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Autotrophic starch production by *Chlamydomonas* species

Imma Gifuni[1] · Giuseppe Olivieri[1] · Antonino Pollio[2] · Telma Teixeira Franco[3] ·
Antonio Marzocchella[1]

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Abstract Microalgal autotrophic cultures may be used as starch feedstocks for a wide spectrum of food and non-food applications, starch-based plastics production included. *Chlamydomonas* is known to accumulate carbohydrates, but only *Chlamydomonas reinhardtii* is widely studied. This is the first paper that analyzes the starch content and production rate of four non-conventional *Chlamydomonas* species and compares their performances to the benchmark *C. reinhardtii*. Two culture systems—shaken flasks and inclined bubble column (IBC) photobioreactors—and nitrogen depletion conditions were characterized. The irradiance was set at 95 μmol photons m^{-2} s^{-1} for flask system and at 220 μmol photons m^{-2} s^{-1} for photobioreactors. CO_2 and light depletion in shaken flasks strongly affected growth rate and starch production. Under these limiting condition, *Chlamydomonas applanata* had the best starch productivity of 1.2 mg L^{-1} day^{-1}. In IBC photobioreactors, the microalgal growth rate and starch production improved with respect to the flask system and nitrogen depletion promoted starch accumulation. The best results of starch productivity and maximum starch fraction were 53 mg L^{-1} day^{-1} and $45\%_{DW}$ for *Chlamydomonas oblonga* and *Chlamydomonas moewusii*, respectively. This was 49 % more than the studied benchmark. A fast and simple method for starch localization in the microalgal cells was also proposed. The starch granules surrounded the pyrenoid under the growth phase, while they fill the whole cell under nutrient depletion.

Keywords Bioplastics · *Chlamydomonas* · Culture systems · Microalgae · Starch

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✉ Giuseppe Olivieri
giolivie@unina.it

[1] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Università degli Studi di Napoli Federico II, Piazzale Tecchio 80, 80125 Naples, Italy

[2] Dipartimento di Biologia, Università degli Studi di Napoli Federico II, Via Foria 223, 80139 Naples, Italy

[3] Faculdade de Engenharia Química–University of Campinas, Av. Albert Einstein, 500, Campinas, SP CEP 13083-852, Brazil

Introduction

The increasing demand of starch, as feedstock for many food and non-food products, has been reported by Srinivas (2007) and by LM International (2002). One of the consolidated starch industrial applications is starch plastic (e.g., thermoplastic starch, TPS) production, which is now the most widespread biobased plastic. Starch-plastic producers (e.g., Novamont) exploit starch accumulated by plants as carbon/energy source (Busi et al. 2014). Plant starch is made up of two polymers of D-glucose: amylose (unbranched) and amylopectin (highly branched). Amylopectin chains may form helical structures that may crystallize promoting the formation of the granule structure (Buléon et al. 1998).

The current main sources of starch are potato, maize, wheat, and sorghum. However, social, environmental, and commercial issues ask for alternative sources characterized by constant supply rate, selected quality, low cost and environmental impact, and production systems that do not compete for arable land.

The interest in microalgae as potential source for starch is related to their fast growth rate—compared with terrestrial

plants—and their wide range of industrial applications including food, biofuels, drugs, cosmetics, and bioremediation. Moreover, microalgal cultures do not compete for arable land, and in autotrophic cultures, they combine the biomass production with CO_2 capture. Regarding microalgae as bioplastic feedstock, the attention of researchers has been mainly focused on the characterization of processing both the whole microalgal biomass and the microalgal proteins for bioplastic production. At Algix, researchers have investigated the capacity of microalgal proteins to form polymers and an algae-to-plastic facility for algae powder extrusion with resin and additives has been built in Missisipi, USA (www.algix.com). However, there is industrial interest concerning also the use of the microalgal starch fraction in bioplastic production as well as in food industries. The process breakthrough is the selection of the optimal culture conditions (i.e., culture strategy and culture systems) to maximize starch accumulation in microalgae.

Ho et al. (2012) reported that it is possible to enhance starch production in microalgae by tuning the microalgal culture conditions such as irradiance, nutrient depletion, temperature variation, pH shift, and CO_2 supply (Brown et al. 1997; Renaud et al. 2002; Khalil et al. 2010; Chen et al. 2013). However, the most common strategy to enhance the accumulation of carbon-rich molecules in microalgae is nitrogen depletion (Dragone et al. 2011; Breuer et al. 2012; Fernandes et al. 2013; Kamalanathan et al. 2016). Under nitrogen depletion, starch is accumulated in the early starvation phase, whereas in late starvation phase, lipid accumulation is promoted. *Chlamydomonas* is known to accumulate a large amount of sugars as starch (Chen et al. 2013), but the most studied species is still *Chlamydomonas reinhardtii* because it is widely characterized from the metabolic and genetic point of view. More than 400 *Chlamydomonas* species have been recognized, and molecular analyses showed that the genus *Chlamydomonas* is polyphyletic and should be divided into eight independent lineages (Demchenko et al. 2012). No information is available about carbohydrates and starch content of other *Chlamydomonas* species. We have investigated four interesting species, in addition to *C. reinhardtii* (used as benchmark):

- *Chlamydomonas pitschmannii*, coming from thermoacidic environment, studied for lipid production and resistance to the presence of metals in the medium (Abou-Shanab et al. 2011; Pollio et al. 2005)
- *Chlamydomonas oblonga* for which only phylogenetic, taxonomic, and structural analyses are reported in literature
- *Chlamydomonas applanata* characterized by a high range of pH tolerance
- *Chlamydomonas moewusii* widely studied for hydrogen production in the last decades (Meuser et al. 2009)

No information is available in the literature about the carbohydrate and starch content of these four species.

