

Microalgae Growth in Winery Wastewater under Dark Conditions

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Wine-making process leads to large amounts of wastewater. Winery wastewaters (WWW) are produced from different activities of wine production: washing, transferring and storage operations. Tank cleaning and filtration equipment are responsible for the release of the largest amount of wastewater, whose polluting power is mainly due to both the large volumes produced and the high organic load. Because of the latter problem, biological WWW treatments are particularly appropriate. Microalgae, often used to treat civil and different industrial wastewaters, are unicellular organisms that can be grown either in autotrophic or heterotrophic mode using various organic and inorganic carbon sources. Their importance is related to their high growth rate, use to produce different biofuels, use in human or animal nutrition, and extraction of chemicals and pharmaceuticals. Purposes of this work were to reduce WWW environmental impact and to find a cheap growth medium able to reduce the microalgae production costs. In this study, three different wastewaters were used, namely WWW from first (1W) and second (2W) washing tanks, and WWW from filtration apparatus (3W). They were 20:80 (v/v) diluted with Bold Basal medium and treated batchwise with a co-culture of *Arthrospira platensis* and *Chlorella vulgaris*. Microalgae were grown under dark conditions in 0.5-L flasks with continuous air supply for 15 days. Biomass concentration was quantified daily by measurements of cell dry weight and optical density at 625 nm and expressed in grams of microalgae per liter (g/L). Chemical Oxygen Demand (COD) and total polyphenols content (by the Folin-Ciocalteu method) of WWWs were quantified daily, in order to evaluate the degradation capability of the co-culture. At the end of cultures, the lipid content of microalgal biomass was also quantified. Biomass grown in the presence of WWW reached final concentrations three times higher than the control. In general, COD was reduced by more than 90 % after 15 days, polyphenols concentration was reduced by 40, 90 and 100 % in 1W, 2W and 3W, respectively, while lipid content of biomass grown in 1W and 3W increased from 7 to 11 and 15 %, respectively. In conclusion, the results of this study demonstrate that microalgae can grow efficiently under dark conditions in media enriched with WWW, hence reducing its environmental impact.

1. Introduction

Wine is produced in many countries, and in 2018 the world wine production was 279 million of hL (OIV, 2018). Wine production requires a large amount of resources (water, fertilizers and energy) and releases huge volumes of wastewater (0.5-14 L per liter of wine produced). Winery wastewaters (WWW) result from different activities of grape treatment: washing, transferring and storage operations (Ioannou et al., 2015). Tank cleaning and filtration equipment are responsible for the release of the largest amounts of wastewaters, whose polluting power is mainly due to the large volumes produced as well as the high organic load resulting from the presence of ethanol, sugars, organic acids, phenolic compounds (Malandra et al., 2003). Because of the latter problem, biological WWW treatments are particularly appropriate (Ioannou et al., 2015). There are several

studies in literature on treatment of civil (Cheah et al., 2016) and industrial (Casazza et al., 2016) wastewaters using microalgae, among them *Arthrospira platensis* and *Chlorella vulgaris* are the commonly used.

Microalgae are unicellular organisms, whose lipid, protein and carbohydrate contents depend on the species and growth conditions (Zhuang et al., 2018). Microalgae, which are attractive because of their high growth rate, find several applications in biofuels production (Brennan and Owende 2010), human or animal nutrition and production of chemicals and pharmaceuticals (Sathasivam et al., 2017). Microalgae are usually grown in phototrophic cultivation fixing the atmospheric carbon dioxide into renewable biomass by photosynthesis process (Zeng et al., 2011). However, phototrophic cultivation is light limited, which leads to low microalgae density and increases the cultivation costs (Song and Pei, 2018). To reduce them, microalgae can be grown under heterotrophic conditions in absence of light using organic carbon source in conventional photobioreactors (Pleissner and Rumpold 2018). There are several other advantages to growing microalgae under heterotrophic conditions such as: a) optimal light supply is no longer required, b) cell density is increased remarkably (from around 2 g/L in open ponds to 10 g/L) and, c) conventional industrial scale equipment can be used, ensuring a better control of pH, temperature, oxygen level and carbon source availability (Hu et al., 2018).

