Kinetics of growth and lipids accumulation in Chlorella vulgaris during batch heterotrophic cultivation : effect of different nutrient limitation strategies

Kinetics of growth and lipids accumulation in *Chlorella vulgaris* during batch heterotrophic cultivation: effect of different nutrient limitation strategies.

Myrsini Sakarika, Michael Kornaros

PII: S0960-8524(17)31014-3
DOI: http://dx.doi.org/10.1016/j.biortech.2017.06.110
Reference: BITE 18347

To appear in: *Bioresource Technology*

Received Date: 2 May 2017
Revised Date: 19 June 2017
Accepted Date: 20 June 2017

Please cite this article as: Sakarika, M., Kornaros, M., Kinetics of growth and lipids accumulation in *Chlorella vulgaris* during batch heterotrophic cultivation: effect of different nutrient limitation strategies., *Bioresource Technology* (2017), doi: http://dx.doi.org/10.1016/j.biortech.2017.06.110

Kinetics of growth and lipids accumulation in *Chlorella vulgaris* during batch heterotrophic cultivation: effect of different nutrient limitation strategies.

Myrsini Sakarika[a,b], Michael Kornaros[a,*]

[a] Laboratory of Biochemical Engineering and Environmental Technology, Department of Chemical Engineering, University of Patras, 1 Karatheodori str., University Campus, 26504 Patras, Greece.

[b] Sustainable Air, Energy and Water Technology Group, Department of Bioscience Engineering, University of Antwerp, Campus Groenenborger, Groenenborgerlaan 171, 2020 Antwerpen, Belgium

Corresponding author at:

Department of Chemical Engineering, University of Patras, 1 Karatheodori str., University Campus, 26504 Patras, Greece

Tel: +30 2610 997418, Fax: +30 2610 969584

E-mail address: kornaros@chemeng.upatras.gr (M. Kornaros).

Abstract

The present study aimed at: (1) determining the effect of sulfur addition on biomass growth and (2) assessing the effect of sulfur, phosphorus and nitrogen limitation on lipid accumulation by *C. vulgaris* SAG 211-11b. The sulfur cellular content was more than two-fold higher under nitrogen and phosphorus limitation (0.52% and 0.54% w w^{-1}, respectively) compared to sulfur requirements (0.20% w w^{-1}) under sulfur limiting conditions. The nitrogen needs are significantly lower (2.81-3.35% w w^{-1}) when compared to other microalgae and become 23% lower under nitrogen or phosphorus limitation. The microalga exhibited substrate inhibition above 30g L^{-1} initial glucose concentration. Sulfur limitation had the most significant effect on lipid accumulation, resulting in maximum total lipid content of 53.43±3.93% g g_{DW}^{-1}. In addition to enhancing lipid productivity, adopting the optimal nutrient limitation strategy can result in cost savings by avoiding unnecessary nutrient additions and eliminate the environmental burden due to wasted resources.

Keywords: *C. vulgaris*; heterotrophic growth; nutrient addition; sulfur, nitrogen, phosphorus limitation; FAMEs;

1. INTRODUCTION

Microalgae favorably compete with soybean, palm, rapeseed, corn, canola and other conventional energy crops in biodiesel production. Although microalgal biodiesel is currently not cost-competitive, the potential of achieving this goal after process optimization and appropriate strain selection is now widely accepted. These microscopic algae present several advantages when compared to agricultural oleaginous crops: (i) their growth cycle lasts few days (Sheehan et al., 1998) in contrast to energy crops where the growth cycle is in the order or magnitude of months; (ii) they are characterized by higher photosynthetic efficiency than terrestrial plants (Chisti, 2007); (iii) the process intensification in compact bioreactors requires minimum land surface area and water and can be achieved with the optimization of operational parameters and reactor design (Wijffels and Barbosa, 2010). For instance, microalgae require 49 up to 132 times less land area than rapeseed or soybean for a 30% g g_{DW}^{-1} of biomass oil content (Chisti, 2007); (iv) their average lipid content ranges from 1 to 70% and can be up to 90% under certain conditions for some strains (Mata et al., 2010), whilst oleaginous energy crops contain about 18-55% (EBTP, 2016) (v) microalgae are highly versatile and can be acclimated to a variety of environmental conditions: it is possible to find species adapted to extreme conditions and/or containing specific growth characteristics which is not possible with plants (Mata et al., 2010); (vi) microalgae are able to grow under harsh conditions and thus they can grow in areas unsuitable for agricultural purposes, and are independent to seasonal weather changes; (vii) wastewater can be used as culture medium and thus they do not require the use of fresh water; (viii) their growth results in simultaneous wastewater

treatment (and thus water recovery) and bio-product formation; (ix) microalgal biodiesel has similar performance to conventional diesel and contains no sulfur thus reduces the emissions of SO_x as well as of particulate matter (PM) and CO (Delucchi, 2003); (x) depending on the microalgal strain there is the potential for co-production of valuable products such as sugars, pigments, antioxidants, and vitamins, as well as biomass for animal feed or fertilizer (Mata et al. 2010); (xi) depending on the lipid extraction method followed, the biomass residue can be further valorized (processed into ethanol, methane, livestock feed, used as organic fertilizer, or burned for energy generation) (Wang et al., 2008); (xii) their growth results in efficient CO_2 sequestration (Wang et al., 2008) thus can be used for CO_2 fixation from industrial gases; (xiii) microalgal lipid profile is more suitable for biodiesel: under nutrient limitation their lipid profile consists mainly of saturated and monounsaturated fatty acids, in contrast to oleaginous crops such as linseed, sunflower seed, soybean and corn which are mainly composed of polyunsaturated fatty acids (PUFAs) (Vesna et al., 2013).

Microalgal photo-production can be intensified and result in higher productivities when artificial light is supplied. Nevertheless, apart from increasing production costs (Blanken et al., 2013), photo-production also involves the limitation of self-shading (Ruiz-Marin, 2010). The increase in biomass concentration causes reduction in light penetration in autotrophic cultures (Chaumont, 1993), usually resulting in biomass density of less than 1 $g_{DW}\ L^{-1}$ (Borowitzka, 1994). This low biomass, and thus lipid, productivity may result to increased costs during microalgal biodiesel production. This limitation however is avoided under heterotrophic cultivation. The present study

focuses on heterotrophic cultivation for the following reasons: (1) no cost of illumination; (2) scaling-up potential; (3) high cell densities; (4) higher metabolite concentrations; (5) higher growth rates (Raven, 1976) and thus faster process compared to autotrophic growth; (6) high nutrient recovery efficiency; (7) applicable to most wastewaters avoiding any pre-treatment stages to remove wastewater color, since wastewaters may still contain respectable amounts of organic compounds, and thus color, even after treatment, such as anaerobic digestion.

Macro-nutrient uptake (such as Nitrogen (N), Phosphorus (P) and Sulfur (S)) results in the formation of structural compounds such as proteins and RNA. However, microalgae can survive and function without the presence of these macro-nutrients. It is widely known that nutrient limitation triggers intracellular storage compound accumulation, such as lipids and starch (Dragone, 2011). It should be noted though that under nutrient limiting conditions lipid accumulation takes place at the expense of biomass productivity. Hence, due to this tradeoff lipid productivity may be compromised, despite the improved lipid content.

The most commonly studied lipid accumulation strategies undoubtedly concern N and then P accumulation. To our knowledge, no data exist in literature in terms of studying the effect of sulfur limitation on *C. vulgaris* growth and its lipid productivity, except our previous work in which the effect of pH on growth and lipid accumulation by *C. vulgaris* was studied (Sakarika and Kornaros, 2016). To this end, the present study

intends to establish (i) the effect of sulfur addition in the form of $MgSO_4 \cdot 7H_2O$ on biomass growth and (ii) the effect of limitation from different nutrients (S, P and N) on lipid accumulation by *C. vulgaris*, under heterotrophic conditions. This is of significant importance, as in many cases, in realistic applications of wastewater treatment using microalgae, mixtures of various wastewater streams are used as cultivation medium. Thus, the potential differences in nutrient concentrations in each wastewater stream and the resulting nutrient limitation could affect the selection of the most appropriate wastewater mixture for *C. vulgaris* cultivation. Finally, the supplementation of culture medium with an inexpensive nutrient source could potentially enhance the performance and lipid productivity of *C. vulgaris*, without substantially increasing the overall costs.