The aim of this work was to investigate starch production by the mentioned non-conventional *Chlamydomonas* species and to select the best starch producer. Moreover, to the authors' knowledge, there is no systematic analysis of culture conditions and culture systems on microalgal starch accumulation. For this reason, the effect of two culture systems has been investigated: shaking flasks and prismatic photobioreactors.

A cytological study about the localization of starch in the cells was also carried out by means of a staining assay coupled with optical microscope observation, in order to localize the amount and the distribution of the granules in the different *Chlamydomonas* species.

Materials and methods

Microorganisms and medium

Chlamydomonas species were from the ACUF collection of the Department of Biology at the Università degli Studi di Napoli "Federico II" (www.acuf.net). Five *Chlamydomonas* species were investigated: *Chlamydomonas reinhardtii* Dangeard (strain number (s.n.) 027); *Chlamydomonas pitschmannii* Ettl (s.n.118); *Chlamydomonas oblonga* Pringsheim (s.n.157); *Chlamydomonas applanata* Pringsheim (s.n.159); and *Chlamydomonas moewusii* Gerloff (s.n.163). The strains were grown in Bold Basal Medium (BBM) as described by Olivieri et al. (2013). The BBM pH was about 6.8.

Analytical methods

Culture samples were collected and characterized in terms of microalgae and medium composition. The samples were centrifuged at 5000×*g* for 20 min (Eppendorf-5804R) to harvest cells from the liquid phase.

Microalgal analysis

The microalgal concentration was measured as optical density at wavelength of 750 and 600 nm (OD_{750} and OD_{600}). The OD values were converted to biomass concentration via appropriate calibration between OD and dry cell weight. The dry cell weight was determined by filtering 50-mL aliquots of culture through a Whatman filter. The filter was dried at 60 °C until constant weight and weighed. This procedure was repeated for several biomass concentrations. The biomass concentration estimated according the conversion factor assessed by processing OD_{750} and OD_{600} did not change within the experimental error.

Total carbohydrates were assayed according to phenol-sulfuric acid method (Miller 2010). The microalgal pellet was dispersed in distilled water to prepare a suspension at 1 g_{DM} L^{-1} cell concentration. One milliliter of the suspension was mixed with 1 mL of phenol (5 %) and 5 mL of sulfuric acid (96 %). The mixture was cooled at 25 °C. The absorbance of the solution was measured at 488 nm. The concentration of carbohydrates was calculated using a calibration curve based on glucose.

Ten milligram of dry microalgae was used to measure the microalgal starch content. Microalgae were suspended in a buffer solution (pH = 7) and processed in a French press for mechanical rupture. The microalgal lysate was centrifuged, re-suspended in an aqueous solution of ethanol (80 %), and incubated at 80–85 °C for 5 min to extract the pigments. Starch content was assayed by an enzymatic kit (Megazyme, Ireland) containing amylase, amyloglucosidase, and oxidase-peroxidase enzymes and adopting the protocol provided by the manufacturers.

The starch granules in microalgal cells were stained with Lugol dye and observed in a microscope (Nikon eclipse E800, bright field). Lugol's dye is a solution of elemental iodine and potassium iodide in water. The solution was mixed with culture samples at a volumetric ratio of 1:3 (Muller et al. 1998). Elemental iodine solutions stain just starches because iodine interacts with the coil structure of the polysaccharide and provides dark-blue/black starch colorization after 10 min of mixing. Simple sugars—such as glucose and fructose—do not interact with Lugol dye. Microscope pictures were analyzed by ImageJ (open sources image processing software by National Institutes of Health, USA) as reported by Schulze et al. (2011), in order to measure the cell area and the cell area filled by starch.

Medium analysis

Liquid phase was characterized in term of pH, nitrate, and total sugar concentration. The pH was measured by a pH meter (InLab Routine series probe, 0–14 pH | FE20 – FiveEasy meter, Mettler Toledo).

Nitrate concentration was determined spectrophotometrically according to Collos et al. (1999). Twenty microliter of 1 M HCl was added to 1 mL of liquid sample to remove carbonate interferences and nitrate content was measured as optical density at 220 nm (OD_{220}) using a UV/VIS spectrophotometer (Varian Cary50). A calibration curve was made vs. $NaNO_3$ to assess the conversion factor between OD_{220} and concentration of NO_3^- ions.

The sugar concentration was assayed using the Phenol-Sulfuric Acid method previously described (see section "Microalgal analysis").

Apparatus, operating conditions, and procedures

Two culture systems were used for *Chlamydomonas* growth: Erlenmeyer flasks and inclined bubble column photobioreactors. All experiments were carried out in triplicates and the standard deviation was calculated on the basis of these tests.

Erlenmeyer flasks of 2 L were set on a Plexiglass shaking plate housed in a thermosted room at ~25 °C. The flasks were lighted from the bottom by fluorescent lamps (Philips, TLD 30W/55) at an irradiance of 95 μmol photons m^{-2} s^{-1}. The irradiance was measured with a LI-COR LI-190 quantum sensor.

