alcohol dehydrogenase preparations and the samples in a total volume of 100 μL and were 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.

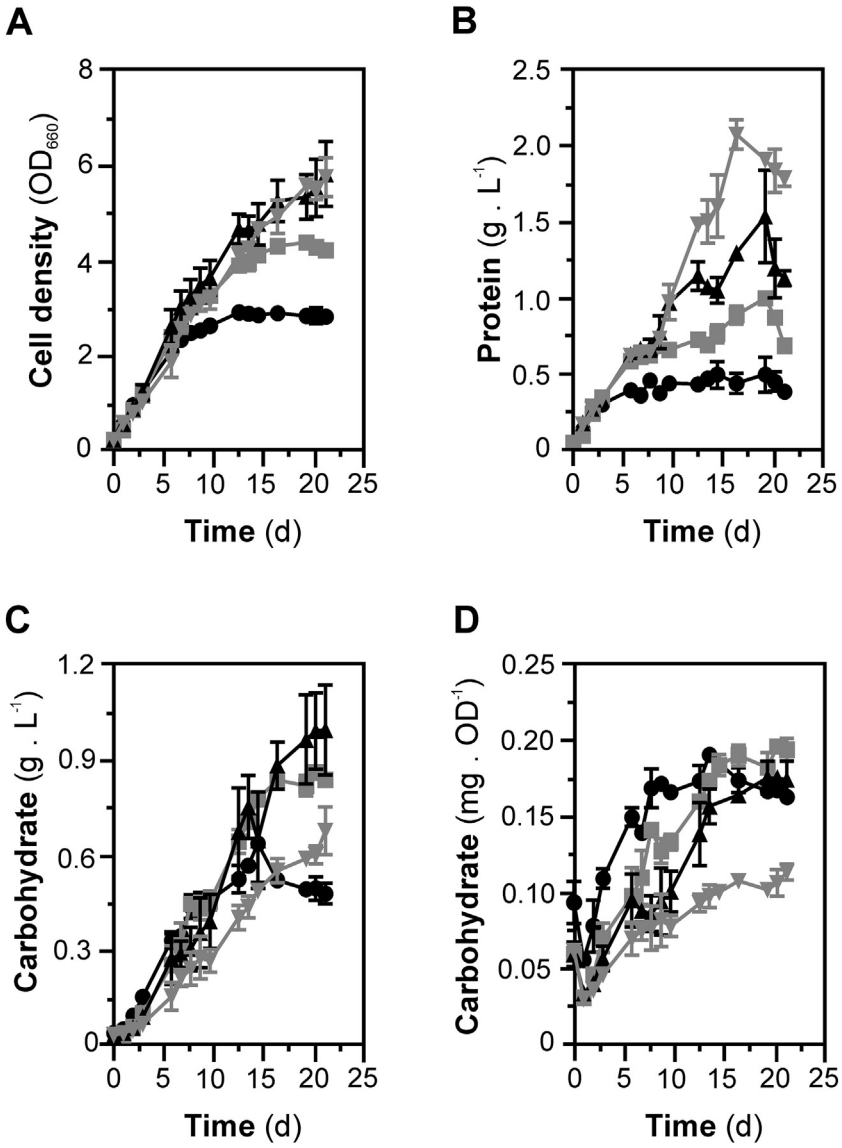

Fig. 3. Effect of different levels of N-deficiency on growth and carbohydrates accumulation of *Desmodesmus* sp. strain FG. (A) Growth curve (OD_{660}); (B) accumulation of proteins; (C) accumulation of carbohydrates; (D) mg carbohydrates·OD_{660}^{-1}. (●) 1.5 mM $NaNO_3$; (■) 3 mM $NaNO_3$; (▲) 6 mM $NaNO_3$; (▼) 12 mM $NaNO_3$. The data represent the mean and standard deviation of two independent experiments.

3. Results and discussion

3.1. Bioprospecting for microalgal strains as an alternative source of fermentable sugars

Our previous efforts for the screening of the strains of our laboratory's collection of native microalgae had been focused towards the accumulation of lipids as an alternative feedstock for the production of biodiesel [20]. From those analyses we also identified seventeen strains as candidates for a more detailed analysis of their potential as an alternative source of sugars for fermentations, for example for the production of bioethanol.

