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Bioprospecting for fast growing and biomass characterization of oleaginous microalgae from South–Eastern Buenos Aires, Argentina

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HIGHLIGHTS

- Thirty-four native-to-Argentina microalgae strains were isolated.
- Novel RNA sequences in the ITS1-5.8S-ITS2 region were reported.
- Some strains accumulated lipids up to 43% of their dry biomass.
- High ratios of mono-unsaturated to poly-unsaturated fatty acids are shown.
- Strains with doubling times up to 6 h were identified.

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ABSTRACT

As part of pioneering efforts to assess the potential of native microalgae as biofuel feedstock in South–Eastern Buenos Aires, 34 monoalgal cultures (corresponding to the Phylum Chlorophyta) were established and 21 were selected for further growth and biomass composition characterization.

Novel RNA sequences in the ITS1-5.8S-ITS2 region were identified. Some strains showed desirable traits as biodiesel feedstock such as (i) apparent maximal doubling times of 6 h, (ii) lipids accumulation of up to 43% of their dry biomass, (iii) high ration of mono-unsaturated to poly-unsaturated fatty acids, (iv) high response to CO_2 supplementation, and (v) complete sedimentation in 4 h. Data of the outdoors performance of some strains suggested they might represent valuable resources for future research towards the regional development of the technology for microalgae-based biofuels.

1. Introduction

To address some of the major challenges of Humanity such as climate change, energy supply and poverty alleviation (Cockerill and Martin, 2008; Tilman et al., 2009). Most countries have made in the last years laws to switch the use of oil or natural gas to biofuels. In this context, the Argentine Congress approved in 2006, the law 26093, promoting the production and use of biofuels in the country, mandating bioethanol and biodiesel account for 5% of all fuel sold by 2010 (Tomei and Upham, 2011). More recently, increased soy production (one of the main sources of biodiesel) and commitment to pursue the goals of the law led to an amendment establishing a minimal 7% biodiesel for blending, with the expectation to reach 10% by the end of 2012 (http://www.energia.gov.ar). Therefore, Argentina has become one of the world's largest producers and exporters of biodiesel in the last few years (Tomei and Upham, 2011). Despite the benefits that this flourishing industry the long term sustainability of first generation biofuels has been questioned by life cycle analysis regarding land use change, impact on food prize and carbon, water and nitrogen footprints (Huo et al., 2011; Searchinger et al., 2008). Thus, a diversification of the energy matrix (beyond and even among biofuels and/or feedstocks) appears to be mandatory for attaining goals of sustainable energy supply and for creating new opportunities towards socio-economical equity and poverty alleviation (Ewing and Msangi, 2009).

During the last years much interest was focused on the research and development (R&D) of third generation biofuels based on microalgae biomass as a feedstock for a diversity of biofuels, especially biodiesel from eukaryotic microalgae lipids. Some of the

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characteristics of microalgae contributing to their potential for biofuels production are: (i) high oil yield per unit of surface and time (up to 100-fold higher in comparison with crop plants currently used as biodiesel feedstock), (ii) non competitiveness for land or food market with crops, (iii) better economy of water and nutrients through effective recycling and (iv) possibility of using residues from industries as source of inexpensive nutrients, especially CO_2, N and P, which in turn might assist in municipal or industrial waste management and help to mitigate climate change (Brennan and Owende, 2010; Chisti, 2007; Hu et al., 2008; Sheehan et al., 1998; Wijffels and Barbosa, 2010). However, up to what extent the potential of biofuels from microalgae could be realized in the future is still a matter of debate (Petkov et al., 2012).

The yield of biodiesel from microalgae depends on the rate of biomass production and the oil of the cells. In general, productivity and lipid content are inversely correlated, and stress conditions such as deprivation of N or P, limit cell growth and promote the accumulations of triacylglycerols (TAG) (Hu et al., 2008; Huo et al., 2011). These TAG can be converted into biodiesel through a standard transesterification reaction. The fatty acid (FA) profile of TAG merits a special consideration since it correlates with most quality parameters of biodiesel (e.g., density, kinematic viscosity, heating value, cetane number and iodine value) (Knothe, 2008). Besides biomass and lipids productivity, as recently reviewed by Wijffels and Barbosa (2010), the "ideal microalga" should combine other desirable characteristics such as large cells with thin walls, flocculation properties, tolerance to high light intensity and oxygen concentration and resistance to contamination with other microorganisms, among others. Although such a strain is currently unavailable, hope is placed in the fact that there are many more species in the wild yet to be isolated and improved by genetic engineering in an integrated fashion with reactors design (Wijffels and Barbosa, 2010).

At a national scale, South–Eastern Buenos Aires might be regarded as strategic for microalgal biotechnology since: (i) average temperatures (and extreme fluctuations) might allow massive outdoors culturing of microalgae for most part of the year, (ii) although the region is characterized for its high soil fertility and agriculture activity, large flat regions of non-productive marginal lands are still available for non-conventional uses (Zelaya and Cabria, 2007), (iii) the region is geographically linked to: (a) Buenos Aires city, one of the largest metropolis in South America, representing both a huge sink for alternative sources of energy and a correspondingly huge source of waste materials to be recycled (CO_2-rich flue gasses and industrial and municipal waste water), (b) to the sea, and (c) to the industrial core comprising the infrastructure for soy-based biodiesel. However, both national and state R&D on microalgae-based biofuels is currently at its infancy, lagging behind the development for the production of first generation biofuels.

This work reports the isolation and characterization of oleaginous microalgae native of Argentina leading to the identification of strains with potential for local production of biodiesel and/or other biotechnological applications of microalgae biomass.

