Manuscript No. BPBSE-19-0206

Organic wastes/by-products as alternative to CO_2 for producing mixotrophic microalgae enhancing lipid production

Davide Veronesi[1], Giuliana D'Imporzano[1], Barbara Menin[2], Silvia Salati[1], Fabrizio Adani[1*]

[1]Gruppo Ricicla – DiSAA, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

* Corresponding author. Tel.: +39 0250316544; fax: +39 0250316521.

E-mail address: fabrizio.adani@unimi.it (F. Adani).

Abstract

In this work, white wine lees (WWL), cheese whey (CW) and glycerol (GLY) were used as carbon (C) sources to mixotrophically support the production of the microalga *Nannochloropsis salina*, replacing CO_2 supply. By doing so algae was allowed to grow on C sources dosed at 2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of C, in the presence and absence of CO_2 supply. WWL and CW were not able to support algae growth due to a fungal contamination that was genomically identified, while GLY gave interesting results in particular with 3 g L^{-1} of C. GLY-C was able replacing CO_2-C completely when this latter was omitted, showing an algal biomass production similar to those obtained in autotrophy. If CO_2-C was provided jointly with GLY-C, biomass production and lipids content increased more than 30% and 23% respectively, compared to autotrophy.

Keywords: Algae production, Carbon dioxide; Mixotrophy, Organic wastes; Organic by-products.

Introduction

Nowadays, the importance that microalgae could play in several sectors such as agriculture, animal feeding, green chemistry, nutraceutics, pharmaceutics and bioenergy production, it is increasingly recognized [1]. To grow and reproduce, these microorganisms require an energy source (light), a carbon source (CO_2 for autotrophic microalgae) and a growth medium (water plus nutrients) [2]. This is the common modality of algae cultivation, i.e. photoautotrophy, in which algal cells use light energy to extract protons and electrons from water, reducing CO_2 to organic molecules. Photoautotrophic cultivation is limited by high production costs; in particular, nutrients and CO_2 supplies are the major production costs in a full-scale microalgae production plant [3]. To reduce these costs, several authors suggested the use of wastewaters as culture medium [4,5]. Some other studies suggested, as feasible alternative to the photoautotrophic cultivation, the exploitation of the ability of some algae strains to grow under mixotrophic conditions using raw materials rich in carbon (C) and nutrients [6,7]. Mixotrophic cultivation is the mode by which microalgae can drive both photoautotrophy and heterotrophy and can utilize both inorganic and organic carbon sources [8]. Inorganic carbon is fixed through photosynthesis which is influenced by illumination conditions, while organic compounds are assimilated through aerobic respiration, which is affected by the availability of organic carbon [9]. Some scientists suggested that the specific growth rate of microalgae under mixotrophic cultivation is approximately the sum of those under photoautotrophic and heterotrophic modes [10] whereas others believed that the specific growth rate in mixotrophy is not the simple combination of the two modes [11,12]. The same authors

indicated that the two metabolic processes (i.e., photosynthesis for photoautotrophy and aerobic respiration for heterotrophy) affect each other under mixotrophic cultivation, determining a synergistic effect that enhances biomass productivity and lipid accumulation. The flexibility of some algal strains to switch their C nutritional mode based on substrate availability and light conditions is one of their inherent evolutionary advantages [13]. Therefore, mixotrophy represents a good solution to obtain both high biomass and high value compounds production, recovering C-rich wastes/by-products [14,15]. In addition, the ability of some algae to recover nutrients (N and P) growing on wastewaters [16], could improve both economic and environmental sustainability of the process. To accomplish viable and cost-effective results, the cheapest carbon enrichment to microalgal medium should be adopted.

The present study focused on the possibility to use alternative carbon sources for the mixotrophic growth of *Nannochloropsis salina*, instead of the usual CO_2 supply to support microalgae production, reusing and valorising different organic carbon wastes/by-products, monitoring the effects on growth and biomass composition of microalgal cultures, grown with and without CO_2 feeding.

Materials and methods

Organism and medium

Nannochloropsis salina - strain SAG 40.85 - was acquired from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut (Universität Göttingen, Germany). The cultures were grown in f/2 medium [17-18], which was enriched with 1.875 g $NaNO_3$ l^{-1} and 0.135 g $NaHPO_4$ l^{-1}. Inocula were prepared batch-wise and were grown under aseptic conditions in Erlenmeyer flasks of 150 mL under constant aeration and mixing by using

filtered air (filter of 0.2 μm) with a continuous illumination of 50 μE m^{-2} s^{-1} provided by fluorescent white tubes, at a controlled temperature of 22 ± 1°C.

Organic carbon sources

Three different C streams were selected: white wine lees (WWL), glycerol (GLY) and cheese whey (CW). WWL were derived from a wine cooperative in Corte Franca (BS), Italy. GLY was obtained from a local biogas plant that uses crude glycerol as co-substrate for biogas production. GLY represents by-product that can be used for many purposes, so that it should be considered that its cost might vary a lot depending on alternative use (e.g. industrial use to produce polymers).

