A novel one-stage cultivation/fermentation strategy for improved biogas production with microalgal biomass

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Highlights:

Combination of algae cultivation under nitrogen limitation and biogas generation
Nitrogen limitation improves biodegradability and C/N ratios of algal biomass
Complete alga disintegration without any pretreatments led to higher biogas yields
~100% increase in biogas and methane yields for *Parachlorella* and *Scenedesmus*
Chlamydomonas biomass optimal substrate for fermentation (478 ± 6 mL methane g^{-1} VS)

Abstract

The use of alga biomass for biogas generation has been studied for over fifty years but until today, several distinct features, like inefficient degradation and low C/N ratios, limit the applicability of algal biomass for biogas production in larger scale. In this work we investigated a novel, one-stage combined cultivation/fermentation strategy including inherently progressing nitrogen starvation conditions to generate improved microalgal biomass substrates. For this strategy, comparable low amounts of nitrogen fertilizers were applied during cultivation and no additional enzymatic, chemical or physical pretreatments had to be performed. The results of this study demonstrate that progressing nitrogen limitation leads to continuously increasing C/N ratios of the biomass up to levels of 24-26 for all three tested alga strains (*Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Scenedesmus obliquus*). Importantly, the degradation efficiency of the algal cells increased with progressing starvation, leading to strain-specific cell disintegration efficiencies of 35% to 100% during the fermentation process. Nitrogen limitation treatment resulted in a 65% increase of biogas yields for *C. reinhardtii* biomass (max. 698 ± 23 mL biogas g^{-1} VS) when compared to replete conditions. For *P. kessleri* and *S. obliquus,* yields increased by 94% and 106% (max. 706 ± 39 mL and 586 ± 36 mL biogas g^{-1} VS, respectively).

From these results we conclude that this novel one-stage cultivation strategy with inherent nitrogen limitation can be used as a pretreatment for microalgal biomass generation, in order to produce accessible substrates with optimized C/N ratios for the subsequent anaerobic fermentation process, thus increasing methane production and avoiding the risk of ammonia inhibition effects within the fermenter.

Keywords: Bioenergy, Microalga, Biogas, Methane, Nitrogen limitation, C/N ratio

Abbreviations: SE, standard error; SD, standard deviation; BMP, biomethane potential; VS, volatile solids; N, nitrogen.

1. Introduction

The development of new processes for stable supply of energy has become increasingly important over the last decades, since the demand for energy is increasing while the finite fossil fuel reserves decline. This trend will continue in the future because today´s renewable energy from wind, water, solar or geothermal sources cover only a very small fraction of the global requirements and often are limited due to the comparably high investment and material costs (Jacobson and Delucchi, 2011).

Microalgae are characterized by potentially fast growth with the ability of high carbon fixation rates (6.2 kg m^{-3} d^{-1}) (Cheng et al., 2006) and concomitant high biomass productivity of up to 40-80 t dry weight ha^{-1} y^{-1} (Wijffels, 2008), which is roughly 10 times higher than conventional agriculture crops (Murphy and Power, 2009). Once algal biomass is produced, it can be converted into methane as a storable energy carrier through anaerobic digestion. The use of algal biomass for biogas generation has been studied since 1957 (Golueke et al., 1957), however the efficiency of the conversion has remained rather low (Passos et al., 2014).

Three main obstacles prevent large-scale application of microalgal biomass for fermentative biogas production. First, the cost of biomass production is still comparably high. Growth of microalgae can be achieved in closed photobioreactors or open pond facilities and much research is ongoing to reduce the currently relatively high biomass production costs (Chisti, 2007; Posten, 2009; Slade and Bauen, 2013; Stephens et al., 2010; Wijffels et al., 2013). Second, many microalgal species are characterized by a high resistance towards degradation by anaerobic microorganisms (Golueke et al., 1957; Mahdy et al., 2014b; Mussgnug et al., 2010; Passos et al., 2014). The reason for the poor degradability is assumed to be based on the protecting outer cell wall of the microalgae, which usually consists of several biopolymer compounds (Passos et al., 2014). For many microalgae, the complex structure and composition is poorly understood (Popper and Tuohy, 2010). In case of the comparably well studied green microalga *C. reinhardtii* (Harris, 2001; Merchant et al., 2007), the cell wall is composed of hydroxyproline-rich glycoproteins (Miller et al., 1972) and does not contain cellulose (Adair and Snell, 1990; Horne et al., 1971). In contrast, many microalgae contain cellulose and hemicellulose (Bisalputra and Weier, 1963; Takeda, 1996), one example being *S. obliquus*, which furthermore was described to contain resilient sporopollenin biopolymers, resulting in an unusually rigid cell wall (Burczyk and Dworzanski, 1988). The cell walls of *C. vulgaris* and other *Chlorella* sp. were shown to contain uronic acids, and a variety of sugar constituents as well as glucosamine as common dominant cell wall breakdown products (Gerken et al., 2013; Huss et al., 1999), indicating the presence of cellulose, hemicellulose, pectin, chitin and chitosan in the cell wall (Gerken et al., 2013; Popper et al., 2011). To access the intracellular cell compounds for biogas production, the cell wall resistance can be overcome by enzymatic, physical or chemical pretreatment methods (Alzate et al., 2012; Keymer et al., 2013; Mahdy et al., 2014a; Mahdy et al., 2014b; Mendez et al., 2014; Schwede et al., 2013), which however are often energy and cost intensive (Passos et al., 2014).

The third obstacle is that microalgal biomass usually contains high amounts of proteins, which is reflected by low carbon to nitrogen (C/N) ratios, usually being in the range of 5-9 (Oh-Hama and Miyachi, 1988; Yen and Brune, 2007) (Lardon et al., 2009; Zhong et al., 2012). Low C/N ratios are detrimental for continuous fermentation processes because the

release of ammonium can lead to high pH levels and the formation of free ammonia, which is highly toxic for the methanogens in the anaerobic community (Chen et al., 2008; Kayhanian, 1994).