2. MATERIALS AND METHODS

2.1 Microorganism and growth conditions

The microalgal strain *C. vulgaris* SAG 211-11b used in this study was obtained from SAG (Sammlung von Algenkulturen der Universität Göttingen), and was grown on BG-11 broth. The maintenance and sub-cultivation of pre-cultures, as well as the preparation of heterotrophic inoculums were performed as described in our previous work (Sakarika and Kornaros, 2016).

2.2 Experimental conditions

All batch experiments were conducted in 1L Erlenmeyer flasks with a working volume of 0.8L. The reactors were covered with black plastic film, in order to avoid light penetration. Aeration was adjusted at 1 vvm under S limiting conditions, whereas 2 vvm were applied under P and N limitation due to increased cell densities. Temperature was controlled at 25±1°C using an orbital shaker bath adjusted at 90 rpm. The evaporation of water caused by aeration was monitored by weighting each reactor before and after sample extraction, and was incorporated in the calculations.

All experiments were carried out at the optimal pH (7.5) for this strain (Sakarika and Kornaros, 2016), with the pH kept constant using the corresponding buffer solution. The synthetic medium used in each experiment was BG-11 supplemented with glucose. As determined in our previous study (Sakarika and Kornaros, 2016), the limiting nutrient for *C. vulgaris* in BG-11 broth was sulfur (S) and thus, in order to determine the effect of other nutrient limitation the addition of an S-source was essential. More specifically, for the investigation of the effect of N and P limitation, the medium was supplemented with 350 mg $MgSO_4 \cdot 7H_2O$ L^{-1} (amount selected to supply excess S, based on N:S ratio in the BG-11 broth). Since the addition of an S-source would result in a higher cell density culture, the medium was supplemented with greater amounts of glucose. Being impossible to achieve phosphorus limitation while using a phosphate buffer, in the case of studying P-limitation the pH stability was realized using 0.1M tris(hydroxymethyl)aminomethane as buffer solution. The substrate composition used in each experiment is summarized in **Table 1**. Finally, the buffer solution and carbon source were sterilized separately, in order to avoid glucose

caramelization (occurring when autoclaving glucose at pH>5.0). After cooling down, the solutions were mixed and prepared for the respective experiment. Each experiment was carried out twice and the mean values are presented.

2.3 Analytical techniques

Cell growth was monitored by measuring the absorbance of algal suspension at 550 nm, after a calibration curve of optical density vs dry weight was constructed (R^2=0.999). Glucose was measured by the L-tryptophan-sulfuric acid-boric acid method (Joseffson, 1983). NO_3^- and SO_4^- were monitored throughout the experimental period using a DIONEX ICS3000 Ion Chromatography system. The estimation of intracellular lipids was carried out on every sample extracted from the cultivation medium using the Sulfo-Phospho-Vanillin method (SPV). The in situ transesterification of microalgal lipids for Fatty Acid Methyl Ester (FAME) analysis was performed as described by Levine et al. (2011). The exact steps of these analytical techniques, as well as the equations used for the calculations of: amount of microalgal lipids, yield of microalgal lipids, maximum specific growth rate (μ_{max}) and maximum biomass productivity (P_{max}) are thoroughly described in Sakarika and Kornaros (2016).

2.3.1 Elemental composition analysis

Elemental composition analysis was performed on freeze-dried algal biomass in order to quantify the content of certain elements (C, O, H, N and S). The elemental analyzer contains a combustion furnace, which was maintained during the analysis at 1020°C.

The combustion furnace is equipped with a quartz column for oxidation and reduction of the solid sample. The carrier gas was helium (constant flow of 100 mL min^{-1}), which was enriched with pure oxygen in order to achieve a strong oxidizing environment for solid material combustion. The solid samples were oxidized to gases CO_2, H_2O, NO_x and SO_3, which after reduction were transformed to a gas mixture of N_2, CO_2, H_2O and SO_2. Finally, the gas mixture was analyzed via Gas Chromatography (GC) equipped with a packed column (Porapack Q) and a Thermal Conductivity Detector (TCD). Based on the elemental analyzer's calibration data, the standard deviation in the reported N, P, S cellular content measurements is lower than 5% of the indicated values.

3. RESULTS AND DISCUSSION

3.1 Biomass growth

Firstly, the effect of the addition of a sulfur (S) source on *C. vulgaris* growth in BG-11 broth, supplemented with glucose under heterotrophic conditions was studied. Apart from the investigation of the growth behavior of this strain, the aim was to result in sulfur, nitrogen and phosphorus (S, N and P) limitation, and investigate the differences in the effect of lipid accumulation kinetics. **Fig. 1** depicts the growth behavior of *C. vulgaris* in all conditions tested.

During S limitation, stationary phase was achieved after 8 days of cultivation, presenting a lag phase of 2 days. The maximum biomass generation was 2.69 g_{DW} L^{-1} and the biomass productivity was 297.6 mg_{DW} L^{-1} day^{-1}, while the exponential growth

phase lasted 6 days. The addition of a S-source (in the form of $MgSO_4 \cdot 7H_2O$) significantly increased the final biomass concentration in the other two experiments (P and N limitation). More specifically, during P limitation the maximum biomass generation was 9.81 g_{DW} L^{-1}, the biomass productivity 828.9 mg_{DW} L^{-1} day^{-1}, while stationary phase was reached in the 10^{th} day of cultivation. The lag phase occuring in this experiment was the same as in S limitation (2 days) and the exponential phase lasted 8 days. It should be noted that the final biomass concentration was increased by a factor of 3.6 compared to S limitation. Next, the supplementation of 350 mg $MgSO_4 \cdot 7H_2O$ L^{-1} combined with the overabundance of P resulted in the desired N limitation. *C. vulgaris* reached stationary phase in the 20^{th} day of cultivation, presenting a lag phase of 6 days. This prolonged lag phase (when compared to S and P limitation) was attributed to nutrient overload (discussed in §**3.2**). The maximum biomass generation in this case was 11.12 g_{DW} L^{-1}, and the biomass productivity 502.5 mg_{DW} L^{-1} day^{-1}, while the exponential growth phase lasted 12 days. In this case, the final biomass concentration was increased by a factor of 4.0 compared to S limitation. These results indicate that biomass productivity is strongly related to nutrient bioavailability, and are summarized in **Table 2**.

3.2 Nutrient uptake

Fig. 2 illustrates the biomass growth as well as the glucose and limiting nutrient consumption of *C. vulgaris* in all experiments. During S and N limitation, P was not monitored because of the use of phosphate buffer. Due to the high concentration of phosphate in the culture medium, no conclusion could be drawn about its

consumption. In all cases, it was verified that the desired limiting nutrient was indeed in limiting concentration whereas the other nutrients were in abundance. The decrease in glucose concentration after achieving stationary phase is ascribed to glucose sequestration by the microalgae towards intracellular lipid formation.