2. Methods

2.1. Isolation and culture of microalgal strains

More than fifty samples were collected 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. The predominant criterion for sample collection was the evident abundance of microalgae (or cyanobacteria) in the water. Each sample was took to the laboratory within the first day of collection and incubated under the following conditions: no additions, half or full strength Bold's basal (0.025 g/L $CaCl_2{\cdot}2H_2O$, 0.075 g/L $MgSO_4{\cdot}7H_2O$, 0.075 g/L K_2HPO_4, 0.0175 g/L KH_2PO_4, 0.025 g/L NaCl, 0.05 g/L EDTA (disodium salt), 0.031 g/L KOH, 4.98 mg/L $FeSO_4{\cdot}7H_2O$, and trace metal mix (11.42 mg/L H_3BO_3, 17.64 mg/L $ZnSO_4{\cdot}7H_2O$, 2.88 mg/L $MnCl_2{\cdot}4H_2O$, 1.42 mg MoO_3, 3.14 mg/L $CuSO_4{\cdot}5H_2O$, and 0.98 mg/L $CoNO_3{\cdot}6H_2O$)) medium or $BG11_0$ (0.04 g/L K_2HPO_4, 0.075 g/L $MgSO_4{\cdot}7H_2O$, 0.036 g/L $CaCl_2{\cdot}2H_2O$, 0.006 g/L citric acid, 0.006 g/L ferric ammonium citrate, 0.001 g/L EDTA (disodium salt), 0.02 g/L Na_2CO_3, and trace metal mix A5 (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2{\cdot}4H_2O$, 0.222 mg/L $ZnSO_4{\cdot}7H_2O$, 0.39 mg/L $NaMoO_4{\cdot}2H_2O$, 0.079 mg/L $CuSO_4{\cdot}5H_2O$ and 0.049 mg/L $Co(NO_3)_2{\cdot}6H_2O$)) medium, both containing either 2 mM $NaNO_3$, 2 mM urea or 2 mM NH_4Cl as the nitrogen source. Other conditions were at 29 ± 1 °C and constant light at 35 μmol photons m^{-2} s^{-1} or at 21 ± 1 °C at 35 μmol photons m^{-2} s^{-1} under a photoperiod of 14 h light and 10 h darkness.

At this stage 25 mL cultures in Erlenmeyer flasks were shaken manually twice a day and 1:25–1:50 dilutions in full strength medium were subcultured according to the previously registered preferences. Most samples were enriched this way for dominant strains for several months until protocols for serial dilution or streaking onto solid medium were used for strains isolation. For each sample, strains enrichment and isolation were followed at the light microscope. Axenicity of the isolates was judged by cultivation on LB medium for the presence of bacteria and observation at the microscope. Strains are maintained by subculturing onto solid medium 21 ± 1 °C at 15 μmol photons m^{-2} s^{-1} under a photoperiod of 14 h light and 10 h darkness every other 3–4 months. For solid medium preparation water-washed agar was supplemented at 1%.

For growth analysis, biomass characterization and lipids induction analysis, microalgae strains were cultivated indoors in sterilized air-bubbled (from the bottom) 250-mL transparent glass-bottles. All strains were acclimated to $BG11_0$ medium containing 1 mM NH_4Cl or 0.5 mM urea (N-deficiency) or 4 mM NH_4Cl or 3 mM urea (N-sufficiency) and 29 ± 1 °C under constant light (70 μmol photons m^{-2} s^{-1}).

For a more detailed analysis of lipids accumulation *Scenedesmus obliquus* strain C1S cells were used at 29 ± 1 °C under constant light (110 μmol photons m^{-2} s^{-1}). When indicated, cells were bubbled with 5% CO_2-supplemented air or ammended with 0.5% sucrose, 0.1 mM NH_4Cl (N-deficiency) and/or 2.5 μM K_2HPO_4 (P-deficiency).

Outdoors cultivation of selected strains was conducted during the summer season using the same media than indoors in 3 L air-bubbled bottles under non-axenic conditions.

2.2. Microalgal strains identification

2.2.1. Morphological determination

During the process of isolation, strains were preliminary identified by observation of morphological characters at the microscope and using taxonomic keys (Bourrelly, 1966).

2.2.2. Ribotypification and sequence analysis

Taxonomic determination was further confirmed by sequencing a ribosomal RNA region comprising ITS1-5.8S-ITS2. Total DNA from the samples was extracted from about 1.10^7 to 1.10^8 microalgal cells in 400 μL of TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) containing 0.5% SDS, 200 μL phenol and 200 μL chloroform. Mixtures were vigorously mixed in a vortex with the aid of a pinch of 0.1 mm glass beads for 1 min and subsequently incubated for 1 min on ice for four times. The aqueous phase was extracted once more with 200 μL phenol and 200 μL chloroform and then incubated with 2 μg of DNAse-free RNAse at 37 °C for 30 min. Preparations were extracted once with phenol and chloroform and then

with chloroform, brought to 0.3 M sodium acetate and precipitated with 1 mL of ice–cold ethanol for 30 min on ice. After centrifugation at 13,000 rpm for 15 min, pellets containing DNA were washed once with 1 mL of ice–cold 70% ethanol and air dryed. Preparations were typically resuspended in 40 μL of deionized water. For PCR amplification, 25 μL reactions containing 2 μL of microalgal-DNA preparation, 1× reaction buffer (Fermentas), 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.62 μM of each primer, and 2.5 U of Taq DNA polymerase (Fermentas) were set. For some samples, different concentrations of $MgCl_2$ and up to 2.5% DMSO were used. Primers for the ribosomal RNA region ITS1-5.8S-ITS2 were 5′-GAAGTCGTAACAAGGTTTCC-3′ (forward) and 5′-TCCTGGTTAGTTTCTTTTCC-3′ (reverse) (Timmins et al., 2009). Cycling temperature program for PCR reaction was: 94 °C, 2 min and 30 cycles at 94 °C, 45 s, 45 °C, 45 s and 72 °C, 45 s and 72 °C, 7 min in an Eppendorf Mastercycler Gradient device (Eppendorf, Hamburg, Germany). For confirmatory purposes, some samples were also amplified in the ribosomal RNA region 18S using the primers EukA (5′-AACCTGGTTGATCCTGCCAGT-3′) and EukB (5′-TGATCCTCCTGCAGGTTCACCTAC-3′) (Medlin et al., 1988) using essentially the same reaction conditions as for the ITS1-5.8S-ITS2 region and the temperature program: 94 °C, 3 min and 30 cycles at 94 °C, 60 s, 50 °C, 45 s and 72 °C, 90 s and 72 °C, 10 min. PCR products were analyzed by electrophoresis on 1% agarose gels and send to the sequencing facility (Macrogen Inc., Seoul, South Korea) for the corresponding analysis. Sequencing results were analyzed with Basic Local Alignment Searching Tool (BLAST) by The National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov) and by multiple sequence analysis using the Clustal W algorithm from The Molecular Evolutionary Genetics Analysis v5 (MEGA5) software (Kumar et al., 2008). Phylogenetic trees were created using the Neighbor Joining algorithm from the MEGA5 software after 500 rounds of bootstrap resampling.

2.3. Lipids methods

2.3.1. Nile Red staining of neutral lipid droplets

Microalgae cells were induced for neutral lipids accumulation by N-deprivation.