In this work, we investigated a strategy of combined alga cultivation, nitrogen limitation and subsequent fermentative biogas generation with the aim of producing microalgal biomass with improved biodegradability and simultaneously higher C/N ratios, therefore addressing two of the three issues mentioned before. The investigation was performed with *Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Scenedesmus obliquus*, which represent three microalgal species of varying levels of natural biodegradability (Mussgnug et al., 2010).

2. Materials and methods

2.1 Strains and growth conditions

Parachlorella kessleri (formerly designated *Chlorella kessleri*, (Krienitz et al., 2004)) was obtained from the Culture Collection of Algae and Protozoa (CCAP, UK) and *Chlamydomonas reinhardtii* strain CC1690 from the Chlamydomonas Center (Duke University, Durham NC, USA). *Scenedesmus obliquus* was obtained from the SAG algae collection (Goettingen University, Germany).

Liquid algal cultures were grown photoautrophically in continuous white light (400 µmol photons m^{-2} s^{-1}; Osram L 36W/865, Osram Germany). Cultivations were conducted in glass bottles (DURAN® max. capacity 3.5 L, outer diameter 110 mm and 450 mm height, Schott Germany) with 2.5 L of algae culture, under continuous agitation on a magnetic stirrer. Carbon supply was achieved by bubbling with carbon dioxide-enriched air (3% v/v) with a flow rate of 10 L * h^{-1}. Nutrients were provided by a Provasoli based minimal medium (Provasoli et al., 1957). For nutrient replete culture conditions, the following components and concentrations were applied: K_2HPO_4 0.57 mM; H_3BO_3 0.16 mM; $MgSO_4$ 4.87 mM; KCl 21.46 mM; $NaNO_3$ 11.77 mM; $CaCl_2$ * $2H_2O$ 2.72 mM; $FeCl_3$ * 6 H_2O 12.2 lM; Na_2-EDTA 12.5 µM; TRIS 8.26 mM; EDTA 103 µM; $ZnCl_2$ 2.2 µM; $MnCl_2$ * 4H20 16.7 µM; $CoCl_2$ * 6 H_2O 50.4 nM; $CuCl_2$ * $2H_2O$ 17.6 nM; Na_2MoO_4-* $2H_2O$ 24.8 nM and NaCl 17.1 mM. For nitrogen-free cultivation, no nitrogen source ($NaNO_3$) was added to the Provasoli based medium. Nitrogen limiting conditions were realized according to Lardon et al. (2009) by applying a limited amount of nitrogen (3.56 mM $NaNO_3$ equals to 50 mg of nitrogen per Liter of culture).

2.2 Determination of algal biomass concentration

The biomass concentration was determined by centrifugation of 15 mL of cell culture (3000*g for 5 min, at least three technical replicates per sample) and drying of the cell pellet in a pre-weighted glass tube at 105 °C for 24 hours. To determine the organic biomass fraction, the

sample tubes were subsequently incubated at 550 °C for 5 hours and the residual ash determined by weighing. The amount of organic biomass (dry weight minus the ash content) was calculated and expressed as volatile solids (VS, g L^{-1}).

2.3 Cell degradation levels

The relative cell disintegration was monitored by determination of the algal cell and cell-shaped particle numbers residing within the anaerobic fermenter via optical microscopy (Motic BA310, Motic, China) and manual cell counting. Cell/particle counts were performed at the start of the incubation period (0 days) and at the end of the fermentation test (50 days). The cell degradability is expressed as percentage of the initial cell count.

2.4 Measurement of elemental N and C content in the biomass (C/N ratio)

Total carbon (C) and nitrogen (N) content of the algal biomass was determined via an element analyzer (VARIO EL III, Elementar Analysesysteme, Hanau, Germany) as described before (Platner et al., 2012)

2.5 Measurement of nitrate in the culture supernatant

Nitrate nitrogen (NO_3-N) content in the cell free supernatant was analyzed using a standardized Hach Lange nitrate cuvette test (LCK339) and measured in a DR 3900 Spectrophotometer (Hach Lange, Germany) according to the manufacturer´s instructions.

2.6 Anaerobic substrate fermentation and biogas analysis

Anaerobic methane potential (BMP) tests were conducted according to the VDI 4630 guideline (VDI, 2004) with a reduced minimal reactor volume, which was possible because of the homogeneous nature of the microalgal biomass substrate. Compared to a previous work (Mussgnug et al., 2010), the reactor volume was reduced to 8 mL (instead of 250 mL), but the ratio of substrate:sludge:gas phase (1:120:480) was kept identical. The fresh algal substrate was obtained by centrifugation of the cultures at 3000*g for 5 min and removal of the supernatant, avoiding freezing or lyophilisation steps. For fermentation, VS amounts were determined as described above and used for respective biogas yield calculations with a substrate:sludge ratio of 1:120 (w:v). In order to achieve anoxic conditions, the reactors were flushed with pure helium before sealing. The fermentation was performed under mesophilic conditions at 38 ± 1 °C in a tempered water bath. Biogas evolution was monitored by a gas pressure measuring device (BMP-Test system 3150, WAL, Germany) equipped with a syringe needle (Sterican®0.4 mm × 20 mm, B. Braun, Germany) by piercing it through the septum in to reactor gas phase. For the calculation of the gas volume evolved during the fermentation process, the equation provided in the guideline (VDI, 2004, chapter 7.3) was

used. Mixing of the reactor tubs were done my manual shaking after each measurement. Fermentation measurements werecarried out until biogas evolution stopped.

