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T. obliquus Cultivation Under Heterotrophic Conditions: Determination of Growth Parameters

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Microalgae are a promising feedstock for the future of the industrial chemistry. Among microalgae, *Tetradesmus obliquus* (generally known as *Scenedesmus obliquus*) is one of the most studied species due to its robustness and its good performances for production of lipids, proteins and carbohydrates. However, the high production costs still limit large part of the possible applications. The exploitation of the heterotrophic metabolism for microalgae cultivation may potentially improve the biomass production sustainability, especially if organic substrates are obtained from wastewaters. However, this latter strategy has technical limits given by the issue of contamination by bacteria, which have growth rates ~ 10 folds higher than microalgae.
The estimation of the microalgae growth parameters could allow optimization of the feeding strategy by which wastewaters are supplied to microalgae culture, and consequently, bacteria contamination minimization.
In this work, an axenic culture of *T. obliquus* was isolated by a sonication pre-treatment followed by an incubation in solid media containing ampicillin (0.1 mg mL^{-1}), penicillin (67 U mL^{-1}) and streptomycin (0.07 mg mL^{-1}). The axenic strain was then cultivated in heterotrophic condition under N-replete and N-starvation. Experimental data of biomass production and nitrogen consumption were well predicted by the Droop model. By means of the model fitting with experimental data, the optimal values for maximum theoretical growth rate ($\bar{\mu}$), maximum nitrogen quota (q_{max}), minimum nitrogen quota (q_{min}) and maximum nitrogen uptake rate (ρ_{max}) were estimated.
By estimating, in the future, the growth parameters also for other substrates and for contaminant microorganisms, a more effective wastewater supply strategy can be developed.

1. Introduction

Microalgae are photosynthetic microorganisms, producing a large variety of different compounds exploitable for applications such as biofuels (Ruiz et al., 2016), biomaterials, food and feed (Spolaore et al., 2006). Although numerous studies have been carried out over the past years, most of the possible applications of microalgae are not economically sustainable yet (Ruiz et al. 2016). The minimum cost for microalgal biomass production has been estimated between 3.2 – 12.4 € kg^{-1} by Tredici et al. (2016) and between 3.4 – 11 € kg^{-1} by Ruiz et al. (2016). The integration of microalgae cultivation with wastewaters treatment is one of the most promising approaches to enhance economic and environmental feasibility of microalgal production (Di Caprio et al., 2019). Wastewaters can provide inorganic nutrients such as P, N, S and others at zero cost, plus organic substrates as sugars and organic acids (Di Caprio et al. 2017). In particular, the organic substrates can be exploited to support the heterotrophic metabolism of microalgae (Visca et al., 2017). The exploitation of heterotrophic metabolism can add a further improvement with respect to conventional photoautotrophic processes, thanks to the higher productivity induced by organic substrate and to the lower surface to volume ratio required in the fermenters (Di Caprio et al., 2019). These factors could potentially reduce both capital and operative costs. However, addition of organic substrate increases considerably the issue of bacteria contamination. This issue is the result of the higher maximum growth rate of bacteria (~ 20 d^{-1}) with respect to microalgae (~ 2 d^{-1}) (Deschênes, 2016). The solution generally adopted to overcome this issue is to work by

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using sterilized wastewaters in a fully axenic environment. However, it is hardly applicable to industrial scale due to the cost increment induced by sterilization.
A possible solution can be found in the optimization of the strategy adopted to supply nutrients to microalgae. For example, by giving nutrients in a strategy by which they are consumed by microalgae before that bacteria contamination becomes relevant. To investigate this possibility, it is required to know the kinetic parameters to describe microalgae growth and the yield of microalgae cells with respect to substrates. *T. obliquus* (generally known as *Scenedesmus obliquus*), the strain investigated in this study, is of the more promising microalgae strains for industrial applications, due to its robustness (Wu et al., 2014), its biochemical composition and its good growth performances (Di Caprio et al., 2018a).
However, there is scarce information in literature about its growth parameters in heterotrophic regime (such as ρ_{max} and $\bar{\mu}$). Major difficulty to derive such parameters is to perform heterotrophic cultivation tests under axenic conditions, i.e., by preventing the contamination of bacteria competing with microalgae for the available nutrients (organic substrates and inorganic nutrients).
The aim of this work was to isolate an axenic strain of *T. obliquus*, and then to perform heterotrophic cultivations in order to estimate the growth parameters by using a conventional unstructured model.