Nile Red was use to monitor neutral lipid accumulation by fluorescence microscopy (Chen et al., 2009) using a Nikon-Eclipse E600 microscope. For this, microalgae cells were incubated in 20% DMSO and 5 μM Nile Red for 30 min in the dark. For quantitative determinations, samples were excited with polarized light at 486 nm using a spectrofluorometer (Shimadzu, model RF-540) and the emission peak at 556 nm emission was recorded. Emission spectra from 500 to 700 nm were routinely analyzed as indicative of neutral lipids.

2.3.2. Gravimetric determination of total lipids

Lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959) with modifications. Cell pellets (0.5–1 g fw) were resuspended in 1,5 mL 1 M NaCl and sonicated at 40% power-output of an ultrasonic processor (Vibra-Cell, model VCX-130, Sonics Inc.) for two cycles of 1 min (10 s on, 1 s off). Then, 4 mL of methanol and 2 mL of chloroform were added and samples were vigorously mixed in a vortex for 10 min in the presence of 10 2–5 mm glass beads. After the addition of 2 mL of chloroform and 1.5 mL of 1 M NaCl, samples were mixed for 2 min and then centrifuged at 7,000 rpm for 10 min. The organic phase was kept and the aqueous phase was extracted two more times with 2 mL of chloroform each time. The pooled organic phases were evaporated at room temperature on previously weighted glass capsules until constant weight. Samples were resuspended in chloroform for further analysis.

2.3.3. Fatty acids methyl esters (FAME) determination

Methylation of fatty acids present in algae lipids was performed following the technique proposed by Sukhija and Palmquist (Sukhija and Palmquist, 1988). Lipid samples in chloroform were incubated with one volume of 5% methanolic acid (acetyl chloride: methanol; 1:10, v/v) for 1 h at 70 °C. After addition of four volumes of 6% potassium carbonate (w/v), preparations were incubated until phase's separation. The organic phase was supplemented with two volumes of chloroform. Fatty acid composition was determined by gas chromatography (GLC) with a Shimadzu GC-2014 equipment (Kyoto, Japan). The injector and detector (FID) temperatures were set at 250 and 275 °C, respectively, while the column temperature was set at 210 °C. A 1 μL sample volume was injected into the column (Omega wax 250, Supelco). The N_2 carrier gas was maintained at a constant pressure of 100 kPa, and the chromatogram data were acquired and processed using Shimadzu GC-solution software.

Fatty acid content was expressed as a percentage of the total fatty acids identified in the oil. Identity of peaks was confirmed by hydrogenation of FAMEs and further GC analysis in a commercial facility (INTI-MdP, Argentina).

2.4. Cells sedimentation

Samples of 10 ml of cultures of *S. obliquus* strain C1S or *Pseudokirchneriella* sp. strain C1D were allowed to sediment by gravity in 15 ml polypropylene tubes (12 cm length and 1.5 cm internal diameter). At different time points (one 10 ml sample for each time point), 1 ml samples were collected from the surface (1 cm from the surface) or the bottom (1 cm from the bottom) and the cells sedimentation was analyzed by counting the cells and chlorophyll *a* determination in the samples.

2.5. Miscellaneous methods

For growth curves analysis, cell number was determined by microscopy using a Neubauer chamber. Data were plotted using the GraphPad PRISM software (Intuitive Software for Science, San Diego, CA) and doubling times were obtained by fitting the experimental data to theoretical curves of exponential growth with $R^2 \geqslant 0.97$. Microalgae biomass dry weight was determined from resuspended cells in the culture medium. Samples were centrifuged at 13,000 rpm for 10 min and the pellets were dried out in an oven at 70 °C until constant weight. For biomass total protein determination, samples were prepared by boiling resuspended cells at 100 °C for 10 min in the presence of 1 N NaOH. Aliquots were subjected to protein determination by the Lowry's method (Lowry et al., 1951) using NaOH-treated BSA as a standard. For biomass total carbohydrates determination, resuspended cells were directly reacted with the Antrona's reagents (Dreywood, 1946). Carbohydrates content was calculated from a standard curve using fructose. Chlorophyll *a* was determined colorimetrically in 90% methanolic extracts (Mackinney, 1941).

3. Results and discussion

3.1. Isolation and identification of South–Eastern Buenos Aires native microalgae

Bioprospecting of indigenous microalgae is very often a starting point in the road map of public and/or private sector efforts towards the development of new technology for biofuels from microalgae biomass (Mutanda et al., 2011; Sheehan et al., 1998; Wijffels and Barbosa, 2010). In Argentina, however, exploration of the potential of microalgae for biodiesel production is at its infancy and

very few studies are available in the international literature (Pérsico et al., 2011; Popovich et al., 2012).

As a contribution to this regard, in the present work more than fifty samples were collected from different continental freshwater environments surrounding Mar del Plata, Buenos Aires, Argentina (38°0′0″S 57°33′0″W). Samples were collected from various water bodies (natural or artificial ponds, swimming pools, streams, lagoons, etc.) during the four seasons from 2009 to 2010. Thirty-four monoalgal cultures were established and about half of them were maintained in an axenic form. The strains with higher growth robustness under standard indoor growth-conditions were selected for further analysis. Fig. 1 shows a neighbor joining phylogenetic tree depicting the relatedness of the native isolates ribosomal RNA region comprising ITS1-5.8S-ITS2 with selected sequences from the public data bases. With the only exception of one strain preliminary designated as *Haematococcus pluvialis* strain HP, according to morphological characters (Bourrelly, 1966), strains presented a good correlation between morphological and genetic assignment. Strains LP1, SP2-15 and C1D (morphologically identified as *Ankistrodesmus* spp, and *Pseudokirchneriella* sp, respectively) were grouped in a sub-clade among the Order Chlorococcales, Family Scenedesmaceae, with no close relative sequences in the public data bases. Strain C1D was further subjected to ribotypification in the RNA region 18S. BLAST analysis of the corresponding sequence indicated a 99% identity with *Monorraphydium* spp. (assigned to the same Family). Thus, it appears that these ITS1-5.8S-ITS2 RNA sequences are the first that have been uploaded sequences of this group in the non-redundant public database and would facilitate further identification of microalgae worldwide.