The purposes of this work were to grow a co-culture of *Arthrospira platensis* and *Chlorella vulgaris* under heterotrophic conditions in order to reduce the environmental impact of WWW and to find, at the same time, a cheap microalgae growth medium able to reduce the microalgae production costs. In this study, three different wastewaters were used: WWW from first (1W) and second (2W) washing tanks, and WWW from filtration apparatus (3W).

2. Materials and methods

2.1 Microalgae strains and culture condition

The co-culture of *Arthrospira platensis* UTEX 1926 (University of Texas Culture Collection, TX, USA) and *Chlorella vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK) was cultivated in Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) with continuous air supply at room temperature (25 °C) under dark conditions. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The three WWWs were provided from a winery cellar located in Piemonte region, Italy.

2.2 Experimental design

The co-culture (inoculum of 0.5 g_{DW}/L) was grown in 500-mL Erlenmeyer flask, under dark conditions, in the presence of 20:80 (v/v) WWWs/BBM for 15 days. WWWs were used without any preliminary treatment. Microalgae concentration was daily determined by dry weight (DW) measurements taking into account the suspended solid content of WWWs (g/100g_{DW}) and by optical density (OD) measurements at 625 nm using an UV-Vis spectrophotometer (Genova, Jenway, Stone, UK). All the measurements were carried out in triplicate, and cell concentration (X) was expressed in grams of dried biomass per liter of medium (g_{DW}/L). The following equation was used to relate dry mass measurements and OD readings:

$$OD = 2.9703 X + 0.0839 \quad (R^2=0.9952) \quad (1)$$

Microalgae growth (C) in absence of WWW, using BBM as culture media, was performed at the same conditions to compare it with those in presence of WWWs.

WWWs under the same above-described conditions, but without any inoculum, were used as growth controls (C1W, C2W and C3W for 1W, 2W and 3W, respectively), whose OD values were subtracted from the final ones of cultivations. After growing, biomass was recovered by centrifugation at 7500 rpm for 15 min (MF20-R, Alliance Bio Expertise, Guipry, France) and freeze dried (Alpha 1-2 LDplus, Martin Christ, Germany), while the exhausted WWWs were collected and frozen at -20°C for subsequent analyses.

2.3 Biomass and winery wastewater characterization

The lipid fraction (C_L) was extracted with a 2:1 (v/v) chloroform/methanol solution as solvent, following a modified version of the Folch method (Casazza et al., 2015).

Chemical oxygen demand (COD) and total polyphenol content (by the Folin-Ciocalteu method) (Pettinato et al. 2017) of WWWs were quantified daily, in order to evaluate the degradation efficiency of microalgae following the methodologies reported by Casazza et al. (2015).

2.4 Kinetic parameters of cultures

Kinetic parameters were evaluated for each culture.

The specific grow rate (μ), expressed in d⁻¹, was calculated by the equation:

$$\mu = \frac{1}{t} \ln \left(\frac{X_f}{X_0} \right) \quad (2)$$

where t is the overall cultivation time (d), while X_0 and X_f are the starting and final biomass concentrations ($\text{g}_{\text{DW}}/\text{L}$), respectively.

The value of μ at maximum biomass concentration (μ_{max}), also expressed in d^{-1} , was calculated by the equation:

$$\mu_{\text{max}} = \frac{1}{t_{\text{max}}} \ln \left(\frac{X_{\text{max}}}{X_0} \right) \quad (3)$$

where X_{max} is the maximum biomass concentration ($\text{g}_{\text{DW}}/\text{L}$) and t_{max} the time (d) needed to reach X_{max} .

