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T. obliquus Mixotrophic Cultivation in Treated and Untreated Olive Mill Wastewater

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Olive mill wastewater (OMW) utilization for microalgae cultivation has been investigated in different studies, in which it is generally strongly pre-treated before use. Here a common pre-treatment method (active carbon) has been carried out for OMW, and its influence on *Tetradesmus obliquus* growth (generally known as *Scenedesmus obliquus*) and phenol and sugar removal has been compared with untreated OMW. Before to carry out test on OMW, nitrate concentration in the media was optimized, finding a modified BG11 media more adequate for our experimental aims. *T. obliquus* used in this work showed a nitrogen requirement of about 4 pg/cell in exponential growth, for a protein content of 34%. Active carbon treatment reduced 90% phenol content and 50% sugar content in OMW. Addition of 9% (v/v) pre-treated OMW to the medium, did not affect microalgae duplication during test. In contrast a cell duplication inhibition was observed after 4-6 days for untreated OMW added at 9% (v/v). Lower final phenol concentration was reached in test with pre-treated OMW (10 mg/L), while no difference was observed in final sugar concentration comparing the two different OMW. Instead higher phenol removal during cultivation was obtained for test with untreated OMW (240 mg/L), indicating the necessity of further tests to better understand mechanism involved in their degradation. A process with active carbon pre-treatment looks more adequate for mixotrophic growth and pollutant removal as main goals, while untreated OMW looks more promising for heterotrophic cultivation.

1. Introduction

Microalgae are microorganisms that can produce a high variety of different compounds industrially interesting. Main applications include biofuels, biomaterials, food, feed and nutraceutical productions. Although numerous studies have been carried out in the last years, most of the possible applications are not still economically sustainable. Development of biorefinery facilities (Markou and Nerantzis, 2013) and process integration with wastewater treatment (in mixotrophy or heterotrophy) are the most promising trends to enhance economic and environmental sustainability in microalgal based processes (Di Caprio et al. 2015a). *T. obliquus* is commonly known as *Scenedemus obliquus*, but it has been recently reclassified (Di Caprio et al. 2018b). It is a species that is particularly promising for an integrated process coupled with a biorefinery facilities. In fact, *T. obliquus* can accumulate until 50 % on dry weight of fatty acids, until 40 % on dry weight of starch (Breuer et al. 2014) and can produce several high value compounds (Di Caprio et al. 2015b). Moreover, it is a robust strain able to grow in typical suboptimal conditions in real industrial processes. *T. obliquus* can grow in presence of different wastewaters (Wu et al. 2014) exploiting both mixotrophic and heterotrophic metabolism (Visca et al. 2017).
Olive mill wastewater (OMW) is produced from olive oil production plants, and its treatment still represents an issue, mainly for high COD and phenol content. OMW contains high amount of phenols, sugars, organic acids, phosphates and potassium, but a low amount of inorganic nitrogen (Di Caprio et al. 2015a). For its utilization in microalgae cultivation, nitrogen should be externally added because in OMW it is present mainly as peptides and proteins, which are difficult to metabolize for microalgae. Different studies have been conducted to investigate microalgae cultivation in media containing OMW (Hodaifa et al. 2013). The effects of different operative parameters such as pH, light intensity, temperature, OMW dilution, mineral salts concentration (Di Caprio et al. 2018a) and different pre-treatments (Cicci et al. 2013) have been investigated. However, in the

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large part of these studies OMW is strongly pre-treated to remove biological contaminants (sterilization), antimicrobial agents (e.g. phenols) and black-dark colour (which limits light penetration). These pre-treatments add further costs and energy consumption, which may be unfeasible at industrial scale, for their economic impact, compromising the advantage theoretically achievable by using wastewaters. Adopted pre-treatment should be chosen on the base of a cost to benefit ratio calculated case by case. Active carbon has been used in some previous works as pre-treatment for OMW before microalgae cultivation, however its effect on OMW composition was not very clear (Hodaifa et al. 2012). In this work in a first phase nitrate concentration in the cultivation medium was optimized, calculating the nitrogen requirement of the used microalgal strain and modifying BG11 composition. In a second phase, the effect of pre-treated and untreated OMW addition at 9% (v/v) to the optimized medium was investigated during the process comparing both cell growth and pollutant (sugars and phenols) removal.