In the study of S limitation, N consumption reached a value of 93.05 mg L^{-1} corresponding to 412.1 mg L^{-1} nitrate (NO_3^-) and in reference to S, the uptake was 4.66 mg L^{-1}, which corresponds to 13.98 mg L^{-1} sulfate (SO_4^{2-}). The overall glucose consumption was 7.143 g L^{-1} from which 3.849 g L^{-1} were removed until reaching S limitation. During the investigation of the effect of P limitation, the P uptake was 13.89 mg L^{-1} corresponding to 42.62 mg L^{-1} phosphate (PO_4^{3-}). Due to the higher biomass density in this case, the nutrient uptake was significantly larger than in S limitation. More specifically, in order to reach final biomass concentration of 9.89 g_{DW} L^{-1} the microalga consumed 24.64 g L^{-1} glucose, 47.18 mg L^{-1} of S (143.9 mg L^{-1} SO_4^{2-}) and 256.0 mg L^{-1} of N (1134 mg L^{-1} NO_3^-). During N limitation, in order to reach final biomass concentration of 11.19 g_{DW} L^{-1}, *C. vulgaris* incorporated 53.66 mg L^{-1} S, which is equivalent to 160.9 mg L^{-1} SO_4^{2-} and 291.8 mg L^{-1} of N, corresponding to 1292 mg L^{-1} NO_3^-. Finally, the overall glucose consumption was 37.63 g L^{-1}. **Table 3** summarizes the nutrient demands by *C. vulgaris* calculated under all experimental conditions.

In the case of S limitation, the elemental composition analysis provided the complete C, H, O, N and S content of *C. vulgaris* and the average biomass composition can be thus presented in the formula below:

$CH_{0.4618}O_{0.5732}N_{0.0537}S_{0.0014}$

Based on the elemental composition and the results shown in **Table 3**, *C. vulgaris* uptakes S in high quantities (0.20 % w w^{-1}), while the need for nitrogen is significantly lower (3.35 % w w^{-1}) when compared to other microalgal strains (Mišurcová et al., 2010). However, the % S and % N content is altered significantly when comparing the S to N and P limiting conditions, under which the cellular S content is more than doubled. Thus, high density cultures can be achieved in low P and/or N concentrations, provided that there is enough S and carbon (C) to support heterotrophic growth of *C. vulgaris*, since the cellular N content is 14% lower when compared to S limited growth. The relatively high S demand of this strain could potentially be attributed to its ability to form S-containing amino acids (Methionine, Cysteine) in higher quantities when compared to other microalgal strains (Becker, 2007). However, the amino acid profile was not determined in the present study.

3.3 Glucose inhibition

Although the increase of available nutrients in general has positive effects on the growth of a microorganism, beyond a certain point it can cause inhibiting effects due to nutrient overload. In the present study, the maximum specific growth rate (μ_{max}) of *C. vulgaris* was found to be related to the initial glucose concentration. As mentioned earlier (**§ 3.1**), during N limitation μ_{max} was significantly lower than the other two experiments (S and P limitation). Hence, comparing the maximum specific growth rate

of *C. vulgaris* under S and P limitation (practicaly the same) with the estimated μ_{max} under N limitation, substrate (glucose) inhibition was observed, which was triggered between 27 and 40 g glucose L^{-1}. This phenomenon (substrate inhibition) also explains the prolonged lag phase during this experiment. In their study, Liang et al. (2009) noticed an inhibiting effect when glucose was provided in concetrations higher than 10 g L^{-1} (1% w v^{-1}), while in the present study inhibitory behaviour became apparent at much higher glucose concentration.

Fig. 3 depicts the specific substrate consumption ($\frac{1}{X} \cdot \frac{\Delta C}{\Delta t}$) by *C. vulgaris* in all experiments. During S and P limitation (initial glucose concentration 10 and 27 g L^{-1} respectively), the specific substrate consumption presented a declining course from the early experimental period, while the rate was lowered through the course of time. This result indicates the normal function of the metabolism of the microalga, from the first days of these experiments. Nevertheless, during N limitation, the specific glucose consumption presented an increasing course during the first days of cultivation, verifying the inhibiting effect of the initial glucose concentration (which in this case was approximately 40 g L^{-1}). The relation between the maximum specific growth rate and the initial glucose concentration is graphically summarized in **Fig. 4**. The concentration of S, P and N did not affect the maximum specific growth rate.

3.4 Intracellular lipid accumulation

Under macro-nutrient (such as N, P or S) limiting conditions, microalgae synthesize intracellular lipids. In this part of the present study, the effect of S, N and P limitation on the lipid synthesis of *C. vulgaris* was investigated. **Fig. 5** depicts the lipid content in g per g dry weight (g g_{DW}^{-1}) of *C. vulgaris* for the nutrient limitations tested. The lipid content was monitored both by FAME analysis and the Sulfo-Phospho-Vanillin (SPV) method (Sanjiv et al., 2014). At this point it should be noted that canola oil presents a similar fatty acid profile to *C. vulgaris* (Vesna et al., 2013), and oleic acid (C18:1) is the main fatty acid produced by this strain, and thus, these compounds were selected as standards for the calibration curve in the SPV method. However, SPV method underestimates the lipid content due to the presence of saturated lipids which interfere with the measurement (Sakarika and Kornaros, 2016). The main fatty acids detected in *C. vulgaris*, arranged in declining order, were oleic (C18:1), linoleic (C18:2), palmitic (C16:0), palmitoleic (C16:1) and stearic acid (C18:0). Other fatty acids were also contained in traces. The FAME profile was mainly composed of monounsaturated fatty acids (MUFAs) with the dominant one being oleic acid (C18:1). It should be noted that the same fatty acid profile was observed in all cases of our previous study (Sakarika and Kornaros, 2016), where different pH values were tested. These results lead to the conclusion that the fatty acid profile is a characteristic fingerprint for a certain microalga at a certain cultivation mode (autotrophic, heterotrophic or mixotrophic).

The intracellular lipid content was highest under S limitation (53.43 ± 3.93 %, 48.17 ± 6.24 % and 32.70 ± 3.27 % g g_{DW}^{-1} in total lipids, unsaturated lipids and oleic acid

respectively), presenting a maximum total lipid productivity of 119.87 ± 9.56 mg L^{-1} day^{-1}. The total lipid productivity was 83.02 ± 5.12 mg L^{-1} day^{-1} during stationary phase. The lipid content in the other two experiments (P and N limitation) was substantially lower. More specifically, S limitation was followed by N limitation (21.48 ± 1.77 %, 20.22 ± 1.02 % and 13.34 ± 0.11 % g g_{DW}^{-1} respectively) with a maximum total productivity of 98.93 ± 8.89 mg L^{-1} day^{-1}. In this case, a declining course was presented after the peak. During stationary phase the total lipid accumulation rate was about 73.91 ± 0.76 mg L^{-1} day^{-1}. Finally, P limitation presented the lowest values of 17.42 ± 6.42 %, 13.66 ± 1.60 % and 10.04 ± 1.95 % g g_{DW}^{-1} in total lipids, unsaturated lipids and oleic acid respectively where the maximum total lipid productivity was 96.47 ± 16.47 mg L^{-1} day^{-1}. As expected due to the low lipid content, during P limitation the mean total lipid productivity during stationary phase presented the lowest value of 66.78 ± 1.16 mg L^{-1} day^{-1}. These findings indicate that the abundance of P has positive effects on lipid accumulation and agree with the findings of Chu et al. (2013) where it was shown that P limitation acts as a suppressing factor for lipid formation. Additionally, after S depletion carbon is mainly accumulated in the form of intracellular lipids instead of other storage compounds (i.e. starch). Hence, the optimal nutrient limitation for biodiesel production using *C. vulgaris* is that of sulfur (S).

Nevertheless, the concentration of lipids was the highest under N limitation, calculated during the 26^{th} day of cultivation. Although, the lipid content of *C. vulgaris* under these conditions was not the highest amongst the three experiments, the high concentration of biomass resulted in high concentration of lipids. However, the concentration of lipids in the other two experiments (S, P limitation) was comparable, even though the

density of *C. vulgaris* cells was significantly higher during P limitation. **Table 4** summarizes the results obtained related to intracellular lipid accumulation.