Within the Order Chlorococcales, a relatively large number of strains (4 out of 10) presented long DNA insertions of 727-bp, 725-bp, 406-bp and at least 318-bp, for strains M1-20, FG, SP2-15 and LP1, respectively, that might represent group I introns. The sequence of these putative group I introns presented low to

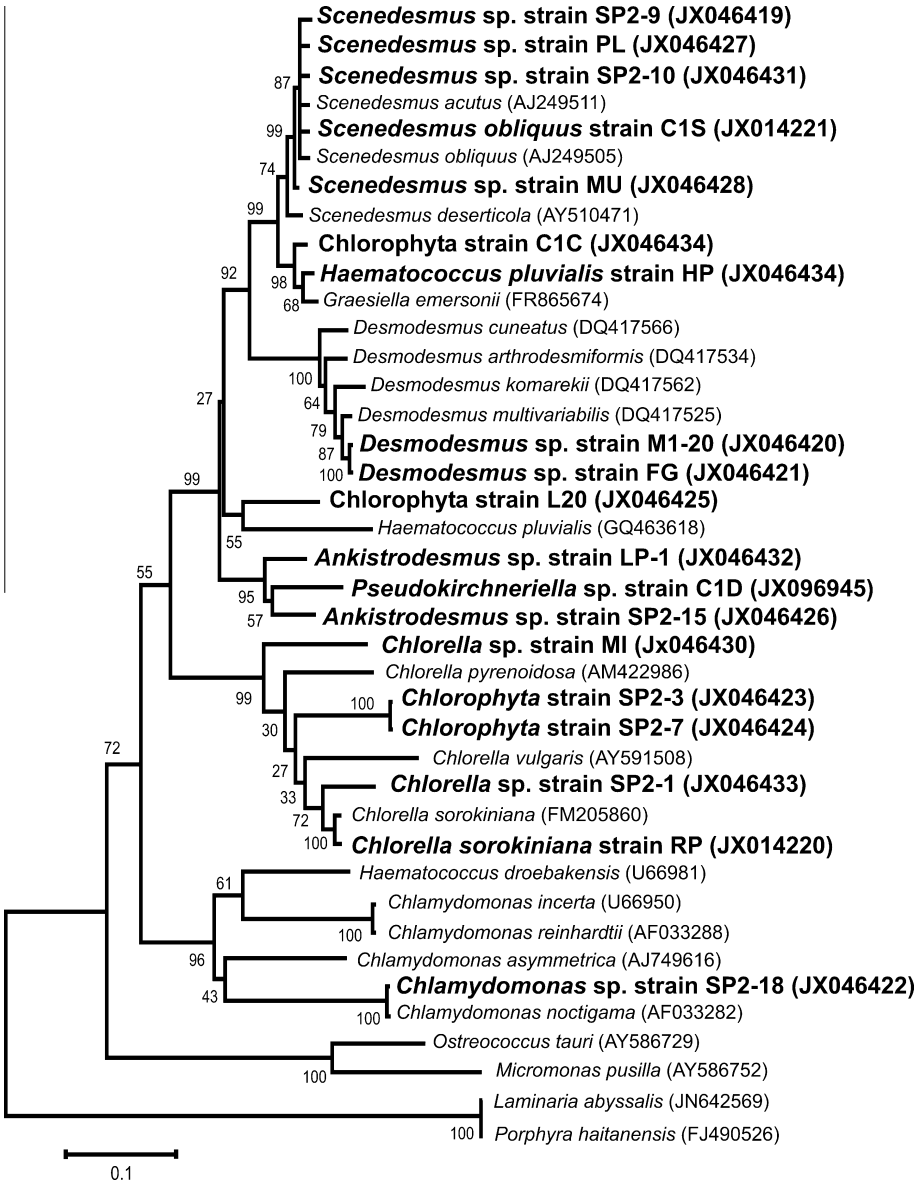

Fig. 1. Phylogenetic analysis of the rRNA ITS1-5.8S-ITS2 region of the native microalgae isolated from South–Eastern Buenos Aires. The sequences corresponding to the strains isolated in this work were indicated in bold case and the corresponding GeneBank accession numbers were indicated. Algae from the Phyla Rodophyta and Heterokontophyta (*Porphyra haitanensis* and *Laminaria abyssalis*, respectively) were included in the analysis to help placing the root of the tree. The multiple sequence alignments were generated using the Clustal W algorithm from The Molecular Evolutionary Genetics Analysis v5 (MEGA5) software. Phylogenetic trees were created using the Neighbor Joining algorithm from the MEGA5 software after 500 rounds of bootstrap resampling.

undetectable sequence identity to available sequences at public data bases.

Nobel native strains can combine several desirable traits and be useful on a regional or broader scale. It is presumed that these strains might be more suitable for outdoors cultivations since they are naturally acclimated to the prevailing environmental conditions and some of them may have acquired strategies to cope with potential competitors sharing a similar ecological niche. Mass cultivation of native strains might ameliorate the potential ecological risk associated with the introduction of exotic (or genetic engineered) strains (Chimera et al., 2010) and could also be favored by the public perception. Native strains could be a very valuable resource of as-isolated algae species and/or as a genetic background or source of genes for selected traits in genetic engineering programs.

3.2. Biomass composition of native microalgae strains

For a preliminary score of the potential of the native strains regarding lipid productivity, a Nile Red fluorometric method was followed. Strains were allowed to grow under conditions of N-deficiency (0.5 mM NH_4^+) and the time course of the fluorescence outcome, indicative of neutral lipids concentration was analyzed (see Supplementary Fig. S1. After this preliminary screening, 21 microalgal isolates were further characterized for their biomass composition and final dry weight yield under either N-replete (4 mM NH_4^+) or N-limiting (1 mM NH_4^+) conditions (Table 1). As expected, most strains displayed a decrease in protein content under nitrogen deficiency. According to data depicted in Table 1, strains could be roughly classified in three groups as strains accumulating predominantly lipids, carbohydrates or intermediate levels of both.

Although this study was primary targeted to explore microalgae oil accumulation, during the course of the analysis it became evident that almost half of the strains of the collection appear to accumulate carbohydrates up to 50% of their dry biomass under conditions of N-deficiency. Thus, some of these strains might be suitable feedstocks for bioethanol or biogass production (Brennan and Owende, 2010) and/or carbohydrates (and N) could be recycled into the lipid production process according to the recently proposed bioconversion of solid by-products with oleaginous microorganisms (Trzcinski et al., 2012).

Table 1
Biomass composition of native strains according to the availability of nitrogen.

