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Microalgae Growth using Winery Wastewater for Energetic and Environmental Purposes

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Winery wastewater (WWW), produced by winemaking activities (cleaning, transferring and storage operations), is an aqueous solution containing ethanol, organic acids, sugars, aldehydes, other microbial metabolites, soaps and detergents. Nowadays, innovative wastewater treatment processes are based on bacterial and yeast species while the role of microalgae is still unclear. *Arthrospira* (*Spirulina*) *platensis* and *Chlorella vulgaris* are unicellular prokaryotic and eukaryotic microorganisms, respectively, which can be easily grown even in non-optimal conditions. Several studies reported that the amount and quality of lipids contained in microalgal cells can differ as an outcome of changes in growth conditions or growth medium characteristics (concentration of carbon, nitrogen, phosphate, iron, etc.). In this study, we investigated the influence of different concentrations of WWW (20, 40 and 60 % v/v of the medium) on the growth and chemical composition of those photosynthetic microorganisms. Microalgae were grown into vertical glass bubblers (250 mL). The biomass concentration was quantified daily by measuring the optical density at 560 and 625 nm for *A. platensis* and *C. vulgaris*, respectively. Total Carbon and total Nitrogen concentrations, both in the media (mg/L) and in microalga biomass (g/100g), were monitored by a CHNS-O analyser. In order to quantify the influence of WWW-enrich media on the lipid concentration and composition, biomass was collected at the beginning of the stationary phase and the lipid fraction was extracted. Results suggested that the two tested microalgae can growth in media enriched with WWW and the total Nitrogen concentrations decreased up to 90 and 100 % for *A. platensis* and *C. vulgaris,* respectively. In conclusion, WWW could be successfully used for the growth of the tested microalgae, leading to a reduction of the environmental impact of this wastewater.

1. Introduction

Wine production is one of the most widespread agro-industrial process, not only in Mediterranean countries, but also in other parts of the world (e.g. Australia, Chile, United States of America and China). Even though winemaking is not consider a polluting activity, the produced wastewater, due to its organic components (sugars, organic acids, esters and polyphenolic compounds), low pH (3-4), salinity and variable nutrient content, has a strong environmental impact (Mosse et al., 2011). Winery wastewater (WWW) are originated from different activities, like washing during the pressing grapes, as well as cleaning operations of the equipments (e.g. fermenters, barrels, bottles). Many problems concerning WWW treatment derive from the large amount of produced effluent (3-5 kL per ton of crushed grapes) and from the seasonality of the production process, which lead to large amounts of wastes in a relatively short time (Kumar and Kookana, 2006). Generally, an ideal treatment system for these wastes should be based on an eco-friendly process, with low operational costs and a good degree of degradation without the need of diluting with water (Malandra et al., 2003). Taking into account that the majority of the WWW organic components are readily biodegradable, biological treatment represents a valuable alternative to the traditional and expensive chemical and physical methods. Microbial bioremediation employing bacteria (e.g. *Pseudomonas* sp., *Enterobacter* sp. and *Klebsiella* sp.) and fungi (e.g. *Penicillium* sp. and *Aspergillus* sp.) leads to a purified effluent through the

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consumption of organic substances, carbon dioxide and volatile acids (Pant and Adholeya, 2007). Usually the concentration of nitrogen and phosphate is low in this type of wastewater and addition of nutrients to improve bacterial and fungal treatments is often required (Kalyuzhnyi et al., 2001). Nevertheless, photosynthetic unicellular organisms, such as microalgae and cyanobacteria, are able to overcome this limit, due to their ability to grow easily even in non-optimal conditions, like nitrogen and phosphate deficiency (Doods et al., 1989), sodium chloride excess (Shalaby et al., 2010) and light intensity limitation (Converti et al., 2009). Several studies have focused their attention on microalgae cultured in wastewater, such as those from textile industry (Lim et al., 2010), farming activities (swine, poultry and cattle) (Markou and Georgakakis, 2011), dairy (Woertz et al., 2009) and municipality (Wang et al., 2010). The relevance of these studies is the use of wastewater as a low cost medium for the production of biomass (Olguín et al., 2001). Microalgal grown in presence of WWW leads to the removal of pollutants, as well as suitable biomass to be used as protein-rich animal feed, biofertilizer and biofuel production. The aim of this work was to establish a new WWW bioremediation method using *Arthrospira* (*Spirulina*) *platensis* and *Chlorella vulgaris*, and to assess the effect of different WWW percentage on the chemical composition of the microalgal species.