CW was sampled from an agro-food industry in northern Italy and successively stored at -20°C. Before being used, CW was subjected to a deproteinization performed by heat treatment at 115 °C for 15 min [19] and successive filtration using 0.2 μm filters. This pre-treatment added cost and this fact should be considered in proposing this C source for algae growth.

Thereafter, CW was hydrolyzed by using β-galactosidase (Sigma-Aldrich, San Luis, Missouri, USA) from *Aspergillus oryzae*. Enzymatic hydrolysis was performed at 30 °C and at pH of 4.5 for 24 h [20] in a shake flask at 200 rpm using 65 U of enzyme per grams of lactose quantified in whey permeate, such as reported [21]. Before their use, all selected carbon sources were chemical characterized in terms of pH, dry weight (DW), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and ammonium nitrogen (N-NH_4^+), according to the methods reported for wastewater sludges [22].

The concentration of organic carbon in the growing media was assessed by COD determination.

Experimental procedures

Nannochloropsis salina SAG 40.85 was grown in batch mode in bubble columns photobioreactors (PBRs) of 0.045 m internal diameter, 0.5 m height with a working volume of 0.5 L. After 15 days of batch culture, C-substrates were added when all the cultures were in exponential phase. Cultures were incubated at 20°C, air-aerated with a flow rate of 5 L min^{-1} and illuminated with a 12:12 light photoperiod with an irradiance of 260 µmol m^{-2} s^{-1}. Cultivation system was equipped with an automatic control of the pH, which remained constant at 8 ± 0.3, by using pure CO_2 injection dosed "on demand" according to pH value. Screening tests were carried out in order to check if *N. salina* was able to mixotrophically grow using all the selected streams, testing them one-by-one at different carbon concentration, as in the following reported: 5.1 g L^{-1}, 7.7 g L^{-1} and 10.2 g L^{-1} of GLY (2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of carbon); 29.4 mL L^{-1}, 44.1 mL L^{-1} and 58.58 mL L^{-1} of WWL (2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of carbon); 36.5 mL L^{-1}, 54.8 mL L^{-1} and 73 mL L^{-1} of CW (2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of carbon). Experiments were carried out in triplicate with a control trial that was performed by cultivating algae under autotrophic condition. Results from the screening tests were used in order to study the effect of CO_2 supply and deprivation on growth and biomass composition of *N. salina*.

CO_2 was supplied during trials on demand according to pH value that was set up to 8.4. Lower pH value caused the injection of pure CO_2 for some seconds in order to adequate pH. One reviewer suggested to quantified total CO_2 consumed. So, we agree with reviewer that this data can help in comparing autotrophy with mixotrophy and in the future should be measured but, unfortunately, this data it difficult to be measured because CO_2 outflow with discharged air.

Analytical methods

Microalgae growth was determined measuring the DW by sampling 5 mL of algae culture from each PBRs every two days. Algae suspension was then centrifuged at 4,000 rpm for 15 minutes. The supernatant obtained was then used for nutrients uptake evaluation by using a spectrophotometric analytical kit (NANNOCOLOR®, Macherey-Nagel, Germany). The algae pellet was washed twice with an equivalent volume of distilled water (in order to avoid any overestimation caused by salt) and then filtered by using pre-weighed Whatman GFC filter of 1.2 μm, previously dried at 65°C overnight. The lipid content of freeze-dry biomass was gravimetrically determined after subsequent solvent extraction (chloroform: methanol 2:1 v/v) and evaporation of the solution this latter obtained by using a rotary evaporator (Büchi R110, Büchi Labortechnik AG., Flawil, Switzerland). Crude protein content was measured by determination of total nitrogen by Kjeldahl method such as previously reported [23]. Total nitrogen value was converted into proteins multiplying by specie-specific nitrogen-to-proteins conversion factors of 4.87 for *Nannochloropsis salina* [23]. Carbohydrate content was determined by the slightly modified phenol-sulphuric acid method of [24]. Briefly, an aliquot of freeze-dry sample was treated with 5 ml of concentrated sulphuric acid (98 % w/w) and 1 ml of phenol (6%, w/v). Samples were cooled to room temperature and the absorbance of the final blend was measured at 490 nm by a Jeneway 7305 UV-visible spectrophotometer; then carbohydrates were quantified by comparative estimation with a calibration curve obtained using glucose.

Carbon (C) content in the medium during trials was determined according to standard methods for wastewaters characterization [22]. This data was used to detect carbon consumption by algae during trials.

