Manuscript Details

Manuscript number	NBT_2018_67
Title	Using agro-industrial wastes for mixotrophic growth and lipids production by the green microalga Chlorella sorokiniana
Article type	Full Length Article

Abstract

In recent years, there has been a growing interest in the use of microalgae for the production of biofuels, but its production cost continues being high to compete with fossil fuel prices. One of the main limitations for photobioreactor productivity is light shielding especially at high cell densities. The growth of the green microalga Chlorella sorokiniana, which is an industrial robust species, has been evaluated under different trophic conditions with traditional carbon sources, such as glucose and sucrose, and alternative low cost carbon sources, such as carob pod extract, industrial glycerol and industrial diluted vinegar. The mixotrophic cultivation of this microalga with industrial vinegar alleviates the problems of light shielding observed in photoautotrophic cultures, improving the specific growth rate (0.052 h-1) in comparison with the other organic sources. The fed-batch mixotrophic culture of Chlorella sorokiniana in a 2L-STR reactor, with optimized nutritional conditions, 100 mM of acetate coming from industrial vinegar and 30 mM of ammonium, allowed obtaining an algal biomass concentration of 11.00 g L-1 with a lipid content of 38 % (w/w). This fed-batch strategy has been found to be very effective to enhance the biomass and neutral lipid productivity.

Keywords	Chlorella sorokiniana; agro-industrial waste; mixotrophic; stirred tank bioreactor; industrial vinegar; fed-batch.
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Suggested reviewers	Alfredo Martinez, E.M. Grima, Lenka Blinova, Alexandra Dubini

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6th of February of 2018

Dear Dr. Mike Taussig

Editor-in-Chief

We are sending you a manuscript entitled “Using agro-industrial wastes for mixotrophic growth and lipids production by the green microalga *Chlorella sorokiniana*” for publication in the Journal New Biotechnology.

The primary goal of this work was the development of a microalga culture using a low-cost carbon source, with the aim to process improvement and to increase the profitability of biomass and lipids production. In this context, biodiesel production using agro-industrial wastes, besides their low cost, have the advantage of the absence of ethical concerns due to the use of food resources and they may compete with products derived from fossil resources in terms of, economical and energetic sustainability, resource availability, supply reliability and environmental friendliness. In this manuscript was evaluated different trophic conditions, with the development of batch and fed-batch strategies, traditional carbons sources and alternative low-cost carbon sources, as agro-industrial wastes. For this reason, we think that the theme focused fits the objectives of this journal.

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Thank you for your attention and we hope the manuscript meets your approval.

Yours sincerely,

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Highlights

- The effect of cellular self-shading is less noticeable in mixotrophic cultures.
- Industrial vinegar from wine waterwastes was the best C source for *C. sorokiniana.*
- Culture medium was optimized for growth and lipids production.
- Fed-batch mixotrophic growth enhances biomass and lipid productivity.

Using agro-industrial wastes for mixotrophic growth and lipids production by the green microalga *Chlorella sorokiniana*

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Abstract

In recent years, there has been a growing interest in the use of microalgae for the production of biofuels, but its production cost continues being high to compete with fossil fuel prices. One of the main limitations for photobioreactor productivity is light shielding especially at high cell densities. The growth of the green microalga *Chlorella sorokiniana*, which is an industrial robust species, has been evaluated under different trophic conditions with traditional carbon sources, such as glucose and sucrose, and alternative low cost carbon sources, such as carob pod extract, industrial glycerol and industrial diluted vinegar. The mixotrophic cultivation of this microalga with industrial vinegar alleviates the problems of light shielding observed in photoautotrophic cultures, improving the specific growth rate (0.052 h^{-1}) in comparison with the other organic sources. The fed-batch mixotrophic culture of *Chlorella sorokiniana* in a 2L-STR reactor, with optimized nutritional conditions, 100 mM of acetate coming from industrial vinegar and 30 mM of ammonium, allowed obtaining an algal biomass concentration of 11.00 g L^{-1} with a lipid content of 38 % (w/w). This fed-batch strategy has been found to be very effective to enhance the biomass and neutral lipid productivity.

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INTRODUCTION

The unique properties of microalgae make them additives of high nutritional value, essential to aquaculture, human food, animal feeding or cosmetic [1,2]. Every year over 5000 tons of dry microalgal biomass are globally produced and marketed with an average value higher than 1 billion Euros [3]. Microalgae are the main natural source of carotenoids [4,5], a potential resource for long-chain polyunsaturated essential fatty acids [6] and, given the diversity of microalgal species that exist and the many that remain to be identified, it is estimated that microalgae can be a potential source of new bioactive compounds still unexplored with importance in the food and pharmaceutical industry [7-9]. Furthermore, in the last years, there has been an increasing interest in microalgae for the production of carbon-neutral biofuels [10-13], and for their ability to mitigate the greenhouse gas emissions, which has stimulated research on the design of cheaper and more efficient photobioreactors [14-16]. However, the cost for microalgae production, harvesting and processing is still very far for being competitive in comparison with the price of fossil fuels [17].

The importance of the bioreactor design is more evident in high-cell density cultures [18], due to the difficulties of dealing with cellular self-shading within the photobioreactor. While the input of carbon, nitrogen or other nutrients in a reactor can be controlled by operational factors (dilution rate, initial concentration), the light input is controlled by the design of the photobioreactor and the avaibility of external light, which changes through the day and the year. In addition to the standard photoautotrophic growth, many microalgae species can be cultured under heterotrophic conditions with an organic source of carbon and/or energy [19], or under mixotrophic conditions, where microalgae are grown with an organic carbon source in the presence of light [20]. Mixotrophic growth is a mixed approach that combines aspects of both photo- and heterotrophic technologies and a promising alternative to grow microalgae, capitalizing on the simultaneous assimilation of carbon dioxide and organic carbon sources such as sugars [21,22], acetate [23] or glycerol [24]. However, to achieve the economically feasible mixotrophic production of microalgae is necessary to choose a cheap organic carbon source. The combination of mixotrophic culture and reusing of industrial waterwastes is an interesting option to reduce costs and improve the overall economics of the process by promoting the circular economy [25].

Different agro industrial wastes have been successfully used as carbon source for yeast and microalgae [26-28], including carob pod extract [29,30], corn powder hydrolysate [31,32], cheese whey effluent [33,34], urban waterwastes [35,36] or apple vinegar [37]. Furthermore, the low price of biodiesel has encouraged intensive research to obtain low cost carbon sources and optimal conditions for oil accumulation in association with the production of high added value co-products, in a sustainable biorefinery approach [38,39].

In this work, the growth of the green microalga *Chlorella sorokiniana* (*C. sorokiniana*), which it is an industrial robust species, able to tolerate high temperatures and levels of solar irradiance [40], has been evaluated under different trophic conditions with traditional carbon sources, such as glucose and sucrose, and alternative low cost carbon sources, such as carob pod extract, industrial glycerol and industrial diluted vinegar. The best carbon source resulted to be the industrial vinegar, which has been chosen to study C, N and light limitations and to establish a fed-batch system where the highest values of biomass and neutral lipids are obtainded.

MATERIALS AND METHODS

Algal strain and standard culture conditions

C. sorokiniana 211-32 was obtained from the culture collection of the Institute of Plant Biochemistry and Photosynthesis, IBVF, (Seville, Spain) and grown in liquid High Salt Sueoka medium (HSM) [41]; containing (g L^{-1}): 0.72 H_2KPO_4, 1.44 HK_2PO_4, 0.02 $MgSO_4\cdot 7H_2O$, 0.01 $CaCl_2\cdot 2H_2O$, 0.50 NH_4Cl and 5 mL L^{-1} of traces solution containing (g L^{-1}): 10.0 EDTA, 2.28 H_3BO_3, 4.40 $ZnSO_4\cdot 7H_2O$, 1.02 $MnCl_2\cdot 4H_2O$, 1.00 $FeSO_4\cdot 7H_2O$, 0.32 $CoCl_2\cdot 6H_2O$, 0.32 $CuSO_4\cdot 5H_2O$ and 0.22 $Mo_7O_{24}(NH_4)_6\cdot 4H_2O$ at 25 °C and bubbled with 3 % CO_2-enriched air. For mixotrophic growth, the medium was supplemented with the organic carbon source indicated in each experiment instead of CO_2-enriched air, and the cultures were agitated at 100 rpm. Pre-inoculums were incubated for three days before the experiments and inoculated to obtain an initial concentration of 0.1 g L^{-1} of dry weight.