2. Materials and methods

2.1 Microalgae strain

A strain of *T. obliquus* isolated from Siracusa (Italy) was maintained in Petri dishes in BG11 solid medium under constant illumination (cool-white fluorescent light) at 80 $\mu mol \cdot s^{-1} \cdot m^{-2}$ and 25 ± 3 °C. The identification of the strain was previously described (Di Caprio et al., 2018b).

2.2 Axenic strain isolation and maintenance

Microalgae were suspended in 30 mL of distilled water, then the suspension was sonicated for 1 min by using Branson Sonifer S 450 (20 kHz) at 50 % amplitude (76 µm, 145 W). Suspension was then centrifuged at 3,000 rpm for 5 min and washed twice with distilled water. The obtained pellet was diluted in distilled water and plated in Petri dishes containing BG11 solid medium enriched with 1 g L^{-1} of glucose and different concentrations of antibiotics (Table 1). The plates were illuminated at 80 µmol s^{-1} m^{-2} at 25 ± 3 °C. The presence of bacteria was evaluated by making replica plating on plates containing LB solid medium. The microalgae strain isolated from the axenic plates were then inoculated in 50 mL glass bottles containing 25 mL BG11 medium enriched with 1 g L^{-1} $NaHCO_3$. The bottles were closed and leaved under constant illumination of 40 µmol s^{-1} m^{-2} at 25 ± 3 °C. The axenic condition was periodically monitored by inoculating an aliquot of the suspension from bottles in both LB and YPD plates. All the handling of the axenic cultures was carried out in fully sterilized conditions.

Table 1: Type and concentration of antibiotics in the different tested conditions

Tested conditions	Ampicillin (mg mL^{-1})	Penicillin (U mL^{-1})	Streptomycin (mg mL^{-1})
Condition 1	0.07	-	-
Condition 2	0.1	-	-
Condition 3	-	67	0.07
Condition 4	0.1	67	0.07

2.3 Heterotrophic cultivation of axenic cultures

Microalgae were harvested from maintenance bottles, centrifuged at 3,000 rpm for 5 min, and the pellet suspended in 150 mL culture medium in 300 mL Erlenmeyer flasks at an initial biomass concentration ranging between 20 – 40 mg L^{-1}. Two different culture mediums were tested, with nutrients concentration all the same as BG11, except for NO_3^-, which was varied at two levels: 0 mg L^{-1} and 200 mg L^{-1} (N = 45 mg L^{-1}). In every flask 5 g L^{-1} of glucose were supplied. All the flasks were covered with aluminum foils to ensure dark environment, closed with cellulose stoppers and continuously shaken at 150 rpm.

2.4 Chemical and biochemical analyses

The concentration of the biomass suspension was measured by reading the optical density at 750 nm by means of a UV-vis spectrophotometer. Biomass concentration (X) was determined by using a calibration line obtained by filtering 10 mL of microalgae suspension on glass microfiber filter (0.7 µm, VWR 698) and by

drying the filters in oven at 105 °C. Glucose concentration was measured by using the Dubois colorimetric method (Di Caprio et al., 2018b), while nitrogen concentration (N) in the culture medium was measured by using a ion-selective electrode (perfectION™, Mettler Toledo) for NO_3^-.

2.5 Growth model

2.5.1 Description of the model

Experimental growth data were described by using the Droop model (Bernard, 2011). Nitrogen was considered as the only limiting nutrient, and its uptake rate ρ ($g_N\ g_X^{-1}\ d^{-1}$) was described as follows:

$$\rho\ (N) = \rho_{max} \frac{N}{N + K_N} \quad (1)$$

where N ($g_N\ L^{-1}$) is the nitrogen concentration in the culture medium, K_N is the half-saturation constant ($g_N\ L^{-1}$) and ρ_{max} is the maximum nitrogen uptake rate ($g_N\ g_X^{-1}\ d^{-1}$). The specific growth rate μ (d^{-1}) of the biomass was a function of the internal nitrogen quota q ($g_N\ g_X^{-1}$):

$$\mu\ (q) = \bar{\mu}\left(1 - \frac{q_{min}}{q}\right) \quad (2)$$

where q_{min} is the minimum internal nitrogen quota and $\bar{\mu}$ is the maximum theoretical growth rate (the growth rate at hypothetical infinite quota).