2. Materials and methods

2.1 Chemicals

Medium salts, chloroform, methanol, hexane and GC standards (methyl miristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, methyl α-linolenate, methyl γ-linolenate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl esters standard solution was prepared with hexane and stored at -20 °C until use. Winery wastewater (pH 5.2) was courteously provided by a winery industry located in Valle d'Aosta region, Italy.

2.2 Microalgae growths

Arthrospira platensis UTEX 1926 (University of Texas Culture Collection, TX, USA) and *Chlorella vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK) were grown in 250 mL-vertical glass bubbler at 80 µmol photons m^{-2} s^{-1} at room temperature (25 ± 1 °C). *A. platensis* and *C. vulgaris* were cultivated in Schlösser (Schlösser, 1982) and in Bold's Basal Medium (Bischoff and Bold, 1963), respectively, at different concentrations of WWW (20, 40 and 60 % v/v of the medium). WWW was used without any preliminary pre-treatments. Total N and C of the media are reported in Figure 2. Biomass concentration (X) was daily determined by optical density measurements at 560 (OD_{560}) and 625 nm (OD_{625}) for *A. platensis* and *C. vulgaris*, respectively, using an UV-visible spectrophotometer, model Lambda 25 (PerkinElmer, Milan, Italy). All measurements were carried out in triplicate, and the concentration X, expressed in milligrams of dried biomass per litre of medium (mg_{DB} L^{-1}), was related to the OD using the following equations:

$$OD_{560} = 0.0024X - 0.1129 \qquad (R^2 = 0.9921) \qquad (1)$$

$$OD_{625} = 4.203X \qquad (R^2 = 0.9900) \qquad (2)$$

Microalgal media and WWW (20, 40 and 60%), without any inoculum, were used as blank runs in order to quantify the growth of possible WWW autochthonous microorganisms by spectrophotometric and gravimetric analysis.The absorbance of the respective blanks at 560 and 625 nm was subtracted to the final absorbance values. After 15 days of growth, cells were separated from the medium by centrifugation at 5000 rpm for 15 min (centrifuge ALC 4226, Milan, Italy). Biomass was lyophilized using a freeze-dryer (Christ model Alpha 1-2 LD_{plus}, Osterode am Harz, Germany) for 24 h, whereas media were collected in test tubes and immediately frozen at -20 °C for later analysis.

2.3 Biomass and medium characterization

Total Carbon and Nitrogen in the media and the elemental analysis (CHNS-O) of microalga biomass were performed using a ThermoQuest FLASH EA1112 CHNS-O Elemental Analyser. The composition of carbon, hydrogen, and nitrogen (expressed as percentage) was determined at the same time in a single test, while the instrument was modified to conduct a specific test for the determination of oxygen. Burning of the sample under controlled conditions, followed by catalytic oxidation and reduction, was done for the determination of C, H, N and S. The produced gases were separated by a gas chromatography and measured using a thermal conduction detector (TCD). The runs were performed following the methodology described by Galvagno et al. (2001).

2.4 Lipid extraction and characterization

In order to recovery the lipid fraction, dried biomass was dispersed in chloroform-ethanol 2:1 v/v (with a solid/liquid ratio equal to 1:10), sonicated in an ultrasonic bath, model UTA 90 (FALC, Treviglio, Italy) for 30 min and then extracted under reflux for 5 h (Casazza et al., 2015). At the end of the extraction time, supernatants were collected by centrifuging at 7500 rpm for 10 min (centrifuge ALC PK 131 Centrifuges, Alberta, Canada) in order to remove cells and debris. All extractions were performed in duplicate. Part of lipid fraction was transesterified through the method described by Ortiz et al. (2014) and fatty acids methyl esters (FAMEs) were qualitatively and quantitatively characterized by a gas chromatograph, model 1000 (Dani Instruments, Milan, Italy) equipped with a Zebron ZB-5 column (Phenomenex, Auckland, New Zealand) and FID detector.

2.5 Growth and yield parameters

The avarage specific growth rate (μ), expressed in 1/d, was calculated by the equation:

$$\mu = \frac{1}{t} \ln\left(\frac{X_m}{X_0}\right) \quad (3)$$

where t (days) is the growth time, X_m and X_0 are the biomass concentration (g_{DB}/L) at the beginning and at the end of the culture, respectively.