The head of the flask was closed by means of a gauze filter to prevent contamination and to allow free gas exchange between the flask and the atmosphere. CO_2 and O_2 exchange between the culture and the gas present in the flask was provided by continuous shaking of the cultures. Flasks with 500 mL medium were autoclaved for 20 min at 121 °C. pH was not controlled during the cultivation. Fifty milliliter of concentrated medium was added each week to the culture to restore the initial volume and nutrient concentration and to prevent nutrient depletion. Sterilized distilled water was supplemented to the flasks for counterbalance the effect of evaporation.

The inclined square bubble column photobioreactors had a volume of 2 L, thickness of 8 cm, and the longitudinal axis was inclined of 30° with respect to the horizontal (Gargano et al. 2013). The working volume was 1.5 L. The photobioreactors were housed in a climate chamber (HeraeusVötsch GmbH; type: HPS 500) at 25 ± 1 °C. Fluorescence lamps (M2M engineering) fixed at the ceiling of the climate chamber continuously illuminated photobioreactors at an irradiance of 220 μmol photons m^{-2} s^{-1}. The volumetric flow rate of the gas stream fed at each photobioreactor was controlled by means of needle valves and was set at 0.22 vvm. The CO_2 concentration in the gas stream was set at 2 %. The photobioreactors with the medium were autoclaved for 20 min at 121 °C. Batch cultures were carried out in these photobioreactors; the pH was maintained at 7 by continuous addition of CO_2 to offset the increase in pH during microalgal growth (Moheimani and Borowitzka 2006). Culture was sampled daily and no medium was added to culture to allow nitrogen depletion.

The biomass-light yield $Y_{X/E}$ was calculated as ratio of the biomass produced and the light energy irradiated over the cultivation time. The produced biomass was assessed as the product of the culture volume, V (L) and the difference of the biomass concentration measured at the culture end, X (g L^{-1}) and the inoculation time X_0 (g L^{-1}). The irradiated energy was assessed as the product of the irradiated surface A (m^2), the irradiance E) (μmol photons m^{-2} s^{-1}) and the culture time t (s). The $Y_{X/E}$ (g_X $mol_{photons}^{-1}$) was:

$$Y_{X/E} = \frac{(X - X_0)V}{AEt} \quad (1)$$

The ratio φ between the area occupied by the starch in a cell and the area of the cell was assessed by processing cell digital images with ImageJ program. Under a set of operating conditions, φ was calculated as the average of the ratio, assessed for *N* observation, with *N* larger than 100:

$$\varphi = \frac{1}{N}\sum_{N}\frac{\text{(starch area)}}{\text{(cell area)}} \quad (2)$$

The standard deviation of ratio was also assessed.

Biomass productivity, r_X (g L^{-1} day^{-1}) was assessed as the ratio between biomass concentration and culture time according to the relationship:

$$r_{X(t)} = \frac{X(t) - X_0}{t - t_0} \quad (3)$$

where $X(t)$ is the biomass concentration (g L^{-1}) at a specific time of the culture, t (d), while t_0 (d) is the beginning of the culture time.

Starch productivity, r_S (g L^{-1} day^{-1}) was assessed as the ratio between starch concentration (g_{starch} L^{-1}) and culture time, $t - t_0$ (d):

$$r_{S(t)} = \frac{\omega_{starch}(t) \cdot X(t)}{t - t_0} \quad (4)$$

where $\omega_{starch}(t)$ is the starch fraction of the dry microalgal dry mass measured at the instant t and t_0 is the inoculation time. The starch mass in the inoculum was not included in Eq. (4) because it was negligible.

Starch and sugar content of microalgae were calculated as the ratio between the starch and the sugar concentration and the dry mass of microalgae analyzed.

Results

Flask culture system

The cultures in flasks were carried out for about 1 month. The culture volume was kept constant during the test and nutrient concentration was never limiting because nutrients were periodically added. The supply of CO_2 to the culture was for dispersion of the CO_2 of the air across the free suspension surface. Culture irradiance was set at 95 μmol photons m^{-2} s^{-1}.

Figure 1 shows the growth curve in the flask system of the five investigated species. The cell concentration (X) increased linearly with time for all species, although there are noticeable differences in growth performances. *Chlamydomonas applanata* and *C. reinhardtii* growth rates are almost comparable, as well as *C. oblonga* and *C. pitschmannii*, while *C. moewusii* is characterized by the slowest growth rate. The biomass-light yield ($Y_{X/E}$) ratio for the investigated cultures (see Fig. S1, supplemental material) according to Eq. (1) decreased with the time and after about 2 weeks they approached a constant value characteristic of each strain. Table 1 reports the $Y_{X/E}$ for the *Chlamydomonas* strains at the end of the culture time (4 weeks). *Chlamydomonas reinhardtii* and *C. applanata* were characterized by the best yields: 0.183 and 0.172 g_x mol^{-1} photons, respectively. Lower values were observed for the others strains.