The variation of q with respect to cultivation time was expressed by Eq (3), as follows:

$$\frac{dq}{dt} = r_N - r_H q = \rho(N) - \mu(q)q \quad (3)$$

Variation of X and N during time were given respectively by Eq (4) and Eq (5):

$$\frac{dX}{dt} = \mu(q)X \quad (4)$$

$$\frac{dN}{dt} = -\rho(N)X \quad (5)$$

The model did not include the influence of glucose because during all the tests the glucose concentration remained quite high (> 1000 mg L^{-1}) to exclude any influence on the growth rate. For comparison, the K_S typically reported in literature for glucose are < 10 mg L^{-1} (Deschênes, 2016).

2.5.2 Estimation of the parameters

The K_N value was fixed at 10^{-5} $g_N\ L^{-1}$, such value was taken from previous literature data (Mairet et al. 2011). The optimal values of $\bar{\mu}$, q_{min} and ρ_{max} were estimated by the minimization of the sum of squared residuals. The value of q_{max} was estimated by using Eq (6), on the base of the parameter values obtained by fitting the model to the experimental data derived in presence of nitrogen (Bernard, 2011).

$$q_{max} = \frac{\rho_{max}}{\bar{\mu}} + q_{min} \quad (6)$$

3. Results and discussion

3.1 Isolation of the axenic strain

Among the different tested strategies, the condition 4 (Table 1) was the only one by which an axenic strain of *T. obliquus* could be obtained. This condition corresponded to the only one in which all the three tested antibiotics (ampicillin, penicillin and streptomycin) were added simultaneously. This result is likely due to the large variability of the bacteria species that live in symbiosis with microalgae (Ramanan et al., 2015), which makes a single antibiotic effective only versus some strains.

3.2 Heterotrophic growth

The evolution of biomass concentration (X) during the cultivation is reported in Figure 1a for both the initial N concentrations tested. In the test with initial N = 45 mg L^{-1}, the biomass grew until reaching 1.1 ± 0.1 g L^{-1} of final biomass concentration at the third day, after that X remained constant and q_{min} was reached (Figure 1c). Instead, in the test in which there was no N in the culture medium at the beginning of the cultivation, the final X was lower and equal to 0.13 ± 0.03 g L^{-1}. The reduced growth was given by the absence of N. While in the presence of N (N-replete) microalgae biomass growth is given by cell duplication, in the absence of N (N-starvation), cell duplication is stopped and biomass growth is mainly given by the limited amount of organic molecules that can be accumulated inside cells. Such accumulation of organic molecules increases cell size and reduces q (Di Caprio et al., 2018a).

In order to determine the growth parameters of the model, a first regression was carried out on experimental data obtained with initial N = 45 mg L^{-1}. As shown in Figure 1a and Figure 1b, the model was able to represent well both data of biomass concentration and data of nitrogen concentration. The parameters estimated by this regression are reported in Table 2. The values of $\bar{\mu}$ and q_{max} were comparable with those previously reported for *I. galbana* (Mairet et al., 2011), for *T. obliquus* (Deschênes and Vande Wouwer, 2016) and for *S. platensis* (Arata et al., 2013) from experiments carried out in photoautotrophic conditions. Instead the value of ρ_{max} is lower than the value of 0.7 ± 0.2 $g_N\ g_X^{-1}\ d^{-1}$ previously reported for the same species (*T. obliquus*) in photoautotrophic condition by Deschênes and Vande Wouwer (2016) and higher than the values reported for *I. galbana*, which were between 0.04 $g_N\ g_X^{-1}\ d^{-1}$ (Mairet et al., 2011) and 0.05 $g_N\ g_X^{-1}\ d^{-1}$ (Bernard, 2011). The q_{min} value found was higher than the one (0.011 ± 0.003 $g_N\ g_X^{-1}$) previously reported for the same species in photoautotrophic conditions by Deschênes and Vande Wouwer (2016) but in agreement with those reported for *I. galbana* (Bernard, 2011).

For the regression of the data obtained in N-starvation, the q_{max} value was fixed as the one estimated from the test with 45 mg L^{-1} of initial N concentration. Such choice was motivated by considering that the microalgae used as inoculum in the N-starvation test came from a photoautotrophic cultivation in a medium fully replete for every nutrient. Therefore, it could be assumed that they started from a $q = q_{max}$ and that this latter was equal to the one determined from heterotrophic test with initial N = 45 mg L^{-1}. The regression was repeated for the data obtained in N-starvation. As shown in Table 2, the values of $\bar{\mu}$ and q_{min} were comparable (the difference were not statistically significant) with those previously estimated in presence of N.