2. Material and methods

2.1 Microalgae strain

A strain of *T. obliquus* was selected in Siracusa (Italy) and maintained in Petri dishes in BG11 solid medium under constant illumination at 80 $\mu mol \cdot s^{-1} \cdot m^{-2}$ and 25 ± 3 °C. The strain was identified by IGA Technology Services by means of genetic analysis based on ITS region. For each test microalgae were first transferred in 800 mL BG11 liquid medium in 1,000 mL Roux bottles under continuous air feeding (0.1 $L \cdot L^{-1} \cdot min^{-1}$) and magnetic stirring, at the same temperature and illumination, and maintained in this condition for 7-15 days, and then inoculated in the new media for test starting.

2.2 Test for different nitrate concentrations

Microalgae from maintenance cultivation were diluted 1 to 10 in BG11 and modified BG11 (MBG11). MBG11 was obtained adding $NaNO_3$ until achieving 0.35 g/L, that is lower than classic BG11 concentration of 1.5 g/L (Carpine et al. 2015). Microalgae were maintained under the same stirring, illumination, temperature and air feeding previously described. Samples were daily taken for chemical and biological analyses. Test was carried out in duplicate.

2.3 Test with OMW

Microalgae from maintenance cultivation were diluted 1 to 10 in MBG11. Three different conditions were compared: a) phototrophic, with only MBG11 as medium, b) mixotrophic with OMW pre-treated with active carbon added at 9 % (v/v) to MBG11 medium and c) mixotrophic with untreated OMW added at 9 % (v/v) to MBG11 medium. OMW was added at the beginning of the test. It was considered untreated OMW that one at which suspended solids were previously removed by means of 2 h of sedimentation. The used OMW was taken by a three phases traditional pressing process from an industrial plant in Velletri (Rome, Italy), stored at – 20 °C until use. Chemical and physical properties of the used OMW have been described in a previous work (Di Caprio et al. 2015a). Microalgae were maintained under the same stirring, illumination, temperature and air feeding previously described. Samples were daily taken for chemical and biological analyses. Test was carried out in duplicate.

2.4 Active carbon treatment

The treatment was carried out following the procedure reported by Hodaifa et al. (2013). For 100 mL of OMW, 6 g of active carbon were added, and the suspension maintained for 30 min at 25 °C. Then HCl 36% was added until reaching pH = 0.5 and maintained for 2-3 h at 25 °C. Finally, pre-treated OMW was filtered with a paper filter and pH corrected to 7 adding NaOH.

2.5 Chemical and biological analyses

Cell concentration was determined by direct counting performed by a Leitz Laborlux 12 optical microscope in a 10^{-4} mL Thoma chamber (Altimari et al. 2014). Biomass dry weight, total lipids and total carbohydrates were measured on biomass obtained at the beginning and at the end of exponential state of growth, as reported in Di Caprio et al. (2015a). Nitrate concentration was measured by using ion-selective electrode (perfectION™, Mettler Toledo). Nitrogen content in biomass was determined measuring variation in nitrate concentration and assuming that all the removed nitrate was incorporated in the biomass. Proteins and nucleic acids content were estimated considering that 12% of the total intracellular nitrogen was given by nucleic acids (Becker 1994), and that 21 % is the average nitrogen content in nucleic acids. The remaining nitrogen was multiplying for 6.25 to determine protein content. Total sugars and phenol in OMW were measured as reported by Di Caprio et al. (2015a).