The biomass productivity (v), expressed in g_{DB}/(L*day) was calculated by the equation:

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

3. Results and Discussions

3.1 Effect of WWW on *A. platensis* and *C. vulgaris* growths

Figure 1 shows the growth of *A. platensis* e *C. vulgaris* with different concentrations of winery wastewater-enriched media. No significant changes in the blanks absorbance (560 and 625 nm) and dry weight were observed during the entire experiments, suggesting non-proliferation of autochthonous microorganisms (data not shown). *A. platensis* growth had a normal trend with low concentration of WWW, while the biomass with a concentration of WWW equal to 40 and 60% tended to decrease during the first days. After a preliminary adaptation phase of about 4 days, the cyanobacterium started growing. Particularly, with a 40% of WWW, the growth rate was comparable to the ones with lower concentration of WWW and also it led to the highest value of final biomass concentration. Otherwise, *A. platensis* grew with a significant lower rate at high concentration of WWW (60%). Regarding *C. vulgaris* (Figure 1B), microalgae resulted in being more sensitive to the WWW in the medium, which was also noticeable by the decrease of the total N in the medium compared to the Bold Basal Medium with a reduction of *v* up to 73 % (Table 2). Shen et al. (2015) observed that the biomass productivity decreased significantly when nitrogen was removed from the culture system (*v* reduction near to 80 %), which indicated that the supply of nitrogen has a substantial effect on the biomass production of *C. vulgaris*. Even though during the first seven days the biomass growth was lower compared to the control but constant, there was no significant increment during the second week, with a final maximum concentration (C_{max}) that is half the control. According to Table 2, the biomass productivity for *A. platensis* grown in media enriched with 20 and 40 % of WWW was similar to the control run, while *C. vulgaris* biomass productivity was significantly lower.

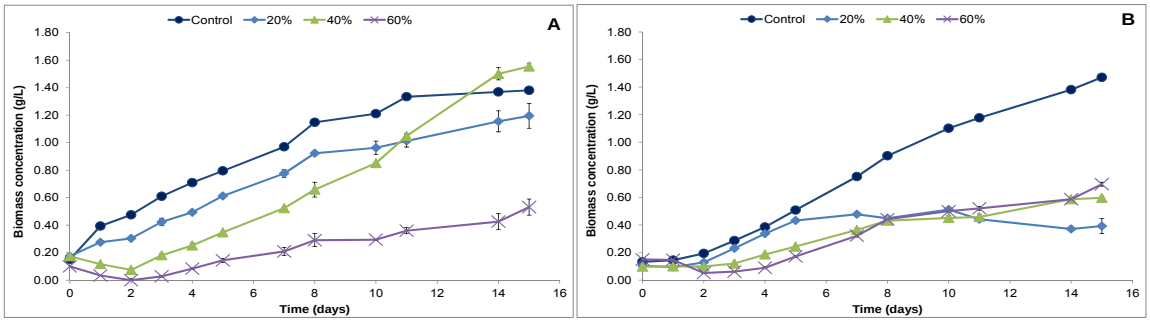

Figure 1. Cell concentration of A. platensis (A) and C. vulgaris (B) versus time during batch cultures with WWW-enriched media

Table 2. Main parameters of growth and biomass production of A. platensis and C. vulgaris at different WWW concentrations in the growth media

	Medium	μ[a] (1/d)	C_{max}[b] (g/L)	v[c] (g/L/d)
A. platensis	Control	0.148±0.006	1.38±0.03	0.092±0.002
	WWW 20%	0.129±0.004	1.20±0.09	0.080±0.006
	WWW 40%	0.147±0.004	1.55±0.02	0.103±0.002
	WWW 60%	0.111±0.007	0.55±0.06	0.035±0.004
C. vulgaris	Control	0.161±0.002	1.47±0.03	0.098±0.002
	WWW 20%	0.089±0.011	0.39±0.06	0.026±0.004
	WWW 40%	0.121±0.002	0.60±0.01	0.040±0.001
	WWW 60%	0.102±0.001	0.70±0.01	0.046±0.001

[a] *Specific growth rate;* [b] *maximum microalga concentration;* [c] *biomass productivity.*

3.2 Effect of WWW on the fatty acid methyl esters composition

The analysis of fatty acid methyl esters (Table 3) yielded high values of methyl palmitate (16:0), which represents about 60 % (w/w) of the overall FAMEs fraction in *A. platensis* and more than 40 % (w/w) in *C. vulgaris.*

In accordance to Piorreck et al. (1984), no significant variation of the amount of palmitic acid was observed as a function of the concentration of total N by adding WWW. In general, the total FAMEs content decrease adding WWW in medium both for *A. platensis* and *C. vulgaris*.