2.7 Methane content measurement via gas chromatography (GC)

The determination of the methane content within the biogas was performed by GC analysis. Biogas from the fermenter headspaces was sampled with a gas tide syringe (500 µL) via the rubber seals and injected into a gas chromatograph (Shimadzu GC-2010 plus, Shimadzu Crop, Japan) equipped with an Agilent GS-Gaspro capillary column (Length: 60 m, inner diameter: 0.32 mm, part # 113-4362) (Agilent Technologies, USA) and a thermal conductivity detector. Helium was used as the carrier gas and the calibration was performed with test gas (Linde, Germany) containing O_2 (0.103%), H_2S (0.208%), H_2 (0.498%), CH_4 (59.4%), CO_2 (34.4%) and N_2 (5.391%), according to DIN EN ISO 6141.

3. Results and discussion

3.1. Nitrogen starvation of microalgal biomass enhances biogas productivity

Microalgal cells undergo drastic changes in their metabolism in response to nutrient deprivation, which is reflected by corresponding changes of the biomass composition (Prochazkova et al., 2014; Timmins et al., 2009). Nitrogen starvation is a frequently used method for triggering the production of neutral lipids and/or starch in algae (Hu et al., 2008; Philipps et al., 2012; Rodolfi et al., 2009). Since lipids and starch are generally regarded as being good substrates for fermentative biogas production, it was our aim to test if the cellular changes in response to nutrient starvation could result in an improved biomass quality for a subsequent fermentative biogas production.

For this purpose we applied nitrogen starvation as a two-stage process (Rodolfi et al., 2009). In the first stage, the algae biomass was grown in nutrient replete conditions, harvested by centrifugation and subsequently washed. In the second stage, the cell pellet was resuspended in nitrogen-free medium and cultured for four days. After the starvation period, the cells were harvested and the cell biomass was subjected to the anaerobic fermentation process. Equal amounts of biomass from nitrogen replete cultures were used as controls. Crystalline cellulose was used as an additional control substrate and yielded a biogas amount of 569 ± 4 mL g^{-1}, which is within the appropriate range (Raposo et al., 2011; VDI, 2004), indicating that the fermenter sludge was composed of an active archaeal and bacterial community. The application of nitrogen starvation for *Chlamydomonas reinhardtii* resulted in 83% higher biogas yields (Fig. 1A) compared to the same amount of biomass from nutrient replete conditions (727 ± 45 mL g^{-1} (-N) versus 398 ± 34 mL g^{-1} (+N)). Similar values of 722 ± 4.6 mL biogas g^{-1} biomass were reported before for fermentation of two-stage mixotrophic cultivated and sulfur deprived *C. reinhardtii* biomass (Mussgnug et al., 2010).

Since this result demonstrated that nitrogen starvation of *C. reinhardtii* biomass indeed resulted in far higher biogas yields, we further investigated whether this effect could also be observed in other algal species. For this purpose, two additional strains, *Parachlorella kessleri* and *Scenedesmus obliquus,* were selected. These strains were chosen because it has been shown before that both species are characterized as being very poorly degradable when grown in standard nutrient replete conditions (Mussgnug et al., 2010).

P. kessleri and *S. obliquus* cell cultures were grown, treated by nitrogen limitation and subjected to fermentation as described for *C. reinhardtii*. Similar to the results obtained with *C. reinhardtii*, the biogas yields were significantly higher after the nitrogen starvation treatment (Fig. 1B, *P. kessleri*: 412 ± 10 mL g^{-1} (-N) versus 283 ± 2 mL g^{-1} (+N) and *S. obliquus*: 504 ± 8 mL g^{-1} (-N) versus 306 ± 3 mL g^{-1} (+N)). These data show that the nitrogen starvation treatment resulted in a 46% increase of biogas production in the case of *P. kessleri* and a 65% increase for *S. obliquus*, thus suggesting that N starvation generally triggers the production of microalgal biomass with a higher potential for biogas generation. In agreement with the biogas yields, the methane yields of the nitrogen starved biomass also were higher compared to the yields from biomass from nutrient replete growth conditions (Fig. 1B, for *C. reinhardtii* 479 ± 6 mL g^{-1} (-N) versus 282 ± 27 mL g^{-1} (+N), for *P. kessleri* 274 ± 5 mL g^{-1} (-N) versus 202 ± 2 mL g^{-1} (+N) and for *S. obliquus* 318 ± 2 mL g^{-1} (-N) versus 215 ± 2 mL g^{-1} (+N)).

These results clearly showed that nitrogen starvation treatment resulted in generation of microalgal biomass with improved fermentation features. However, for large scale cultivation or industrial application it is not feasible to perform a two-stage process to generate the improved biomass, since this setup would involve energy-intensive, sequential biomass centrifugation/resuspension steps (Collet et al., 2011). Therefore, we designed an alternative, one-stage production process for sequential growth and nitrogen starvation treatment, avoiding intermediate centrifugation steps. This was achieved by initial supplementation of the growth medium with only a defined, limiting amount of nitrogen, calculated to be sufficient to promote cell growth for a limited time before automatic transition to the nitrogen starvation stage, similar to a strategy described before (Yeh and Chang, 2011).

3.2. Production of algal biomass in N limiting conditions in a one-stage cultivation process with improved C/N ratios

To achieve automatic transition from N replete to N deplete conditions in a one-stage process, the growth-media for the cultivation of algal biomass were supplemented with only 50 mg nitrogen (supplied as $NaNO_3$). This N concentration in the medium was calculated to

be sufficient to promote photoautotrophic growth to up to 1 g of biomass (dry weight), before entering the nitrogen limitation stage (Lardon et al., 2009).