Strain	Nitrogen	Cell dwt (mg/L)	Protein (% dwt)	Carbohydrate (% dwt)	Lipid (% dwt)
RP	+N	581.50 ± 0.71[a]	45.11 ± 3.89	9.30 ± 2.27	14.89 ± 2.51
	−N	475.50 ± 19.09	33.78 ± 2.89	22.20 ± 0.19	30.03 ± 0.34
FG	+N	581.50 ± 45.96	41.58 ± 0.52	9.04 ± 0.36	11.71 ± 1.04
	−N	454.50 ± 16.26	20.65 ± 7.10	18.70 ± 0.71	22.28 ± 6.68
PI	+N	635.78 ± 52.54	40.62 ± 5.76	25.36 ± 0.94	5.87 ± 1.6
	−N	429.48 ± 27.75	25.32 ± 0.42	29.10 ± 0.14	43.82 ± 0.02
C1D	+N	451.50 ± 47.38	44.39 ± 1.80	12.20 ± 1.47	15.02 ± 1.83
	−N	336.00 ± 14.14	28.65 ± 1.30	22.07 ± 1.51	41.10 ± 2.54
C1S	+N	442.00 ± 4.24	48.92 ± 2.55	12.43 ± 1.67	13.75 ± 2.59
	−N	405.00 ± 7.07	20.11 ± 1.02	23.20 ± 3.18	32.05 ± 0.45
C1C	+N	331.00 ± 3.87	53.10 ± 0.70	17.77 ± 6.21	12.89 ± 4.77
	−N	252.00 ± 0.40	28.26 ± 4.03	37.08 ± 1.29	32.87 ± 14.20
L20	+N	284.70 ± 169.40	44.55 ± 7.42	12.77 ± 2.72	14.39 ± 6.27
	−N	169.44 ± 4.50	21.22 ± 9.17	47.28 ± 3.36	25.50 ± 0.56
MI	+N	451.82 ± 16.46	44.20 ± 8.20	13.25 ± 1.21	12.54 ± 0.07
	−N	289.45 ± 21.47	25.51 ± 1.42	46.96 ± 7.56	18.81 ± 2.02
SP2-1	+N	453.40 ± 30.50	59.50 ± 6,36	15.75 ± 4.70	14.44 ± 1.21
	−N	409.95 ± 24.40	37.00 ± 3.40	29.00 ± 2.16	17.26 ± 2.39
SP2-3	+N	216.13 ± 26.70	51.00 ± 8.73	25.80 ± 1.55	18.63 ± 0.04
	−N	232.50 ± 4.52	30.92 ± 3.09	28.87 ± 2.98	32.00 ± 6.50
SP2-9	+N	490.00 ± 74.00	47.96 ± 1.23	28.42 ± 2.91	8.80 ± 0.74
	−N	420.70 ± 22.91	16.50 ± 3.95	46.17 ± 4.34	32.60 ± 3.78
SP2-10	+N	138.50 ± 27.29	52.12 ± 5.54	17.66 ± 1.74	21.84 ± 2.29
	−N	167.87 ± 6.83	25.10 ± 2.33	50.45 ± 0.90	22.40 ± 2.30
SP2-15	+N	242.06 ± 1.02	52.25 ± 1.48	20.88 ± 0.94	35.31 ± 5.65
	−N	299.32 ± 5.54	25.12 ± 1.32	41.13 ± 3.22	32.20 ± 4.24
CH	+N	430.65 ± 177.27	46.30 ± 13.43	19.83 ± 1.08	11.77 ± 0.74
	−N	559.70 ± 19.21	21.25 ± 0.63	45.62 ± 2.31	12.16 ± 0.09
LP1	+N	287.25 ± 11.66	59.59 ± 4.01	28.80 ± 8.62	19.16 ± 3.36
	−N	233.35 ± 47.16	23.25 ± 0.04	39.95 ± 5.58	27.97 ± 8.00
HP	+N	270.30 ± 11.30	37.70 ± 2.76	34.00 ± 3.21	13.54 ± 2.11
	−N	274.30 ± 15.40	28.50 ± 2.10	36.19 ± 2.31	22.62 ± 0.78
M1-20	+N	ND[b]	ND	ND	12.75 ± 1.04
	−N	ND	ND	ND	26.28 ± 3.38
MH[a]	+N	194.90 ± 39.03	43.82 ± 2.86	35.13 ± 6.60	12.34 ± 1.91
	−N	460.73 ± 24.26	27.10 ± 0.98	48.13 ± 4.00	14.78 ± 2.48
L2[b]	+N	472.22 ± 117.27	41.93 ± 1.07	32.50 ± 9.19	2.00 ± 0.43
	−N	400.60 ± 26.02	19.41 ± 3.41	33.16 ± 9.24	37.51 ± 13.57
RD[b]	+N	433.20 ± 21.49	51.25 ± 6.71	24.77 ± 7.70	14.25 ± 6.22
	−N	424.32 ± 59.73	15.26 ± 3.48	41.33 ± 6.13	36.14 ± 2.48
C2P[c]	+N	512.88 ± 15.03	42.25 ± 0.37	22.20 ± 2.26	14.54 ± 1.91
	−N	497.37 ± 23.60	29.13 ± 1.78	35.86 ± 5.89	21.90 ± 4.24

[a] Data represent de mean ± SD of two independent experiments.
[b] Not determined.

3.3. Fatty acids profiles of native microalgae strains

A key aspect of strains selection and/or improvement, although until now less considered, is the FA composition of the microalgae oleaginous biomass. Differences in chemical and physical properties among biodiesel fuels can be explained largely by the fuels FA profiles. Two critical features of biodiesel quality are the FA size distribution and the degree of unsaturation within the FA structures (Hoekman et al., 2012). Although it is not possible to define a single FA composition for optimum performance, it is suggested that quality biodiesel should contain relatively low concentration of both long-chain saturated FAME and poly-unsaturated FAME for satisfactory low temperature operability and oxidative stability (Hoekman et al., 2012).

In the present study, total lipids from either induced (N-deficient) or non-induced (N-sufficient) cells of previously selected strains were subjected to methylation and analysis of FAME (Table 2 and Supplementary Fig. S2). The most abundant fatty acids found (90% of total FAME) were palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). Fig. 2 shows the relative percentages of saturated FA (SAF), mono-unsaturated FA (MUFA) and poly-unsaturated FA (PUFA) of the isolated native microalgae. The comparison of these values with those of the main current feedstocks for biodiesel worldwide, rapeseed, palm and soy oils, suggested that most of these microalgae oils might be of acceptable quality for biodiesel production (rounded symbols in Fig. 2). The rest of the strains presented characteristics consistent with a recent comparison of some microalgae FA profiles with those of vegetable oils, showing that they have broader carbon-chain lengths distribution (C16 and C18 species are less dominant) and higher levels of PUFA (Hoekman et al., 2012; Hu et al., 2008; Paik et al., 2009), raising concerns about the usefulness of them as feedstock for quality biodiesel (Hoekman et al., 2012).

