CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649032

Lipid Content and Productivity of *Arthrospira platensis* and *Chlorella vulgaris* under Mixotrophic Conditions and Salinity Stress

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This study aims at evaluating the influence of mixotrophic growth and salinity stress in the lipid content and productivities of microalgae *Arthrospira platensis* (*A. platensis*) and *Chlorella vulgaris* (*C. vulgaris*). For comparison purposes, both microalgae were also cultivated in the standard autotrophic conditions: modified Zarrouk's medium for *A. platensis* and Bold's basal medium for *C. vulgaris*. The mixotrophic conditions were created by adding 1.00 g/L of glucose to their standard media, and the salt stress was induced by introducing sodium chloride (NaCl) in different amounts. As expected, the biomass concentration and productivity increases under mixotrophy but decreases with salinity stress. Therefore, although the lipid content increased with the salinity stress under mixotrophy, reaching maximum values of 15.4 and 23.0 % dry weight (dwt) respectively, for *A. platensis* (in 0.428 M of NaCl) and *C. vulgaris* (in 0.0214 M of NaCl), the biomass productivity reached minimum values. Consequently, the maximum biomass and lipid productivities were obtained with an intermedium lipid content and without the salinity stress. The maximum biomass and lipid productivities are 99.7 and 9.7 mg/L/day for *A. platensis* and 227.2, and 37.7 mg/L/day for *C. vulgaris,* respectively. This study also revealed that *C. vulgaris* adapts faster to salinity stress, whereas *A. platensis* is able to tolerate higher salinity concentrations.

1. Introduction

For the past 50 years, extensive research has been performed on microalgae and how they can be used in a wide variety of processes, or to manufacture many practical and economic important products (Ribeiro et al., 2015). Microalgae have huge potential, when compared to other feedstocks, as source of chemicals and biochemicals for food ingredients (Chen and Zhang, 1997), feed proteins, polymers, cosmetics, pharma and nutraceuticals or even for renewable energy instead of vegetable oils (Caetano et al., 2012), animal fats (Mata et al., 2011), or other residual lipid sources (Caetano et al., 2013) that have limited supply and may have significant environmental, economic and societal impacts (Mata et al., 2013a). In the food industry, for instance, there is a growing demand for natural pigments, proteins and polyunsaturated fatty acids, which are rare in plant and animal sources but can be easily obtained from microalgae (Mata el al., 2010), representing a promising means of reducing Europe's dependence on imports (e.g. vegetable oils, proteins, and other ingredients for food and feed), diminishing the pressure on land resources (Mata et al., 2013b).
The first large-scale production of microalgae started in the early 1960s in Japan by Nihon Chlorella Inc., with the culture of *Chlorella*. It aimed at supplying a cheap protein source for food or feed in protein-deficient areas of the world. In the early 1970s a harvesting and culturing facility for *Arthrospira* was established in Mexico by Sosa Texcoco S.A., and it is used in human nutrition because of its high protein content and its excellent

Please cite this article as: Mata T., Martins A., Oliveira O., Oliveira S., Mendes A., Caetano N., 2016, Lipid content and productivity of arthrospira platensis and chlorella vulgaris under mixotrophic conditions and salinity stress, Chemical Engineering Transactions, 49, 187-192 DOI: 10.3303/CET1649032

nutritive value. As animal feed, microalgae have found their widest use in poultry and aquaculture industries. In particular, aquaculture hatcheries absorb about one-fifth of the commercial microalgae biomass to nourish fish and shellfish (Richmond, 2004).

Microalgae are primarily cultured autotrophically for large-scale production, in open ponds or closed photobioreactors, although with low culture density and low biomass productivity (Mata et al., 2014a). On the other hand, the mixotrophic cultivation is more costly, but allows to attain relatively high lipid yields and biomass productivity (Liang et al., 2009). One way to make it more economical is by recovering the biggest possible amount of different and valuable products, in a biorefinery concept (González-Delgado and Kafarov, 2012; Mata et al., 2012; Ribeiro et al., 2015), or by using low-cost carbon sources such as industrial by-products (Mata et al., 2013c) or even a waste stream (Mata et al., 2014b). Also, salinity may be used as a source of stress or for contamination prevention in microalgae cultures (Vonshak et al., 1988). Hence, this study aims at evaluating the influence of mixotrophic growth conditions and salinity stress in the lipid content and productivities of microalgae *A. platensis* and *C. vulgaris*. For comparison purposes, both microalgae were also cultivated in autotrophic conditions.