Contaminant identification

Additionally to microscopic examination, a molecular analysis was performed to identify the fungal contaminant mycelium. Genomic DNA was extracted and purified from 250 mg of freeze-dry fungal mycelium using the NucleoSpin® Soil (Macherey- Nagel, Düren, Deutschland) extraction kit, following manufacturer's instructions. DNA quantity and quality were assessed by means of NanoDrop 1000 Spectrophotometer. Fungal-specific PCR primers developed for analysis of the ITS region were used for the DNA amplification: the forward primer ITS1f CTTGGTCATTTAGAGGAAGTAA and the reverse primer ITS2 GCTGCGTTCTTCATCGATGC. PCR amplification was performed in a total volume of 25 µL containing 1.5 µL of total genomic DNA normalized at 5ng/ ul, 0.25 µL of forward and reverse primer at 10µM 2X GoTaq® Hot Start Colorless Master Mix (Promega, Fitchburg, United States) containing GoTaq® Hot Start DNA Polymerase supplied in 2X Colorless GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM MgCl2. Amplification was run in a GeneAmp PCR system 2700 (Applied Biosystems, Massachusetts, USA) as follows: 1 cycle of 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C and a 30 s extension step at 72 °C plus final extension of 5 minutes at 72°C. The PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel stained with 2 µl of Midori Green DNA Stain solution (Nippon Genetics Europe GmbH, Düren; Germany). Expected band size of 230 bp was sequenced by Sanger sequencing on both strands and manually checked.

Statistical analysis

All statistical analyses were performed using analysis of variance (ANOVA) with the Tukey test used to compare means (SPSS statistical software, SPSS Chicago IL).

Results and Discussion

Effect of carbon source on growth and molecular identification of the contaminant

Preliminary results shown that *N. salina* was unable to growth mixotrophically on WWL and CW: algal cells did not consume the organic C provided and the algae culture was affected by a strong fungal contamination. This data was in contrast with those coming from previous work [16] that showed that *Chlorella sp.* was able to use both CW and WWL with good growth performance producing proteins, indicating that the capacity of algae to growth on particular C-source, probably, it is species-specific.

The culture contaminant was studied by molecular analysis and the consensus ITS sequence obtained (see paragraph 2.5), was aligned and compared with published reference strains using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov). The alignment between the query and reference sequences showed the best match (97% of identity) with a *Fusarium sp.* SSU rRNA sequence, this species being recognized as a typical contaminant of marine culture [25].

Regarding GLY substrate, it was assimilated by algal cells at all the concentrations tested, allowed higher biomass production (5.30-6.59 g L^{-1} DW) than that obtained under autotrophic condition (4.40 g L^{-1} DW) Table 1.

Mixotrophic growth on glycerol without CO_2 supply

This aimed, also, to investigate the effect of GLY on the growth of the selected alga as an alternative C source to CO_2.

Doing so and taking into consideration the results obtained in previous trials, it was decided to start the PBRs culture in autotrophy with CO_2 supply (control) provided during the exponential growth phase until the 15^{th} day, i.e. when organic carbon was added at different concentration (section 2.3) and CO_2-feeding was stopped in mixotrophic PBRs.

The best concentration of GLY was 3 g L^{-1} of carbon with which *N. salina* has reached a maximum biomass-DW similar to that obtained in autotrophic conditions, i.e. biomass DW of 4.20 ± 0.17 g L^{-1} and of 4.30 ± 0.08 g L^{-1} for autotrophy and mixotrophy, respectively (Table 1). Productivity values were also very similar for different PBRs, i.e. 0.16 ± 0.01 g DW L^{-1} d^{-1} for autotrophy as well as mixotrophy with 2 g L^{-1} and 3 g L^{-1} of carbon, while the trial with 4 g L^{-1} of C had a productivity of 0.14±0.01 g DW L^{-1} d^{-1}. These values are in according with Gim et al. [26] that found a daily biomass productivity of 0.17 g L^{-1} d^{-1} in mixotrophic conditions. Both biomass DWs produced and daily biomass productivity measured for the trial performed under mixotrophy condition and fed with 4 g L^{-1} of carbon, were slightly lower than correspondent values measured for autotrophy trials. However, as highlighted in Figure 1b, organic carbon was completely absorbed by *Nannochloropsis salina* at all concentrations provided. These results suggested that mixotrophic metabolism was activated and that glycerol was used as carbon source for algae growth instead of CO_2.

The fact that simple carbon compounds are able to support mixothrophic growth is well documented in literature (Table 1). Mixotrophic growth of *Nannochloropsis* strain on glucose, glycerol, sodium acetate and ethanol have been reported previously [27, 28, 29,

29, 31, 32, 33] and resumed in the Table 1. Data collected showed that mixotrophic growth represents a good solution to obtain both higher biomass and lipid production than photoautotrophic cultivations, and that glycerol and glucose were the most common C sources used for *Nannochloropsis* cultures under mixotrophic way. In general, biomass production obtained under mixotrophic conditions were much lower than that obtained in this work [26, 27, 28, 30] (Table 1). Interesting was the work of Sforza et al. [27] which tested different organic substrates available at industrial scale. They found that glycerol was able to support the mixotrophic growth of *Nannochlopsis salina* and that using glycerol at 10 g L^{-1} they observed a lipid productivity similar to the best results obtained in this work by using only 3 g L^{-1} of glycerol. Interesting was, also, the fact that mixotrophic growth was triggered only when CO_2 was supplied under day–night cycle and that the excess of CO_2 inhibited the assimilation of the organic substrate.