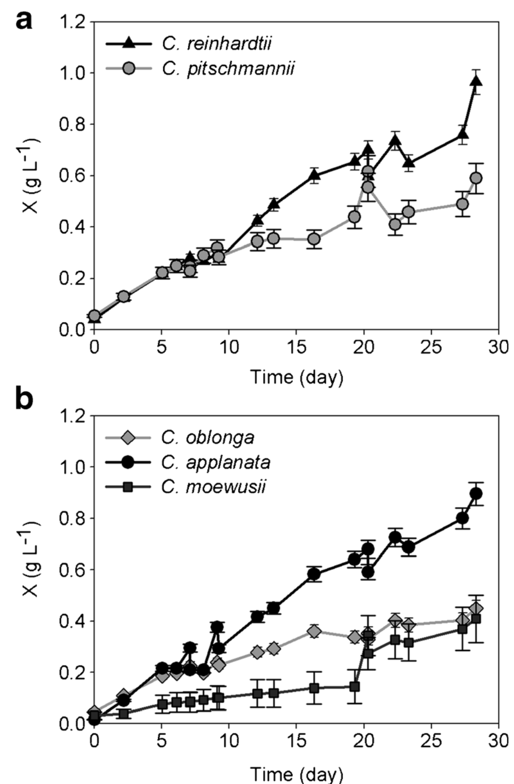

Fig. 1 *Chlamydomonas* species growth in flasks: **a** *C. reinhardtii, C. pitschmannii*; **b** *C. oblonga*, *C. applanata*, *C. moewusii*. Operating conditions: volume 500 mL; temperature 25 °C; pH 6.7; irradiance 95 μmol photons m^{-2} s^{-1}. All points represent the average ± s.d. of three independent experimental replicates

Table 1 also reports the starch fraction of *Chlamydomonas* species and the starch productivity for the flask system. *Chlamydomonas applanata* and *C. reinhardtii* were characterized by the best biomass and starch productivity: 0.034, 0.032 g L^{-1} day^{-1} and 1.1, 1.2 x 10^{-3} g starch L^{-1} day^{-1}. Although the starch contents were not the highest among the five strains, these strains were characterized by the highest growth rate and biomass productivity under the operating conditions tested. The highest starch content (5 %) for cultures in the flask system was measured for *C. oblonga*.

The concentration of sugar secreted in the medium is also reported in Table 1. Secreted sugar concentration increased with the time for all species and was as high as 0.2 g L^{-1}.

Table 1 Main data assessed for the cultures of the investigated *Chlamydomonas* species

Chlamydomonas species	$Y_{X/E}$ (g_x $mol_{photons}^{-1}$)	Biomass productivity (g L^{-1} day^{-1})	Starch productivity (g L^{-1} day^{-1})	Total sugar concentration in biomass ($\%_W$)	Starch concentration in biomass ($\%_{DW}$)	Secreted sugar (g L^{-1})
C. reinhardtii	0.183 ± 0.009	0.034 ± 0.004	1.1 ± 0.1 x 10^{-3}	27.21 ± 0.72	3.1 ± 0.28	0.108 ± 0.002
C. pitschmannii	0.119 ± 0.006	0.021 ± 0.002	0.5 ± 0.1 x 10^{-3}	22.18 ± 0.08	4.6 ± 0.56	0.198 ± 0.007
C. oblonga	0.104 ± 0.005	0.019 ± 0.005	0.8 ± 0.2 x 10^{-3}	27.85 ± 0.09	5.2 ± 1.15	0.157 ± 0.008
C. applanata	0.172 ± 0.009	0.032 ± 0.002	1.2 ± 0.3 x 10^{-3}	19.91 ± 0.23	3.7 ± 1.03	0.190 ± 0.009
C. moewusii	0.086 ± 0.003	0.015 ± 0.007	0.6 ± 0.2 x 10^{-3}	14.84 ± 0.68	4.04 ± 0.97	0.083 ± 0.008

Cultivation system: flasks. CO_2 feeding: diffusion of air at the culture surface. Irradiance 95 μmol photons m^{-2} s^{-1}. All points represent the average ± s.d. of three independent experimental replicates

Cell growth and starch production rate in photobioreactors

Figure 2 reports the concentration of microalgal cells vs. time for cultures in inclined square bubble column photobioreactors (IBCPs) irradiated with cold white fluorescent lamps at 220 μmol photons m^{-2} s^{-1}. The photobioreactors were fed with 2 % CO_2 air streams. A two-phase pattern of the microalgal cell growth was recognized: a 5–6 day growth phase followed by a stationary phase. The lag phase was almost absent because of the high inoculum size.