As mentioned earlier, in all experiments the dominant fatty acid was oleic acid. Moreover, the oleic acid content in total lipids presented the same pattern through time, in all nutrient limitations tested. **Fig. 6** illustrates the oleic acid content in total lipids during the experimental period for S limitation (all experiments presented the same pattern). Oleic acid content presented an increasing trend during growth phase and remained constant at the highest value (60% of total lipids) during stationary phase. This conclusion was also drawn in our previous study (Sakarika and Kornaros, 2016) where different pH values were tested. This fact indicates that fatty acids are not accumulated in the same rate during the cell cycle and was also supported by studies focusing on the metabolic pathways of lipid synthesis. For example Hu et al. (2008) thoroughly explained the mechanisms of *de novo* lipid synthesis, indicating that saturated fatty acids are synthesized first and double bonds are introduced later by the soluble enzyme stearoyl ACP desaturase.

Tables 5 and **6** present a comparison between the data found in literature, although estimated under different cultivation conditions, and the results of the present study, concerning N and P limitation, respectively. It should be noted that, to our knowledge there is no other available study investigating the effect of S limitation. As shown in **Table 5**, although the lipid content observed in the present study under N limitation

remained in low levels (21.48 ± 1.77 % g g_{DW}^{-1}) compared to other studies, the lipid productivity presented the second highest value of 98.93 ± 8.89 mg L^{-1} day^{-1}.

Examining in depth the available literature, Piorreck et al. (1984) studied the effect of different N regimes on *C. vulgaris* under autotrophic conditions. At low N concentrations, the green alga contained 45% of total lipids mainly consisting of oleic and palmitic acid (C18:1 and C16:0) and only minor portions of PUFAs (C16:2, C16:3, C18:2 and C18:3). Therefore, the lipid content obtained under these conditions was applicable for biodiesel production. At high N concentrations (greater than 0.003% NH_4CI or 0.001% KNO_3), the lipid accumulation dropped at 20% with the alga producing substantially lower amounts of C18:1, but larger proportion of PUFAs. In their study, Converti et al. (2009) autotrophically grew *C. vulgaris* in various N concentrations. When they reduced the N concentration by 75%, from 1500 mg $NaNO_3$ L^{-1} to 375 mg $NaNO_3$ L^{-1}, they observed an increase in the lipid content from 5.90% to 15.31% with the lipid productivity increasing from 8.16 to 20.30 mg L^{-1} day^{-1}. The lipid content was mainly composed of palmitic acid (C16:0, about 60% mol mol^{-1}). Additionally, the low concentration of the PUFA linolenic acid (C18:3) constituted the lipid profile suitable for biodiesel according to the European standards (EN 14214 and EN 14213). Another observation was the increase in the ratio lipid/protein as the lack of NO_3^- limited the protein biosynthesis.

Feng et al. (2011) grew the microalga mixotrophically in a column aeration photobioreactor providing artificial wastewater containing 0.41 g L^{-1} glucose. Under N

limiting conditions (20 mg NH_4^+-N L^{-1}), the lipid content in the stationary phase was 34.1% during batch cultivation, while the biomass concentration achieved was 1.58 to 1.72 g_{DW} L^{-1}. Griffiths et al. (2014) cultivated *C. vulgaris* in airlift photobioreactors. The authors tested a variety of initial NO_3^- concentrations. The best tradeoff between biomass and lipid production was presented in starting nitrate concentration of 170 mg L^{-1} resulting in a lipid productivity of 790 mg L^{-1} day^{-1}; a value more than double that under N-replete conditions. When sufficient N was supplied the lipid content was 54%, in contrast to 65% for N-deplete conditions. A strong correlation was observed between N content and biomass and lipid accumulation, as well as the pigment and protein content. Finally, the authors stated that N limitation not only does enhance the lipid productivity but also improves the lipid profile for biodiesel production and reduces the requirement for N addition, resulting in cost and energy savings. Liang et al. (2009) grew *C. vulgaris* autotrophically, heterotrophically and photoheterotrophically. The highest lipid content of 38% g g_{DW}^{-1} was observed under autotrophic growth. However, due to the low biomass concentration the lipid productivity remained at low levels (4 mg L^{-1} day^{-1}). When the strain was grown photoheterotrophically with 1% glucose, although the lipid content was not the highest (21% g g_{DW}^{-1}) the relatively high biomass concentration (1696 mg_{DW} L^{-1}) resulted in high lipid productivity (54 mg L^{-1} day^{-1}). Under heterotrophic conditions the lipid content was 23% g g_{DW}^{-1} resulting in lipid productivity of 35 mg L^{-1} day^{-1} whilst the biomass density was 1206 mg_{DW} L^{-1}. In the study of Lv et al. (2010), *C. vulgaris* was grown autotrophically with 1.0% CO_2. When KNO_3 was supplied at concentrations of 0.2, 1.0, 3.0 and 5.0 mM the total lipid content was 22.5, 20.0, 18.5 and 15.9% g g_{DW}^{-1} respectively. Thus, the lipid content was decreased with the increase of KNO_3

concentration. Although the highest biomass concentration was obtained at the KNO_3 concentration of 5 mM, the maximum lipid productivity of 40 mg L^{-1} day^{-1} was observed at 1.0 mM KNO_3. Since the highest biomass concentration was compromised by the lowest lipid content, a relatively lower (compared to the maximum) lipid productivity of 35 mg L^{-1} day^{-1} was obtained at 5 mM KNO_3. Stephenson et al. (2010) investigated the most effective N-limitation regime and found that rather than transferring cells to a medium without N, the maximal triacylglyceride (TAG) productivity (46 mg L^{-1} day^{-1}) was achieved by allowing the cells to deplete the N naturally. Moreover, in this case, the major fraction (over half) was TAGs, whilst the proportion of phospholipids and glycolipids was reduced. In addition, the fatty acid (FA) composition of the TAGs was also altered under N limitation, so that the major fatty acid was now oleic acid (C18:1, 32–37% g g_{DW}^{-1}), while the proportions of the more highly unsaturated fatty acids, C18:2, C18:3 and C16:2, were all reduced compared to the levels under nutrient-sufficient conditions. The authors also observed that stearic acid (C18:0) was also reduced under N limitation, indicating that the alterations were not simply due to lower rates of unsaturation, but rather due to a shift in overall fatty acid production. The authors commented that although under nutrient sufficient conditions the total lipid production can be high, it consists predominantly of phospholipids and glycolipids. They observed that under these conditions the “appropriate lipids for biodiesel production” comprise about 0.5% of the total lipid content, thus drastically lowering the useful lipid productivity. Yeh and Chang (2011) investigated the effect of N starvation strategies on the autotrophic lipid production of *C. vulgaris*. When they compared single-stage and two-stage N starvation strategies they found that single stage cultivation with low initial N

concentration (313 mg L^{-1} KNO_3) was the most effective approach to enhance microalgal lipid production, attaining a lipid productivity of 78 mg L^{-1} day^{-1} and a lipid content of 55.9% g g_{DW}^{-1}. Over 65% of fatty acids in the microalgal lipid were saturated (i.e. palmitic (C16:0) and stearic acid (C18:0)) and monounsaturated (MUFA) (i.e. oleic acid (C18:1)).

On the other hand, the lipid content of *C. vulgaris* under P limitation in the present study was comparable to the one reported by Mutlu et al. (2011) (**Table 6**). After screening 10 *Chlorella* and *Parachlorella* strains, Přibyl et al. (2012) found that the most effective lipid producer was *C. vulgaris*. When provided with 40mM NO_3^- and 5mM PO_4^{3-} the strain presented the highest lipid productivity of 604 mg L^{-1} day^{-1}, with a total lipid content of 57.25% g g_{DW}^{-1}. When they reduced the nitrate and phosphate concentration by 75%, resulting in 10mM and 0.25mM NO_3^- and PO_4^{3-} respectively, they obtained the highest lipid productivity of 1425 mg L^{-1} day^{-1}. The authors concluded that the availability of N and P has a strong effect on growth and accumulation of lipids in cells by affecting cell division. When Mutlu et al. (2011) investigated the effect of P limitation they found that the lipid content was increased from 12.29% g g_{DW}^{-1} (control) to 16.7% g g_{DW}^{-1} when they reduced the P content by 50%, from 18.2 to 9.1 mg PO_4^{3-} L^{-1}. However, when they also reduced by 50% the initial N supply, from 58.4 to 29.2 mg NO_3^- L^{-1} they observed a further increase in lipid content (20.5% g g_{DW}^{-1}), noticing thus a synergistic effect on simultaneous N and P limitation.