2. Materials and methods

2.1 Preparation of culture mediums

In this study *A. platensis* UTEX LB 2340 from University of Texas at Austin, USA (http://web.biosci.utexas.edu/utex/bulkcultures.aspx) and *C. vulgaris* ACOI 879 from University of Coimbra, Portugal (http://acoi.ci.uc.pt/) were used. The macronutrient solutions for cultivating these microalgae in standard autotrophic conditions were prepared according to the modified Zarrouk's culture medium (Parada, 1998) for *A. platensis* and the Bold's basal medium (Bischoff and Bold, 1963; Andersen, 2005) for *C. vulgaris*, which composition is presented in Table 1.

Table 1: Modified Zarrouk's and Bold's basal mediums for A. platensis and C. vulgaris, respectively

Component	Modified Zarrouk's medium (g/L)	Bold's basal medium (g/L)
NaCl	1.000	2.500×10^{-2}
$MgSO_4.7H_2O$	2.000×10^{-1}	7.500×10^{-2}
$CaCl_2.2H_2O$	4.000×10^{-2}	2.500×10^{-2}
EDTA-Na_2	8.000×10^{-2}	5.000×10^{-2}
$FeSO_4.7H_2O$	1.000×10^{-2}	5.000×10^{-3}
H_3BO_3	2.860×10^{-3}	1.140×10^{-2}
$ZnSO_4.7H_2O$	2.220×10^{-4}	1.412×10^{-3}
$MnSO_4.4H_2O$	1.810×10^{-3}	2.320×10^{-4}
$CuSO_4.5H_2O$	7.900×10^{-5}	2.520×10^{-4}
$Co(NO_3)_2.6H_2O$	4.400×10^{-5}	8.000×10^{-5}
$NaNO_3$	2.500	2.500×10^{-1}
$Na_2MoO_4.2H_2O$	1.800×10^{-5}	1.920×10^{-4}
K_2HPO_4	5.000×10^{-1}	7.500×10^{-2}
K_2SO_4	1.000	-
KH_2PO_4	-	1.750×10^{-1}
$NaHCO_3$	$1.600 \times 10^{+1}$	-
Na_2CO_3	2.000	-
KOH	-	3.100×10^{-2}

2.2 Routine microalgae culturing and acclimatization

As initial inoculum, test tube cultures were prepared for both microalgae under autotrophic conditions. After 2 weeks from the initial inoculum, about 25 mL of the dense test tube cultures were transferred to 250 mL Erlenmeyer flasks and supplemented with fresh culture medium. This was done both for the mixotrophic and autotrophic growth, starting the acclimatization in both culture conditions. After 2 more weeks, 150 mL of the 250 mL cultures were transferred to 1000 mL Erlenmeyer flasks and supplemented with fresh culture medium. Finally, after 2 further weeks, mother cultures were prepared using 750 mL of these pure dense cultures and supplemented to 5000 mL with fresh culture medium. These were allowed to grow until reaching dense cultures and entering in the stationary phase, i.e. up to the stabilization of the absorbance value of culture solution. After these successive inoculation and cultures, the microalgae cells were fully adapted to the autotrophic and mixotrophic growth conditions, as demonstrated by their rapid and repeatable growth rates

observed in the experiments performed.
All test cultures were subjected to a light/dark (L/D) photoperiod of 12/12 h, at 28 ± 2 °C of room temperature, and illuminated by fluorescent lamps (36 W, Sylvania Aquastar T8), positioned laterally to the cultures and providing an illuminance of about 4500 Lux, measured with a luxmeter (Lutron LX-1102) in fixed positions of the benches. Air sterilized by filtration (0.2 µm pore diameter filter) was supplied to the microalgae cultures (in the 250 mL, 1000 mL and 5000 mL Erlenmeyer flasks) at an average rate of 4 mL/s by air pumps (Pacific AP6), through the perforated rubber stopper of the Erlenmeyer flasks. Before each sub-culturing, the culture purity was verified by visual inspection, by using an optical microscope (Trinocular microscope 3B Scientific Physics, Model 400, U30712), and an improved Neubauer counting chamber with 0.100 mm depth (Hirschmann, EM Techcolor, Germany). Also, in order to prevent contamination by other microorganisms, all the laboratorial material and glassware (test tubes, and glass flasks) were washed with water and detergent, rinsed with distilled water, autoclaved at 121 ºC for 20 minutes, and placed in a laminar flow cabinet (CRUMA, 870-FL), under UV radiation for 60 minutes. Disposable pipettes and sterile loops were used.