Most strains presented 20–30% of saturated FA (SAF), mainly 16:0. The cumulative percentage of MUFA was more variable, ranging from 7% to 60%, with many strains accumulating around 50%. In *Pseudokirchnerilella* sp. strain C1D, oleic acid reached a 60% relative level. The abundance of PUFA was between 12% and 45%, while the highest values (40–45%) corresponded to the *Chlorella* sp. strains (RP, MI, SP2-1 and CH).

C. sorokiniana strain RP (as most other *Chlorella* strains analyzed in this study) presented a FA profile quite similar to that of soy, characterized by a high content of 18:2 (Hoekman et al., 2012). On the other hand, *S. obliquus* (strains C1S and PL) and *Pseudokirchnerilella* sp. strain C1D had a FA composition more similar

Table 2
Fatty acids profiles of native strains according to the availability of nitrogen.

Strain	Nitrogen	Fatty acid composition (%)								
		C16:0	C16:1	C18:0	C18:1	C18:1ω7	C18:2	C18:3	C18:4ω3	Others
RP	+N	20.90 ± 1.66[a]	11.02 ± 0.06	2.98 ± 0.55	3.72 ± 0.09	2.45 ± 0.24	25.71 ± 0.90	18.06 ± 0.12	ND[b]	14.53 ± 1.28
	−N	23.96 ± 2.61	2.63 ± 0.41	2.95 ± 0.02	22.9 ± 2.75	0.86 ± 0.03	33.09 ± 1.76	4.45 ± 0.65	ND	8.75 ± 1.26
FG	+N	27.81 ± 2.43	10.70 ± 2.40	7.31 ± 0.86	13.56 ± 3.76	ND	15.96 ± 0.74	20.37 ± 2.56	ND	4.10 ± 1.59
	−N	25.36 ± 1.00	3.20 ± 0.00	7.73 ± 0.53	35.73 ± 1.56	ND	14.73 ± 0.11	9.03 ± 0.14	ND	4.13 ± 0.06
PL	+N	26.88 ± 0.77	5.05 ± 0.35	3.15 ± 0.48	36.52 ± 4.28	ND	9.92 ± 0.97	11.85 ± 2.54	ND	8.23 ± 0.55
	−N	20.62 ± 0.21	1.81 ± 0.10	3.03 ± 0.14	49.08 ± 0.57	0.62 ± 0.12	10.53 ± 1.18	3.83 ± 0.36	0.34 ± 0.48	8.26 ± 0.11
C1D	+N	26.92 ± 1.43	1.39 ± 1.96	3.97 ± 0.11	17.56 ± 0.21	ND	9.47 ± .074	18.93 ± 1.42	4.97 ± 0.65	15.98 ± 0.90
	−N	21.03 ± 0.82	0.53 ± 0.53	3.89 ± 0.16	59.60 ± 1.91	ND	1.72 ± 0.09	7.41 ± 0.21	2.00 ± 0.07	3.63 ± 0.09
C1S	+N	23.41 ± 1.43	3.73 ± 0.21	4.24 ± 0.32	12.84 ± 1.11	ND	9.94 ± 0.36	26.76 ± 0.06	1.21 ± 1.71	17.06 ± 1.52
	−N	25.85 ± 1.61	1.77 ± 0.15	2.96 ± 0.13	46.5 ± 0.10	ND	7.14 ± 1.69	9.27 ± 0.10	1.43 ± 0.07	4.83 ± 0.05
RD	+N	26.55 ± 3.46	3.36 ± 3.58	3.20 ± 0.84	37.63 ± 1.84	1.40 ± 1.26	10.54 ± 4.73	9.75 ± 5.73	0.34 ± 0.48	5.84 ± 1.98
	−N	34.47 ± 0.75	1.56 ± 0.05	4.14 ± 0.35	35.75 ± 0.17	0.43 ± 0.01	11.03 ± 1.03	8.06 ± 0.43	0.66 ± 0.08	3.68 ± 0.22
C2C	+N	23.15 ± 0.79	3.26 ± 0.22	1.40 ± 0.42	21.12 ± 2.97	ND	8.18 ± 0.41	17.68 ± 0.45	7.27 ± 0.92	17.94 ± 1.68
	−N	22.23 ± 1.60	3.71 ± 0.55	2.40 ± 0.23	38.92 ± 5.38	0.28 ± 0.40	7.88 ± 0.82	10.75 ± 1.34	3.90 ± 0.28	9.70 ± 0.28
L20	+N	27.96 ± 1.52	2.84 ± 0.08	5.33 ± 1.24	18.38 ± 1.42	ND	13.13 ± 1.40	17.46 ± 5.04	ND	14.85 ± 0.65
	−N	26.86 ± 0.82	3.73 ± 0.10	3.43 ± 0.21	28.86 ± 1.91	ND	12.12 ± 0.42	16.32 ± 2.17	0.60 ± 0.60	7.99 ± 0.07
SP2-1	+N	38.26 ± 12.67	0.59 ± 0.84	4.49 ± 0.45	17.75 ± 1.69	3.49 ± 0.47	14.72 ± 3.28	4.26 ± 1.65	ND	16.34 ± 7.67
	−N	19.83 ± 1.00	2.12 ± 0.16	1.34 ± 0.19	3.54 ± 0.59	1.93 ± 0.23	16.66 ± 0.59	20.91 ± 1.47	2.41 ± 0.77	30.04 ± 1.22
MI	+N	23.75 ± 0.54	10.10 ± 3.53	1.35 ± 0.70	5.50 ± 1.55	1.23 ± 0.23	15.94 ± 0.45	28.52 ± 0.65	ND	12.20 ± 0.77
	−N	31.03	4.31	2.81	12.14	0.88	31.60	8.50	ND	9.26
C1C	+N	28.05 ± 3.17	9.29 ± 0.09	3.21 ± 0.56	12.14 ± 0.27	ND	14.63 ± 1.50	26.75 ± 1.43	ND	6.30 ± 3.66
	−N	27.18	2.61	4.10	31.94	0.78	11.83	11.36	ND	10.13
SP2-9	+N	32.31	1.39	2.62	21.67	ND	18.22	6.86	ND	17.15
	−N	25.54	0.32	4.77	48.43	ND	10.88	3.20	ND	6.03
SP2-10	+N	51.79	ND	9.58	28.66	ND	5.25	5.24	ND	0.00
	−N	24.34	3.68	4.54	33.73	2.39	17.13	8.76	ND	5.42
SP2-15	+N	27.79	1.26	3.38	23.67	2.40	4.53	15.74	7.74	13.42
	−N	26.5	1.82	4.96	40.21	0.77	2.90	11.92	5.12	5.73
LP1	+N	22.69	1.35	5.32	21.61	ND	8.54	12.26	4.94	23.12
	−N	27.69	1.04	5.75	22.07	1.43	16.55	6.52	ND	18.69
L2	+N	24.82	ND	7.20	30.14	ND	22.35	5.10	ND	10.39
	−N	21.65	3.40	2.22	35.17	0.47	19.54	7.38	0.43	9.20
CH	+N	25.15	9.76	1.95	5.07	1.00	19.07	24.64	ND	13.36
	−N	27.28	5.59	3.00	8.26	ND	33.81	11.88	ND	10.18