Mixotrophic growth on glycerol with CO_2 supply

In this second experiment, glycerol was added to the media of the mixotrophic PBRs on day 15 while continuing CO_2 supply. All mixotrophic trials showed higher biomass production than that for autotrophic control (Figure 1c). The best performance was obtained for the trial performed by adding 3 g L^{-1} of GLY-C with a biomass DW of 4.40 ± 0.21 g L^{-1} and 6.59 ± 0.32 g L^{-1} for autotrophy and mixotrophy, respectively (Table 1). Daily biomass productivities were higher for all the mixotrophic PBRs than those obtained under autotrophy condition (Table 1). Both algal biomass DWs and productivities were higher than those of trials without CO_2 supply. This is likely due to the dual metabolisms, i.e. photosynthesis plus aerobic respiration of carbon substrates, which may have had a synergistic effect enhancing biomass production [33,11]. Again, glycerol was completely used for all the C concentrations considered after 12 days (Figure

1d). By comparing carbon uptake (Figures 1b and 1d), it was clear that when CO_2 was supplied, the carbon absorption (glycerol-C) was faster than when CO_2 was not supplied. Interesting was the fact that more than half of the total carbon dosed was consumed after only 7 days in trials with GLY and CO_2. This fact can be explained by considering the higher growth rate of algal cells when both photoautotrophic and heterotrophic metabolisms are efficiently performed simultaneously [34].

Comparing data obtained in this work with other reported for similar studies (Table 1) it is easy to note that data obtained in this work reported higher performance in terms of biomass production (DW) than those of previous ones. This fact could be ascribed to the higher light intensity used in this work i.e. 260 μmol m^{-2} s^{-1}, that is almost two folds higher by the maximum value reported and used by Das et al. [30]. In addition, none of the previous works reported (Table 1) used a system for CO_2 supply based on the microalgal demand according to pH value, which is the better system to support the photosynthetic activity of microalgal cells minimizing any leak of CO_2. In this way pH registered during trials were as in the following: autotrophic (control) of 8.4, mixotrophic without CO_2 of 8.9 and mixotrophic plus CO_2 of 8.4.

Biomass composition

The biochemical compositions of the lyophilized biomass obtained for each experiment is reported in Table 2. In both tests performed with and without CO_2 supply, trials grown under mixotrophic condition showed some changes in the final biomass composition with particular references to lipid content. It is well know that the addition of organic carbon source stimulates the lipids [35,36] and pigments [37] accumulation in several microalgal strains. In this study higher lipids amounts were recorded for mixotrophic trials where 3

and 4 g L^{-1} of GLY-C was added. Regardless of CO_2 feeding, when 2 g L^{-1} of C was provided, the total lipid content was very similar to that of autotrophic control (35.6 ± 0.2 % DW and 34.6 ± 0.2 % DW for autotrophic and mixotrophic condition, respectively). The dose of 2 g L^{-1} C was probably not sufficient to trigger to mixotrophic metabolism changing, also, biochemical composition. As reported in Table 2, the highest lipid content was obtained in PBRs with 3 g L^{-1} and 4 g L^{-1} of C (biomass of 45.7 ± 1.8 % DW and of 46.6 ± 1.6 % DW for experiment with and without CO_2, respectively). Therefore, the higher content of GLY-C was able to affect algal biomass and lipid accumulation. Liang et al. [38] observed an increase in lipid content of *Chlorella vulgaris* cultured with an increasing concentration of glycerol, i.e. the lipid content increased from 22% DW with 1% (w/v) glycerol addition, to 32% DW with 2% (w/v) glycerol supplementation. The same authors reported that the additions of 5% (w/v) and 10% (w/v) of glycerol exerted inhibition. Andruleviciute et al. [39] stated that compared to autotrophic conditions, the use of glycerol for microalgal cultivation increased the lipid content for several algal species, but also found that the continuous addition of glycerol cause a decrease in lipid content. Results presented in this work and data from literature seem to indicate that substrate inhibition is strain-dependent [38]. In particular, in this study the inhibition threshold for *N. salina* was found to be 4 g L^{-1} of C (Figures 1a) as biomass produced was the lowest under mixotrophic condition compared to 3 g L^{-1} of GLY-C that was the best concentration for the mixotrophic growth of the tested strain, both in presence and absence of CO_2. Regarding the crude protein content, no differences were evident for mixotrophic condition at all GLY-C concentrations tested and autotrophic condition, except for the PBRs with 3 g L^{-1} of GLY-C plus CO_2 in which the final proteins content, i.e. 16.3 ± 0.6 % DW, was slightly higher than the others (Table 2). Carbohydrate contents

were higher in algal culture cultivated autotrophically than mixotrophically (Table 2). Li et al. [40] showed that accumulation of lipids in algal cell occurred through the conversion of either starch or carbohydrates to lipids, but conversion depended by microalgal strains, i.e. different strains have different mechanism to switch from carbohydrate pathway to lipids production [41]. Furthermore, in this study, all trials performed without CO_2 addition reached final carbohydrate content slightly lower than that obtained with mixotrophic condition with CO_2 addition. This fact may be explained taking into consideration that under autotrophic condition the cell efficiency to store light energy as carbohydrates is often limited by the availability of inorganic carbon [42].