All cultures were grown, under continuous white light irradiation of 100 μE m^{-2} s^{-1}. For high irradiance experiments light was provided with halogen adjustable lamps and light intensity measured by a Delta OHM quantum photo radiometer HD 9021 equipped with a LP 9021 PAR sensor (Delta OHM, Italy). Carob pod extract is a rich-sugar extract by-product, obtained from Portuguese processing carob industry, as described in Lima-Costa et al [42], with (w/w) 56 % sucrose, 26 % glucose and 18 % fructose. Industrial glycerol was obtained from BioVegetal SA Company and the industrial vinegar was obtained from wine waste lees kindly provided by the Wine Cooperative “Ntra Sra de la Estrella” Chucena (Huelva, Spain). Wine waste lees were converted to vinegar by oxidative incubation with *Acetobacter acetii* at 28 ºC and 200 rpm. *Acetobacter* was cultured in 50 mL of YPD medium (20 g L^{-1} peptone, 10 g L^{-1} yeast extract and 20 g L^{-1} mannitol). After 48 h, *Acetobacter* was collected by centrifugation at 4500 rpm for 5 min and resuspended in 250 mL of filtered and autoclaved wine waste lees, where it was cultured until reaching a final acetate concentration between 3 and 5 % (v/v) and no significant quantities of ethanol. After that, the cells were harvested by centrifugation at 450 rpm and 20 min; and the supernatant was used as carbon source.

Fed-batch culture

For the fed-batch culture, *C. sorokiniana* was grown for three days in a 500 mL erlenmeyer flask with 250 mL of Sueoka medium supplemented with 100 mM acetate and 30 mM ammonium, at 25 °C and agitation of 150 rpm. After that, 10 % (v/v) of the culture was transferred to a 2 L-STR jacketed vessel (Applikon Biotechnology, England), equipped with a Rushton-type turbine (radial flow) and a microporous sparger, responsible for the aeration, connected to a controller (Bio Controller ADI 1030, Apllikon, Holland). The dissolved oxygen, temperature and pH were measured with specific probes, polarographic DO_2 sensor, Pt-100 sensor in thermowell in topplate and classic pH sensor respectively, and the values were frequently registered. The temperature of the reactor was maintained at 30±2 °C, pH between 6.5 and 7, and the agitation at 550 rpm. Culture was continuously mixed with air at a flow rate of 0.75 vvm and the light intensity was 100 μE m^{-2} s^{-1}. During the fed-batch experiment, carbon and nitrogen sources were added to the reactor at 72, 144, 216 and 288 h of culture, so that the concentration in the vessel did reached 100 mM of acetate and between 20 and 30 mM of ammonium after each addition. The volume of the additions was 200 mL of medium, and a sample was collected before and after each addition. The routine samples were taken every 24 h.

Analytical determinations

For dry weight determination, 5 mL sample of culture was filtered through pre-tared Whatman GF/F Filter paper (Whatman International Ltd, Maidstone, UK), the filters were oven-dried overnight at 100 °C, cooled in a desiccator and weighted in an analytical balance. Dry weight was obtained from the difference between initial and final weight. All measures were done in triplicate.

Neutral lipid content was quantified by fluorescence spectrometry, using Nile Red, as was described by Kimura et al [43] and optimized according to Chen et al [44] for this microalga. Fluorescence was measured at an excitation wavelength of 510 nm and an emission wavelength ranging between 500 and 800 nm. A 200 µL aliquot of *C. sorokiniana* cell suspension, 1800 µL of PBS buffer and 100 µL of DMSO (Dimethylsulfoxide) were added to the fluorescence cuvette and the spectrum was recorded after 20 minutes of stirring at 40 °C. Thereafter, 10 µL of Nile Red solution (0.1 mg of Nile Red in 1 mL acetone) were added and the spectrum newly recorded after 15 minutes of stirring at 40 °C. The fluorescence value corresponds to the difference spectrum with and without dye, and the results expressed in fluorescence intensity (FI). A calibration curve was performed using triolein, representing the fluorescence intensity versus the triolein concentration (Equation 1). This standard curve was performed according to Bertozzini et al [45]:

Fluorescence intensity (FI) = 3645.8 x Triolein concentration (g L^{-1}) **Equation 1**

Acetate was determined using a high-performance liquid chromatography (Hitachi LaChrom Elite HPLC, Japan) equipped with a refractive index detector (Hitachi L-2490, Japan). A Sugar-Pak column (Waters) was used with Milli-Q water as eluent at 83 °C and flow rate of 0.5 mL min^{-1}. The supernatant obtained by the filtration of culture, for the determination of dry weight, was centrifuged at 13,400xg for 10 min (Eppendorf Centrifuge 5415D, Germany).

Ammonium in the culture medium was determined with an ammonium ion selective electrode (Crison Instruments S. A. Spain). The total ionic strength was adjusted with ISA buffer solution (1 M $MgSO_4$). For each measurement, 1 mL of sample was mixed with 1 mL ISA solution and 8 mL of deionized water at 25 °C.

All the tests were carried out in triplicate and the results were the mean of six values (three replicates of the process and two replicates of the analysis).

Determination of culture efficiency and kinetics parameters

The specific growth rates (h^{-1}) were calculated in exponential phase of growth, by as the slope, fitted by linear regression of the ln (dry weight) as a function of time, using the DMFIT modeling tool (http://modelling.combase.cc).

Duplication time (t_D) was calculated according to the next equation:

$$t_D(h) = \frac{\ln 2}{\mu} \qquad \textbf{Equation 1}$$

where $\mu(h^{-1})$ is the specific growth rate was calculated as described above.

The lipid content was calculated according to the following formula:

$$Y_{L/X} = \frac{L_i - L_0}{X_i - X_0} \qquad \textbf{Equation 2}$$

where X_i and L_i are the dry cell weight and lipid concentration on day t_i, respectively and X_0 and L_0 are the dry cell weight and lipid concentration on day t_0 (the first day), respectively.

The lipid yield was calculated according to the following formula:

$$Y_{L/S} = \frac{L_i - L_0}{S_0 - S_i} \qquad \textbf{Equation 3}$$

where S_i and S_0 are the acetate concentration on day t_i and t_0 (the first day), respectively and L_i and L_0 are the lipid concentration on day t_i and day t_0 (the first day), respectively.

The biomass productivity (P_{biom}) was calculated as the slope, fitted by linear regression of the biomass produced (dry weight) as a function of time.

The lipid productivity (P_{lipid}) was calculated by the equation:

$$P_{lipid}(g\ L^{-1}\ day^{-1}) = \frac{L_{max}}{t_i} \qquad \textbf{Equation 4}$$

where L_{max} is the maximum lipid concentration on day t_i.

RESULTS AND DISCUSSION

Comparison of the influence of the effective light on the growth of C. sorokiniana microalgae, cultivated in different trophic conditions

The dependence of productivity of photoautotrophically grown microalgal cultures on the availability of light is well known. If no other nutrient is limited and all operational parameters are optimal, specific growth rate depends of light intensity until a maximum saturating value [46,47]. Due to the shading effects of one cells upon others, the design of photobioreactors is especially important to ensure the adequate distribution of light, which is one of the most difficult parameters to handle when scaling up photobioreactors [48,49] because it depends not only on external irradiance, but also on the diameter of the reactor and the cell density of the cultures. We have evaluated the effect of self-shading on the effective light in *C. sorokiniana* cultures and compared the influence of effective light on photo- and mixotrophycally grown cultures of this microalga.