Biomass productivity at the end of cultivation (v) and its value at X_{max} (v_{max}), both expressed in ($\text{g}_{\text{DW}}/\text{Ld}$), were calculated as follows:

$$v = \frac{X_f}{t} \quad (4)$$

$$v_{\text{max}} = \frac{X_{\text{max}}}{t} \quad (5)$$

Defining the lipid content of biomass (C_L , $\text{g}_L/100\text{g}_{\text{DW}}$) as the fraction of lipid mass referred to 100 g of dry biomass, the lipid productivity (v_L), expressed in $\text{g}_L/100\text{g}_{\text{DW}}\text{d}$, was calculated as:

$$v_L = \frac{C_L}{t} \quad (6)$$

3. Results and Discussion

3.1 Effect of the type of WWW on the co-culture growth

The growth curves of co-cultures on the different WWWs, determined by dry weight and optical density, are shown in Figures 1a and 1b, respectively. The growth curves obtained with both analytical methods exhibited almost the same trend for WWWs from the first washing tank (1W), the filtration apparatus (3W) and the control (C), while for that from the second washing tank (2W) the concentration determined with the former were always higher.

During the whole experiments, no significant changes in both dry weight and absorbance were observed for the three controls (C1W, C2W and C3W), suggesting no proliferation of autochthonous microorganisms. The co-culture grown on 1W and 2W quickly entered the exponential phase of growth (within only 2 days) and reached, at the end of the runs, biomass concentrations about 2 times higher than the control. Instead, 3W presented the same trend as the control, probably because of the low content of organic carbon source.

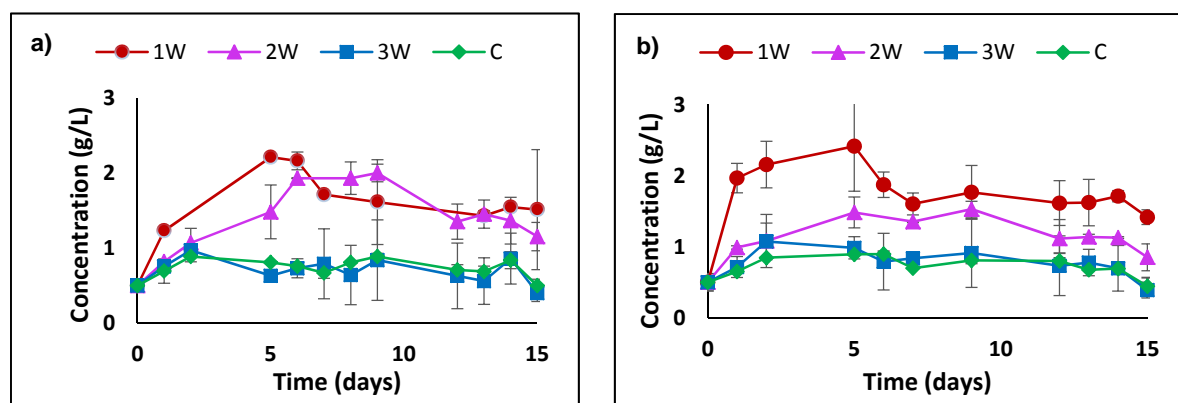


Figure 1: Microalgae growth curves obtained by a) dry weight, b) optical density.

Because of the lower standard deviation values shown in Table 1, only the main parameters of microalgae growth obtained by dry weight will be discussed herein. A maximum biomass concentration of $2.22 \pm 0.01 \text{ g}_{\text{DW}}/\text{L}$ was reached after 5 days on 1W, while a comparable value ($X_{\text{max}} = 2.01 \pm 0.12 \text{ g}_{\text{DW}}/\text{L}$) required no less than 9 days on 2W. On the other hand, it achieved only twice the starting biomass concentration either on 3W or C.

Since the specific growth rate at the end of cultures (μ) was quite low on all WWWs (0.24 ± 0.10 and 0.22 ± 0.03 d⁻¹ on 1W and 2W, respectively) because of the decrease in concentration occurred after the stationary phase, μ_{\max} values were more than twice those of μ . A different trend could be observed for biomass productivity, in that it was on 1W (0.41 ± 0.05 g_{DW}/Ld) about 41% higher than on 2W, while the lipid content of biomass increased in presence of WWWs from 7.55 ± 0.00 to 11.71 ± 0.59 , 10.15 ± 0.00 and 15.38 ± 5.89 g_{DW}/100g_{DW} on 1W, 2W and 3W, respectively.