The organic biomass accumulation was monitored via measurement of VS (volatile solids) in an interval of two days (Fig. 2A). All three strains tested showed similar growth kinetics and reached biomass densities of more than 2 g L^{-1} VS after ten days (*C. reinhardtii*: 2.3 ± 0.12 g L^{-1}, *P. kessleri* 2.2 ± 0.03 g L^{-1}, *S. obliquus* 2.11 ± 0.05 g L^{-1}). However *C. reinhardtii* reached the maximal biomass density already after six days, whereas biomass accumulation of *P. kessleri* and *S. obliquus* was significantly slower, reaching the maximal density after ten days. The concentration of the nitrogen source $NaNO_3$ was determined during the cultivation period in the culture supernatant and the nitrogen bound in the biomass was determined by quantitative elemental analysis. Surprisingly, the major portion of the nitrogen in the culture media was absorbed by the algal cells already after two days of cultivation, indicating that the increase of the biomass after day two was mainly due to carbon-based metabolite accumulation, instead of protein synthesis. The measurement of the elementary nitrogen within the cells is in good agreement with this assumption (Fig. 2B).

Additionally, the C/N-ratio of the biomass was determined since this factor is known to be very important for the biomass fermentation process. Low C/N-ratios of around 5-9 have previously been described as being key bottlenecks for the fermentation process (Lardon et al., 2009; Oh-Hama and Miyachi, 1988), since inhibitory effects due to ammonia accumulation can lead to inhibition of the microbial community within the fermenters and subsequent fermenter failure (Chandra et al., 2012; Chen et al., 2008). C/N-values in the range of 20-30 are reported to be optimal for the microbial performance (Parkin and Owen, 1986). In order to reach such optimal C/N ratios, algal biomass is often used as a mixture with carbon rich material (co-digestion), which then results in higher biogas productivities and decreased ammonia concentrations during fermentation (Yen and Brune, 2007; Zhong et al., 2012), however, increasing the level of complexity of the overall fermentation process management.

As shown in Fig. 2C, the C/N ratios of all three strains grown in nutrient replete conditions are very low, between 5 and 6 (Fig. 2C, time point t_0), which is in good agreement with previous reports (Lardon et al., 2009; Yen and Brune, 2007) but falls within the range of unfavorable substrates. During the course of the one-stage cultivation treatment, a significant and continuous increase of the C/N-ratio of all three strains was detected. After day ten, the C/N-ratios reached values of 26.3 ± 3.6 for *C. reinhardtii*, 23.8 ± 3.4 for *P. kessleri* and, 26.4 ± 0.3 for *S. obliquus*, respectively. As a conclusion, this result demonstrates that the one-stage treatment with a concomitant relative accumulation of carbon-based metabolites led to biomass concentrations of approximately 2 g L^{-1} and a shift of the C/N ratios from 5-6 to 23-

26, therefore from potentially inhibiting towards the optimal C/N range for the fermentation process.

3.3. Cellular disintegration levels improved with ongoing starvation

An efficient conversion of algae biomass into biogas is dependent on the complete disintegration of all cellular components. Therefore, the progress of cell disintegration was investigated by optical microscopy and cell counting (Fig. 3). Fresh algal substrate from each two-day harvesting time point was added to batch fermenters and the cell number was determined before and after anaerobic fermentation process (BMP test).

Continuing nitrogen limitation led to morphological changes of *C. reinhardtii* (Fig. 3, prior ferm.). The intracellular granulation appeared to increase, which most likely is due to the accumulation of starch and neutral lipids, a well described effect occurring during nutrient starvation in *C. reinhardtii* (Goodson et al., 2011; Prochazkova et al., 2014). Compared to *C. reinhardtii*, less obvious intracellular morphological changes were detected for *P. kessleri* and *S. obliquus* (Fig. 3, prior ferm.).

Particle counting revealed that only ca. 8 % of *C. reinhardtii* cells harvested at day zero of the one-stage treatment were disintegrated after the fermentation period. However when cells were harvested only two days later, the relative amount of cell-shaped particles after fermentation decreased to 41 ± 5 %. Close to 100% of the cell material were disintegrated after fermentation when cells were harvested after six or more days of the one-stage treatment (Fig. 3), indicating a full accessibility of the algal biomass to the anaerobic microorganisms. Disintegration efficiency was generally lower for both, *P. kessleri* and *S. obliquus* compared to *C. reinhardtii*, however a similar tendency was observed in that degradability significantly increased as a consequence of the nitrogen limitation treatment (Fig. 3). In detail, when biomass from nutrient replete cultivations was subjected to batch fermentation, no significant decrease of the cell number was observed after anaerobic incubation (Fig. 3, *P. kessleri*, *S. obliquus* t_0), similar to what was described in a previous work (Mussgnug et al., 2010). In contrast, ca. 35% of the *P. kessleri* and ca. 52% for *S. obliquus* cells harvested after 6-10 days of limitation treatment were found to be completely disintegrated after fermentation. Although cell-shaped particles were still detectable within the reactors (Fig. 3, t_6-t_{10}), it should be noted that many of these particles appeared to be partially or completely empty. This observation suggests that these particles do not represent intact and living cells, but more likely are remains of the cell wall which could not be degraded by the microbial community within the fermenter. A possible reason for incomplete disintegration of the *P. kessleri* and *S. obliquus* cell material, in contrast to *C. reinhardtii*, could be that the compositions of the protective cell walls of strains differ substantially. The *C. reinhardtii* cell wall is mainly composed of hydroxyproline-rich glycoproteins (Miller et al.,

1972) and these proteins seem to be more accessible to the anaerobic community. *Chlorella* and *Scenedesmus* species generally possess several more robust cell wall components. *Chlorella* strains are known to contain cellulose (Northcote et al., 1958) and intermediates of cellulose and mannan or cellulose with mannan co-polymers as well as pectin (Loos and Meindl, 1982; Takeda, 1991), but also chitosan and chitin-like glycans (Gerken et al., 2013; Kapaun and Reisser, 1995; Mihara, 1961) within their cell walls. The cell wall of *S. obliquus* is reported to be rigid and, since in addition to cellulose it contains resilient sporopollenin biopolymers (Burczyk and Dworzanski, 1988).