To evaluate the effect of cellular self-shading on the effective light in different points of a photobioreactor, we prepared cultures of *C. sorokiniana* with cell densities ranging between 0.1 to 2 g L^{-1} and preformed two different experiments. On the one hand, the cultures were exposed to an external irradiance of 850 µE m^{-2} s^{-1} and the effective light was measured at different distances from the surface of the reactor (Fig. 1A). On the other hand, the cultures were exposed to increasing irradiance, with values ranging from 200 to 1200 µE m^{-2} s^{-1} at the surface of the bioreactor, and the effective light was measured at 3.5 cm from the surface of the bioreactor (Fig. 1B).

As it is shown in Figure 1A, for a cell density of 1 g L^{-1}, at 2 cm from the surface, the effective light is reduced in about 90 %. Increasing light intensity at the surface of the reactor causes an increase in the effective local light (Fig. 1B), but for high density cultures the effective light measured at only a few centimeters from the surface continues being extremely low. For cultures with cell densities of 1 g L^{-1}, increasing the light on the surface to 1200 µE m^{-2} s^{-1}, provided a local effective light of only 60 µE m^{-2} s^{-1} at 3.5 cm from the reactor surface. The reached light intensity was only 11 µE m^{-2} s^{-1} for cultures with cell densities of 2 g L^{-1} DW in the same conditions. This means that more than 99% of the light applied at the surface of the reactor was absorbed across the bioreactor due to shading effect for this cell density.

Fig. 1: Influence of cell density on cellular self-shading in *C. sorokiniana* cultures. The light intensity was measured at different distances from the surface for a fixed surface light of 850 µE m^{-2} s^{-1} (A) and for different light intensities at the reactor surface at a fixed distance of 3.5 cm from the surface (B). In both

cases *C. sorokiniana* cultures of 0.1 (▪), 0.2(Δ), 0.3 (x), 0.7 (●), 1(+), 1.3 (-) and 2 g L^{-1} ( ) of dry weight were used.

Difficulties to obtain high-density cultures due to self-shading have been extensively studied for different photoautotrophically grown microalgae [50,51]. Although efficient agitation can guarantee that all the cells will be temporally near the surface receiving the maximum irradiance, this is not enough to compensate shelf-shading effect and light gradients within the reactor. Moreover, it is necessary to remember that over certain values, excess of light is inhibitory [52], so increasing light on the surface is not the solution to overcome self-shading in high cell-density photoautrophic cultures.

Since the growth of microalgae in mixotrophic conditions is less dependent of photosynthesis than growth in phototropic conditions, it is expected that the productivity of a mixotrophic culture is not as strongly influenced by the availability of light as is the productivity of a photrophycally grown culture. In order to check this hypothesis, *C. sorokiniana* was cultured both in photoautotrophic (CO_2 + light) and mixotrophic (acetate + light) conditions in cylindrical bottles of 1.25, 2.5, 5 and 9 centimeters of radius with an initial cellular concentration of 0.1 g L^{-1}. The growth was followed over the time in each culture and the specific growth rate and the duplication time were calculated in the middle of the exponential phase as indicated in Materials and Methods (Fig. 2; Table 1).

Fig. 2: Influence of recipient diameter in duplication time with different mixotrophic (continuous line) and photoautotrophic (dash line) cultures of *C. sorokiniana*, with a light of 100 µE m^{-2} s^{-1} and an agitation of 100 rpm. The duplication time of the different cultures was calculated with equation 1, as described in Materials and Methods. The carbon concentration was 16 mM of acetate in mixotrophic and similar concentration of CO_2 in photoautotrophic.

As it was expected, productivity of the cultures decreased in an inversely proportional way to the radius of the reactor and, what is more interesting, the reduction of growth rate is much less acute in the case of mixotrophic cultures. When plotting the duplication time against the radius of the recipient, a slope of 1.12 h cm^{-1} is obtained in the case of mixotrophic cultures, while the slope reaches a value of 2.40 h cm^{-1} for photoautotrophically growth cultures. The slope for mixotrophic cultures is less than half the value observed in photoautotrophic conditions.

It is also interesting to note that mixotrophic growth is, in all cases, higher than photoautotrophic growth, being both the specific growth rate and the final biomass reached higher in the mixotrophic cultures. These values have been summarized in Table 1, which also includes data of heterotrophic cultures tested in the same conditions than the mixotrophic ones excepting light. The growth rate of mixotrophic cultures is more than two-fold higher than that of photoautotrophic ones (Fig. 2; Table 1), and is also higher that the growth rate of heterotrophic cultures (Table 1). The differences between photoautotrophic and mixotrophic cultures are more pronounced when the radius of the recipient is bigger, confirming that the light-dependence is lower in the case of mixotrophic growth, which combines advantages of photoautotrophic and heterotrophic cultures. In mixotrophic cultures both the external organic carbon source and the endogenously generated CO_2 are metabolized, contributing to an important increase in productivity and to a lower dependence of external light.

Similar results have been shown when comparing mixo and photoautotrophic growth in other green algae, such as *Chlamydomonas reinhardtii* [53], *Chlorella vulgaris* [54] or *Chlorella protothecoides* [55]. There are also previous reports comparing the growth of *C. sorokiniana* in photoautotrophic and mixotrophic conditions, with organic carbon sources such as glucose [5,56] or acetate [57]. All these studies conclude that μ_{max} in mixotrophic cultures is much higher than for photosynthetic conditions, in agreement with our data for acetate-based mixotrophic cultures. Furthermore, Kim et al [56] determined the inorganic carbon concentration in different trophic conditions and observed that the inorganic carbon solved in the culture medium decreased in photoautotrophic, increased in heterotrophic and remained approximately constant in mixotrophic cultures. This indicates that the CO_2 released due to the respiration process was photosynthetically assimilated by *C. sorokiniana*.

Table 1: Maximum biomass produced and specific growth rate for the *C. sorokiniana* microalgae, cultivated in different trophic conditions.

Different carbon sources for C. sorokiniana

The choice of an adequate carbon source is essential for the optimal mixotrophic growth of microalgae. To evaluate the carbon source preferences of *C. sorokiniana* in terms of biomass productivity, the microalga was grown in Sueoka HSM supplemented with different organic carbon sources, at an initial concentration of 20 g L^{-1} (Fig.3). The carbon sources tested were glucose, sucrose, industrial glycerol, vinegar and carob pod extract, which is rich in sucrose (56 %, w/w), glucose (26 %, w/w) and fructose (18 %, w/w), prepared as previously described in Lima-Costa et al [42]. The industrial vinegar was obtained from the wine waste lees, after treatment with *Acetobacter acetii*, as indicated in Materials and Methods section. In all cases, the final pH of the medium was adjusted to 6.5-7 before sterilization. A control grew in the same conditions without any carbon source was also included. All cultures were carried out in 500 mL erlenmeyer flasks (5 cm of radius), at 25 °C of temperature, with agitation (100 rpm) white light irradiation (100µE m^{-2} s^{-1}).

Fig. 3: Growth curve of *C. sorokiniana* cultivated with different carbon sources. All the cultures were grown at 25 ˚C, 100 rpm and a light intensity of 100 µE m^{-2} s^{-1}, with 20 g L^{-1} of the indicated carbon source. A control without carbon source (continuous line) has been also included. The carbon sources tested were different sugars (dashed line) like glucose (Gluc) or sucrose (Suc); or different types of wastes (point line) like carob pod extract (Carob), industrial glycerol (Glycerol) or industrial vinegar (Vinegar).