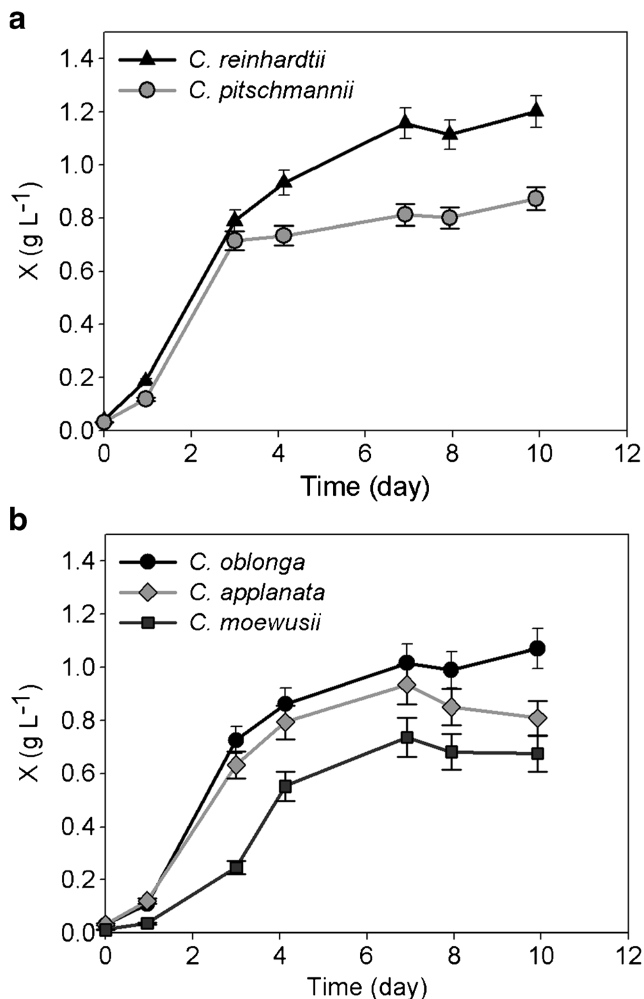

Fig. 2 Growth of the *Chlamydomonas* species in photobioreactors. **a** *C. reinhardtii, C. pitschmannii*; **b** *C. oblonga*, *C. applanata*, *C. moewusii*. Operating conditions: inoculum size 0.1 g L^{-1}; BBM; volume: 1.6 L; temperature 25 °C; pH 6.7; air flow 0.22 vvm; CO_2 concentration 2 %; irradiance 220 μmol photons m^{-2} s^{-1}. All points represent the average ± s.d. of three experimental replicates

Figure 3 reports the biomass productivity—assessed according to Eq. 3—as a function of culture time. The maximum productivity was measured at about day 3 after the inoculation and the value depended on the species. The total carbohydrate fraction, the starch fraction of the biomass and the starch productivity—assessed according to Eq. 4—are reported in Table 2 and Fig. 4, respectively. Both total carbohydrate and starch fractions of the biomass increased from early growth phase (day 1 in Fig. 2) to early N-starvation phase (day 7 in Fig. 2). As N-starvation was extended (day 10 in Fig. 2), the total carbohydrate fraction decreased mainly as a consequence of the reduction of the non-starch components. The highest biomass productivity was reached by *C. reinhardtii*, but the non-conventional strains showed the highest starch content and starch productivity. In particular, *C. moewusii* sugar content reached 73 % of microalgal DW and the starch fraction was 45 % of DW, while the highest starch productivity was measured for *C. oblonga* and was 0.53 g L^{-1} day^{-1}.

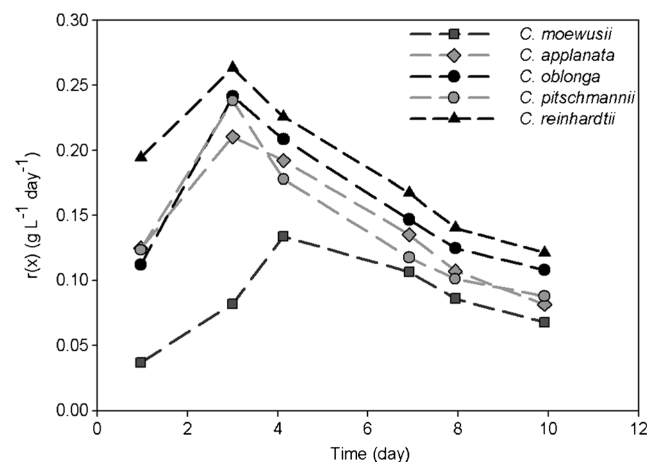

Fig. 3 Biomass productivity of the *Chlamydomonas* species during cultivation time in photobioreactors

Table 2 Carbohydrate content and starch content of the biomass and secreted sugars measured during the cultures of the investigated *Chlamydomonas* species

	Total carbohydrates in biomass (% of $_{DW}$)	Starch in biomass (% of $_{DW}$)	Secreted sugar (g L^{-1})
Early growth phase (day 1 in Fig. 2)			
C. reinhardtii	10.01 ± 0	4.83 ± 0.32	34.45 ± 5.24 x 10^{-3}
C. pitschmannii	17.93 ± 0.69	7.09 ± 0.16	41.90 ± 1.51 x 10^{-3}
C. oblonga	28.4 ± 3.61	12.07 ± 0.62	31.77 ± 1.16 x 10^{-3}
C. applanata	33.64 ± 5.81	22.58 ± 0.19	25.37 ± 0.81 x 10^{-3}
C. moewusii	47.84 ± 0.98	6.46 ± 0.85	21.07 ± 2.44 x 10^{-3}
First day under N-depletion (day 7 in Fig. 2)			
C. reinhardtii	48.31 ± 1.98	22.14 ± 0.49	54.59 ± 5.90 x 10^{-3}
C. pitschmannii	50.34 ± 2.51	29.58 ± 0.68	70.25 ± 4.62 x 10^{-3}
C. oblonga	72.58 ± 1.46	37.73 ± 1.12	57.21 ± 5.62 x 10^{-3}
C. applanata	50.34 ± 1.34	31.76 ± 2.24	51.62 ± 5.47 x 10^{-3}
C. moewusii	72.76 ± 5.72	44.65 ± 3.33	48.65 ± 7.72 x 10^{-3}
Third day under N-depletion (day 10 in Fig. 2)			
C. reinhardtii	43.83 ± 2.5	26.36 ± 0.44	126.77 ± 2.23 x 10^{-3}
C. pitschmannii	48.78 ± 3.61	30.38 ± 0.13	83.69 ± 5.59 x 10^{-3}
C. oblonga	74.56 ± 4.95	43.89 ± 0.57	71.76 ± 3.87 x 10^{-3}
C. applanata	55.47 ± 5.41	29.94 ± 1.82	59.19 ± 2.04 x 10^{-3}
C. moewusii	79.04 ± 1.01	36.04 ± 1.01	88.00 ± 5.70 x 10^{-3}