3.4. Fermentation of biomass generated in nitrogen limiting conditions results in higher biogas and methane yields

In order to verify that higher disintegration levels of algal cells (Fig. 3) also lead to increased biogas and methane yields, we analyzed biogas and methane amounts, which resulted from the fermentation tests. To ensure the consistent quality of the anaerobic sludge and the reproducibility of the fermentation performance throughout the experiments, a standard substrate (microcrystalline cellulose, (VDI, 2004)) was included to each fermentation set-up. The yields of the biogas and methane from the standard substrate did not differ significantly between the set-ups (Fig. 4, cellulose) ensuring that all subsequent fermentation results from the different harvesting time points can directly be compared.

C. reinhardtii biomass from t_0 (nutrient replete conditions) reached a maximal biogas yield of 422 ± 18 mL g^{-1} VS (Fig. 4), which is similar to the data in Fig. 1B. Significant lower values were observed for *S. obliquus* t_0 data (Fig.4, 284 ± 2 mL g^{-1} VS) and *Parachlorella kessleri* (Fig. 4, 364 ± 5 mL g^{-1} VS). These values are in good agreement with previously published results (Table 1). In analogy with the two-stage nitrogen limitation experiments, one-stage nitrogen limitation treatment of the biomass resulted in a significant progressive increase of the biogas evolution potential for all three strains (Fig. 4). For *C. reinhardtii* and *S. obliquus*, eight days of treatment were sufficient to yield maximal biogas production (698 ± 23 mL g^{-1} VS and 586 ± 36 mL g^{-1} VS, respectively). A continuous rise of the biogas production amount until ten days of treatment was observed in case of *P. kessleri* to yield a final value of 706 ± 39 mL g^{-1} VS (Fig. 4). These results show that the one-stage cultivation and nitrogen limitation treatment resulted in a 65%, 94% and 106% increase of the biogas potential for *C. reinhardtii*, *P. kessleri* and *S. obliquus*, respectively.

In good agreement with the rise of the biogas yield in the one-stage nitrogen limitation treatment process, the maximal methane yields also increased to 478 ± 6 mL g^{-1} VS for *C. reinhardtii,* 449 ± 8 mL g^{-1} VS for *P. kessleri* and 406 ± 21 mL g^{-1} VS for *S. obliquus*, respectively (Fig. 4). It is noticeable that these values are significantly higher than previous published yields for untreated algal biomass (Table1). For different *Chlorella* species, the

previously published methane yields range between 166 to 265 mL methane per gram VS (Mussgnug et al., 2010; Prajapati et al., 2014), which correlate with our results from nutrient replete conditions, but are approximately 2-fold lower compared to the nitrogen starved biomass. Similar values were described in literature for *Scenedesmus* species, with methane yields of 178 to 258 mL g^{-1} VS (Frigon et al., 2013; Keymer et al., 2013; Mussgnug et al., 2010; Zamalloa et al., 2012).

Nevertheless, biogas and methane data from pretreatment trails are available, which are in a similar range to the maximal yields presented within this work. For instance, Mahdy et al. detected 479 mL methane g^{-1} VS and 460 mL methane g^{-1} VS after enzymatic hydrolyzation of *C. reinhardtii* and *C. vulgaris* biomass, respectively (Mahdy et al., 2014b). For *Scenedesmus* sp. biomass, 325 mL methane g^{-1} VS could be obtained after application of hydrothermal (170°C, 8 bar) pretreatment (Keymer et al., 2013). However, the use of enzymatic, chemical or physical pretreatments of biomass are extremely expensive and/or energy intensive, and are therefore most likely not profitable for biofuel generation (Passos et al., 2014).

Based on the data described above, *C. reinhardtii* performed best of the three strains tested in this study, since it showed complete cell disintegration (Fig. 3A), an optimal C/N-ratio and high methane yield of 478 ± 6 mL g^{-1} VS already after eight days of the one-stage nitrogen limitation treatment. These data confirm theoretical considerations (Sialve et al., 2009) that microalgal biomass can have a greater biogas potential than maize silage, which is commonly used for biogas generation today and typically produces methane in the range of 345 ± 7 mL g^{-1} VS (Bauer et al., 2010).

Conclusions

In this work, we systematically investigated the biodegradability and the fermentative biogas production potential of microalgal biomass generated after specific nitrogen limitation treatment. Two distinct methods to generate the nitrogen limited biomass were investigated, a classical two-stage and a novel one-stage cultivation strategy, which should theoretically be the preferred option, since less energy intensive intermediate biomass harvesting steps have to be performed.

Three algal species were selected for the investigation, one described to be efficiently degradable (*C. reinhardtii*) and two strains which were shown to be very resistant against microbial degradation (*S. obliquus* and *P. kessleri*) (Mussgnug et al., 2010).

For all three strains, a significantly increased biodegradability and concomitant biogas production was observed, which was ca. 1.7-fold higher for *C. reinhardtii* and approximately 2-fold higher for *P. kessleri* and *S. obliquus* biomass, when compared to biomass harvested from nutrient replete conditions. These data clearly indicate that nutrient limitation could in

general be a very useful strategy to increase the biogas production potential for microalgal biomass.

C. reinhardtii was found to be the overall best substrate for fermentation in this work, with 478 ± 6 mL methane g^{-1} VS being produced from the biomass harvested after eight-day treated algal culture.