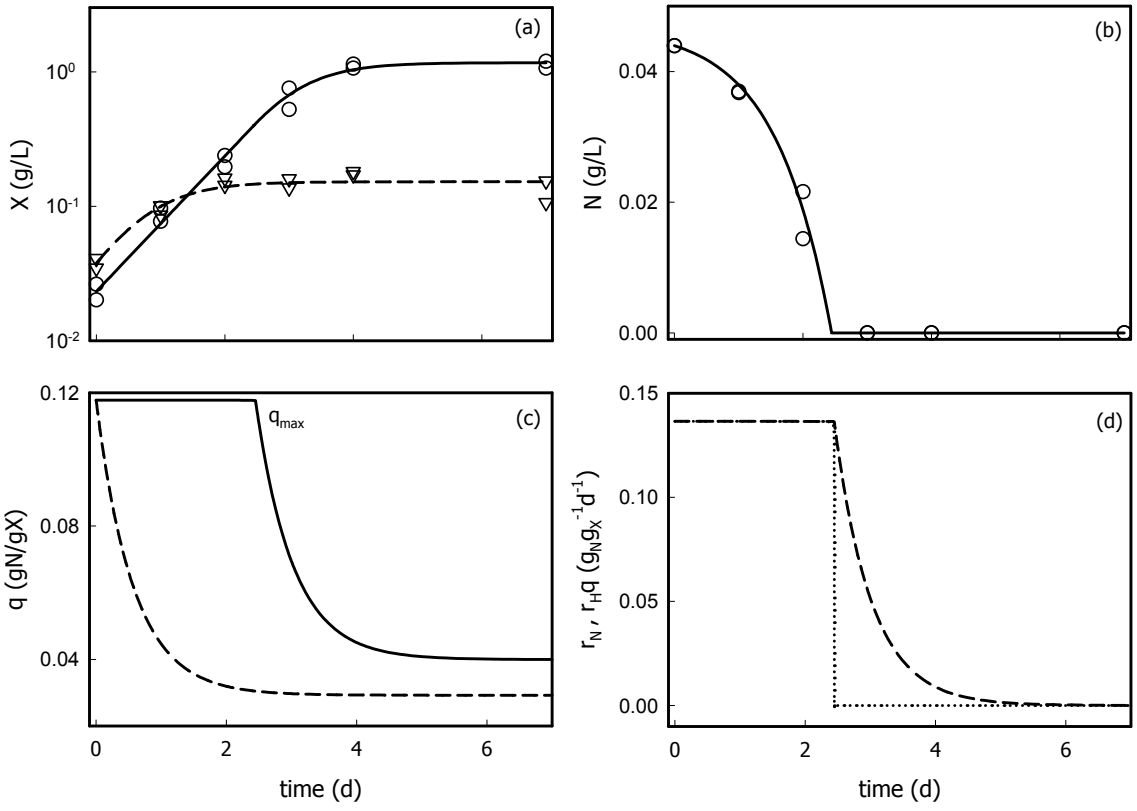

Figure 1: Comparison between model predictions and experimental values; (a) biomass concentration; (b) nitrogen concentration in the culture medium; (c) nitrogen quota; (d) r_N (dotted line) and $r_H q$ (dashed line) for 45 mg L^{-1} initial nitrogen concentration. In (a), (b) and (c), solid and dashed lines describe model predictions attained with 45 mg L^{-1} and 0 mg L^{-1} initial nitrogen concentration in the medium, respectively.

The Figure 1c and Figure 1d give further information about the dynamic of N uptake inside microalgae biomass. For the test without N in the medium, microalgae started from a $q = q_{max}$. During cultivation the internal quota of N gradually decreased until q_{min}, because ρ(N) was maintained equal to 0 (see Eq(1) and Eq(3)) during all cultivation.

Instead, in the test with initial N concentration equal to 45 mg L^{-1}, there was a phase of about 2 days, in which q remained constant. During this phase there was an equilibrium between the rise of q given by the N uptake rate (r_N) and the reduction of q due to the biomass production rate ($r_H q$).