As Fig.3 shows *C. sorokiniana* can actively use glucose, sucrose, glycerol and acetate as carbon sources. Industrial diluted vinegar not only was no toxic to *C. sorokiniana*, but also provided higher growth rate than equivalent concentrations of acetate (data not shown) and resulted to be the best organic carbon source for *C. sorokiniana*. Growth of the microalga with carob pod extract was higher than in control conditions, but slightly lower than growth with sucrose, glucose or industrial glycerol (Table 1). This is an expected result because, both glucose and sucrose concentrations are lower in carob pod extract (26 % and 56 %, w/w, respectively) than in the trials with the respective single sugar. Besides, in agreement with the studies described by Pérez-García et al [58], in the presence of a substrate with more than one sugar, as is the case, the microalgae assimilate the sugar with higher affinity.

Industrial diluted vinegar was chosen as organic carbon source for mixotrophic growth of *C. sorokiniana* because it provided the highest growth rate, with a μ value of 0.036 h^{-1}, about 7-fold the specific growth rate value of the control culture with no carbon source (Table 2).

Table 2: Specific growth rate for *C. sorokiniana* cultivated with different carbon sources. The cultures were grown at 25 ºC, 100 rpm and a light intensity of 100 μE m^{-2} s^{-1}, with 20 g L^{-1} of the indicated carbon source.

Vinegar could be an optimal carbon source with multiple advantages, like lower cost and lower susceptibility to contamination than other widely used carbon sources like glucose [59] and the possibility of obtaining industrial wastewater rich in acetate from the wine, as is the case, the textile industries, from the hydrolysis processes or effluent of anaerobic digestors [21].

Optimization of initial acetate and ammonium concentrations for C. sorokiniana culture

C. sorokiniana was cultured with increasing concentrations of industrial vinegar, which were adjusted to provide acetate concentrations ranging from 16 to 100 mM (Fig. 4). Samples were periodically withdrawn to determine the dry weight and acetate concentration in the culture medium. No inhibitory effect of vinegar was observed in *C. sorokiniana* until concentrations equivalent to 300 mM Ac (data not shown). Increasing vinegar concentration causes an increase in the final biomass obtained until concentrations of acetate about 80 mM, however growth is saturated for higher acetate concentrations (Fig. 4A).

Acetate in the culture medium is completely exhausted only in cultures with an initial concentration of 16 mM. Strong acetate consumption is observed in the first 72 h of culture (Fig.4B), as can be deduced from the initial slope of consumption kinetics, which increases slightly with the initial concentration of acetate, from 0.315 to 0.544 mM h^{-1}. However, after 72h, acetate assimilation is practically stopped although there is still acetate available in the medium (Fig. 4B).

Fig. 4: Growth curve (A) and acetate consumption (B) for *C. sorokiniana* cultivated in mixotrophic conditions with increasing concentrations of acetate. Cultures were grown at 25 ˚C, 100 rpm and a light intensity of 100 μE m^{-2} s^{-1}, in standard medium with 8 mM of ammonium and increasing additions of industrial vinegar, that provide acetate concentrations between 16 and 100 mM. Initial pH was adjusted to 6.5-7 in all the cultures.

The drastic reduction of the growth observed in Fig.4B indicates that another limiting nutrient exist in these conditions. To check the possible limitation of nitrogen, *C. sorokiniana* cultures with an initial vinegar concentration of 100 mM were incubated with different ammonium concentrations, ranging from 8 to 100 mM (Fig.5).

Fig. 5: Growth curve (A) and ammonium consumption (B) for *C. sorokiniana* cultivated in mixotrophic conditions with increasing concentrations of ammonium. Cultures were grown at 25 ˚C, 100 rpm and a light intensity of 100 μE m^{-2} s^{-1}, in mixotrophic medium with 100 mM of industrial vinegar and increasing ammonium concentrations ranged between 8 to 100 mM.

The highest dry weight was observed with initial ammonium concentrations between 20 mM and 30 mM. For ammonium concentrations of 50 mM or higher, there is an important growth inhibition, probably due to certain inhibitory effect of high ammonium concentrations (Fig. 5A), as was previously described for other microalgae [60,61]. After 48 h, ammonium is practically exhausted for initial ammonium concentrations below 30 mM.

Increasing the concentration of ammonium allowed getting higher biomass densities, which increased from 2.8 g L^{-1} in six days, with 8 mM, to almost 4 g L^{-1} in the same time, with 20 mM (Fig. 5A). Increasing ammonium concentration represents an increase of 3-fold of the biomass obtained in mixotrophic medium (16 mM acetate and 8 mM ammonium), which is about 1.5 g L^{-1} (Fig. 4B).

Further experiments were done with initial concentrations of 100 mM of acetate, supplied with industrial vinegar, and 30 mM of ammonium.

Influence of acetate concentration on neutral lipids productivity.

To investigate the influence of acetate concentration on neutral lipids accumulation, mid log *C. sorokinia*na cultures were transferred to nitrogen deprived culture medium, with industrial vinegar, adjusted to provide initial acetate concentrations of 16, 50 mM and 100 mM. Samples were collected every 24h after nitrogen deprivation to determine the content of neutral lipids, using Nile red staining (Fig. 6).

This experiment has allowed us to confirm that there is an important accumulation of neutral lipids under -N deprivation, as has been previously described in *C. sorokiniana* and other microalgae [59,62,63].

Furthermore, increasing the acetate concentration to 100 mM leads to reach neutral lipids values of 0.94 g L^{-1}, 6-fold the values reached at 16 mM of acetate. These results, together with the increase of biomass observed for high acetate concentrations (Fig. 4) indicate that mixotrophically grown *C. sorokiniana* cultures with vinegar as carbon source can provide high neutral lipids content of 0.39 g g^{-1}.

Our observations are in agreement with recent reported studies about the effect of other carbon sources, such as glucose [64], inorganic carbonates [51] or glycerol [24] on lipid productivity in *C. sorokiniana* strain. Kumar et al [22] also found that acetate worked as an elicitor for lipid enrichment in *C. sorokiniana,* cultured with glucose as C source.

Fig. 6: Concentration of neutral lipids cultivated with –N starvation and with different concentrations of acetate. Cultures were grown at 25 ˚C, 100 rpm and a light intensity of 100 µE m^{-2} s^{-1}, in standard medium with the concentrations of 16, 50 and 100 mM of acetate.

Fed-batch Strategy on biomass and neutral lipids productivity

The fed-batch culture of *C. sorokiniana* was carried out in a 2L-STR reactor, equipped with a Rushton-type turbine and a microporous sparger. The experiment was performed with the optimized nutritional conditions, 100 mM of acetate, in the form of industrial diluted vinegar, and 30 mM of ammonium. Other parameters were an agitation rate of 550 rpm, a flow rate of 0.75 vvm and a continuous light of 100 µE m^{-2} s^{-1}. Kinetics parameters of *C. sorokiniana* growth in 2L-STR are presented in Table 3. Two phases were differentiated: firstly, a three-batches period for biomass production, with periodical additions of vinegar and ammonium as detailed in Materials and Methods section (0-72 h, 72 – 144 h and 144 – 216 h); followed by a period to induce the production of lipids (216 – 288 h, 288 – 360 h) during which the fresh medium added only contained vinegar. Produced biomass and specific growth rate were determined for each fed-batch stage (Table 4). Comparing these values with those of Tables 1 and 2 it can be concluded that the biomass produced in mixotrophic STR is significantly higher than the obtained in erlenmeyers flasks cultivated in mixotrophic, heterotrophic or photoautotrophic conditions.

Table 3: Kinetics parameters of *C. sorokiniana* growth in 2L-STR fed-batch culture.

Table 4: Maximum biomass produced and specific growth rates in fed-batch STR culture.

The specific growth rate obtained in the STR during the first 72 hours of cultivation (0.055 h^{-1}) was slightly higher (0.052 h^{-1}) than that observed in erlenmeyer flasks with an equivalent diameter, as shown in Table 1. In this first fed-stage there was a high consume of acetate and complete depletion of the nitrogen source. The growth rate and the values of acetate and ammonium consumption decrease progressively in each cycle over the culture time (Fig 7A, Table 4). The average specific growth rate observed in the photobioreactor during the whole process was 0.039 h^{-1}. However, the total biomass produced in the 2-STR was approximately 10-fold higher than that produced in erlenmeyer flasks, increasing from 1.28 g L^{-1} in the flask to 11.00 g DW L^{-1} in the STR, with an equivalent radius of 5 cm.