2.3 Microalgae growth evaluation

For evaluating the microalgae growth, calibration curves of absorbance measured at 680 nm versus biomass concentration were traced for both microalgae, *A. platensis and C. vulgaris*, according to the following procedure: (1) For both microalgae, 1000 mL Erlenmeyer flasks cultures were prepared in triplicate with about 200 mL from the mother cultures (prepared as described in session 2.2) and allowed to grow until reaching dense cultures; (2) Absorbance measurements were done daily in a UV-Vis spectrophotometer (Shimadzu UV-160A) by taking 6 mL samples from the 1000 mL cultures for performing the reading in duplicate (with about 3 mL for each reading). The stabilization of the absorbance values indicated that the cultures reached the stationary phase as dense cultures; (3) Biomass was harvested by centrifugation (in a Super-speed Automatic Centrifuge SORVALL SS-3) at 4000 rpm for 20 minutes and then lyophilized (in a BT6K EL Virtis freeze dryer); (4) Eight standard solutions of known concentration were prepared by weighing different amounts of the lyophilized biomass (in a Kern ALJ 220-4 digital balance, with ± $5x10^{-5}$ g accuracy), and adding distilled water; (5) The absorbance of these eight solutions was read in triplicate at 680 nm (maximum absorbance peak) by using a cuvette with a light path length of 1 cm (in a Shimadzu UV-160A UV-Vis spectrophotometer); (6) Finally, the calibration curves were traced for each microalgae. Second, the assays were performed in triplicate in 1 L flasks inoculated with 200 mL of the mother culture. The growth of all cultures was evaluated by daily reading the absorbance in the UV-Vis spectrophotometer at 680 nm. The biomass concentrations were obtained on the basis of the calibration curve of absorbance versus biomass dry weight concentration. The stationary phase was reached when the absorbance values stabilized, then it was followed by the biomass harvesting and centrifugation at 3000 revolutions per minute (rpm), for 15 minutes (using a Super-speed Automatic Centrifuge SORVALL SS-3). Then, the biomass was catalogued and stored in cold environment (at -20 ± 5 ºC) until the lipids extraction was carried out.

2.4 Lipids extraction and quantification

For the lipids extraction and quantification a modified Bligh and Dyer (1959) method was used, as follows: (1) The biomass sample obtained by centrifugation was weighed in a pre-weighed glass tube; (2) The co-solvents were added in ratios of 1, 2 and 0.8 (v/v) for chloroform (Riedel de Haën, p.a.), methanol (Riedel de Haën, p.a.) and distilled water, respectively. Since it was used a centrifuged biomass with 70 % dwt of water content and not lyophilized biomass, these proportions were corrected assuming the water fraction in biomass; (3) The centrifuge tube containing the biomass sample with co-solvents was subjected to ultrasounds for 30 minutes in a Baldelin Sonorex TK30 equipment; (4) A second extraction step was then performed by adding the co-solvents at ratios of 2, 2 and 1.8 (v/v) of chloroform, methanol and distilled water respectively; (5) The sample was again subjected to ultrasounds for 30 more minutes and then centrifuged at 3000 rpm, for 15 minutes in a ECCO Tvp 25 No. 8601 centrifuge. (6) After centrifugation three layers became visible: an upper layer rich in water and methanol, a middle layer consisting of extracted biomass, and a lower layer rich in lipids and chloroform. The upper layer was discarded and the lower layer was recovered to a previously weighed glass tube; (7) The chloroform was evaporated in a laboratorial hood at room temperature of about 25 ºC, and the purified lipids extract remained in the glass tube; (8) The tube containing the lipids (pre-weighed when empty) was weighed again to determine the microalgae lipid content by gravimetry.

2.5 Microalgae biomass and lipid productivities

The maximum dry weight biomass productivity (P_{max}, mg/L/day) was calculated from Eq. (1), where C_2 and C_1 are the average biomass concentration (g/L) at day t_2 and t_1, respectively, during the microalgae exponential growth.

$$P_{\max} = (C_2 - C_1)/(t_2 - t_1) \quad (1)$$

The maximum lipid productivity (L_{max}, mg/L/day) was calculated from Eq. (2), where $L_{content}$ is the maximum accumulated lipid content at the end of cultivation (dwt %), and P_{max} is the maximum dry weight biomass productivity (mg/L/day) calculated from Eq. (1).