Our data show that ongoing limitation of nitrogen results in two beneficial effects, the progressively increasing accessibility to microbial degradability and a continuously increasing C/N-ratio of the biomass, from potentially inhibiting towards optimal levels for fermentation, resulting in higher biogas and methane yields. An additional potential advantage of the nitrogen limitation treatment is that lowering of nitrogen supplementation for algal culturing equals to reduction of the demand for growth fertilizers, thereby minimizing the costs for biomass generation. Similar methane yields as achieved with our simple one-stage nitrogen limitation strategy have only been reached before after sophisticated physical, chemical or enzymatic pretreatments of the algal biomass (Mahdy et al., 2014a; Mahdy et al., 2014b; Schwede et al., 2013). However, these pretreatments are inherently costly and therefore it seems unlikely that the application will be feasible for larger scale trials. In conclusion, we suggest that the pretreatment strategy presented in this work represents a significant step towards generating highly biodegradable microalgal substrates for anaerobic fermentation and could be a cheap alternative to previously described options, since the requirements for energy, material or additional chemicals are significantly lower.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: Biogas and methane production of microalgal substrates generated by photoautotrophic or mixotrophic cultivation.

Microalga species	**Biogas mL g^{-1} VS**	**CH_4 mL g^{-1} VS**	**Cultivation mode**	**Reference**
C. reinhardtii	587 ± 8.8 (SE)	387 ± 5.8 (SE)	mixotrophic,	Mussgnug et al. 2010
C. reinhardtii	422 ± 18 (SE)	290 ± 16 (SE)	photoautotrophic, r. c.	This work
C. reinhardtii	698 ± 23 (SE)	478 ± 6 (SE)	photoautotrophic, l. N.	This work
Chlorella kessleri	335 ± 7.8 (SE)	218± 5 (SE)	mixotrophic,	Mussgnug et al. 2010
Chlorella vulgaris	369 ± 67	196 ± 35	Photoautotrophic	Prajapati et al. 2014
Chlorella minutissima	340 ± 114	166 ± 56	Photoautotrophic	Prajapati et al. 2014
Chlorella pyrenoidosa	464 ± 66	265 ± 38	Photoautotrophic	Prajapati et al. 2014
Parachlorella kessleri	364 ± 5 (SE)	240 ± 2 (SE)	photoautotrophic, r. c.	This work
Parachlorella kessleri	706 ± 39 (SE)	449 ± 8 (SE)	photoautotrophic, l. N.	This work
Scenedesmus obliquus	287 ± 10.1 (SE)	178 ± 6.3 (SE)	Photoautotrophic	Mussgnug et al. 2010
Scenedesmus sp.	No data	258 ± 7	Photoautotrophic	Frigon et al. 2013
Scenedesmus obliquus	No data	210 ± 30	Photoautotrophic	Zamalloa et al. 2012
m. c.+ Scenedesmus	No data	180 ± 10 (SE)	Photoautotrophic	Keymer et al. 2013
Scenedesmus obliquus	284 ± 2 (SE)	213 ± 4 (SE)	photoautotrophic, r. c.	This work
Scenedesmus obliquus	586 ± 36 (SE)	401 ± 21 (SE)	photoautotrophic, l. N.	This work

r. c.: replete conditions; l. N.: low Nitrogen; m.c.: mixed culture; VS: volatile solids

Figure captions:

Fig. 1: Anaerobic BMP batch fermentation of algal biomass generated by nutrient replete cultivation or by two-stage nitrogen depletion treatment. The standard substrate microcrystalline cellulose (cellulose) was used as a fermentation process control. (**A**) Cumulative biogas evolution during the fermentation of the *C. reinhardtii* biomass and the control substrate. (**B**) Maximal amounts of biogas (white bars) and biomethane (black bars) produced by fermentation of biomass of three microalgal strains (*C. reinhardtii*, *P. kessleri* and *S. obliquus*) and the control substrate cellulose, respectively. Error bars represent standard deviation (SD, n=3).

Fig. 2: Photoautotrophic accumulation of algal biomass and nitrogen assimilation in a one-stage nitrogen limitation process. Cultivation was performed at 400 µmol photons m^{-2} s^{-1} (white light) and by aeration with air enriched with 3% (v/v) of CO_2 for ten days. **A.** Biomass accumulation monitored by determination of volatile solids. **B.** Nitrogen amounts detected in the media supernatant and accumulated in the algal biomass. The nitrogen concentration in the culture media is represented as NO_3-N, whereas the nitrogen amount in the cells represents the elemental nitrogen bound in algal biomass. **C.** C/N-ratio of the algal biomass, determined by elemental analysis. The area highlighted in gray indicates the typical range described for microalgal biomass produced under nutrient replete conditions (Oh-Hama and Miyachi, 1988, Yen and Brune, 2007). Error bars represent standard deviation (SD, n=6).

Fig. 3: Algae cell appearance and disintegration levels after anaerobic fermentation process. Microalgal cell morphology during the one-step nutrient limitation treatment before fermentation (prior ferm.) and level of disintegration of the cells within the fermenter sludge after fermentation for 50 days in darkness at mesophilic temperatures (38°C) (after ferm.) were determined by optical microscopy. The percentage of cell disintegration was calculated by division of the number of cells added to the fermenter by the number of cell-shaped particles visible in the fermenter sludge after the fermentation period. Scale bars represent 10 µm. Error bars represent standard errors (SE, n=6).

Fig. 4: Maximal biogas and methane production potential of the algal substrates during the one-step nutrient limitation treatment processes. Cellulose was used as a control substrate and digested in parallel to each fermentation trial in order to ensure equal activity of the microbial communities within the fermenters. Maximal amounts of biogas (white bars) and methane (black bars) are shown for each algae cultivation harvesting time point. Error bars represent standard error (SE, n=6 for biogas, and n=4 for biomethane).