Table 3: Percentages of individual fatty acid methyl ester (FAME) over the total FAMEs (g/100 g_{FAME}) in A. platensis and C. vulgaris at different WWW concentrations in the growth media

FAME ($g/100g_{FAME}$)	*A. platensis*				*C. vulgaris*			
	Control	WWW 20%	WWW 40%	WWW 60%	Control	WWW 20%	WWW 40%	WWW 60%
14:0	1.1	-	-	-	0.4	0.3	0.4	0.4
16:0	65.6	58.6	63.7	60.5	39.4	39.8	40.0	47.3
16:1	4.8	-	-	-	5.8	9.2	12.4	1.2
Unknown 1	-	-	-	-	7.6	6.2	1.6	2.9
Unknown 2	-	-	-	-	4.0	3.4	2.1	4.6
18:0	4.9	1.4	8.3	8.6	1.5	5.3	3.4	2.5
18:1	6.8	0.9	5.8	8.6	7.3	8.2	14.0	23.3
18:2	2.2	1.5	5.7	7.8	10.8	5.4	6.9	5.5
18:3 ω3	3.0	5.2	3.2	3.0	11.9	6.7	9.3	12.3
18:3 ω6	0.7	-	0.6	-	11.2	15.4	10.0	-
Unknown 3	10.9	32.4	12.8	11.6	-	-	-	-
Total FAME ($mg_{FAME}/100g_{DB}$)	22.3	12.3	13.6	16.4	21.4	19.7	12.7	14.1

No evidence of methyl myristate (14:0) and methyl palmitoleate (16:1) has been found for *A. platensis* grown with WWW, while the same FAMEs appear in the control growth.

Only for *C. vulgaris*, the increment of WWW concentration in the Bold Basal Medium, from 20 to 60%, led to an increased content of oleic acid, from 7 % of the control run to 23 % for the run enriched with 60% of WWW. The polyunsaturated fatty acids trend of *C. vulgaris* (18:2, 18:3 ω3 and 18:3 ω6) was noticeable, which decreased with increasing WWW amount (from 34 to 18 % increasing WWW from 0 to 60 %, respectively), while *A. platensis* showed the opposite trend (from 6 to 11 % increasing WWW from 0 to 60 %, respectively).

3.3 Effect of WWW on microalgae elemental composition

Total N and C in microalgal media were analysed at the beginning of the growths (day 0), and after 8 and 16 days of growth (Figure 2).

In *A. platensis* medium, the initial total C concentration (Figure 2 A1) decreased by the addition of WWW due to the low quantity of carbon in the winery wastewater (349 mg/L) compared to the Schlösser medium (3047 mg/L). Otherwise, for *C. vulgaris*, the total C concentration (Figure 2 B1) augmented by the increasing WWW percentage with respect to BBM (16 mg/L). However, the total N concentration decreased by the addition of WWW in Schlösser (Figure 2 A2) and Bold Basal (Figure 2 B2) media.

During 16 days of growth, the total N concentration decreased up to 90 and 100 % in *A. platensis* and *C. vulgaris* media, respectively, while the total C concentration remained relatively high due to the CO_2 provided by the air bubbling.