Conclusions

Nannochloropsis salina displayed the inability to growth mixotrophically on WWL and CW, while it grew very well on GLY improving biomass and lipid production. Results shown that when CO_2 was not provided, GLY-C was able to replace CO_2 when it was dosed at 3 g L^{-1} C. The use of C-rich by-products may be a viable way to produce microalgal biomass at low cost, increasing the productivity of final biomass and target compouds e.g. lipids, but not all substrates are suitable and the adaptability to some C-substrates is species-specific. However, the possible contamination by other microorganism e.g. fungi, that can affect the microalgal growth and the final quality of the biomass, is a real risk related to mixotrophic way. More in depth analysis should consider the cost of C sources, as pre-treatment (e.g. WL) and/or alternative uses (e.g. glycerol for industrial purpose such as polymer production, biogas production etc.) might affect it.

Acknowledgments The Cariplo Foundation, Italy, financially supported this research in relation to the DANCE (2014-0587) project.

Compliance with ethical standards Conflict of interest The authors declare that they have no conflict of interest.

References

1. Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 65: 635–648
2. Blair MF, Kokabian B, V.G. Gude (2014) Light and growth medium effect on Chlorella vulgaris biomass production. J Environ Chem Eng 2: 665–674
3. Acién FG, Fernàndez JM, Magàn JJ, Molina E (2012) Production cost of a real microalgae production plant and strategies to reduce it. Biotechnol Adv 30: 1344–1353
4. Park J, Jin HF, Lim BR, Park KY, Lee K (2010) Ammonia removal from anaerobic digestion effluent of livestock waste using green alga Scenedesmus sp. Bioresour Technol 101: 8649–8657
5. Ledda C, Romero Villegas GI, Adani F, Acién Fernández FG, Molina Grima E (2015) Utilization of centrate from wastewater treatment for the outdoor production of Nannochloropsis gaditana biomass at pilot-scale. Algal Res 12: 17–25
6. Wan M, Liu P, Xia J, Rosenberg JN, Oyler GA, Betenbaugh MJ, Nie Z, Qiu G (2011) The effect of mixotrophy on microalgal growth, lipid content, and

expression levels of three pathway genes in Chlorella sorokiniana. Appl Microbiol Biotechno. 91: 835–844

7. Xiong W, Gao C, Yan D, Wu C, Wu Q (2010) Double CO_2 fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production. Bioresour Technol 101: 2287–2293.
8. Kang CD, Lee JS, Park TH, Sim SJ (2005) Comparison of heterotrophic and photoautotrophic induction on astaxanthin production by Haematococcus pluvialis. Appl Microbiol Biotechnol 68: 237–241
9. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant J 54: 621–639
10. Marquez FJ, Sasaki K, Kakizono T, Nishio N, Nagai S (1993) Growth characteristics of Spirulina platensis in mixotrophic and heterotrophic conditions. J Ferment Bioeng 76: 408–410
11. Vonshak SM, Chen CF (2013) Mixotrophic growth modifies the response of Spirulina (Arthrospira) platensis (Cyanobacteria) cells to light. J Phycol 36: 675–679
12. Yu H, Jia S, Dai Y (2009) Growth characteristics of the cyanobacterium Nostoc flagelliforme in photoautotrophic, mixotrophic and heterotrophic cultivation. J Appl Phyco. 21: 127–133
13. Venkata Mohan S, Rohit MV, Chiranjeevi P, Chandra R, Navaneeth B (2015) Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: Progress and perspectives. Bioresour Technol 184: 169–178.

14. Ogbonna JC, Yoshizawa H, Tanaka H, (2000) Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms. J Appl Phycol 12: 277–284
15. Yang C, Hua Q, Shimizu K (2000) Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochem Eng J 6: 87–102
16. Salati S, D'Imporzano G, Menin B, Veronesi D, Scaglia B, Abbruscato P, Mariani P, Adani F (2017) Mixotrophic cultivation of Chlorella for local protein production using agro-food by-products, Bioresour Technol 230: 82–89
17. Guillard RRL (1975) In: Smith WL, Chanley MH (eds) Culture of phytoplankton for feeding marine invertebrates, Culture of Marine Invertebrate Animals. Plenum Press, New York
18. Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea Cleve. Can J Microbiol 8: 229-239
19. Dragone G, Mussatto SI, Almeida JB, Teixeira JA (2011) Optimal fermentation conditions for maximizing the ethanol production by Kluyveromyces fragilis from cheese whey powder. Biomass and Bioenergy 35: 1977–1982 doi:10.1016/j.biombioe.2011.01.045.
20. Abreu AP, Fernandes B, Vicente AA, Teixeira J, Dragone G (2012) Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. Bioresour Technol 118: 61–66