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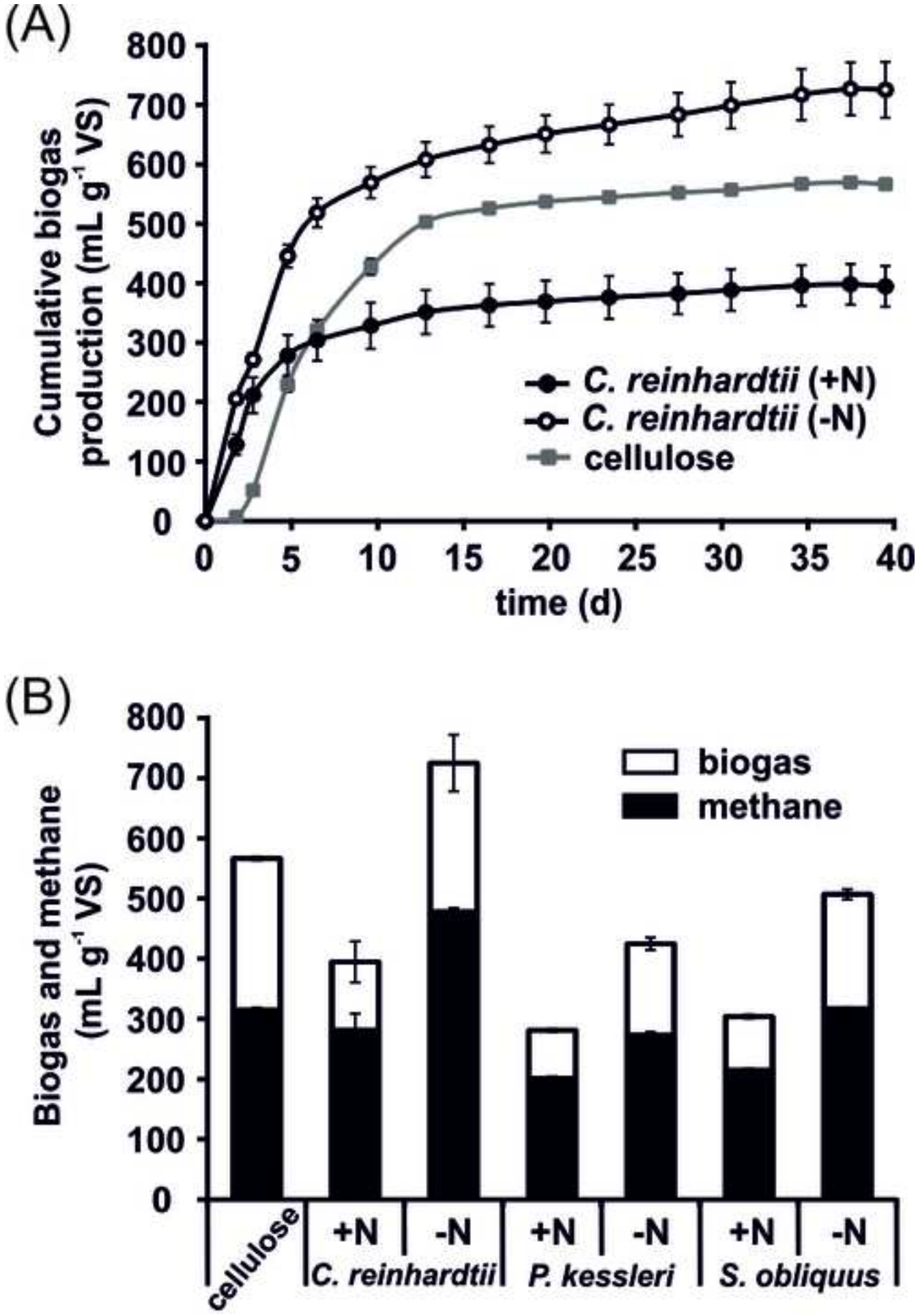

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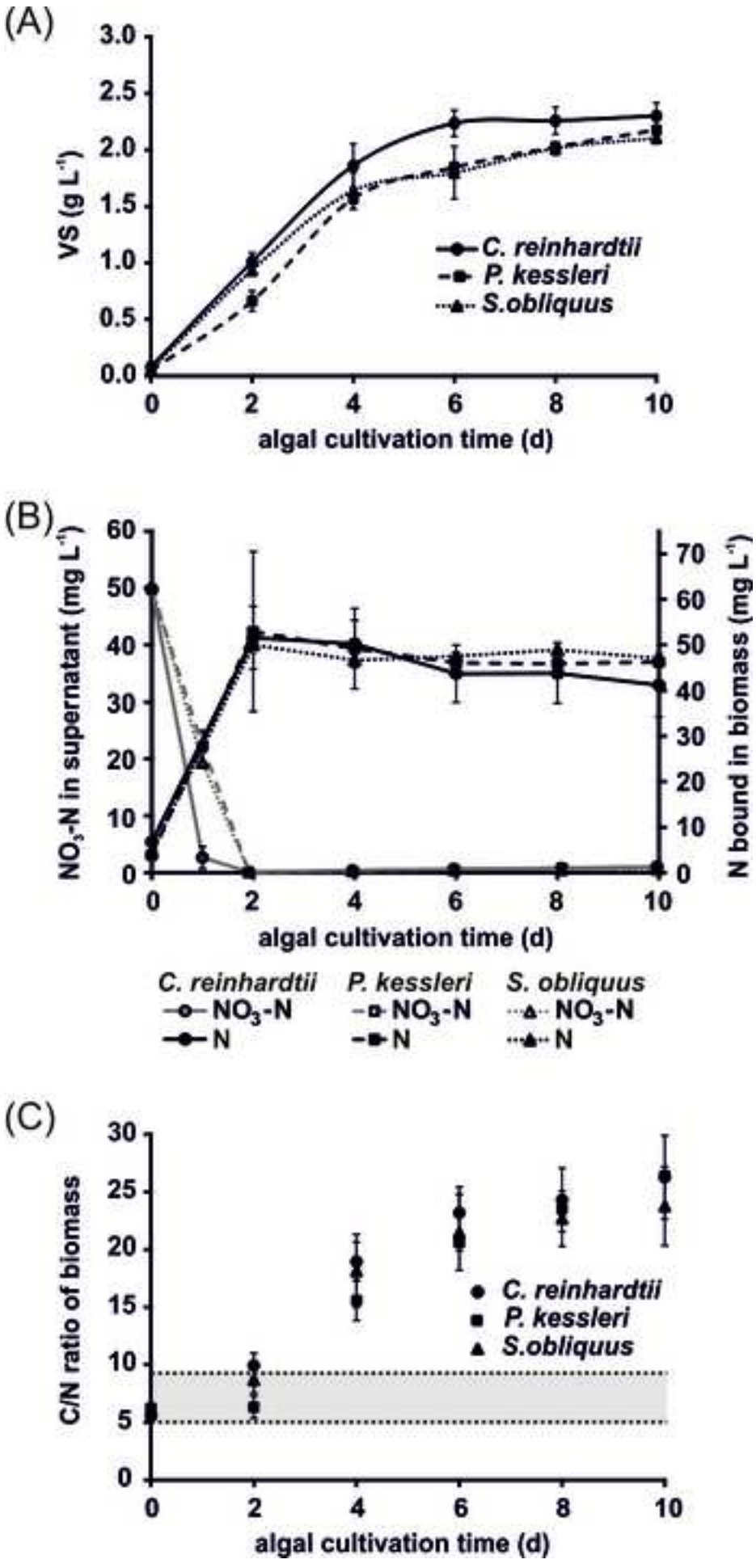