Table 1 shows the accumulation of total carbohydrates of microalgal strains cultivated in mineral medium containing a limiting amount of nitrogen (1 mM $NaNO_3$), under constant light (100 μmol photons m^{-2}·s^{-1}) and temperature (28 ± 1 °C), for 10 days. Carbohydrates accumulation ranged from 40% (w/w) to over 70% (w/w) on a dry weight basis. As a confirmation, we show that a subset of strains that accumulated the highest levels of carbohydrates did not accumulate lipids, while under identical growth conditions the reference strain *Scenedesmus* sp. strain C1S [20] displayed the opposite trend reaching up to 50% total lipids and low levels of carbohydrates.

Strains SP2-3, *Chlorella* sp. MI, and *Desmodesmus* sp. FG, accumulating total carbohydrates at 70.4% (w/w), 57.8% (w/w), or 53.5% (w/w), respectively (Table 1), were selected for further analysis.

A detailed time-course analysis of the increase in biomass (OD_{660}), and accumulation of total carbohydrates and proteins indicated that *Desmodesmus* sp. strain FG grew more robustly than the other two strains under the conditions used (Fig. 1A) and that, regardless of the difference in total carbohydrates richness of its biomass, it presented the largest carbohydrates productivity of this analysis (Fig. 1D). Proteins accumulation was severely halted upon N-deficiency in the three strains (Fig. 1). Thus, ratios of carbohydrates to protein over 2 indicated maximum carbohydrates accumulation.

To compare the biomass of these strains as a feedstock for bioethanol, a set of fermentation assays with baker's yeast *S. cerevisiae* were run. The three strains were inoculated at the same initial OD_{660} (0.2) in 250-mL N-limiting mineral medium for 10 days (enough for each strain to reach the maximum carbohydrates accumulation (Fig. 1B–D)) and then the whole biomass was collected, hydrolyzed in

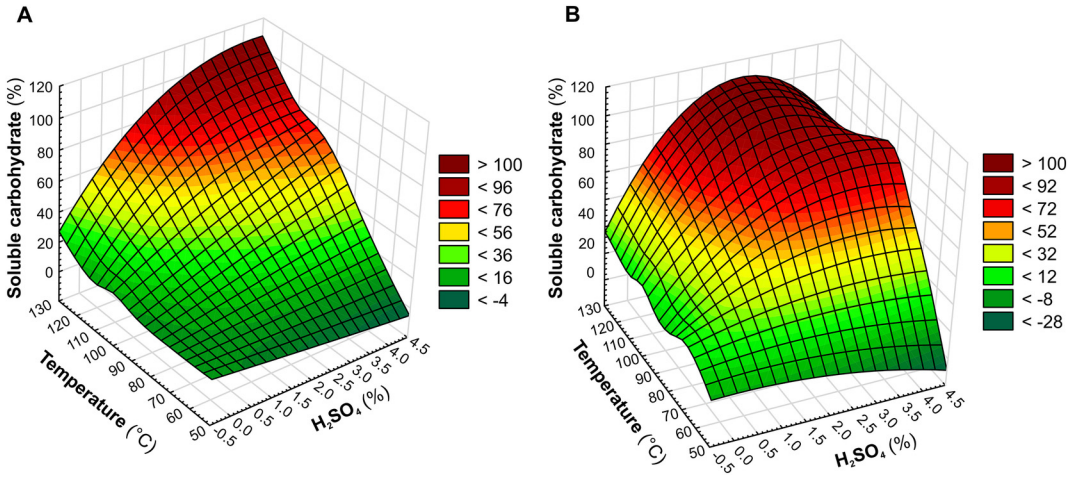

Fig. 4. Surface plot analysis of sugars release from microalgal biomass. Representative plots are shown for the treatment of 10% (w/v) *Desmodesmus* sp. strain FG biomass at different temperatures and H_2SO_4 concentrations for 30 min (A) or 60 min (B). The complete analysis including lower biomass loads are included as Supplementary Fig. S1. Each data point represents the mean of technical duplicates.

diluted acid and then fermented with *S. cerevisiae* cells. *Desmodesmus* sp. strain FG, strain SP2-3, *Chlorella* sp. strain MI yielded ethanol at 98 ± 30; 61 ± 37; or 37 ± 20 mg·L^{-1} of the original microalgal culture or 0.134; 0.091; or 0.064 g·g biomass^{-1} (Table 2). These screening test led us to select *Desmodesmus* sp. strain FG as the most productive of the microalgae analyzed in this work and strain SP2-3 as the one that accumulated the highest levels of fermentable carbohydrates in its biomass for a more detailed analysis of their potential as an alternative sources of fermentable sugars.