Nitrogen deprivation in the second period of the experiment induces the accumulation of lipids and the deceleration of the growth (Fig.7B and Table 4). The lipid content was determined at 216 h, at which the last addition of nitrogen source was done. It was newly registered at regular intervals from the end of the fourth batch cycle, when the nitrogen source was almost exhausted. The content of neutral lipids increased continuously during the last phase of the experiment, reaching a concentration of 38 % (w/w; Fig.7B) at the end of the fifth batch. The lipid productivity obtained in this study (193.37 mg L^{-1} day^{-1}; Table 3) was higher than that obtained in previous studies with *C. sorokiniana* grown with glucose or sodium acetate [22,65-67] or with other microalgal species grown in mixotrophic conditions [68,69].

The accumulation of neutral lipids grown in fed-batch systems with sodium acetate or glucose as carbon source has been recently described in *C. sorokiniana* [70] and other microalgal species grown in mixotrophic conditions [71]. In agreement with our results with wine waterwastes, these authors describe higher production of lipids in mixotrophic conditions and corroborate that the higher availability of acetate results in an elevated flux towards lipid biosynthesis as it integrates into the central metabolism as acetyl Co-A [72], which is the key precursor for lipid biosynthesis in microalgae. Attempts to culture *C. sorokiniana* with industrial water [35] or other industrial wastes [33] have had different degree of success and have provided biomass and lipid productivities lower than the obtained with industrial vinegar in the present study.

Fig. 7: Dry weight evolution, acetate and ammonium consumption of *C. sorokinana* cultivated in 2 L-STR reactor (A) and neutral lipid content (% DW) determined at the indicated time. Culture was grown at 30 °C, 550 rpm and a light intensity of 100 µE m^{-2} s^{-1}, in mixotrophic medium with the optimal concentration of acetate and ammonium. Periodical additions of vinegar and ammonium (72, 144 and 216 h) or only vinegar (288 h) were done at indicated time to recover the initial concentrations of ammonium and/or acetate.

CONCLUSIONS

We have demonstrated, using reactors of different radius, that the influence of effective light is less pronounced when *Chlorella sorokiniana* is cultured in mixotrophic conditions. However, to guarantee the economical feasibility of mixotrophic cultures it is necessary to find an appropriate economical carbon source. Industrial vinegar obtained from wine waste was chosen, among other agro-industrial wastes as the best carbon source for *C. sorokiniana*. The fed-batch strategy and the medium optimization, with nutrient supplementation, have been found to be very effective to enhance biomass and neutral lipid productivity, suggesting that this is promising strategy for production of microalgal biomass.

Acknowledgements Part of this work has been supported by research grants from the Spanish (AGL2016-74866-C32R-AEI/FEDER) and the European governments (INTERREG VA-POCTEP-2014-2020; 0055_ALGARED_PLUS_5_E). We thank Dr. Molinari from the University of Milan, for kindly providing the *Acetobacter acetii* strain. The help of CEIMAR University Excellence Campus is also acknowledged.

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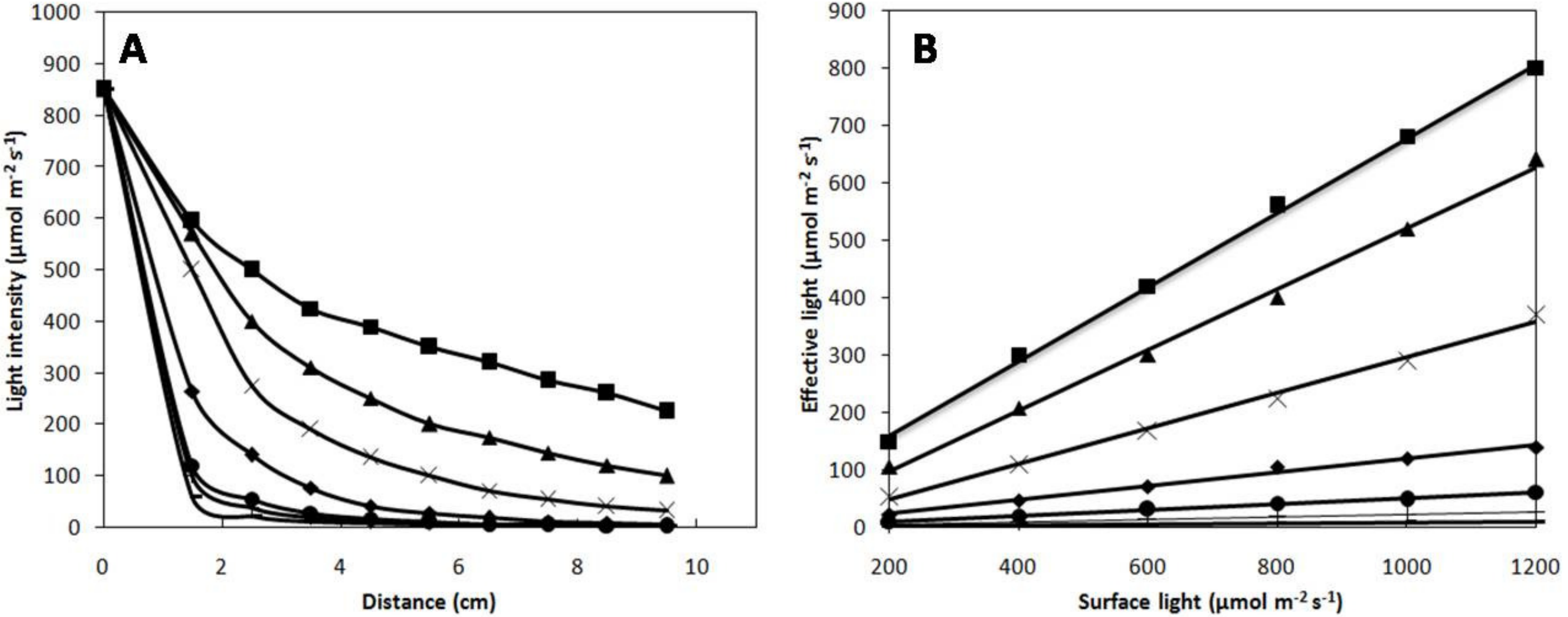
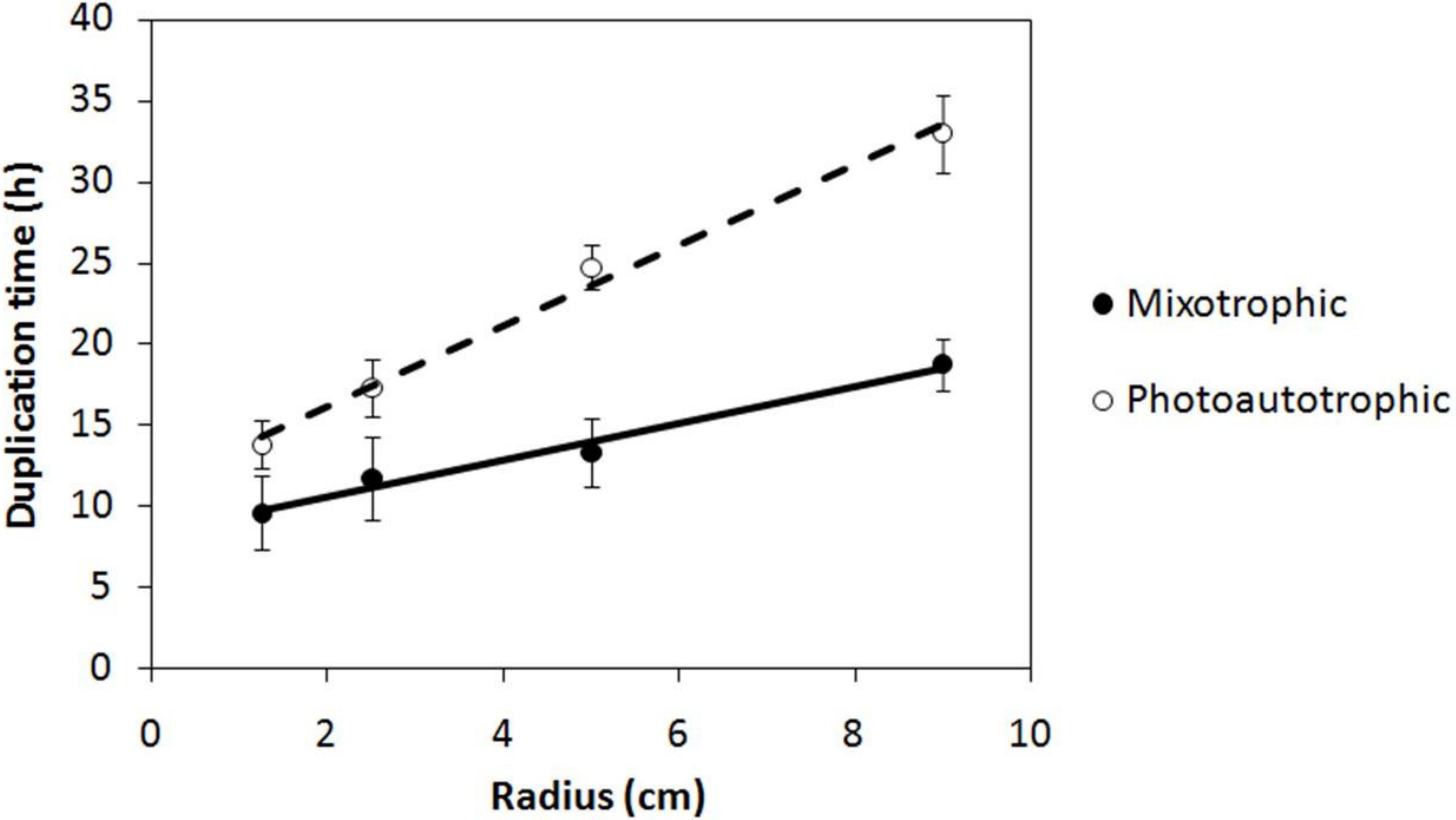