See caption of Fig. 2 for operating conditions and culture system. All points represent the average ± s.d. of three independent experimental replicates

The secreted sugar concentration in the medium increased with the time for all the investigated species.

Starch distribution in microalgal cells grown in photobioreactors

Figure 5 shows microscope pictures of *C. oblonga* cells stained with Lugol dye. The cells were harvested in the growth phase (Fig. 5a) and in the N-starvation phase (Fig. 5Bb). The dark regions represent the pyrenoids surrounded by starch granules. The microscope observation of the cell highlighted that the pyrenoid was in the central-basal position for *C. reinhardtii* and *C. moewusii*, in a sub-apical position against the cell wall in *C. pitschmannii* and *C. applanata* (see Fig. S2, supplemental material), and in the central position in *C. oblonga* (Fig. 5a). Under nitrogen starvation conditions, the starch filled the cell in all the strains (Fig. 5Bb).

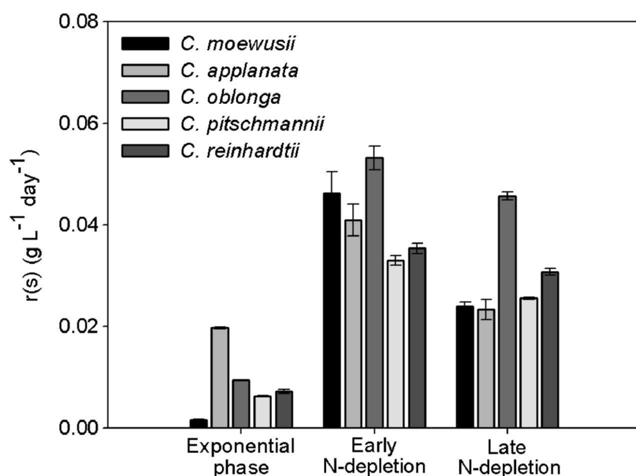

Fig. 4 Starch productivity of the *Chlamydomonas* species investigated (see Fig. 2 for operating conditions). Data refer to the three phases of growth: phase, early N-depletion, after 3 days in N-starvation. All *bars* represent the average ± s.d. of three experimental replicates

Table 3 reports the ratio φ between the area occupied by the starch in a cell and the area of the cell, assessed according to Eq. 2 in the growth phase and the N-starvation phase. It can be seen that during N-starvation, the area of the cells and the fraction of cell area occupied by starch increase with respect to the exponential phase. Cell fattening was observed for all the investigated *Chlamydomonas* species under N-depletion conditions.

Discussion

Flask system vs. IBC for starch production studies The linear growth observed for the test carried out in flask system (Fig. 1) may be due to some nutrient limitations that prevented exponential growth. The biomass productivity reported in Table 1 was constant for each species throughout the culture time because the biomass concentration increased linearly with the time. The growth-limiting factors did not include N- and P-sources because they were periodically

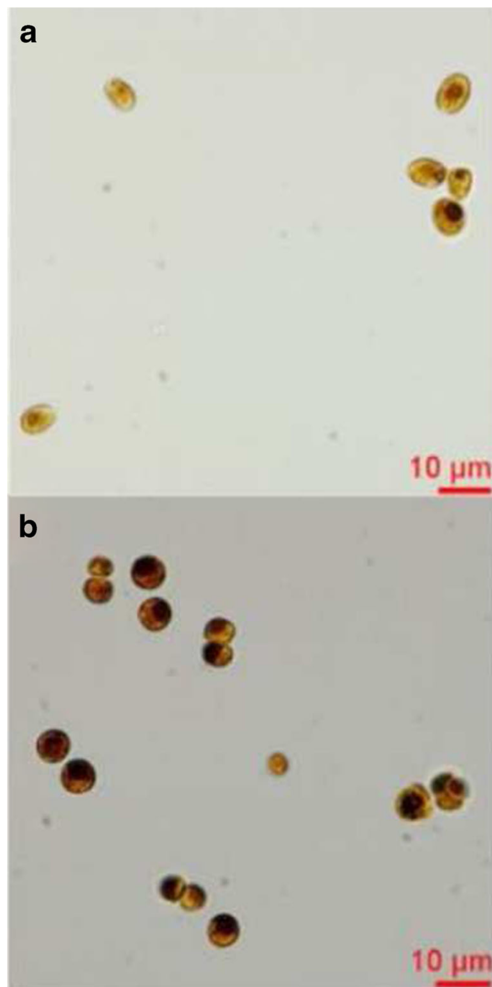

Fig. 5 Light micrographs of *C. oblonga* cells stained with Lugol dye to highlight the starch granules (*dark region*). Cell harvested under **a** growth phase and **b** N-starvation phase