3. Results and discussion

3.1 Test for different nitrate concentration

Results reported in this work, make part of a project aiming to produce lipids and carbohydrates from microalgae cultivated with OMW. However, before to carry out tests with OMW, a preliminary experiment was conducted to optimize nitrate content in the medium, for two main reasons: nitrate concentration in BG11 is quite high, and a batch process should be conducted for a long period before to reach a N-starvation in our cultivation conditions; N source is the main nutrient absent in OMW, that should be added, representing a cost which should be minimized (Di Caprio et al. 2015a). Comparing the two media, it is observable that the cell concentration is the same for the first 12 days (Figure 1A), after that microalgae in MBG11 stopped to duplicate, while an increased cell concentration was observed in BG11. Nitrate were exhausted in MBG11 within the 9th day, while in BG11 there was still an abundant concentration at the end of the cultivation (Figure 1B). Time between nitrate depletion and reduced cell growth, observed for MBG11, can be explained considering a Droop model for growth (Lemesle and Mailleret, 2008) instead of a Monod model (Pagnanelli et al. 2014). After 10-12 days of cultivation, microalgae in BG11 showed a reduced rate in cell duplication, although no nitrate depletion was reached (Figure 1B). This effect was probably given by a reduced light penetration in the bottles because of the increased cell concentration (Di Caprio et al. 2016).

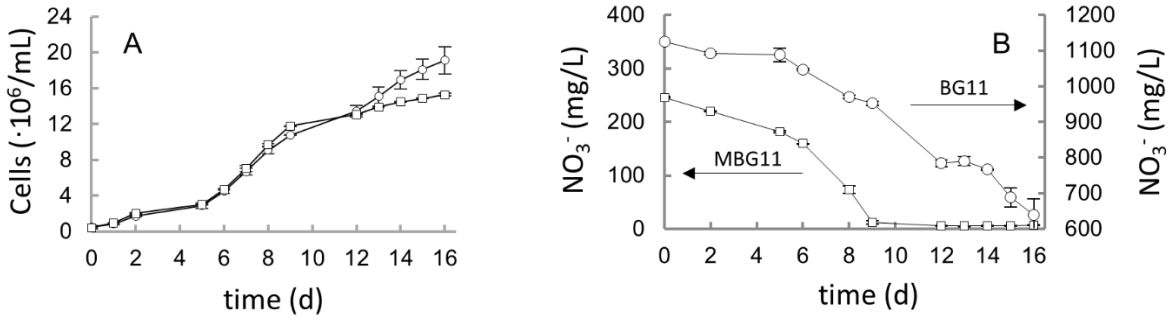

Figure 1: Evolution of cell (A) and NO_3^- (B) concentration during time for T. obliquus cultivated in phototrophic batch condition. BG11: circles, MBG11: squares). Data reported as mean ± standard deviation (n=2).

Table 1: Cell yield to consumed nitrate ($Y_{cell/nitrate}$), nitrogen cellular content (N/cell) and nitrogen quota (%) for T. obliquus cells determined during exponential growth (0-8th day) for the two different initial nitrogen concentrations. Data reported as mean ± standard deviation (n=2).

test	$Y_{cell/nitrate}$ ($\cdot 10^6$/mg)	N/cell (pg/cell)	N (%)
BG11	47 ± 16	4.0 ± 0.2	6.1 ±0.1
MBG11	48 ± 8	4.7 ± 0.8	6.3 ±0.1

Cell yield with respect to nitrate consumed was determined for both tested conditions during the phase in which there was nitrate in solution (0-8th cultivation day). The same value was found for both conditions, that was equal to about $50 \cdot 10^6$ cells produced for each mg of consumed NO_3^- (Table 1), for an average cell weight of 70 ± 10 pg/cell. Nitrogen quota and nitrogen cellular content were also determined in this phase of the cultivation, without finding any relevant difference. The obtained values agree with a previous measurement carried out on the same strain with an elemental analyzer (Di Caprio et al. 2015b), proving the validity of the assumption that all nitric nitrogen was incorporated in microalgal biomass. Considering the cell growth and the nitrogen consumption, which were equal for the two conditions, it could be deduced that microalgae grew in the same physiological state during the first 8 days of cultivation. Consequently, by using a MBG11 in place of BG11, a time reduction to reach N-starvation condition could be achieved, without affecting *T. obliquus* growth during N-replete phase.