In their research, Chia et al. (2013) investigated the effect of P limitation by providing PO_4^{3-} in concentrations of $2.3x10^{-4}$, $2.3x10^{-6}$ and $6.0x10^{-7}$ mg L^{-1}. The total lipid content was 0.80, 7.79 and 9.14 pg $cell^{-1}$ respectively. In addition, TAG content exhibited an increasing trend with decreasing phosphate concentration. The authors also observed a decrease in phospholipid (PL) fractions with decreasing PO_4^{3-} concentrations, whereas the total saturated fatty acids (SAFA) increased at limiting PO_4^{3-}. MUFA concentration was increased with lowering PO_4^{3-} concentration. They also observed an increase in C14:0, C16:0 and C18:0 with the decrease in P concentration. A general remark was that the lipid profile of *C. vulgaris* was dominated by C16 and C18 chain length fatty acids. Hence, the degree of fatty acid saturation is strongly related to the P supply. Many authors (Chia et al., 2013; Villar-Argaiz et al., 2009; Spijkerman and Wacker, 2011) have observed that when PO_4^{3-} is sufficiently supplied, *C. vulgaris* presents higher ω-3 fatty acids as well as total PUFA, when compared to P limitation. Additionally, Leu et al. (2006) found that the pathway for C18 PUFA biosynthesis starts at C18:0. Sufficient PO_4^{3-} concentrations subsequently trigger the activation of this pathway towards the formation of C18 PUFAs (after double bond insertion) at the expense of the C18:0 precursor (Leu et al., 2006; Villar-Argaiz et al., 2009). Finally, even if P is an obligate requirement for growth, microalgae can lower their physiological P demand up to 50% in response to P limitation. When P availability is lowered they reduce either the phospholipid or nucleic acid synthesis rate, as a mechanism to support cell duplication (Van Mooy et al., 2009).

Another conclusion drawn in the present study is related to the optimal time of cell harvesting. More specifically, when the microalga has achieved stationary phase, it possesses the most desirable characteristics for biofuel production. At this time, the intracellular lipids (and the most suitable for biodiesel production, oleic acid) are accumulated at the highest rate and their concentration has reached a value that is comparable to the maximum one. More specifically, based on our results the lipid content increases approximately 5% during the period between early stationary phase and the end of each experiment. If the microalga remains further in the medium without external nutrient supply, not only will the lipid content not be substantially increased, but the assimilation of storage lipids could be triggered if there is not enough carbon in the medium. Additionally, in terms of nutrient uptake viewed as a wastewater post-treatment process, the highest rate is observed during the exponential growth phase. Hence, in order to harness the desired characteristics of *C. vulgaris* at the greatest extent, both from biofuel production and wastewater treatment efficiency point of view, the harvest should be performed during early stationary phase. In continuous systems, the microalgal specific growth rate is related to the hydraulic retention time (HRT). HRT is a parameter strongly related to the energy requirements and operational costs of the plant. Thus, HRT should be adjusted so as to achieve both nutrient uptake and biofuel production, without increasing the operational costs.

4. CONCLUSIONS

C. vulgaris was grown heterotrophically at pH 7.5. The addition of sulfur significantly increased (4-fold) the final biomass concentration. FAME profile was found to be unrelated to the nutrient causing growth limitation, while oleic acid was the predominant fatty acid. The highest lipid content (53.43 ± 3.93 % g g_{DW}^{-1}) was obtained under sulfur limitation. The results of this study can be viewed as a step towards sustainable biodiesel production through *C. vulgaris* cultivation as adopting the optimal nutrient limitation strategy can result in high concentration of value compounds, without compromising the high growth rates and biomass concentration.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25; 207–210.
2. Blanken, W., Cuaresma, M., Wijffels, R.H., Janssen, M., 2013. Cultivation of microalgae on artificial light comes at a cost. Algal Res. 2; 333–340.
3. Borowitzka, M.A., 1994. Large-scale algal culture systems: the next generation. Australas. Biotechnol. 4, 212–215.
4. Chaumont, D., 1993. Biotechnology of algal biomass production – a review of systems for outdoor mass-culture. J. Appl. Phycol. 5, 593–604.
5. Chia, M.A., Lombardi, A.T., Melão, M.G.G., Parrish, C.C., 2013. Lipid composition of Chlorella vulgaris (Trebouxiophyceae) as a function of different cadmium and phosphate concentrations. Aquat. Toxicol. 128– 129, 171– 182.
6. Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv., 25, 294–306.
7. Chu, F.F., Chu, P.N., Cai, P.J., Li, W.W., Lam, P.K.S., Zeng, R.J., 2013. Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency. Bioresour. Technol. 134, 341–346.

8. Converti, A., Casazza, A.A., Ortiz, E.Y., Perego, P., Del Borghi, M., 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem. Eng. Process. 48, 1146-1151.
9. Delucchi M.A., 2003. A Lifecycle Emissions Model (LEM): lifecycle emissions from transportation fuels; motor vehicles, transportation modes, electricity use, heating and cooking fuels. Main report UCD-ITS-RR-03-17.
10. Dragone, G., Fernandes, B.D., Abreu, A.P., Vicente, A.A., Teixeira, J.A., 2011. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl. Energy 88, 3331–3335.
11. EBTP, 2016. European Biofuels Technology Platform. Last accessed: 26/04/2017. <http://biofuelstp.eu/oil_crops.html>.
12. Feng Y., Li C., Zhang D., 2011. Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. Bioresour. Technol. 102, 101–105.
13. Griffiths, M.J., van Hille, R.P., Harrison, S.T.L, 2014. The effect of nitrogen limitation on lipid productivity and cell composition in *Chlorella vulgaris*. Appl. Microbiol. Biotechnol., 98, 2345–2356.
14. Joseffson, B., 1983. Rapid spectrophotometric determination of total carbohydrates In: Grasshoff, K., Ehrhardt, M., Kremling, K. (Eds.), Methods of Seawater Analysis. Verlag Chemie GmbH, pp. 340–342.
15. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., et al., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621–639.

16. Leu, E., Falk-Petersen, S., Kwasniewski, S., Wulff, A., Edvardsen, K., Hessen, D.O., 2006. Fatty acid dynamics during the spring bloom in a high Artic fjord: importance of abiotic factors versus community changes. Can. J. Fish. Aquat. Sci. 63, 2760–2779.

17. Levine, R.B., Costanza-Robinson, M.S., Spatafora, G.A., 2011. Neochloris oleoabundans grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production. Biomass Bioenerg, 35, 40–49.

18. Liang, Y., Sarkany, N., Cui, Y., 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol. Lett. 31, 1043–1049.

19. Lv, J.M., Cheng, L. H., Xu, X.H., Zhang, L., Chen, H.L., 2010. Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. Bioresour. Technol., 101, 6797–6804.

20. Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renew. Sustain. Energy Rev. 14, 217–32.

21. Mišurcová, L., Kráčmar, S., Klejdus, B., Vacek, J., 2010. Nitrogen content, dietary fiber, and digestibility in algal food products. Czech J. Food Sci. 28: 27–35.

22. Mutlu, Y.B., Isık, O., Uslu, L., Koç, K., Durmaz, Y., 2011. The effects of nitrogen and phosphorus deficiencies and nitrite addition on the lipid content of *Chlorella vulgaris* (Chlorophyceae). Afr. J. Biotechnol. 10, 453-456.

23. Piorreck, M., Baasch, K.H., Pohl, P., 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue– green algae under different nitrogen regimes. Phytochemistry 23, 207–216.