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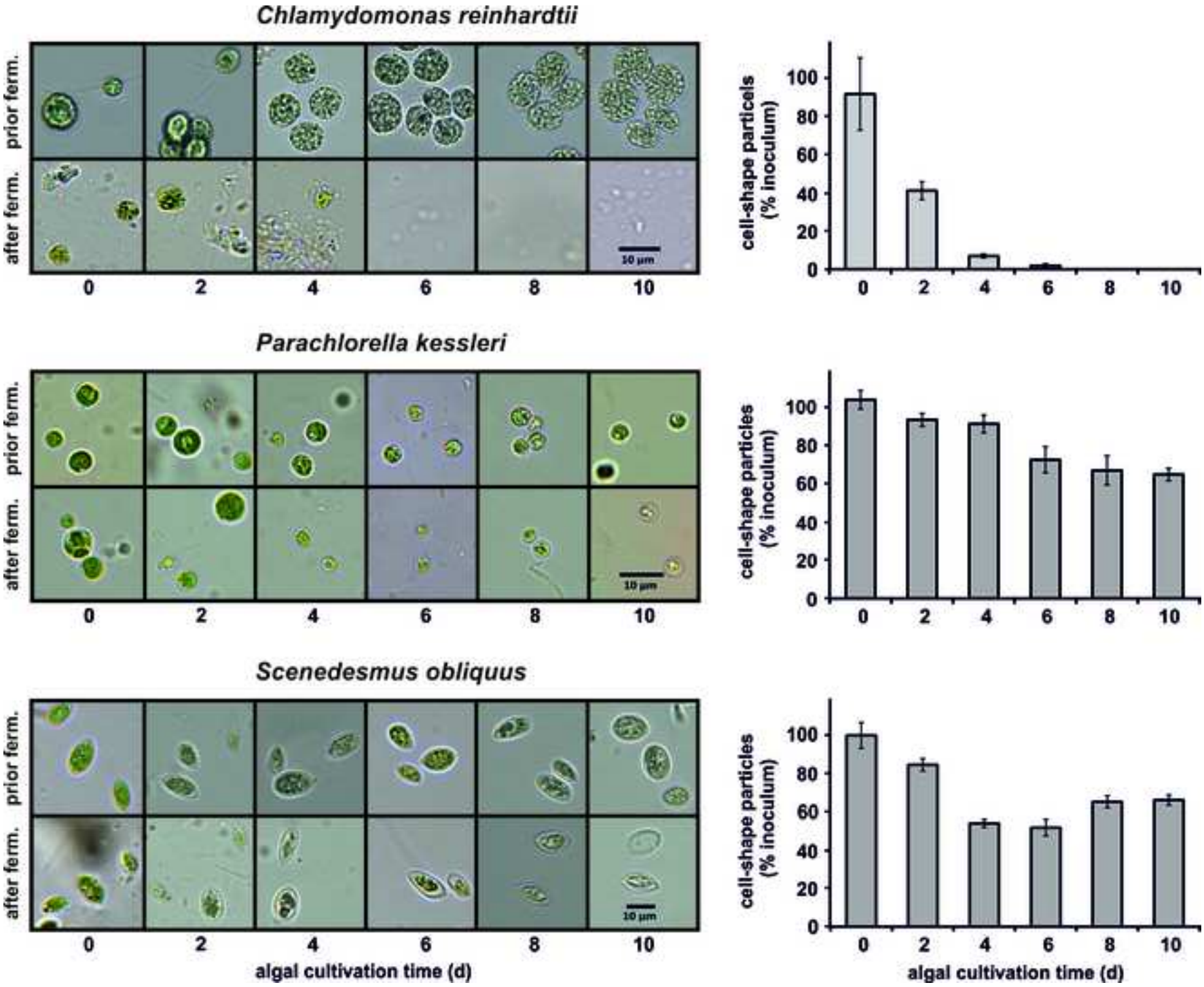

Figure 4

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