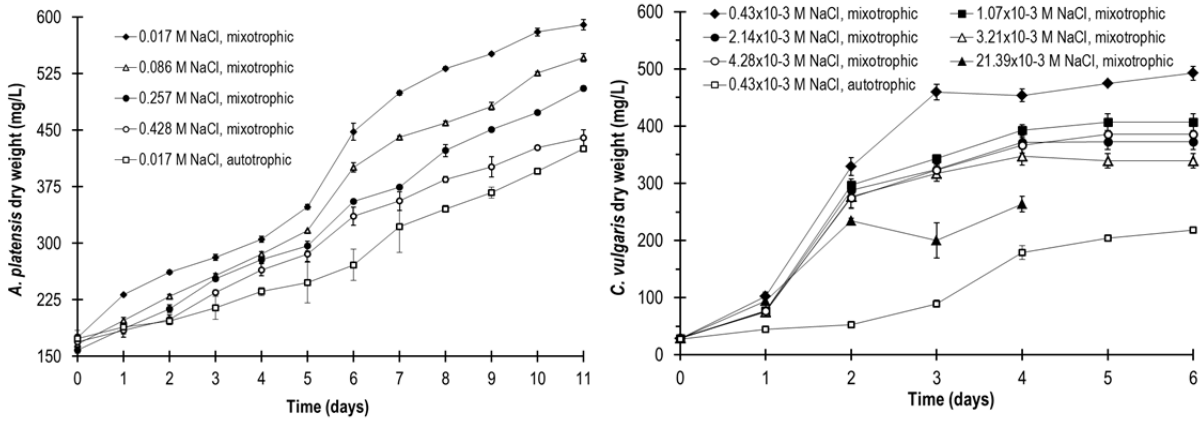
$$L_{max} = P_{max} \times L_{content} \quad (2)$$

The growth experiments and the analytical methods were applied at least in triplicate to confirm the reproducibility of the data reported in this work, and to obtain a measure of the experimental error. Thus, for each data generated, the mean and standard deviations were calculated.

3. Results and discussion

3.1 Evaluation of the microalgae growth

Growth curves were traced for *A. platensis* and *C. vulgaris* under mixotrophy and salinity stress and compared with control conditions, autotrophic and mixotrophic (Figure 1). The temporal evolution of these growth curves shows a lag phase, followed by an exponential growth phase (representing the maximum growth rate under the experimental specific conditions), a stationary phase for *C. vulgaris* and the beginning of a stationary phase for *A. platensis*. The decline phase is not visible in these graphs, which would correspond to the microalgae cells death and reduction of the overall biomass concentrations.



(a) (b)

Figure 1: Growth curves of (a) A. platensis and (b) C. vulgaris under mixotrophic and autotrophic conditions, with salinity stress.

As shown in Figure 1, the exponential growth phase was favored by mixotrophic conditions and lower salinity. The maximum biomass concentration was achieved for both microalgae under mixotrophic growth in the control culture with the lowest salinity. In the control culture under autotrophic conditions, although with the lowest salinity, the growth rate for both microalgae was lower than in the mixotrophic cultures.

Both microalgae showed different growth rates and adaptation (halotolerance and sensitivity) to the salinity stress, probably due to differences in their internal metabolism and specific wall features. Although *C. vulgaris* adapted faster to the salinity stress, *A. platensis* tolerated higher salinity concentrations.

Under mixotrophic conditions, *C. vulgaris* growth was significantly reduced at 4.28 x10^{-3} M NaCl and greatly inhibited at 21.39 x10^{-3} M NaCl and above, indicating that there is a critical NaCl concentration for this microalga adaptive process, and that it is sensitive to high salinity. In this regard, Alyabyev et al. (2007) showed that after 5 days under different salinities the growth of *C. vulgaris* was higher in the low salt concentration. Also, Ismail et al. (2011) showed that the photosynthetic pigments in *C. vulgaris* markedly decreased at the higher salinity levels. This is because the Na^+ ions inhibit metabolic rates, and under high NaCl concentrations cells need to adapt themselves to maintain the intracellular concentration of these ions lower than the toxic values (Alyabyev et al., 2007). Concerning *A. platensis* some studies demonstrated that cells can adapt to extreme salinity conditions, maintaining growth and photosynthetic activity, although this process becomes slower as NaCl concentration increases (Lu and Vonshak, 2002).

3.2 Influence of salinity stress on microalgae productivities

Tables 2 and 3 present the maximum biomass and lipid productivities of *A. platensis* and *C. vulgaris* respectively, for the two growth regimens and different salinity conditions. As expected, under mixotrophic conditions, biomass productivity is generally higher than under autotrophic conditions, in some cases more

than double when compared with the values obtained under autotrophic conditions, as also verified both for *C. vulgaris* (Liang et al., 2009) and *A. platensis* (Vonshak et al., 1988).