The average cell composition was determined for biomass harvested during exponential phase of growth (0-8th cultivation day). The determined composition is reported in Figure 2. Our *T. obliquus* strain showed to be composed for 56% by carbohydrates and lipids, already in nitrogen replete condition, proving to be a good strain for lipid and carbohydrate production. Protein content that we found (34 %) is lower than those reported by other authors for the same microalgal species, which is until 50-60% (Breuer et al. 2012).

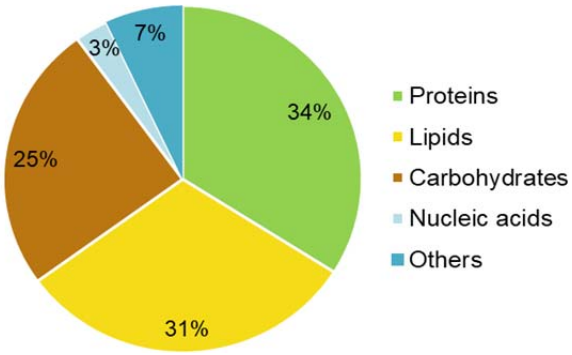

Figure 2: Average T. obliquus composition during exponential phototrophic growth.

It is possible that the difference is given by the specific strain used, or by the analytic method used (Bradford method is often used instead of measurement of N content). The remaining 7% required to close the mass balance could be imputed to residual water, mineral salts, not reducible sugars, some residual lipids and some other minor biochemical components. The low protein (and nitrogen) content found in exponential growth is a positive characteristic for production of carbohydrates and lipids by cultivation in OMW, in which there is a low amount of nitrogen available for microalgae.

3.2 Cultivation with OMW

Cell growth curves obtained for microalgae cultivated with OMW are reported in Figure 3A. It was observed that for phototrophic test and for pre-treated OMW, the same growth curve was obtained. It is possible that the positive effect inducible by the organic compounds in OMW (Table 3) as added energy source, was balanced by the negative effect given by OMW light adsorption. It is also possible that both the factors (light adsorption and organic compounds) gave too little effects to be detected. Differently, untreated OMW induced an inhibition of cell duplication after about 6 days of cultivation. Considering that the same nitrate concentration was added in all the tests, it is deducible that with untreated OMW cell stopped to duplicate before nitrogen depletion. Consequently, the main factors responsible for the inhibition could be the higher concentration of phenols and/or the darker colour (Di Caprio et al. 2015a) with respect to pre-treated OMW. Probably initial nitrate concentration can be further reduced using untreated OMW in the tested conditions, to minimize costs and avoid further pollutant in residual water. Although light was furnished, in test with untreated OMW the cultivation could be assumed heterotrophic. An indication of the heterotrophic metabolism was given by the pH measured during cultivation, that was already 11 after 2 days of cultivation for both phototrophic test and test with pre-treated OMW, while remained around 7 for untreated OMW (Figure 3B). In this latter case, the increase of pH in the first days could be a consequence of organic acids consumption (Di Caprio et al. 2015a).

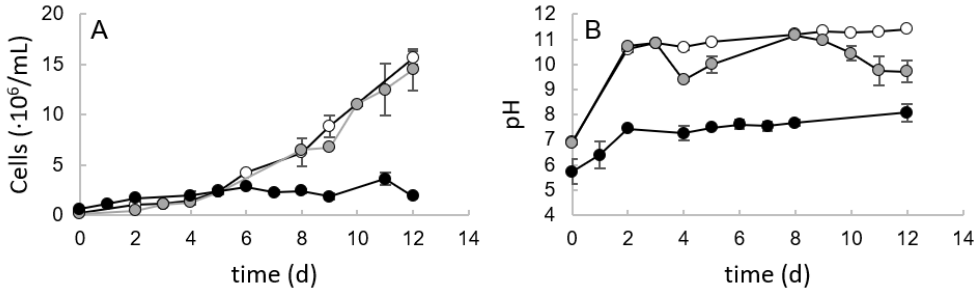

Figure 3: Evolution of cell concentration (A) and pH (B) during time for T. obliquus cultivated phototrophically (white) and mixotrophically with 9% (v/v) OMW pre-treated with active carbon (grey) and untreated OMW (black). Data reported as mean ± standard deviation (n=2).