21. Espinosa-Gonzalez I, Parashar A, Bressler DC (2014) Heterotrophic growth and lipid accumulation of Chlorella protothecoides in whey permeate, a dairy by-product stream, for biofuel production. Bioresour Technol 155: 170–176
22. IRSA, CNR (1994) Analytical methods for Water Analysis (in Italian). Quaderni N. 100 Istituto Poligrafico e Zecca dello Stato, Rome, Italy.
23. Templeton DW, Laurens LML (2015) Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel. Algal Res 11: 359–367
24. DuBois M, Gilles KA, Hamilton JK, Rebers PA (1956) Smith F, Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356
25. Palmero D, Iglesias C, de Cara M, Lomas T, Santos M, Tello JC (2009) Isolated from River and Sea Water of Southeastern Spain and Pathogenicity on Four Plant Species. Plant Dis 93: 377–385
26. Gim GH, Ryu J, Kim MJ, Il Kim P, Kim SW (2016) Effects of carbon source and light intensity on the growth and total lipid production of three microalgae under different culture conditions. J Ind Microbiol Biotechnol 43: 605–616
27. Sforza E, Cipriani R, Morosinotto T, Bertucco A, Giacometti GM (2012) Excess CO2 supply inhibits mixotrophic growth of Chlorella protothecoides and Nannochloropsis salina. Bioresour Technol 104: 523–529
28. Xu F, Cai ZL, Cong W, Ouyang F (2004) Growth and fatty acid composition of Nannochloropsis sp. grown mixotrophically in fed-batch culture. Biotechnol Lett 26: 1319–1322

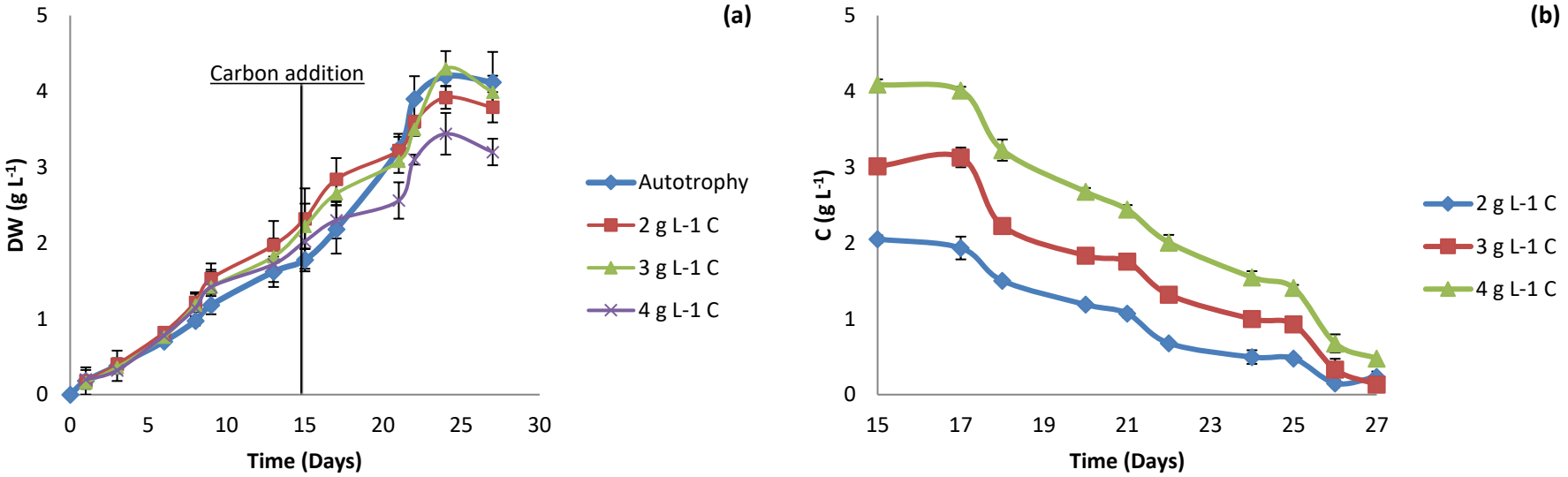
29. Xu F, Hu H, Cong W, Cai Z, Ouyang F (2004) Growth characteristics and eicosapentaenoic acid production by Nannochloropsis sp. in mixotrophic conditions. Biotechnol Lett 26: 51–53
30. Cheirsilp B, Torpee S (2012) Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. Bioresour Technol 110: 510–516
31. Das P, Aziz SS, Obbard JP (2011) Two phase microalgae growth in the open system for enhanced lipid productivity. Renew Energy 36: 2524–2528
32. Hu H, Gao K (2003) Optimization of growth and fatty acid composition of a unicellular marine picoplankton Nannochloropsis sp., with enriched carbon sources. Biotechnol Lett 25: 421–425
33. Park KC, Whitney C, McNichol JC, Dickinson KE, MacQuarrie S, Skrupski BP, Zou J, Wilson KE, O'Leary SJB, McGinn PJ (2012) Mixotrophic and photoautotrophic cultivation of 14 microalgae isolates from Saskatchewan, Canada: Potential applications for wastewater remediation for biofuel production. J Appl Phycol 24: 339–348
34. Wan M, Liu P, Xia J, Rosenberg JN, Oyler GA, Betenbaugh MJ, Nie Z (2011) The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in Chlorella sorokiniana. Appl Microbiol Biotechnol 91: 835–44
35. Cerón García M C, Sánchez Mirón A, Fernández Sevilla JM, Molina Grima E, García Camacho F (2005) Mixotrophic growth of the microalga Phaeodactylum