A
Light intensity (µmol m-2 s-1)
1000
900
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
Distance (cm)
B
Effective light (µmol m-2 s-1)
900
800
700
600
500
400
300
200
100
0
200
400
600
800
1000
1200
Surface light (µmol m-2 s-1)


Duplication time (h)
40
35
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25
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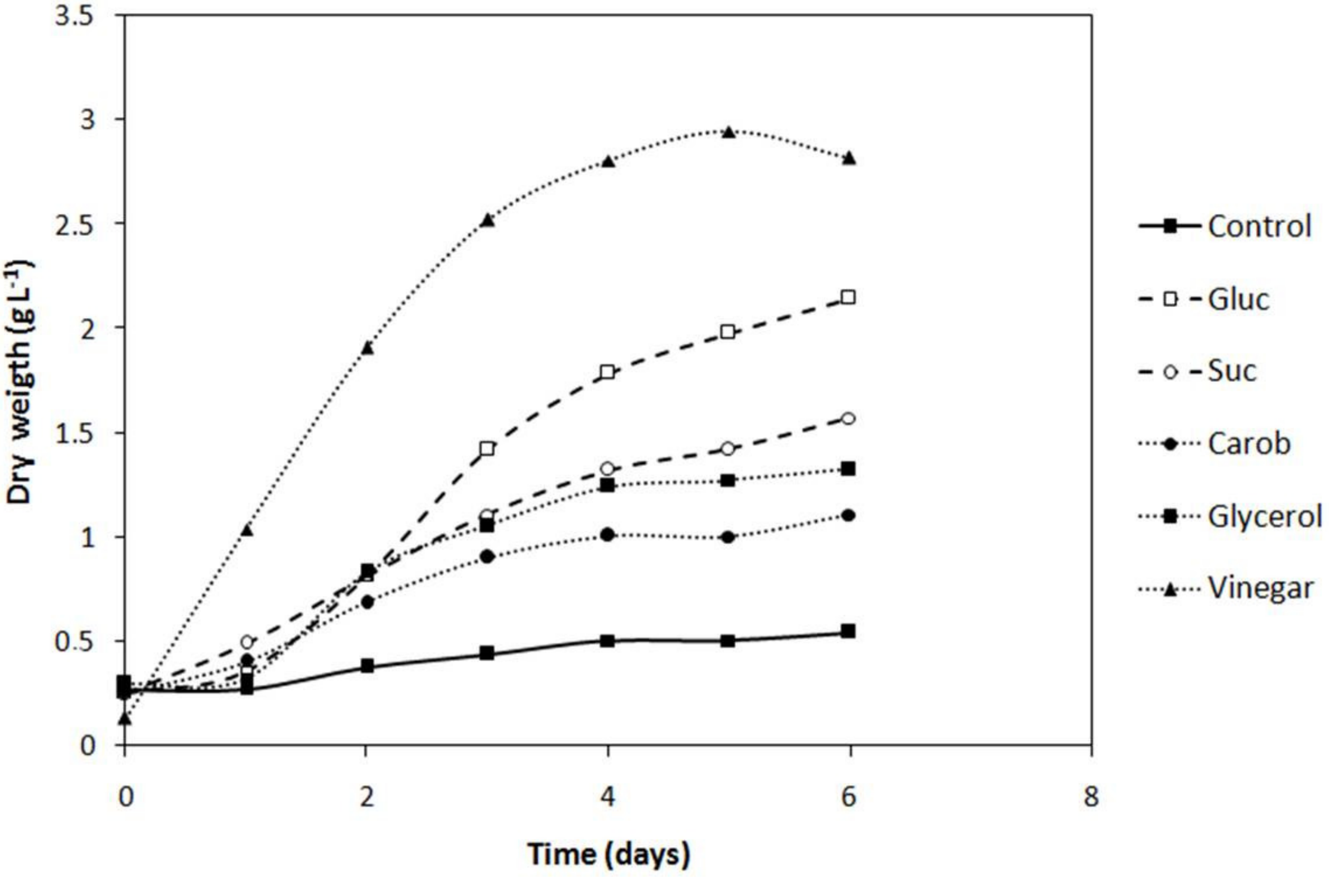
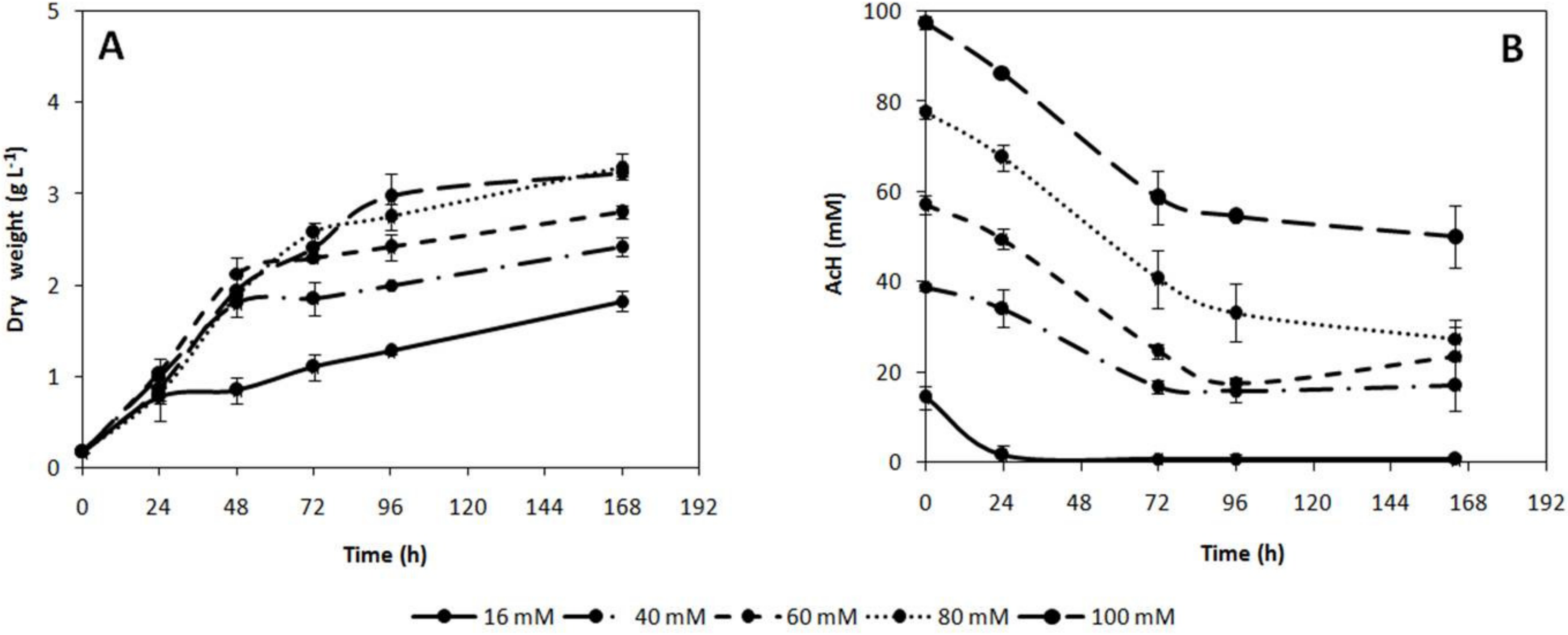
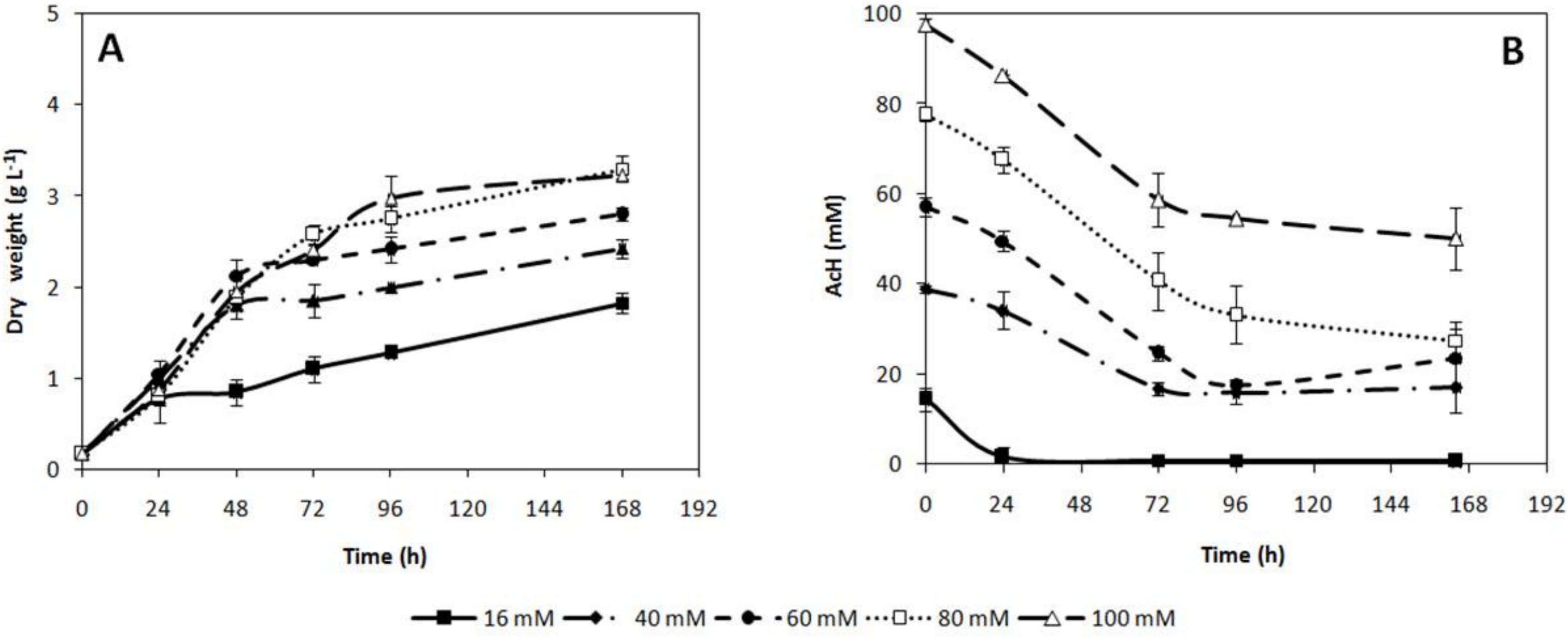
Radius (cm)
● Mixotrophic
○ Photoautotrophic


3.5
3
2.5
2
1.5
1
0.5
0
Dry weigth (g L-1)
0
2
4
6
8