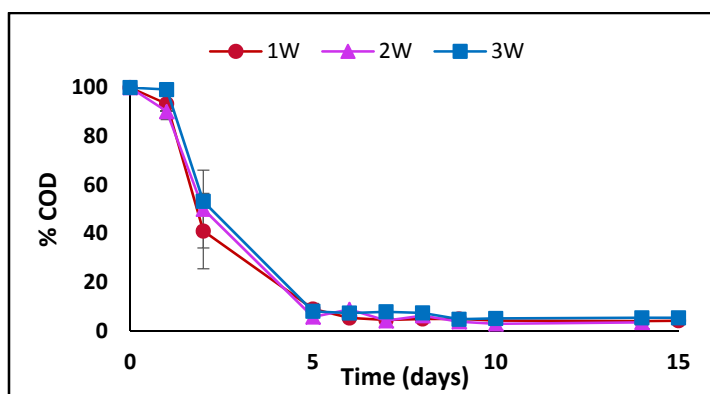
Table 1: Growth parameters and lipid and biomass productivities, determined by dry weight or optical density, in the presence of winery wastewaters from the first (1W) and second (2W) washing tanks and from the filtration apparatus (3W).

	X_f^a (g _{DW} /L)	X_{\max}^b (g _{DW} /L)	μ^c (day ⁻¹)	μ_{\max}^d (day ⁻¹)	v^e (g _{DW} /Ld)	v_{\max}^f (g _{DW} /Ld)	C_L^g (g _L /100g _{DW})	v_L^h (g _L /100g _{DW} d)
Dry weight methodology								
1W	1.51±0.80	2.22±0.01	0.07±0.04	0.24±0.10	0.10±0.05	0.41±0.05	11.71±0.59	0.01±0.00
2W	1.16±0.19	2.01±0.12	0.06±0.01	0.22±0.03	0.08±0.01	0.29±0.11	10.15±0.00	0.00±0.00
3W	0.40±0.11	0.97±0.07	-0.02±0.02	0.08±0.17	0.03±0.01	0.02±0.23	15.38±5.89	0.00±0.00
C	0.50±0.08	0.89±0.07	0.00±0.01	0.06±0.01	0.03±0.01	0.10±0.00	7.55±0.00	0.00±0.00
Optical density methodology								
1W	1.42±0.10	2.42±0.32	0.07±0.00	0.31±0.03	0.09±0.01	0.48±0.07	11.71±0.59	0.01±0.00
2W	0.85±0.11	1.53±0.27	0.04±0.01	0.14±0.00	0.06±0.01	0.27±0.11	10.15±0.00	0.00±0.00
3W	0.39±0.11	1.07±0.37	-0.02±0.02	0.08±0.16	0.03±0.01	0.02±0.24	15.38±5.89	0.00±0.00
C	0.45±0.12	0.90±0.04	-0.01±0.02	0.11±0.01	0.03±0.01	0.16±0.02	7.55±0.00	0.00±0.00

^a final biomass concentration, ^b maximum biomass concentration, ^c specific growth rate, ^d specific growth rate at X_{\max} , ^e mean biomass productivity, ^f biomass productivity at X_{\max} , ^g lipid content of biomass, ^h lipid productivity.

3.2 COD content in WWWs

The reduction of polluting power of WWWs was evaluated in terms of chemical oxygen demand (COD) decrease versus time (Figure 2). The COD curves showed the same trend in the three different WWWs, in that this parameter decreased quickly during the first 4 days of cultivation and then more slowly up to the achievement of a minimum threshold value. At the end of runs, the COD was reduced as much as 97% in all WWWs, even if its content in 1W and 2W was three times higher than in 3W. As shown in Table 2, final COD values in 2W and 3W (0.50 and 0.39 g/L, respectively) were lower than the limit concentration (0.5 g/L) allowed for discharge of WWWs into the sewer system. Such a COD removal efficiency is comparable or even better than those of conventional WWW treatment. To give only a few examples, Ioannou et al. (2015) reported values of WWW COD removal by Advanced Oxidation Processes (AOPs) from 50 to 95%, while the combination of biological processes and AOPs lead to removals in the range 30 to 96%.