[a] When available, data represent the mean ± SD of two independent experiments, otherwise represent the data from a single experiment.
[b] Not detected.

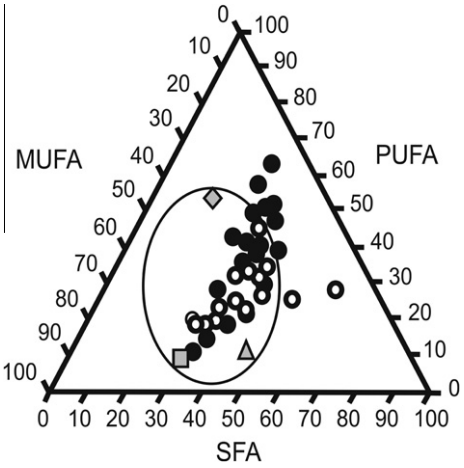

Fig. 2. Comparison of fatty acids saturation in native microalgae oils and those of main biodiesel feedstock. The triangular plot represents the percentual distribution of SFA, MUFA and PUFA. FA classes were forced to sum 100% by considering only the FA that could be identified, which corresponded to about 90% (Table 2). (●) N-deficient microalgae cells; (○) N-replete microalgae cells; (▲) palm oil; (■) rapeseed oil; and (♦) soybean oil (Hoekman et al., 2012).

to that of rapeseed and palm oils, with 18:1 as the most abundant FA (Hoekman et al., 2012) (see Supplementary Fig. S3). These microalgae strains presented low percentages of PUFA (around 10%), distinguishing them from the characteristic FA profiles of the microalgae *Nannochloropsis* spp. (up to 30% of PUFA, mostly 20:5), a model of marine microlgae for sustainable production of microalgae-based biodiesel (Khozin-Goldberg and Boussiba, 2011). Like most fresh water strains (Paik et al., 2009), the strains reported herein contained low levels of 16:1, another major difference with *Nannochloropsis* spp., presenting values close to 30% (Khozin-Goldberg and Boussiba, 2011; Paik et al., 2009).

3.4. Biomass and lipid accumulation properties of selected native microalgae strains

Indoors culturing under controlled conditions were used for a screening of the most promising strains according to their potential for fast growth, high biomass concentration, high lipid content and FA profile. Although a quite continuous score could be assigned for the biotechnological potential of the strains of the collection, four strains (RP, C1D, C1S and PL) were selected for a more detailed growth characterization either indoors under controlled conditions or outdoors under environmental conditions (Table 3).

For indoors experiments 300 mL cultures were set in air-bubbled bottles and continuously illuminated at 70 μmol photons m^{-2} s^{-1} and 29 ± 1 °C. These strains attained maximal cell densities of 10^7–10^8 cells/mL and about 0.5 g dw/L with maximal doubling times of 6–11 h.

For outdoors cultivation of these native strains, the summer season was originally targeted according to a previous report (Pérsico et al., 2011) indicating that that was the less favorable season for *Nannochloropsis oculata* cultivation in the city of Mar del Plata. Thus, we challenged strains RP, C1D, C1S and PL to outdoors

Table 3
Growth characteristics of selected native strains.

Strain	Indoors			Outdoors		
	Cellular density[a] (10^6 cell/mL)	Cell dwt (mg/L)	Doubling time (h)	Cellular density (10^6 cell/mL)	Cell dwl (mg/L)	Doubling time (h)
RP	64.50 ± 10.10[b]	581.50 ± 0.70	5.90 ± 0.90	23.30 ± 4.50	741.9 ± 115.00	33.50 ± 10.20
C1D	51.20 ± 8.10	451.00 ± 47.30	7.70 ± 1.40	58.20 ± 2.10	613 ± 94.40	26.30 ± 5.50
C1S	11.30 ± 0.42	442.00 ± 7.07	10.40 ± 1.20	15.90 ± 1.60	881.1 ± 34.90	41.60 ± 6.00
PL	9.80 ± 0.21	635.70 ± 52.50	6.10 ± 0.90	10.90 ± 2.50	830.5 ± 94.30	41.70 ± 8.60

[a] At stationary phase of growth.
[b] Data represent the mean ± SD of three to six independent experiments.

environmental conditions from December to March in 3,000 mL cultures in air-bubbled bottles under partial shade. Reference light and temperature parameters for the location on the corresponding season are, on average, 900 μmol photons m^{-2} s^{-1} at noon and 14 h light, average maximal temperatures of 25 °C and average minimal temperatures of 13 °C (Pérsico et al., 2011). As expected, each of the four strains performance during outdoors cultivation differed significantly from the potential they displayed during indoors growing conditions with doubling times of at least 1 day. Maximal biomass yields became higher and resulted from similar or lower maximal cell densities. A corresponding increase in cell size for most strains was confirmed by optical microscopy (not shown). Strains RP and C1D appeared to be more sensitive to extreme maximal temperatures over 35 °C than the two *S. obliquus* strains C1S and PL.

A critical aspect of microalgae biotechnology is cell collection that is currently an energy and infrastructure-demanding process (Chisti and Yan, 2011; Hu et al., 2008; Wijffels and Barbosa, 2010). Thus we compared the cell sedimentation properties of the relatively larger cells of strain C1S, as representative of the *S. obliquus* strains, and the smaller cells of strain C1D. While most of the C1S biomass could be recovered in one-tenth of the original culture volume (10-fold concentration) in as soon as 4 h, only roughly one-fifth of the C1D cells could be recovered with a concentration factor of twofold in the same time (Fig. 3).