24. Přibyl, P., Cepák, V., Zachleder, V., 2012. Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. Appl. Microbiol., Biotechnol. 94, 549–561.

25. Raven, J.A., 1976. The quantitative role of dark respiratory processes in heterotrophic and photolithotrophic plant growth. Ann. Bot. 40, 587–602.

26. Ruiz-Marin, A., Mendoza-Espinosa, L.G., Stephenson, T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresour. Technol., 101, 58–64.

27. Sakarika, M., Kornaros, M., 2016. Effect of pH on growth and lipid accumulation kinetics of the microalga *Chlorella vulgaris* grown heterotrophically under sulfur limitation, Bioresour. Technol., 219, 694–701

28. Sanjiv, K.M., William, I.S., Wasif, F., Myounghoon, M., Anupama, S., Park, M.S., Yang, J.W., 2014. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresour. Technol., 155, 330-333.

29. Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A look back at the U.S. Department of Energy's aquatic species program: biodiesel from algae. NREL/TP-580-24190, National Renewable Energy Laboratory, USA.

30. Spijkerman, E., Wacker, A., 2011. Interactions between P-limitation and different C conditions on the fatty acid composition of an extremophile microalga. Extremophiles 15, 597–609.

31. Stephenson, A.L., Dennis, J.S., Howe, C.J., Scott, S.A., Smith, A.G., 2010. Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks. Biofuels 1, 47–58.

32. Van Mooy, B.A.S., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblížek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappé M.S., Webb, E.A., 2009. Phytoplankton in the Ocean Use Non-Phosphorus Lipids in Response to Phosphorus Scarcity. Nature 458, 69-72.

33. Vesna, K., Shaban, M., Biljana, B., 2013. Fatty acid composition of edible oils and fats. JHED 4, 112-116.

34. Villar-Argaiz, M., Medina-Sanchez, J.M., Bullejos, F.J., Delgado-Molina, J.A., Perez, O.R., Navarro, J.C., Carrillo, P., 2009. UV radiation and phosphorus interact to influence the biochemical composition of phytoplankton. Freshw. Biol. 54, 1233–1245.

35. Wang, B., Li, Y., Wu, N., Lan, C.Q., 2008. CO_2 bio-mitigation using microalgae. Appl. Microbiol. Biotechnol.; 79:707–18.

36. Wijffels, R.H., Barbosa, M.J., 2010. An Outlook on Microalgal Biofuels. Science 329, 796-799.

37. Yeh, K.L., Chang, J.S., 2011. Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implications for biofuels. Biotechnol. J. 6, 1358–66.

Figure and Table Captions

Fig. 1: Growth of *C. vulgaris* in medium compositions tested.

Fig. 2: Biomass growth, glucose and liming nutrient uptake by *C. vulgaris* during various nutrient limiting conditions.

Fig. 3: Specific Glucose Consumption ($\frac{1}{X} \cdot \frac{\Delta C}{\Delta t}$) by *C. vulgaris* during the experimental period, under S, P and N limitation respectively.

Fig. 4: Relation between maximum specific growth rate (μ_{max}) and initial glucose concentration.

Fig. 5: Lipid content of *C. vulgaris* under S, P and N limitation, respectively.

Fig. 6: Oleic acid content, as % of total lipids, during the S limitation experiment.

Table 1: Composition of cultivation medium used in the experiments of this study.

Table 2: Main features of *C. vulgaris* growth cultivated under different medium compositions at pH=7.5.

Table 3: Nutrient content of *C. vulgaris* cultivated under different nutrient limited conditions at pH=7.5.

Table 4: Main features of *C. vulgaris* lipid accumulation, under different nutrient limitations at pH=7.5 (maximum values).

Table 5: Comparison of final biomass concentration, total lipid content and total lipid productivity of *C. vulgaris* reported in literature under N limitation.

Table 6: Comparison of final biomass concentration, total lipid content and total lipid productivity of *C. vulgaris* reported in literature under P limitation.