3.2. Optimization of carbohydrates accumulation by culture conditions

We compared the effect of the deficiency of nutrients other than N (P and S) on growth and accumulation of carbohydrates by *Desmodesmus* sp. strain FG. Final carbohydrates contents were 56.3 ± 3% (w/w), 49.5 ± 3% (w/w) or 38.4 ± 2% (w/w) for total biomass accumulation of 0.62 ± 0.03, 0.46 ± 0.04, or 0.92 ± 0.04 g biomass·mL^{-1} culture for cells deprived of N, S, or P, respectively. Sulfur deprivation was a very good trigger of carbohydrates accumulation, even higher than N at shorter times. However, conversely to N-deprivation, S-limited cells consistently tended to decline their carbohydrates content at longer times. On the other hand P-limited cultures continued accumulating proteins up to considerable higher levels but carbohydrates accumulation was only modest (Fig. 2). Thus N-deprivation was the trigger of choice since in addition to the previous results, managing its dosification might be beneficial for the economics of microalgae massive culture towards the production of low value commodities [30,31].

Since N-deprivation triggered accumulation of carbohydrates and slowed growth at the same time, we analyzed in more detail the effect of different levels of N-deficiency in order to maximize carbohydrates productivity and biomass enrichment. It was observed that *Desmodesmus* sp. strain FG cells accumulated protein up to nearly 2 g·L^{-1} of culture, proportionally to the availability of N up to the maximum level analyzed of 12 mM. While the overall maximum volumetric productivity of carbohydrates of 1 g·L^{-1} was observed for cultures at the expense of 6 mM $NaNO_3$, maximum carbohydrates accumulation in biomass of 57% corresponded to cultures in the presence of 3 mM $NaNO_3$ (Fig. 3). Final biomass yields were 1.00 ± 0.07; 1.49 ± 0.04;

Table 3
Comparison of ethanol yield after saccharification and fermentation of microalgal biomass.

Strain	Hydrolysis treatment	Biomass load (g·L^{-1})	Sugar concentration (g·L^{-1})	Fermenting microorganism	Ethanol (g·L^{-1})	Ethanol (g·g^{-1} biomass)	% of theoretical yield	Year/reference
Chlamydomonas reinhardtii	H_2SO_4	50	28.5	*S. cerevisiae*	14.6	0.29	100.0	2009 [32]
Spirogyra sp.	Enzymatic	50	12.5	*S. cerevisiae*	NA	0.08	78.4	2010 [33]
Chlorococum sp.[b]	Supercritical CO_2	10	NA[d]	*S. bayanus*	3.8	0.38	NA	2010 [34]
C. reinhardtii	Enzymatic	50	NA	*S. cerevisiae*	11.7	0.24	NA	2010 [16]
Chlorococcum infusionum	NaOH	50	NA	*S. cerevisiae*	NA	0.26	NA	2011 [35]
Chlorella vulgaris	H_2SO_4/enzymatic	5	5.5	*Escherichia coli*	1.7	0.40	61.0	2011 [36]
Scenedesmus obliquus	H_2SO_4	500	63.2	*Kluyveromyces marxianus*	11.7	0.023	36.3	2012 [37]
C. vulgaris	Enzymatic (SHF)[a]	20	7.8	*Zymomonas mobilis*	3.6	0.18	87.6	2013 [17]
C. vulgaris	Enzymatic (SSF)[a]	20	NA	*Z. mobilis*	4.3	0.21	87.1	
C. vulgaris	H_2SO_4	50	23.6	*Z. mobilis*	11.7	0.23	96.7	
S. abundans	H_2SO_4/enzymatic	50	10.8	*S. cerevisiae*	4.7	0.10	85.5	2013 [38]
Mychonastes afer	H_2SO_4/enzymatic	50	6.0	*S. cerevisiae*	2.8	0.06	92.0	
C. reinhardtii	H_2SO_4	NA	NA	*S. cerevisiae*	8.7	0.15	86.0	2013 [39]
C. vulgaris	Enzymatic	10	1.2	*S. cerevisiae*	0.6	0.07	89.0	2014 [40]
Chlamydomonas mexicana	Enzymatic (SHF)[a]	38	22.5	*S. cerevisiae*	8.5	0.41	72.0	2016 [41]
C. mexicana	Enzymatic (SSF)[a]	38.1	22.5	*S. cerevisiae*	10.5	0.50	88.2	
Desmodesmus sp.	H_2SO_4	100	55.3	*S. cerevisiae*	23.0	0.23	81.4	This study
SP2-3	H_2SO_4	100	72.9	*S. cerevisiae*	23.6	0.24	63.7	
SP2-3[c]	H_2SO_4	100	137.2	*S. cerevisiae*	61.2	0.31	87.4	

[a] SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation (SSF).
[b] Defatted microalgal biomass.
[c] Concentrated hydrolysate.
[d] Not available.