Time (days)
Control
Gluc
Suc
Carob
Glycerol
Vinegar

A
Dry weight (g L-1)
0
1
2
3
4
5
0
24
48
72
96
120
144
168
192
Time (h)
B
AcH (mM)
0
20
40
60
80
100
0
24
48
72
96
120
144
168
192
Time (h)
16 mM
40 mM
60 mM
80 mM
100 mM


A
B
Dry weight (g L-1)
AcH (mM)
Time (h)
Time (h)
0
1
2
3
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5
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20
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60
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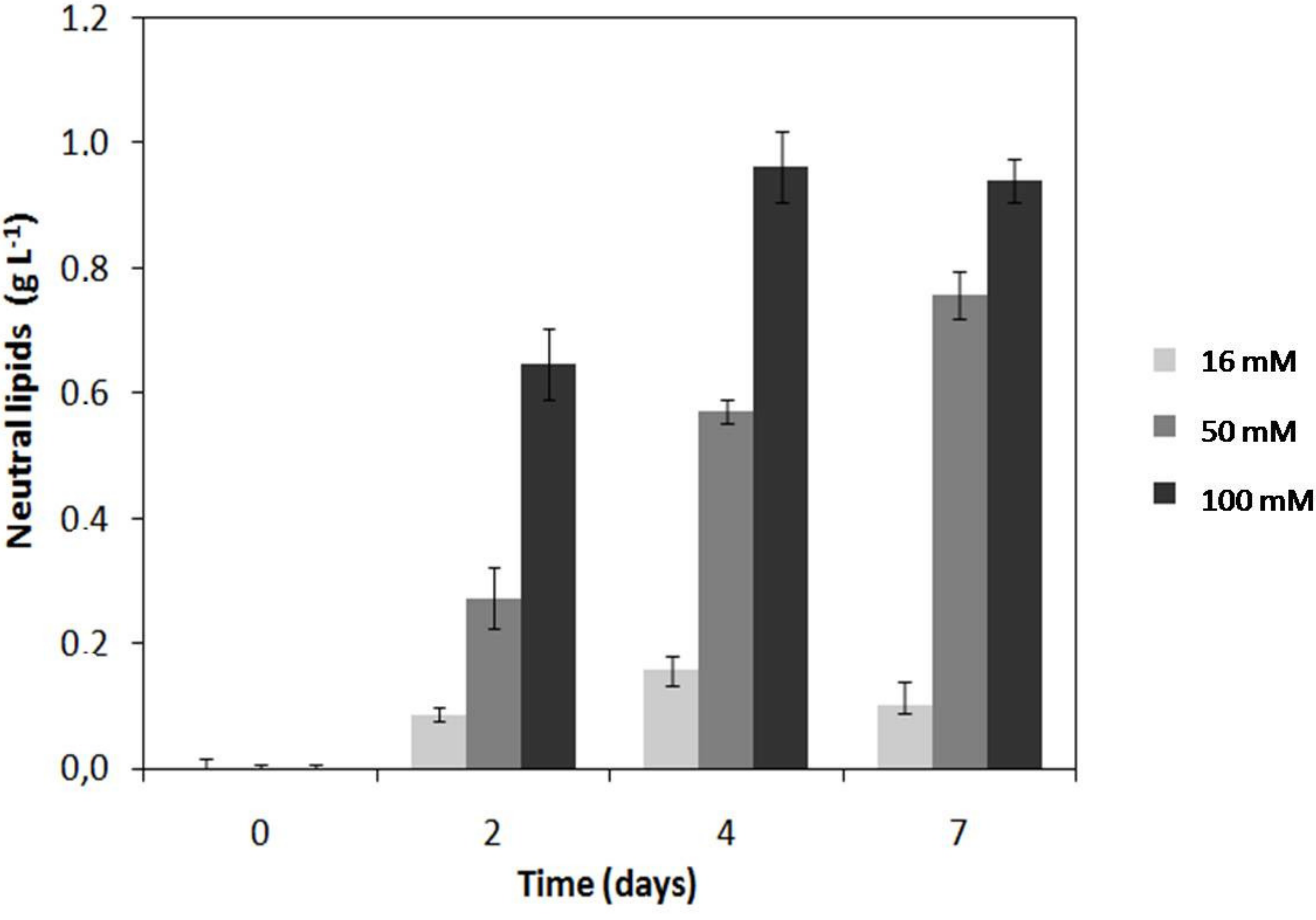
24
48
72
96
120
144
168
192
16 mM
40 mM
60 mM
80 mM
100 mM