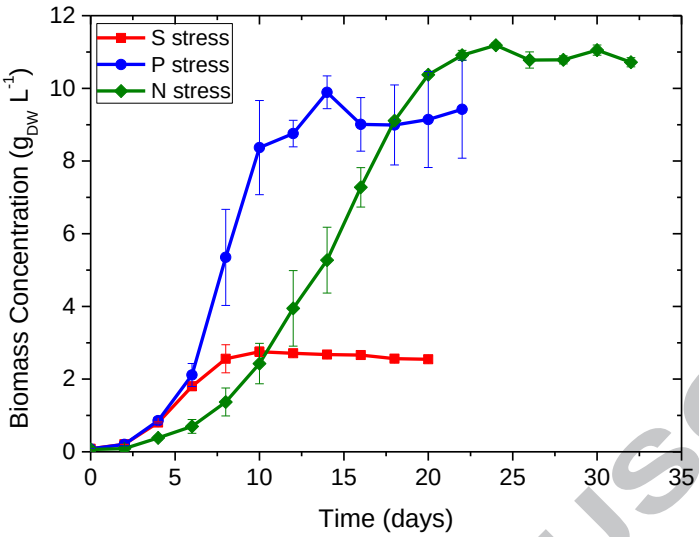


Fig. 1

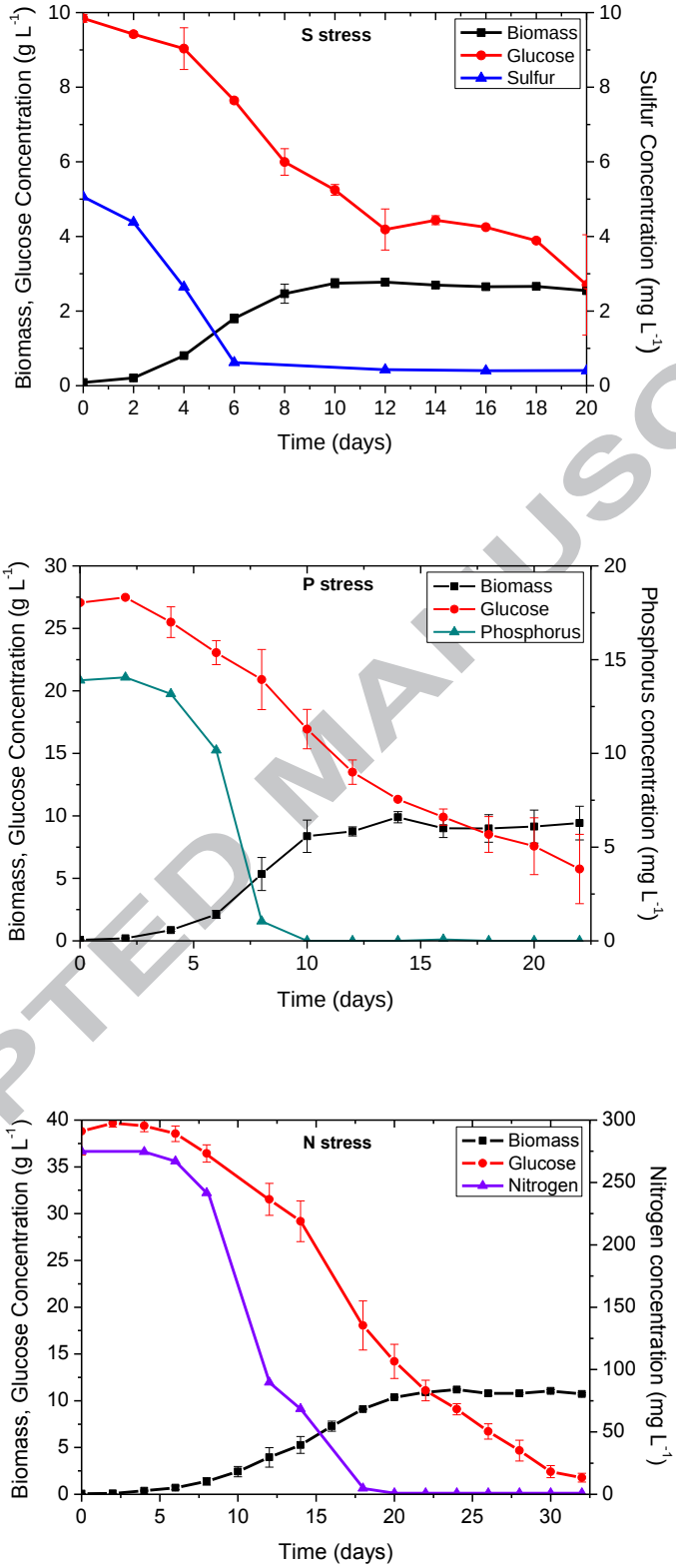


Fig. 2

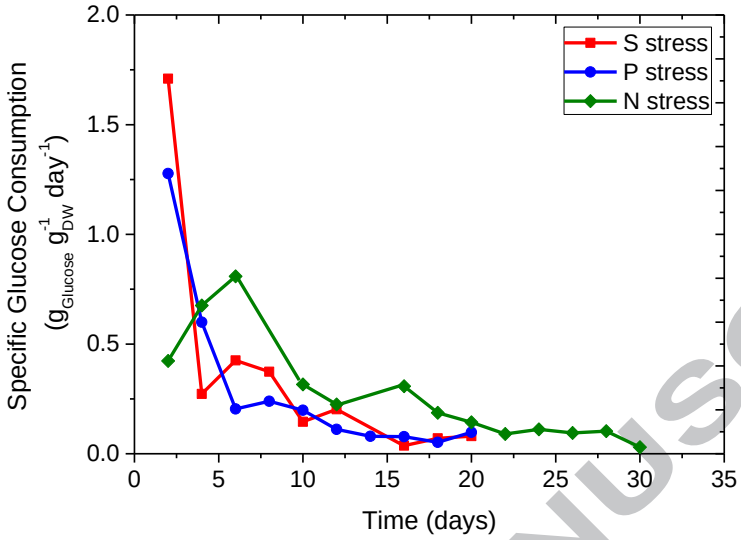


Fig. 3

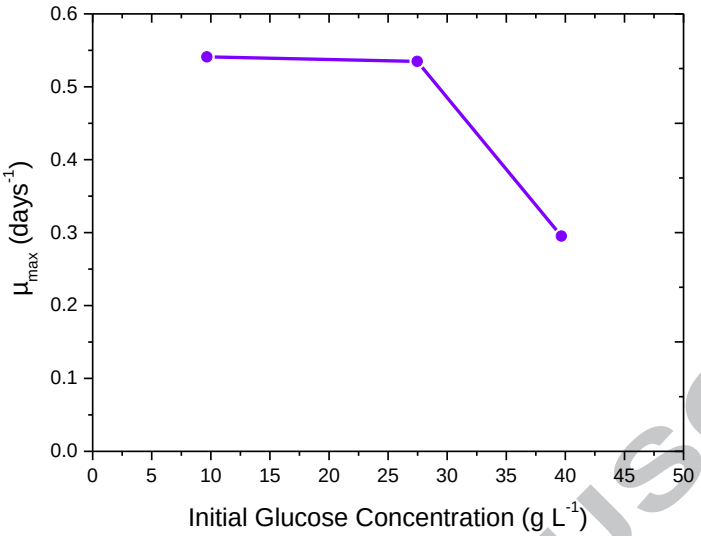
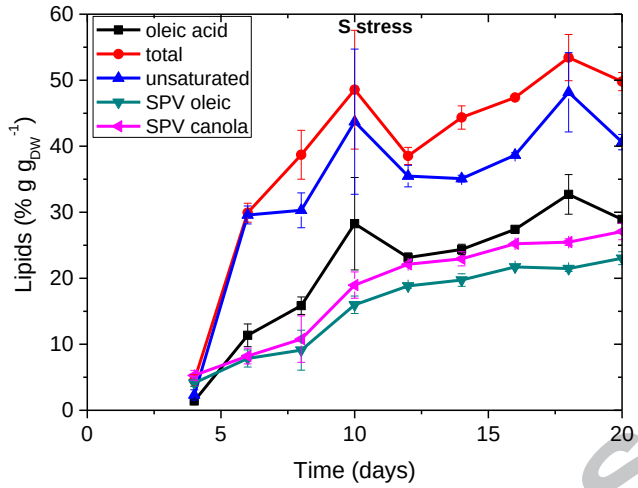
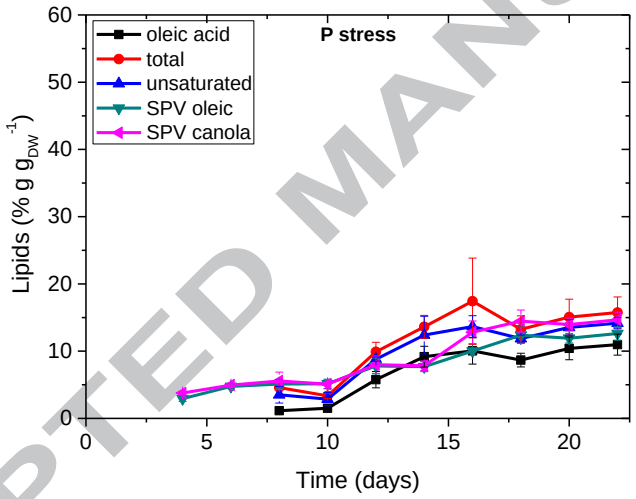


Fig. 4





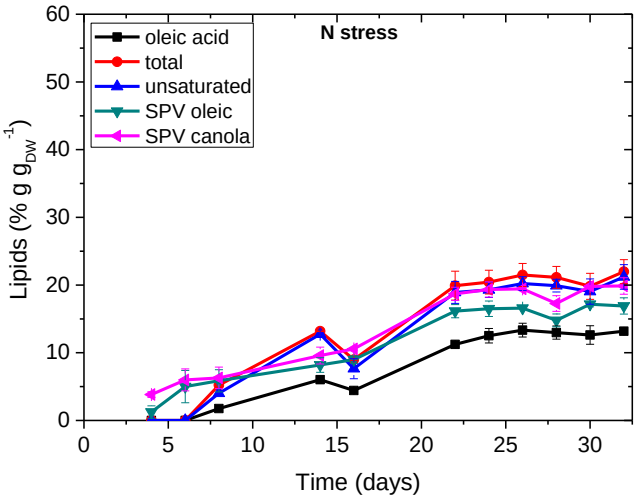


Fig. 5

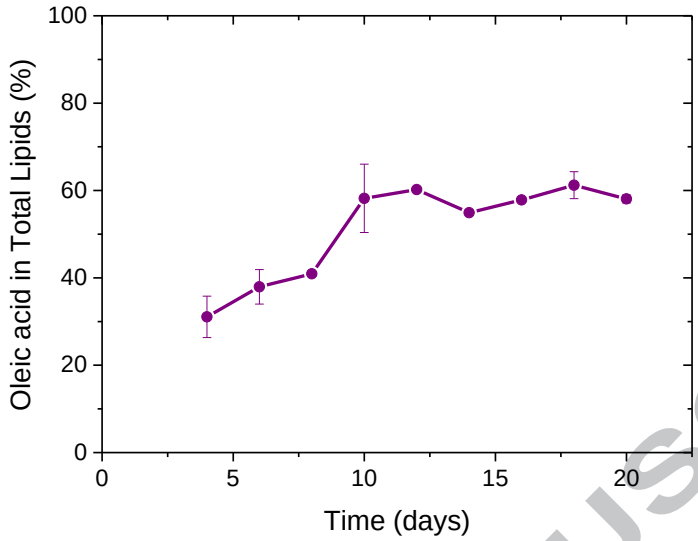


Fig. 6

Table 1

	S limitation	P limitation	N limitation
Initial glucose	10 g L^{-1}	27 g L^{-1}	40 g L^{-1}
$MgSO_4 \cdot 7H_2O$ addition	-	350 mg L^{-1}	350 mg L^{-1}
Buffer solution	Phosphate buffer	Tris(hydroxymethyl) aminomethane	Phosphate buffer
Basic broth	BG-11	BG-11	BG-11