Table 2: Lipid content, biomass and lipid productivities of A. platensis under mixotrophic and autotrophic conditions, with and without salinity stress

Salinity stress	NaCl (M)	Glucose (g/L)	Growth regimen	Period (day n°.)	Biomass productivity (mg/L/day)	Lipid content (%)	Lipid productivity (mg/L/day)
No	0.017	0.00	Autotrophic	6-7	50.8±23.3	7.1±0.3	3.6±0.3
No	0.017	1.00	Mixotrophic	5-6	99.7±6.7	9.7±0.3	9.7±0.3
Yes	0.086	1.00	Mixotrophic	5-6	84.1±3.6	10.9±0.3	9.2±0.3
Yes	0.257	1.00	Mixotrophic	5-6	59.0±3.9	12.4±0.4	7.3±0.4
Yes	0.428	1.00	Mixotrophic	5-6	50.1±9.0	15.4±1.7	7.7±1.7

Table 3: Lipid content, biomass and lipid productivities of C. vulgaris under mixotrophic and autotrophic conditions, with and without salinity stress

Salinity stress	NaCl (M)	Glucose (g/L)	Growth regimen	Period (day n°.)	Biomass productivity (mg/L/day)	Lipid content (%)	Lipid productivity (mg/L/day)
No	$0.43x10^{-3}$	0.00	Autotrophic	3-4	89.5±8.0	16.7±0.3	14.9±0.3
No	$0.43x10^{-3}$	1.00	Mixotrophic	1-2	227.2±10.3	16.6±0.3	37.7±0.3
Yes	$1.07x10^{-3}$	1.00	Mixotrophic	1-2	219.9±6.6	14.2±0.3	31.2±0.3
Yes	$2.14x10^{-3}$	1.00	Mixotrophic	1-2	212.1±0.4	15.5±0.4	32.9±0.4
Yes	$3.21x10^{-3}$	1.00	Mixotrophic	1-2	202.3±12.0	17.4±0.3	35.2±0.3
Yes	$4.28x10^{-3}$	1.00	Mixotrophic	1-2	198.1±10.6	18.5±0.5	36.6±0.5
Yes	$21.39x10^{-3}$	1.00	Mixotrophic	1-2	140.4±6.8	23.0±0.8	32.3±0.8

Under mixotrophy, biomass productivity increases as salt concentration decreases, reaching the highest value at the lowest salinity (in the control cultures). The maximum biomass productivity of *A. platensis* was obtained during the exponential growth phase, between day 5 and 6 under mixotrophy, and between day 6 and 7 under autotrophy. The maximum biomass productivity of *C. vulgaris* occurred during the exponential growth phase, between day 1 and 2 in the cultures under mixotrophy, and between day 3 and 4 in the culture under autotrophy. On the other hand, although the salinity stress induced the lipid accumulation in both microalgae, verified by the lipid content increase, with the maximum value at the highest salinity, the lipid productivities decreased with the salinity increase. This is because the lipid content increase is not enough to overcome the reduction in biomass productivity at high salinities. For *A. platensis*, the maximum lipid content is 15.4 % dwt (at the highest NaCl concentration of 0.428 M) that is 59 % higher than the lipid content for which the highest biomass productivity was obtained (in the control mixotrophic culture with the lowest salinity). Similarly, for *C. vulgaris* the maximum lipid content is 23.0 % dwt (at the highest NaCl concentration of $21.39x10^{-3}$ M) that is 39 % higher than the lipid content for which the highest biomass productivity was obtained (in the control mixotrophic culture with the lowest salinity).

4. Conclusions

This work evaluated the influence of mixotrophic growth and salinity stress on the growth, lipid content, and biomass and lipid productivities of both microalgae *A. platensis* and *C. vulgaris*, in comparison with the autotrophic growth. Results showed that the mixotrophic conditions favored the growth and biomass productivity of both microalgae in comparison with autotrophic conditions, even at higher NaCl concentrations. The salinity stress under mixotrophic growth induced the lipid accumulation in both microalgae although accompanied by a decrease in biomass productivity, which contributed to the decrease of lipid productivity. Although *C. vulgaris* acclimated faster to the salinity stress, *A. platensis* could tolerate higher salinity concentrations.

Acknowledgments
Teresa Mata would like to thank Fundação para a Ciência e Tecnologia (FCT) for their support through provision of a research grant Ref. IF/01093/2014. This work was financially supported by: Project UID/EQU/00511/2013-LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy – EQU/00511) by FEDER funds through Programa Operacional Competitividade e Internacionalização – COMPETE2020 and by national funds through FCT.

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