2.25 ± 0.35; or 2.12 ± 0.11 g·L^{-1}, for cells cultivated in the presence of 1.5; 3.0; 6.0; or 12 mM $NaNO_3$, respectively for 22 days.

3.3. Optimization of sugars release from microalgal biomass acid hydrolysis

Next we optimized the hydrolysis of microalgal biomass using *Desmodesmus* sp. strain FG cells according to the biomass load, temperature, acid concentration and incubation time. For these assays microalgal cells were cultivated in 5-L air lift photobioreactors according the optimized growth conditions for carbohydrates productivity and content. It was found that the mildest and more productive conditions for the release of up to 95% sugars from the biomass was a biomass load of 10% dry w/v, 2% H_2SO_4 (v/v), and 120 °C for 30 min (Fig. 4 and Supplementary Fig. S1). Optimized parameters for biomass hydrolysis fell in the range of previous studies which had been mostly conducted at biomass loads up to 5% (w/v) (Table 3 and references therein). Importantly, in this study we pushed forward the biomass load up to 10% (w/w) which is nearly at the limit for convenient handling of the biomass as a very concentrated slurry (fresh biomass of *Desmodesmus* cells normally contained 15% solids (w/v)). This result was very satisfactory towards increasing the concentration of sugar, without the need of additional energy-intensive steps for drying the biomass or concentrating the hydrolyzates.

3.4. Ethanol production by S. cerevisiae fermentation of saccharified microalgal biomass

For a more detailed analysis of ethanol production from *Desmodesmus* sp. strain FG and strain SP2-3 cells, biomass at 100 g dry weight·L^{-1} was hydrolyzed according to the optimized conditions to yield soluble carbohydrates preparations at 55.3 g·L^{-1} or 72.9 g·L^{-1}, respectively. These preparations were inoculated with *S. cerevisiae* cells and accumulated up to 23.0 ± 1.8 or 23.6 ± 0.7 g ethanol·L^{-1}, respectively. These values represented 81.4% or 63.7% of the maximum theoretical values assuming full conversion of glucose into ethanol (0.51 g ethanol per g glucose) and ethanol yields of 0.23 and 0.24 g ethanol·g algal biomass^{-1}, respectively. Fermentation of concentrated strain SP2-3 hydrolysates to 137.2 g soluble carbohydrates·L^{-1} accumulated ethanol up to 61.2 g·L^{-1} (87.4% of theoretical value). Fig. 5 shows a time-course analyses of representative fermentation runs of ethanol accumulation and sugars depletion from the fermentation broth. These results indicated that microalgae biomass could be converted into ethanol by baker's yeast *S. cerevisiae* as efficiently as commercial grade dextrose and that other nutrients, usually used to improve fermentation, such as the N-source, were already present in the hydrolyzed microalgal biomass. Both, almost complete exhaustion of carbohydrates from the fermentation broth and high conversion efficiency of carbohydrates into ethanol indicated very high enrichment of fermentable sugars in the biomass of the strains selected in this study and in their corresponding hydrolysates. It also indicated that sugar loss and/or generation of fermentation inhibitors, if present, remained at negligible levels after the optimized saccharification treatment.

Very optimistic estimations had been made a few years ago for microalgal-derived bioethanol productivities of 46,760–140,290 L·ha·year^{-1}, that would be several orders of magnitude larger than yields obtained from other feedstocks [41]. However, despite the advances made in recent years, commercialization of algal bioethanol remains challenging because of the techno-economic constraints [42]. More recent data on realistic microalgal-biomass productivities around 27 and up 61 Tn·ha^{-1}·year^{-1} for open ponds or close culture systems in southern Spain, respectively [43], at achievable ethanol yields of 0.25 g·g biomass^{-1} (Table 3 and references therein) would yield 8500–19,500 L·ha·year-1, which is still higher but in the range of common plant crops currently used as bioethanol feedstocks [41]. Since the feedstock's cost has the greatest impact on the cost of producing bioethanol [44], the expected decrease of current costs of producing microalgal biomass from € 3.4 to € 0.5 in the next 10 years [43] would be mandatory for large scale production and commercialization.