Table 2

Parameter	Symbol	Unit	S limitation	P limitation	N limitation
Maximum Specific Growth Rate	μ_{max}	$days^{-1}$	0.5409	0.5348	0.2953
Lag phase	-	days	2	2	6
Stationary phase[a]	-	day	8	10	20
Maximum Biomass Generation	X_{gen}	$g_{DW}\ L^{-1}$	2.689	9.809	11.12
Maximum Biomass Productivity[b]	P	$mg_{DW}\ L^{-1}\ day^{-1}$	296.7	828.9	502.5

[a] day of achievement; [b] calculated from the mean biomass concentration during stationary phase

Table 3

Nutrient	Unit	S limitation	P limitation	N limitation
Sulfur (S)	% mg_S mg_{DW}^{-1}	0.20	0.54	0.52
Phosphorus (P)	% mg_P mg_{DW}^{-1}	-[a]	0.16	-[a]
Nitrogen (N)	% mg_N mg_{DW}^{-1}	3.35	2.95	2.81

[a] P not monitored due to the use of phosphate buffer

Table 4

Parameter[a]		S limitation	P limitation	N limitation
Lipid Content (% g g_{DW}^{-1})	Total Lipids	53.43 ± 3.93	17.42 ± 6.42	21.48 ± 1.77
	Unsaturated Lipids	48.71 ± 6.24	13.66 ± 1.60	20.22 ± 1.02
	Oleic acid	32.70 ± 3.27	10.04 ± 1.95	13.34 ± 0.11
Productivity (mg L^{-1} day^{-1})	Total Lipids	119.87 ± 9.56	96.47 ± 16.47	98.93 ± 8.89
	Unsaturated Lipids	93.78 ± 17.33	76.56 ± 23.7	89.96 ± 5.96
	Oleic acid	49.04 ± 8.27	65.12 ± 13.91	58.33 ± 5.38
Concentration (mg L^{-1})	Total Lipids	1422.3 ± 172.1	1469.1 ± 5.1	2317.5 ± 231.1
	Unsaturated Lipids	1283.1 ± 312.0	1329.6 ± 110.5	2181.3 ± 155.0
	Oleic acid	870.8 ± 148.9	1023.5 ± 55.6	1439.6 ± 139.8

[a] Maximum values obtained

Table 5

Strain	Light	Carbon source	Temperature	Aeration	Initial N concentration	Final biomass concentration	Total lipid content[a]	Total lipid productivity[a]	Reference
	µmol m^{-2} s^{-1}		°C			g_{DW} L^{-1}	% g g_{DW}^{-1}	mg L^{-1} day^{-1}	
***C. vulgaris* Beijerinck**	~11.2 (~800 lux)	CO_2 (air)	22	air (flow rate n/a)	KNO_3: 0.1%	0.287	22.6	n/a	Piorreck et al., 1984
					KNO_3: 0.03%	0.293	21.8		
					KNO_3: 0.01%	0.212	22.0		
					KNO_3: 0.003%	0.077	42.7		
					KNO_3: 0.001%	0.057	**62.9**		
					KNO_3: 0.0003%	0.017	57.9		
					NH_4Cl: 0.03%	0.205	11.8		
					NH_4Cl: 0.01%	0.190	14.1		
					NH_4Cl: 0.003%	0.160	20.2		
					NH_4Cl: 0.001%	0.095	41.8		
					NH_4Cl: 0.0003%	0.038	52.8		
***C. vulgaris* #259**	continuous fluorescent light	CO_2 (air)	room temp.	air (0.2 L min^{-1})	0 mg $NaNO_3$ L^{-1}	0.315	33	4	Liang et al., 2009
	continuous fluorescent light	1% glucose			250 mg $NaNO_3$ L^{-1}	1.696	21	**54**	
	No light	1% glucose				1.206	23	35	
***C. vulgaris* CCAP 211**	70 (continuous)	CO_2 (air)	30	air (flow rate n/a)	1500 mg $NaNO_3$ L^{-1}	n/a	5.9	8.16	Converti et al., 2009
					750 mg $NaNO_3$ L^{-1}		14.37	**20.44**	
					375 mg $NaNO_3$ L^{-1}		**15.31**	20.30	
***C. vulgaris* (strain n/a)**	40 (continuous)	CO_2	25	air with 1.0% CO_2 (flow rate n/a)	5 mM KNO_3	1.2	15.9	35	Lv et al., 2010
					3 mM KNO_3	1.05	18.5	n/a	
					1 mM KNO_3	0.77	20	**40**	
					0.2mM KNO_3	0.4	**22.5**	n/a	
***C. vulgaris* 211/11B**	165 (continuous)	CO_2	20-23	10%$_{vol}$ CO_2; 71%$_{vol}$ N_2; 19%$_{vol}$ O_2 (flow rate n/a)	550 mg NO_3^- L^{-1}	5.21	18	24	Stephenson et al., 2010
					200 mg NO_3^- L^{-1}	3.11	**46**	**111**	
					100 mg NO_3^- L^{-1}	2.17	39	65	
					10 mg NO_3^- L^{-1}	0.84	22	21	

					0 mg NO_3^- L^{-1}	0.72	20	20	
***C. vulgaris* (FACHB1068)**	42 (3000 lux)	0.4125 g glucose L^{-1}	30	air (0.5 vvm)	20 mg $N\text{-}NH_4^+$ L^{-1}	1.58-1.72	34.1	n/a	Feng et al., 2011
***C. vulgaris* (strain not provided)**	80 (16h light: 8h dark)	CO_2, $NaHCO_3$	22	air (flow rate n/a)	80 g $NaNO_3$ L^{-1}	0.18	12.29	n/a	Mutlu et al., 2011
					40 g $NaNO_3$ L^{-1}	0.19	17.5	n/a	
					0 g $NaNO_3$ L^{-1}	0.18	**35.6**	n/a	
***C. vulgaris* ESP-31**	60	CO_2	25	CO_2 2% (0.2 vvm)	1250 mg KNO_3 L^{-1}	5.0	20.9	50	Yeh and Chang, 2011
					313 mg KNO_3 L^{-1}	n/a	**55.9**	**78**	
					156 mg KNO_3 L^{-1}	1.1	41.3	n/a	
***C. vulgaris* UTEX 395**	250 (continuous)	CO_2	25	air enriched with 0.29% CO_2 (2 L min^{-1})	2,000 mg NO_3^- L^{-1}	0.08	14	31	Griffiths et al., 2014
					1,200 mg NO_3^- L^{-1}	0.48	12	29	
					570 mg NO_3^- L^{-1}	0.83	28	48	
					420 mg NO_3^- L^{-1}	1.19	35	55	
					170 mg NO_3^- L^{-1}	1.48	54	**57**	
					100 mg NO_3^- L^{-1}	1.87	52	53	
					70 mg NO_3^- L^{-1}	1.68	56	48	
					40 mg NO_3^- L^{-1}	2.35	60	30	
					0 mg NO_3^- L^{-1}	2.30	**65**	3	
***C. vulgaris* SAG 211-11b**	**No light**	**40 g glucose L^{-1}**	**25**	**air (2 vvm)**	**1.5 g $NaNO_3$ L^{-1}**	**11.12**	**21.48**	**98.93**	**This study**

[a] Highest values from each reference are presented in bold

Highlights:

- *C. vulgaris* has higher S and lower N requirements compared to other microalgae.
- Substrate inhibition was exhibited at glucose concentration higher than 30 g L^{-1}.
- Sulfur limitation results in highest intracellular lipid accumulation.
- Phosphorus limitation results to maximum biomass productivity.
- Fatty acid profile is dominated by oleic acid (C18:1).