Growth rate was measured for the three different conditions investigated, by linear regression of data plotted as $\ln(X_t)$-$\ln(X_0)$ vs time. Only data in exponential growth phase were used in such regression. While phototrophic test and test with pre-treated OMW showed the same growth rate, a relevant lower value was obtained for untreated OMW (Table 2).

Table 2: Growth rate calculated during exponential growth for microalgae cultivated phototrophically and mixotrophically with 9% (v/v) OMW pre-treated with active carbon and with untreated OMW. Data reported as mean ± standard deviation (n=2).

test	μ (d^{-1})
Phototrophic	0.45 ± 0.01
Pre-treated OMW	0.47 ± 0.02
Untreated OMW	0.31 ± 0.02

Phenols and sugar content in OMW was measured before and after each step of the different investigated phases, as indicator of the pollutant removal (Table 3). Active carbon treatment reduced 10 times phenol content and halved sugar content; consequently, the same relative difference in phenol and sugar content was found for initial concentrations in test with pre-treated OMW with respect to untreated OMW (Table 3). The lower final phenol concentration was achieved in test with pre-treated OMW, and it was equal to 10 mg/L, corresponding to 80% removal with respect to the beginning of the cultivation. Final phenol concentration was 260 mg/L for test with untreated OMW, corresponding to 48% reduction during cultivation. Instead, considering the amount of phenol removed during cultivation, the higher amount was 240 mg/L, obtained with untreated OMW, with respect to 40 mg/L obtained with pre-treated OMW. These values indicate that phenol removal observed for the two conditions was not related to *T. obliquus* cell duplication. Two hypotheses, which need further investigation, could explain this different phenol removal: 1) the bioenergetic strategy of microalgae lead to an increase in phenol degradation when there is low energy available for growth (Papazi et al. 2017); 2) bacteria growing in symbiosis with microalgae can be the main responsible for phenol removal, and they activity should be related to organic carbon concentration. Further tests in axenic conditions (for example with antibiotics) will be required to understand influence of these bacteria in phenol removal in OMW. The higher sugar removal was observed for untreated OMW as well, in which 540 mg/L were removed, corresponding to 75%, with respect to pre-treated OMW, in which 180 mg/L were removed, corresponding to 50%. For both conditions, the same final concentration was reached, indicating, as previously observed (Di Caprio et al. 2018a), that there is a fraction of non-biodegradable sugars for microalgae in OMW.

Table 3: Phenol and sugar content in culture medium containing pre-treated and untreated OMW at 9 % (v/v). Concentration before microalgae growth (t_0) and after microalgae growth (t_f) are shown. Data reported as mean ± standard deviation (n=2).

OMW	Phenols (g/L)	Sugars (g/L)
$t_{0,\ untreated}$	0.50 ± 0.01	0.71 ± 0.02
$t_{0,\ pre\text{-}treated}$	0.050 ± 0.001	0.36 ± 0.03
$t_{f,\ untreated}$	0.26 ± 0.02	0.17 ± 0.02
$t_{f,\ pre\text{-}treated}$	0.010 ± 0.001	0.18 ± 0.02

4. Conclusions

Nitrogen yield for *T. obliquus* during exponential phase of growth was determined and a modified BG11 (MBG11), with a reduced $NaNO_3$ concentration, was used for the further tests with OMW. *T. obliquus* average composition during exponential growth phase showed a lower protein content (34 %) than those reported in previous studies, indicating to be promising for lipids and carbohydrates production by cultivation in OMW. OMW pre-treated with active carbon did not lead to any negative effects on microalgae growth in the tested conditions, showing to be the most promising strategy for mixotrophic cultivation and for pollutant removal. Instead growth with untreated OMW is recommended for heterotrophic processes, because the dark colour. More studies are required to better understand the mechanisms involved in phenol degradation during microalgae growth.

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