*Figure 2: Efficiency of WWW Chemical Oxygen Demand removal by a co-culture of *Arthrospira platensis* and *Chlorella vulgaris*.*

In general, the COD content in WWWs, after treatments with microalgae co-culture, was four times lower than in controls (Table 2), which confirms that microalgae are able to use the organic load of WWWW as carbon source for their metabolism (Huy et al., 2018).

Table 2: COD values in WWWs and controls after 1, 7 and 15 days of heterotrophic treatment with a co-culture of *Arthrospira platensis* and *Chlorella vulgaris*.

Time (d)	1W (g/L)	2W (g/L)	3W (g/L)	C1W (g/L)	C2W (g/L)	C3W (g/L)
1	23.26±0.00	23.86±0.00	7.38±0.00	23.26±0.00	23.86±0.00	7.38±0.00
7	1.02±0.31	0.98±0.08	0.57±0.08	8.70±0.00	7.47±0.00	3.38±0.00
15	0.95±0.07	0.50±0.00	0.39±0.03	5.55±0.00	4.25±0.00	1.85±0.00

3.3 Polyphenol content in WWWs

Polyphenol content of WWWs was determined either before or after treatment with the microalgae co-culture by the Folin-Ciocalteu method. As illustrated in Figure 3, polyphenols were removed following a similar trend for the three WWWs, in that their content decreased quickly during the first 5 days of growth and then slowly achieved an almost constant minimum value. At the end of treatments, polyphenol content, expressed as gallic acid equivalents (GAE) per liter, was reduced from 0.29±0.02 to 0.13±0.01 g_{GAE}/L in 1W and to 0.08±0.00 g_{GAE}/L in 2W, while total removal occurred in 3W.

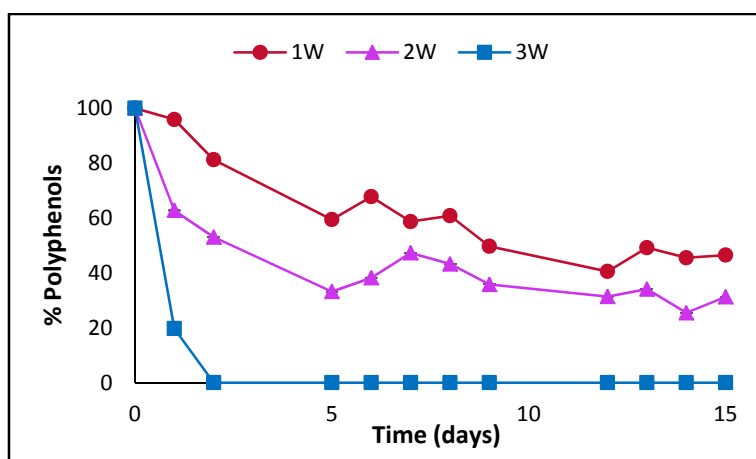


Figure 3: Time behaviour of polyphenol removal efficiency during WWWW treatment with a co-culture of *Arthrospira platensis* and *Chlorella vulgaris*

4. Conclusion

The results obtained in this study demonstrated that winery wastewater (WWW) may be used to cultivate microalgae under dark conditions. A co-culture of *Arthrospira platensis* and *Chlorella vulgaris* was able to effectively grow on WWWs from the first and second washing tanks as culture media, achieving maximum biomass concentrations after 5 and 9 days, respectively, a COD reduction as high as 95% and quite low polyphenol content. Therefore, the growth could be stopped when the maximum concentration is reached. This could give a profit in terms of specific growth rate, lipid accumulation and productivity.

The use of winery wastewater as a culture medium for microalgae growth in the absence of light could significantly reduce the microalgae production costs. The resulting microalgae biomass may then be used for biodiesel production, for extraction of value-added compounds and/or as protein-rich animal feed.

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