Strain C1S cells showed a robust response to CO_2 supplementation attaining a four to fivefold dry biomass yield after 3 days of cultivation in comparison to air-bubbled cells in medium containing 4 mM N- and P-sufficiency. Since the dry biomass percentage of lipids was similar in both conditions, the lipids yield was fourfold after 6 days of culture. No significant differences in total biomass or lipids were observed in sucrose amended cells (Fig. 4A). Under N-, and especially N- and P-deficiency, increases in the lipidic

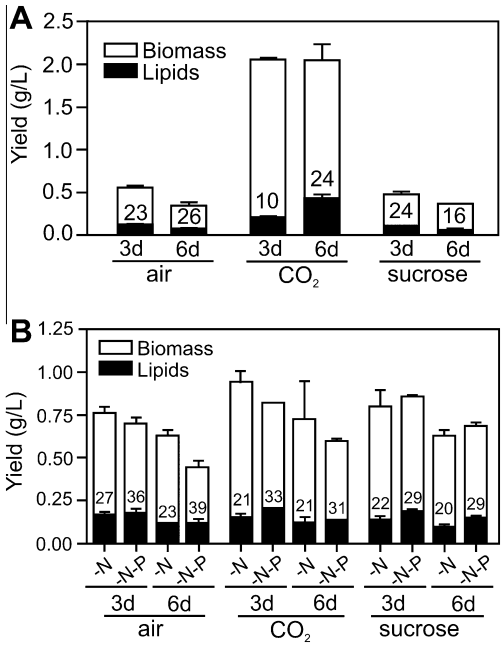

Fig. 3. Carbon source and N- and P-deficiency effect on biomass and lipid productivity of *S. obliquus* strain C1S. (A) Cells were cultivated in nutrients replete medium (4 mM NH_4^+) for 6 d supplemented with the indicated carbon sources. (B) Cells cultivated in nutrients replete medium (4 mM NH_4^+) for 6 d were transferred to the indicated N- or N–P-deficiency for 3 or 6 d. Empty bars represent the dry biomass yield, full bars the total lipids yield and numbers within the empty bars the lipid content as a percentage of the dry biomass. Data represents the mean and standard deviation of two independent experiments.

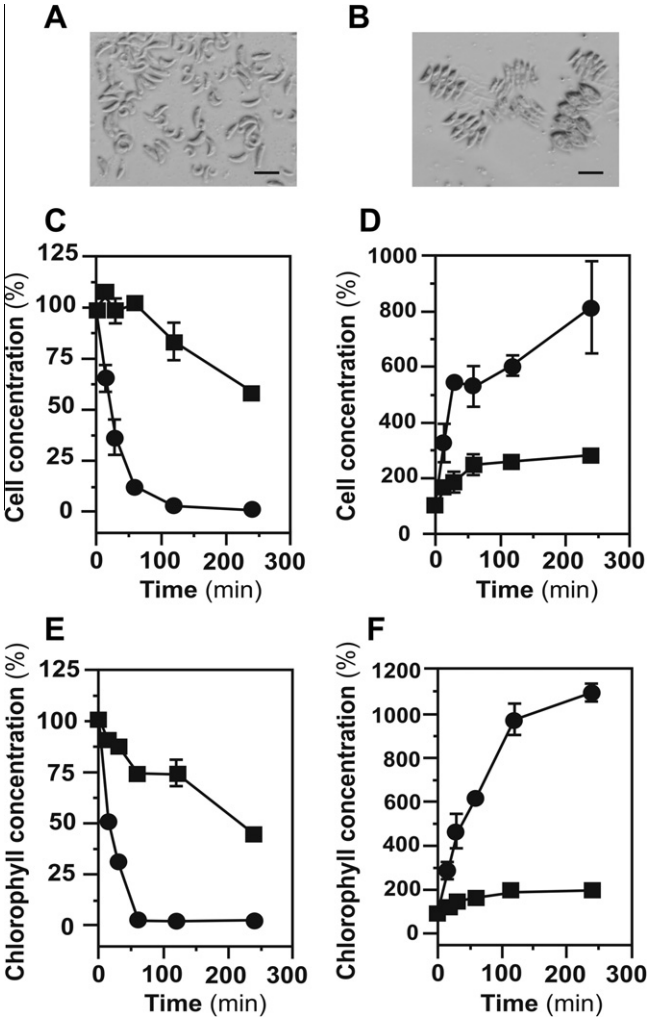

Fig. 4. Time course of sedimentation of selected native strains. Differential phase-contrast images of *Pseudokirchneriella* sp. strain C1D (A) or *S. obliquus* strain C1S (B). The bars represent 10 μm. Determination of (■) *Pseudokirchneriella* sp. strain C1D; or (●), *S. obliquus* strain C1S cells number (C and D) and chlorophyll (E and F) were determined from the 10% of the culture volume at the surface (C and E) or the 10% of the culture volume at the bottom (D and F). In C–F each data point represents the mean and standard deviation of two independent experiments.

content of the cells were observed for all the conditions. However lipid productivity was offset by the lower yields in biomass resulting from the imposed nutrients limitations. Thus the final lipid productivity remained similar regardless the nature of the limiting nutrient and insensitive to CO_2 or sucrose supplementation (Fig. 4B). This inverse relationship in biomass and lipid productivity appears to be a common trend for microalgae (Feng et al., 2011) and led researchers to proposed different alternatives such as a microalgae-protein-based process for the production of biofuels (Huo et al., 2011). According to this concept the most attractive feedstocks should be fast growing strains with protein rich and lipid poor (10%) biomass cultured under nutrients (especially N) sufficiency (Huo et al., 2011). Also the controlled feeding of nitrogen has been shown to promote lipid productivity in the microalga *Isochrysis zhangjiangensis* (Feng et al., 2011) and an alternative strategy for the N fertilization of microalgae was also recently proposed. It was shown that three of the strains selected in this work (RP, C1S and C1D) engage in artificial symbiosis with a diazotrophic and ammonium-excreting *Azotobacter vinelandii* mutant-strain, suggesting a potential for controlled N-delivery from the air (Ortiz-Marquez et al., 2012).

4. Conclusions

In this work, 34 native-to-Argentina microalgae strains were isolated; some of them presenting novel RNA sequences. Desirable traits as biodiesel feedstock such as doubling times of 6 h, lipids accumulation of up to 43% of their dry biomass, appropriate FA profile, and complete sedimentation in 4 h were observed. *S. obliquus* strain C1S, presented one of the best combination of desirable traits, what makes it a good candidate for further assessment of the techno-economical feasibility of developing a microalgae-based industry in the region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.08.057.

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