supplemented at sufficient mass rate to avoid N- and P-depletion. The two factors that could have limited the cell growth are CO_2 and light. The flask irradiation was definitively low and this kind of culture system did not allow efficient conversion of the light provided into biomass as shown by the $Y_{X/E}$ values. Kliphuis et al. (2012) reported $Y_{X/E}$ for *C. reinhardtii CC1690* in steady-state cultures irradiated at constant irradiance for 24/24 h. The irradiance was changed between 80 and 1000 (μmol photons m^{-2} s^{-1}). They found that the $Y_{X/E}$ decreased from 1.25 to 0.51 g_x mol^{-1} photons when the irradiance increased between 80 and 1000 μmol photons m^{-2} s^{-1}. The yield assessed in the present study was about one order of magnitude less than that observed by Kliphuis et al. (2012) at approximately equal irradiance. It may be inferred that some factors limited the light conversion into biomass. Indeed, Kliphuis et al. (2012) sparged the cultures with air at 5 % of CO_2, while the CO_2 concentration in the present tests was about 0.035 %. Nascimento et al. (2013) reported a biomass productivity of 0.24 g L^{-1} day^{-1} when *Chlamydomonas* species were grown in shaken flasks, at 80 μmol photons m^{-2} s^{-1} with air supplied at 2 % CO_2, much higher than those obtained in our experiment (Table 1). Analysis of the data in Table 1 and those reported by Kliphuis et al. (2012) and Nascimento et al. (2013) suggests that CO_2 diffusion from air to liquid promoted by flask shaking was not adequate for supplying the cultures.

The presence of limiting factors also affects starch accumulation and productivity in flasks system. Indeed, the obtained results of 5 % of starch on DW and 1.1x10^{-3} g L^{-1} day^{-1} are definitely not attractive for a mass production.

The biomass and starch productivities measured in the IBC photobioreactors were higher than those measured in the flasks (Table 2). The high performance in the IBCs with respect to the performance in the flasks is probably due to the improved supply of CO_2 and to the light availability. Indeed, the mixing induced by the bubble flow promoted cell movement between the dark and the light zones (Olivieri et al. 2013). Moreover, as reported by Yao et al. (2012), for the microalga *Tetraselmis subcordiformis*, the increase in light energy causes a superior starch accumulation, as well as increased biomass productivity. According to Johnson and Alric (2013) and Chen et al. (2013), appropriate irradiance offers energy that may be stored as carbohydrates. Indeed, *C. reinhardtii* can accumulate carbohydrates up to 60 % of dry biomass, and about 55 % of these carbohydrates is starch. Fernandes et al. (2013) reported that the starch content in *Chlorella vulgaris* increased from 8.5 % (dry weight basis) to 40 % when the light intensity was increased from 215 to

Table 3 Microalgal cell area and fraction of area occupied by starch (φ)

	Growth phase		N-starvation phase	
	Cell area (μm^2)	φ	Cell area (μm^2)	φ
C. reinhardtii	23.4 ± 8.1	25.0 ± 11.8	44.8 ± 8.4	40.1 ± 18.6
C. pitschmannii	32.3 ± 8.1	22.8 ± 15.5	47.5 ± 22.5	47.0 ± 22.5
C. oblonga	29.1 ± 7.9	33.3 ± 11.9	35.8 ± 10.2	47.4 ± 21.4
C. applanata	23.6 ± 9.1	31.4 ± 10.6	35.8 ± 6.1	43.5 ± 22.2
C. moewusii	43.5 ± 16.5	27.6 ± 16.1	26.5 ± 7.6	46.4 ± 16.3

All points represent the average ± s.d. of 100 independent analytical replicates

330 μmol photons m^{-2} s^{-1}. Therefore, the optimization of the operating conditions—e.g., light irradiance and CO_2 availability—may remarkably improve starch content and production. The analysis of the results of the present tests proves that a simple growth system as that used in the present investigation (shaking flasks) is not well designed for the study of microalgal starch production, because CO_2 supply is limited to the diffusion of the fraction present in the air and there is a limitation in light availability.

Regarding the secreted sugars, their concentration in the medium for cultures carried out in the IBC photobioreactors (Table 2) is lower than that measured in cultures carried out in flasks for most of the strains (Table 1). The high concentration of secreted sugars measured in the flasks could be due to cell lysis and/or due to the stress conditions (CO_2-depletion): microalgae secrete sugars to make a protective habitat. However, secreted sugar concentrations of 0.05–0.1 g L^{-1} were reached in the PBR system. Bafana (2013) pointed out that *Chlamydomonas*-secreted sugars are EPS (exopolysaccharides) and that these sugars are characterized by significant antioxidant activity typically utilized in food, cosmetics, and pharmaceuticals. These sugars can be considered as byproducts of starch production, and their utilization may improve the economics of the starch production process.

Starch accumulation in non-conventional *Chlamydomonas* species In the flask system, where limiting factors occur, *C. reinhardtii* and *C. applanata* showed the best growth and the highest starch productivity of about 0.001 g L^{-1} day^{-1}. Therefore, these two species can be considered the strains characterized by the highest robustness and ability to convert light into biomass (Table 1).