*Table 2: Parameters values computed by fitting model predictions to the experimental biomass and nitrogen concentrations values attained during microalgae growth. Errors are reported as ± confidence interval (95 %). *Parameter maintained constant during the optimization.*

Parameter	N 0 mg L^{-1}	N 45 mg L^{-1}
$\bar{\mu}$ (d^{-1})	1.7 ± 0.4	1.8 ± 0.3
q_{min} ($g_N\ g_X^{-1}$)	0.030 ± 0.005	0.040 ± 0.004
q_{max} ($g_N\ g_X^{-1}$)	0.1 ± 0.3	0.1 ± 0.3
ρ_{max} ($g_N\ g_X^{-1}\ d^{-1}$)	-	0.14 ± 0.02
K_N ($g_N\ L^{-1}$)	-	10^{-5}*

As shown in Figure 1d, these terms maintained the same value when N was available in the culture medium. Only when N in solution finished (N = 0 mg L^{-1}), r_N instantaneously dropped to zero. This change was so sudden because of the lower value of K_N (0.01 mg L^{-1}) with respect of the ΔN induced by microalgae growth in the time intervals considered (Figure 1b). The value of $r_H q$ decreased progressively after the end of N in solution, because of the gradually reduction of q (that was given by the stopped N uptake) until to 0, when q = q_{min} was reached.
The experimental data obtained were used to determine the yield factors for the two main substrates, which were NO_3^- and glucose. The yield factors were determined according to Eq(7) and Eq(8):

$$Y_{X/NO_3^-} = \frac{X_f - X_0}{[NO_3^-]_0 - [NO_3^-]_f} \quad (7)$$

$$Y_{X/\mathrm{glu}} = \frac{X_f - X_0}{[\mathrm{glu}]_0 - [\mathrm{glu}]_f} \quad (8)$$

Where X_0 and X_f are the initial and final biomass concentration measured during the cultivation respectively. $[NO_3^-]_0$ and $[NO_3^-]_f$ are the initial and final NO_3^- concentration measured during the cultivation respectively. $[glu]_0$ and $[glu]_f$ are the initial and final glucose concentration measured during the cultivation respectively.
The yield factors obtained for glucose are comparable with those obtained for the same strain in previous studies conducted by Camacho Rubio et al. (1989) and by Di Caprio et al. (2018c). The lower value obtained in N-starvation condition can be a consequence of a higher percentage of glucose used for maintenance with respect to biomass production. The value determined for Y_{X/NO_3^-} (Table 3) is higher than those previously reported for other microalgae species cultivated in photoautotrophic condition, which were 3.2 g g^{-1} (Del Río et al., 2005) and 3.3 g g^{-1} (Del Rio-Chanona et al., 2017). The difference was given by the cultivation conditions. While the previous data from literature were obtained by using microalgae cultivated in a medium replete in N, the Y_{X/NO_3^-} determined in this work includes the biomass produced during N-starvation, which makes the yield factor higher.
Such effect was described also in Di Caprio et al. (2019), showing that, during a batch which includes N-starvation, the Y_{X/NO_3^-} of *T. obliquus* was ~ 2 folds higher than the value achieved in the only N-replete condition, due to the biomass growth given by the sole organic carbon accumulation during N-starvation.

Table 3: Yield factors for biomass with respect to NO_3^- and glucose obtained from T. obliquus cultivated in heterotrophic condition. Errors are reported as ± standard deviation.

N	Y_{X/NO_3^-}	$Y_{X/glu}$
0 (mg L^{-1})	-	0.13 ± 0.05
45 (mg L^{-1})	5.7 ± 0.5	0.3 ± 0.1

4. Conclusions

The results obtained prove that the Droop model can be successfully used to predict *T. obliquus* growth under heterotrophic conditions. The model parameters $\bar{\mu}$ and q_{max} estimated were comparable with those previously reported in literature for other different microalgae cultivated in phototrophic conditions, while ρ_{max} and q_{min} were respectively lower and higher than those previously estimated for the same species in phototrophic

growth. By coupling these results with the values that can be estimated in future by using other substrates and other microorganisms (including contaminant bacteria), it will be possible to predict the optimal strategies to cultivate microalgae in wastewaters by exploiting their heterotrophic metabolism.

References

Arata S., Strazza C., Lodi A., Del Borghi A., 2013, Spirulina platensis culture with flue gas feeding as a cyanobacteria-based carbon sequestration option, Chemical Engineering Technology, 36, 91-97.