Regarding downstream processing, microalgal carbohydrates content (quantity and quality) and pretreatment are among the most critical variables towards competitive production of bioethanol. It is estimated that pretreatment would account for as much as 33% of the total cost in the production of algae bioethanol. It has been suggested that dilute sulfuric acid treatment for biomass saccharification would be the most effective strategy for industrial applications and that to further decrease the cost of the pretreatment it would be essential to increase solids concentration as high as possible and to minimize sugar losses [42]. Also, for economically-competitive ethanol production a minimum of 40 g ethanol·L^{-1} of fermentation broth would be needed to reduce distillation costs [45]. Our study shows microalgal strains containing up to 70% fermentable carbohydrates and an optimized protocol for saccharification at a biomass load of 10% (w/v) for hydrolyzates at 7.3% (w/v) fermentable sugars yielding 24–30 g ethanol·L^{-1}. These results represent an improvement over yields obtained in previous studies so far (Table 3 and references therein).

It is broadly accepted that microalgal-based biofuels economics would be largely improved if obtained in the frame of biomass biorefineries for the production of multiple commodities and higher value products [46]. Fermentations run in this study yielded as co-products 0.06 kg dry edible yeast *S. cerevisiae* per 1 kg dry *Desmodesmus* sp. strain FG biomass and the spent fermentation broth that would be used as animal feed supplements or other biotechnological applications [44,46]. Although not confirmed in this work, it is presumed that CO_2 produced as a fermentation product (at least 0.22 kg·kg of dry *Desmodesmus* biomass) could be recycled into microalgal culture as shown before by others [47], to increase productivity and reduce the C-footprint of bioethanol production.

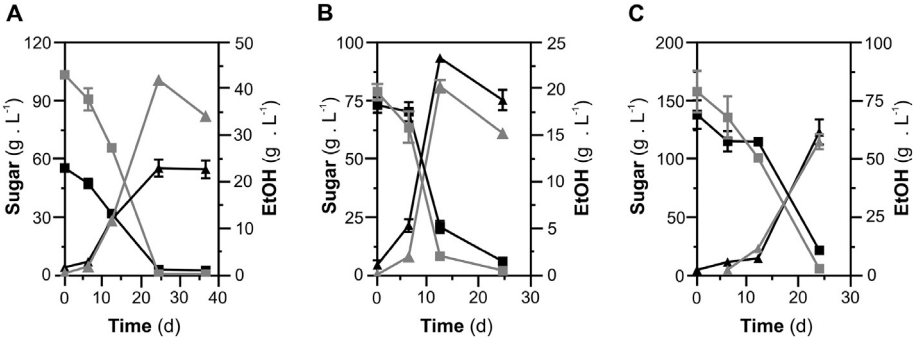

Fig. 5. Time-course of ethanol accumulation and sugars consumption during fermentation of hydrolyzed microalgal biomass by *S. cerevisiae*. (A) *Desmodesmus* sp. strain FG hydrolyzed-biomass; (B) strain SP2-3 hydrolyzed-biomass; (C) strain SP2-3 concentrated hydrolyzed-biomass. (■) Sugars depletion from YPD medium; (▲) ethanol production from YPD medium; (■) sugars depletion from microalgal hydrolyzed-biomass; (▲) ethanol production from microalgal hydrolyzed-biomass. The data represent the mean and standard deviation of two (A–C) or four (B) independent experiments.

4. Conclusions

In this work we have identified two novel strains *Desmodesmus* sp. FG and the still unidentified green microalga, strain SP2-3 that produced copious amounts of carbohydrates of 57% and 70% on a dry weight basis, under nitrogen deficiency at laboratory growth conditions.

After optimization of strain selection, microalgae culture conditions, saccharification and fermentation we were able to show both a high algal biomass-to-ethanol conversion efficiency of 0.24 g ethanol·g algal biomass^{-1} and high alcohol concentration after fermentation 24 g ethanol·L hydrolysate^{-1}, for up to 87.4% of the theoretical. These results represent an improvement of previously demonstrated yields and might encourage further research worldwide. Additionally, these strains represent good candidates for outdoors studies to further explore the potential of native strains as an alternative feedstock for bioethanol in southeastern Buenos Aires as part of a microalgal biomass biorefinery.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.12.021.

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