In the IBC, photobioreactors characterized by substantial improvement of light and CO_2, *C. reinhardtii* still had the best growth, but similar growth was recorded by *C. oblonga* which had the highest starch productivity of 0.053 g L^{-1} day^{-1}. The highest starch content of 45 % of DW was reached by *C. moewusii* in this culture system.

The comparison of the maximum biomass productivity (Fig. 3) and the maximum starch productivity (Fig. 4) for a given species deserves some comments. The expected starch productivity at the instance of the maximum biomass productivity—assuming the starch fraction is constant under exponential growth phase—was typically less than half the maximum starch productivity assessed during early N-depletion. This result is in agreement with the conclusion of Yao et al. (2012) about starch accumulation in *T. subcordiformis*: starch accumulation is uncoupled with growth and the reduction of nitrogen sources concentration causes an increase in starch content and productivity at the expense of biomass productivity.

In particular, the analysis of Fig. 3 points out that the prolonged nitrogen starvation was associated to a reduction in starch productivity for all species. Moreover, data in Table 2 confirm that the decrease of starch productivity is species dependent and it is remarkable mainly for *C. moewusii*. Msanne et al. (2012) proposed that the reduction of starch content during prolonged N-starvation is the result of a microalgal metabolism shift to TAG synthesis. Results reported by Siaut et al. (2011) for *C. reinhardtii* cultures support the hypothesis that starch is converted to reserve lipids. On one hand, the production rates of TAG and carbohydrates are affected by the competition for common precursors: intermediates of the central carbon metabolism, e.g., glyceraldehyde-3 phosphate or acetyl coenzyme A (Breuer et al. 2014). On the other hand, the metabolism responsible for cellular growth and maintenance uses starch as energy and carbon sources under nutrient depletion. The result of both phenomena is starch content reduction during N-starvation.

The present investigation confirms the effectiveness of the nitrogen starvation to drive the carbohydrate increase, and the differences among species in the amount of carbohydrate accumulated during N-starvation prove that the accumulation process is species dependent.

Although the model species *C. reinhardtii* exhibited the best growth performances and biomass productivity in both culture systems, a significant improvement of 49 % in starch productivity was obtained by the lesser known species, *C. oblonga*. A real advantage could come from the use of unconventional species for starch production.

Advantages of a simple localization method of starch inside the cells Typically, the pyrenoid is located in the centre of the chloroplast and as starch concentration increases a variable number of starch granules wraps the pyrenoid (Barsanti et al. 2013; Gorelova et al. 2015). This study highlights that there are small differences in starch localization in *Chlamydomonas* species and that nitrogen concentration influences the area occupied by the starch granules inside the chloroplast.

It is known that, as the starch is synthesized, it forms a sheath around the pyrenoid, a subcellular microcompartment where RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) enzymes and CCM (CO_2-concentrated mechanisms) enzymes act. The observations of the starch-cell system, reported in the literature, make use of advanced diagnostics (e.g., scanning electron microscope, transmission electron microscope, fluorescent microscopy) (Brányiková et al. 2011; Tanadul et al. 2014). The comparison between our observation and those reported in the literature (Muller et al. 1998) proves that the diagnostic tool adopted—staining with Lugol dye coupled with optical microscope observation and image analysis—is a simple, fast, and cheap method to highlight the starch distribution in microalgal cells.

The picture analysis by ImageJ program does not provide quantitative data because the standard deviation of the measured data was not negligible (10–20 %),

notwithstanding the quite high number of cells observed under each operating condition. The high value of the standard deviation is a figure of the wide variability in the cell populations. However, this analysis provides information about the physiological condition of growing and starved cells. The increase of cell area, simultaneously with the increase of the area occupied by starch, is in agreement with the well-known observation that microalgae do not duplicate under nutrient depletion conditions, but accumulate high-energy compounds and increase their average size (Dragone et al. 2011; Fernandes et al. 2013; Johnson and Alric 2013; Gorelova et al. 2015). The observed phenomenon was particularly obvious for *C. applanata* and *C. oblonga* (see Table 2).

Concluding remarks Batch cultures of *Chlamydomonas* species in flasks proved that simple culture systems that do not provide adequate light and CO_2 supply to the cultures (i.e., open pounds) are not suitable for starch production investigation.

The tests of *Chlamydomonas* species in inclined bubble column photobioreactors identified two non-conventional *Chlamydomonas* species characterized by promising starch productivity. *Chlamydomonas oblonga* and *C. moewusii* showed the highest starch productivity with respect to the investigated species (0.053 and 0.046 g L^{-1} day^{-1}, respectively) during early nitrogen depletion. Moreover, a significant increase of 49 % in starch productivity can result from the use of *C. oblonga* instead of the known benchmark *C. reinhardtii*. Interesting results are also related to *C. moewusii*, characterized by high carbohydrate ($73\%_{DW}$) and starch content ($45\ \%_{DW}$), features that facilitate product recovery and reduce downstream process costs.

A method based on Lugol dye starch staining coupled with optical microscope observation and image analysis provided a simple, fast, and cheap method to highlight the presence of starch in microalgal cells and its distribution during different growth phases.

Further studies about the physical and chemical characterization of starch (e.g., amylose and amylopectin content) are needed to verify the real industrial application of microalgal starch.

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