Bernard O., 2011, Hurdles and challenges for modelling and control of microalgae for CO2 mitigation and biofuel production, Journal of Process Control, 21,1378-1389.

Camacho Rubio F., Martínez Sancho M.E., Sánchez Villasclaras S. Delgado Pérez A., 1989, Influence of pH on the kinetic and yield parameters of Scenedesmus obliquus heterotrophic growth, Process Biochemistry, 40, 133-136.

Del Rio-Chanona E.A., Ahmed N. R., Zhang D., Lu Y. Jing K., 2017, Kinetic modelling and process analysis for Desmodesmus sp. lutein photo-production, AIChE Journal, 63, 2546-2554.

Del Río E., Acién F.G., García-Malea M.C., Rivas J., Molina-Grima E. Guerrero M.G., 2005, Efficient one-step production of astaxanthin by the microalga Haematococcus pluvialis in continuous culture, Biotechnology and Bioengineering, 91, 808-815.

Deschênes J.S., 2016, A Bacteriostatic Control Approach for Mixotrophic Cultures of Microalgae, IFAC-PapersOnLine, 49, 1074-1078.

Deschênes J.S. Vande Wouwer A., 2016, ScienceDirect Parameter Identification of a Dynamic Model of Cultures of Microalgae Scenedesmus obliquus – An experimental study, IFAC-PapersOnLine, 49, 1050-1055.

Di Caprio F., Altimari P. Pagnanelli F., 2017, Effect of Ca2+ concentration on Scenedesmus sp. growth in heterotrophic and photoautotrophic cultivation, New Biotechnology, 40 B, 228-235.

Di Caprio F., Altimari P., Iaquaniello G., Toro L. Pagnanelli F., 2018b, T. obliquus mixotrophic cultivation in treated and untreated olive mill wastewater, Chemical Engineering Transactions, 64, 625–630.

Di Caprio F., Altimari P. Iaquaniello G., Toro L., Pagnanelli F., 2019, Heterotrophic cultivation of T. obliquus under non-axenic conditions by uncoupled supply of nitrogen and glucose, Biochemical Engineering Journal, 145, 127-136.

Di Caprio F., Pagnanelli F., Wijffels R.H. Van der Veen D., 2018a, Quantification of Tetradesmus obliquus (Chlorophyceae) cell size and lipid content heterogeneity at single-cell level, Journal of Phycology, 54, 187-197.

Di Caprio F., Scarponi P., Altimari P., Iaquaniello G. Pagnanelli F., 2018c, The influence of phenols extracted from olive mill wastewater on the heterotrophic and mixotrophic growth of Scenedesmus sp., Journal of Chemical Technology & Biotechnology, 93, 3619-3626.

Mairet F., Bernard O., Masci P., Lacour T. Sciandra A., 2011, Modelling neutral lipid production by the microalga Isochrysis aff. galbana under nitrogen limitation, Bioresource Technology, 102, 142–149.

Ramanan R., Kanga Z., Kim B.H., Cho D.H., Jin L., Oh H. M., Kim H. S., 2015, Phycosphere bacterial diversity in green algae reveals an apparent similarity across habitats, Algal Research, 8, 140-144.

Ruiz J., Olivieri G., De Vree J., Bosma R., Willems P., Reith J.H., Eppink M.H.M., Kleinegris D.M.M., Wijffels R.H., Barbosa M.J., 2016, Towards industrial products from microalgae, Energy & Environmental Science, 9, 3036-3043.

Spolaore P., Joannis-Cassan C., Duran E. Isambert A., 2006, Commercial applications of microalgae, Journal of Bioscience and Bioengineering, 101, 87-96.

Tredici M.R., Rodolfi L., Biondi N., Bassi N. Sampietro G., 2016, Techno-economic analysis of microalgal biomass production in a 1-ha Green Wall Panel (GWP®) plant, Algal Research, 19, 253-263.

Visca A., Di Caprio F., Spinelli R., Altimari P., Cicci A., Iaquaniello G., Toro l. Pagnanelli F., 2017, Microalgae Cultivation for Lipids and Carbohydrates Production, Chemical Engineering Transactions, 57, 127-132.

Wu Y.H., Hu H.Y., Yu Y., Zhang T.Y., Zhu S.F., Zhuang L.L., Zhang X. Yun L., 2014, Microalgal species for sustainable biomass/lipid production using wastewater as resource: a review, Renewable & Sustainable Energy Reviews, 33, 675-688.