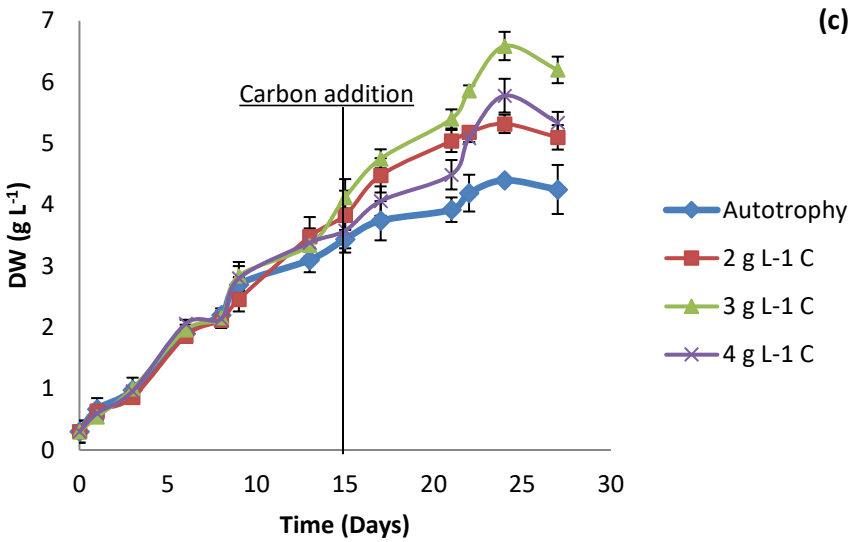
tricornutum: influence of different nitrogen and organic carbon sources on productivity and biomass composition. Process Biochem 40: 297–305

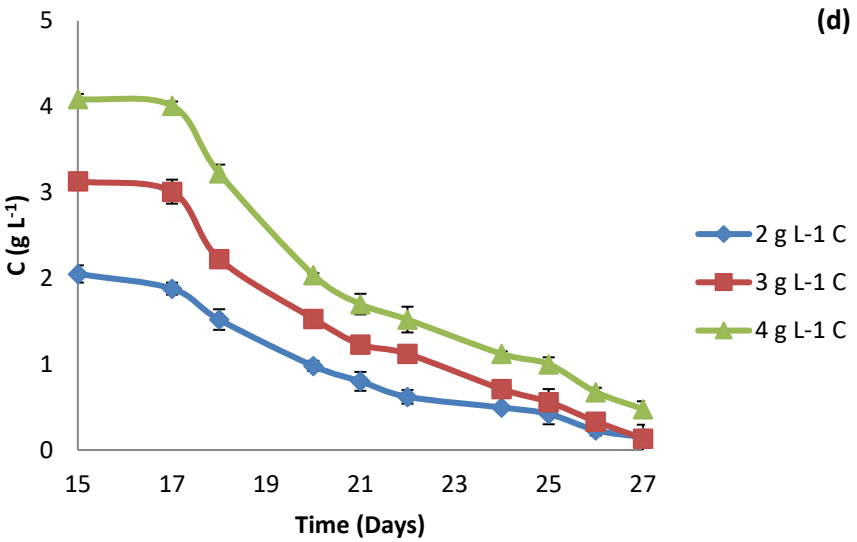
36. Heredia-Arroyo T, Wei, R. Ruan W, Hu B (2011) Mixotrophic cultivation of Chlorella vulgaris and its potential application for the oil accumulation from non-sugar materials. Biomass and Bioenergy 35: 2245–2253
37. Ip PF, Wong KH, Chen F (2004) Enhanced production of astaxanthin by the green microalga Chlorella zofingiensis in mixotrophic culture. Process Biochem 39: 1761–1766
38. 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
39. Andruleviciute V, Makareviciene V, Skorupskaite V, Gumbyte M (2014) Biomass and oil content of Chlorella sp., Haematococcus sp., Nannochloris sp. and Scenedesmus sp. under mixotrophic growth conditions in the presence of technical glycerol. J Appl Phycol 26: 83–90
40. Li Z, Sun M, Li Q, Li A, Zhang C (2012) Profiling of carotenoids in six microalgae (Eustigmatophyceae) and assessment of their carotene productions in bubble column photobioreactor. Biotechnol Lett 34: 2049–2053. doi:10.1007/s10529-012-0996-2.
41. Minhas AK, Hodgson P, Barrow CJ, Adholeya A (2016) A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids, Front Microbiol 7: 1–19

42. Blifernez O (2012) Molecular mechanisms behind the adjustment of phototrophic light-harvesting and mixotrophic utilization of cellulosic carbon sources in Chlamydomonas reinhardti. P.h.D Thesis pp 91.

Figure 1. *Nannochloropsis salina* growth and organic carbon consumption (**a**) growth curve without CO_2 supply; (**b**) organic carbon uptake of *Nannochloropsis salina* without CO_2 supply; (**c**) growth curve with CO_2 supply; (**d**) organic carbon consumption of *Nannochloropsis salina* with CO_2 supply (DW = dry weight; Autotrophy = CO_2 control).