1,2
1.0
0.8
0.6
0.4
0.2
0,0
Neutral lipids (g L-1)
0
2
4
7
Time (days)
16 mM
50 mM
100 mM

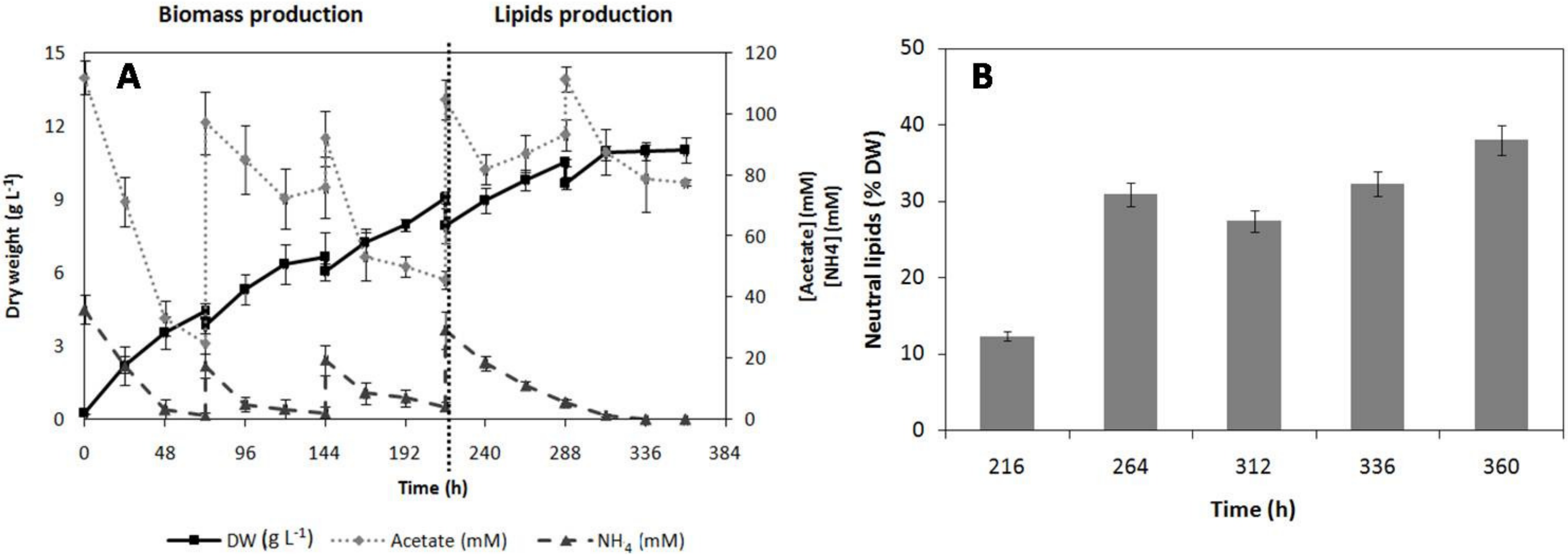

Biomass production
Lipids production
A
Dry weight (g L⁻¹)
15
12
9
6
3
0
120
100
80
60
40
20
0
[Acetate] (mM)
[NH4] (mM)
0
48
96
144
192
240
288
336
384
Time (h)
DW (g L⁻¹)
Acetate (mM)
NH₄ (mM)
B
Neutral lipids (% DW)
50
40
30
20
10
0
216
264
312
336
360
Time (h)

Table 1: Maximum biomass produced and specific growth rate for the *C. sorokiniana* microalgae, cultivated in different trophic conditions

	Radius of the bioreactor (cm)	**μ (h^{-1})**	**Maximum biomass (g L^{-1})**
Mixotrophic	1.25	0.072	1.32
	2.50	0.059	1.47
	5.00	0.052	1.28
	9.00	0.037	1.08
Photoautotrophic	1.25	0.050	1.16
	2.50	0.040	1.18
	5.00	0.028	0.66
	9.00	0.021	0.39
Heterotrophic	5.00	0.034	0.77

The value of μ was determined in exponential phase of the cultures and maximum biomass was determined at 48 h of culture. μ- Specific growth rate

Table 2: Specific growth rate for *C. sorokiniana* cultivated with different carbon sources. The cultures were grown at 25 ºC, 100 rpm and a light intensity of 100 μE m^{-2} s^{-1}, with 20 g L^{-1} of the indicated carbon source.

	CONT -	**Gluc**	**Suc**	**Carob**	**Glycerol**	**Vinegar**
μ (h^{-1})	0.005	0.027	0.016	0.011	0.016	0.036

A control without carbon source (Cont-) has been also included. The carbon sources tested were glucose (Gluc), sucrose (Suc), carob pod extract (Carob), industrial glycerol (Glycerol) and industrial vinegar (Vinegar).

Table 3: Kinetics parameters of *C. sorokiniana* growth in 2L-STR fed-batch culture

μ (h^{-1})	**X_{max}(g L^{-1})**	**$Prod_X$(g L^{-1}day^{-1})**	**L_N(g L^{-1})**	**$Y_{L/X}$(g g^{-1})**	**$Prod_L$ (mg L^{-1}day^{-1})**
0.039 ±0.008	11.00	1.39	1.16	0.38	193.37

μ- Specific Growth rate, calculated with DMFIT software; X_{max} - Maximum Biomass; $Prod_X$ - Biomass Productivity; L_N – Neutral Lipids; $Y_{L/X}$ - Lipid Content; $Prod_L$ - Lipids productivity

Table 4: Maximum biomass produced and specific growth rates in fed-batch STR culture

Time (h)	**0 - 360**	**0 - 72**	**72 - 144**	**144 - 216**	**216 - 288**	**288 - 360**
μ (h^{-1})	0.039	0.055	0.011	0.005	0.004	0.002
ΔX (g L^{-1})	10.75	4.20	2.83	3.00	2.60	1.35

μ- Specific Growth rate; ΔX – Maximum biomass produced in fed-batch, calculated as the difference between the maximum and the initial biomass in each phase.