(c)
Carbon addition
DW (g L-1)
Time (Days)
Autotrophy
2 g L-1 C
3 g L-1 C
4 g L-1 C


(d)
C (g L-1)
Time (Days)
2 g L-1 C
3 g L-1 C
4 g L-1 C

6

7

Table 1. Culture conditions and growth results of *Nannochloropsis* strain obtained in this work, in comparison with literature data.

Microalgae strain	Trophic way	Carbon source	Carbon amount (g L^{-1})	Cultivation system	CO_2 supply	Light intensity (µmol m^{-2} s^{-1})	Productivity (g L^{-1} d^{-1})	DW (g L^{-1})	Lipid (% DW)	References
Nannochloropsis salina	*Mixotrophy*	Glycerol	10	Batch	Air flow	100 (Continuous)	-	0.43	-	[26]
	Autotrophy	-	-	Batch	5% (v/v)			2.53		
	Mixotrophy	Glycerol	10	Semibatch				2.1		
	Autotrophy	-	-	Semibatch	5% (v/v) light - 0% night	100 (12:12 h day-night)		1.3		
	Mixotrophy	Glycerol	10	Semibatch				2.05	50	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	Air flow	80 (Continuous)	-	0.8	27	[27]
	Mixotrophy	Glucose	5	Fed batch				1.1	31	
Nannochloropsis salina	*Autotrophy*	-	-	Batch	Not reported	73 (Continuous)	-	0.36	5.6 (EPA % DW)	[28]
	Mixotrophy	Glucose	5.4					0.51	4.5 (EPA % DW)	
		Ethanol	1.4					0.45	-	
		Sodium acetate	2.5					0.32	-	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	Air flow	42 (16:8 h day-night)	-	0.35	25	[29]
	Mixotrophy	Glucose	2					1.2	25.5	
			10					3.8	19.3	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	Air flow	140 (12:12 h day-night)	0.03	0.59	14.74 (FAME % DW)	[30]
	Mixotrophy	Glucose	2				0.09	0.77	15.00 (FAME % DW)	
	Mixotrophy	Sucrose	2				0.1	0.82	14.76 (FAME % DW)	
	Mixotrophy	Glycerol	2				0.11	0.8	19.06 (FAME % DW)	

Nannochloropsis oculata	*Autotrophy*	-	-	Batch	No	80 (12:12 h day-night)	-	0.54	26.5	[31]
	Mixotrophy	Glucose	3.6				0.17	1.69	37.3	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	air flow	50 (Continuous)	-	0.46	7	[32]
		-	-		0.28%			0.63	9	
	Mixotrophy	Sodium acetate	0.16		air flow			0.5	7	
	Mixotrophy				0.28%			0.6	8	
Nannochloropsis salina	*Autotrophy*	-	-	Batch	Yes	260 (12:12 h day-night)	0.16	4.20 ± 0.17b[a]	35.6	This work
	Mixotrophy	Glycerol	2		No		0.16	3.92 ± 0.19a	34.6	
			3				0.16	4.30 ± 0.08b	45.7	
			4				0.14	3.44 ± 0.32a	44.8	
	Autotrophy	-	-		Pure CO_2 on-demand according to pH value		0.18	4.40 ± 0.21a	35.5	
	Mixotrophy	Glycerol	2				0.22	5.32 ± 0.19b	34.4	
			3				0.24	6.59 ± 0.32d	46.6	
			4				0.23	5.78 ± 0.12c	42.5	

[a]Values in the same column followed by the same letter are not statistically different at $p < 0.05$ according to Tukey test.

Table 2. Biomass composition of *Nannochloropsis salina* grew on glycerol with and without CO_2 supply.

CO_2 supply	Carbon ($g\ L^{-1}$)	Lipid content (% DW)	Protein content (% DW)	Carbohydrates content (% DW)
+	0	$35.6 \pm 0.2^{a*}$	13.3 ± 0.7^{a}	17.7 ± 0.8^{b}
-	2	34.6 ± 0.3^{a}	13.1 ± 0.1^{a}	16.1 ± 0.1^{b}
-	3	45.7 ± 1.8^{b}	14.1 ± 0.2^{a}	5.2 ± 0.7^{a}
-	4	44.8 ± 3.4^{b}	12.3 ± 0.6^{a}	6.3 ± 1^{a}
+	0	35.5 ± 2.1^{a}	12.5 ± 0.9^{a}	18.9 ± 2^{b}
+	2	34.4 ± 1.7^{a}	13.7 ± 0.5^{a}	18.5 ± 1.4^{b}
+	3	46.6 ± 1.8^{b}	16.3 ± 0.6^{b}	6.5 ± 0.5^{a}
+	4	42.5 ± 1.1^{b}	13.8 ± 0.5^{a}	7.5 ± 0.7^{a}

*Values in the same column followed by the same letter are not statistically different at $p < 0.05$ according to Tukey test.