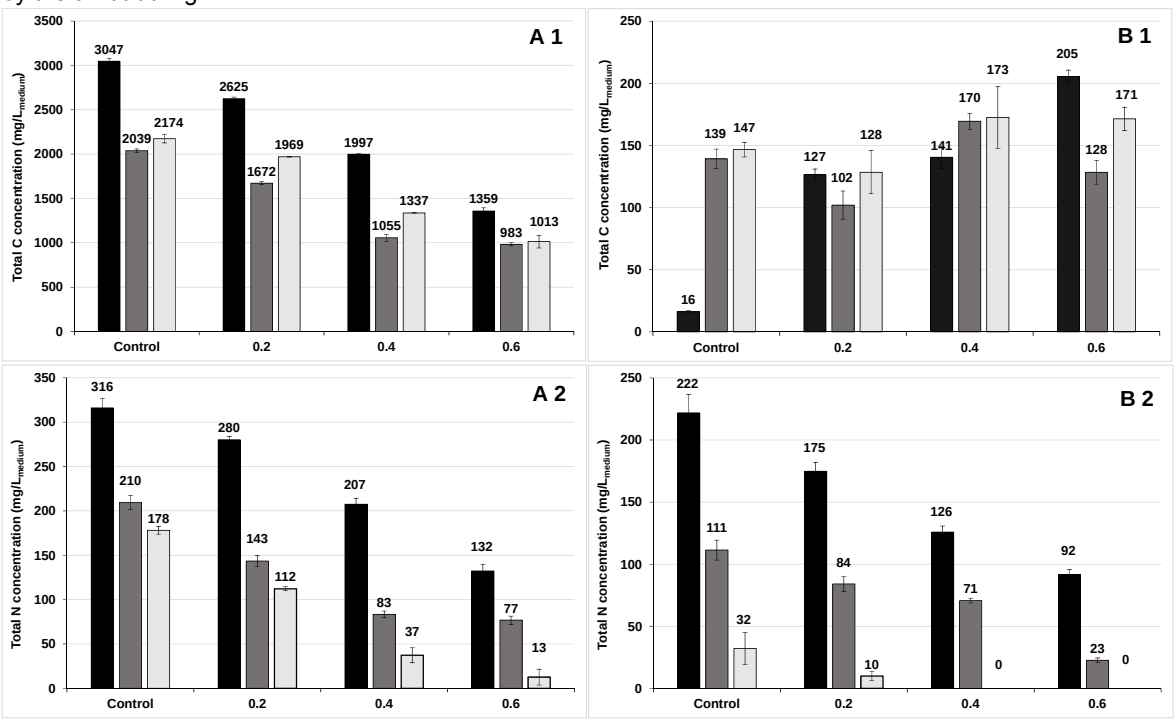

Figure 2. A. platensis total Carbon (A1) and Nitrogen (A2) and C. vulgaris total Carbon (B1) and Nitrogen (B2) concentrations in media. ■ day 0; ▒ day 8 and □ day 16.

According to Figure 3A, total Carbon composition of *A. platensis* had no significant variation depending on the medium composition, while total N resulted in decreasing with an increment of the WWW content, with a maximum corresponding to a WWW concentration of 20 %. On the contrary, Sulfur was not observed in the samples.

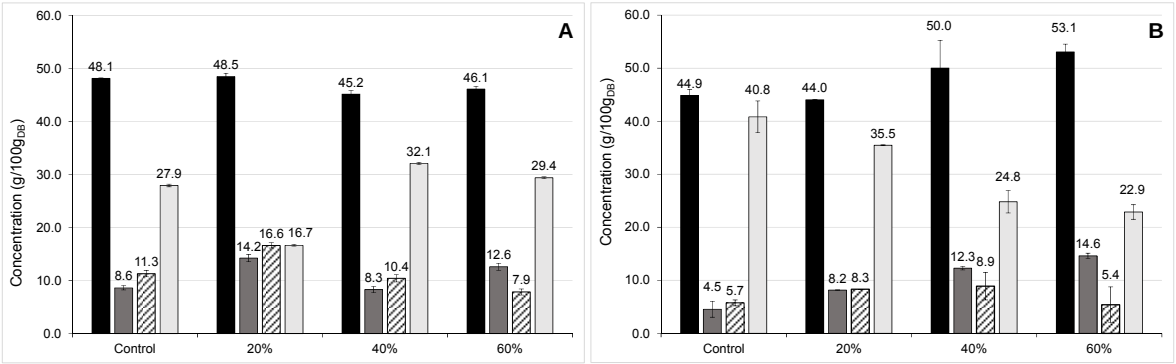

Figure 3. A. platensis (A) and C. vulgaris (B) growths in WWW-enriched media. ■ Carbon; ▒ Hydrogen; ▨ Nitrogen; □ Oxygen.

Regarding *C. vulgaris*, Oxygen content tended to decrease with higher amount of WWW (from 41 to 23 g/100g of dried biomass), while Carbon and Hydrogen content increased from 44.9 to 53.1g/100g_{DB} and from 4.5 to 14.6g/100g_{DB}, respectively.

4. Conclusions

Winery wastewater can be successfully used for the growth of microalgae. *A. platensis* in presence of medium enriched with 40% of WWW can grow similarly to the control run. The fatty acid composition, in terms of

methyl esters, and the elemental composition do not undergo substantial changes. In addition, *A. platensis* and *C. vulgaris* could be used to remove pollutant species from WWW (the total nitrogen decreased in media up to 90 and 100 % for *A. platensis* and *C. vulgaris,* respectively). These growths lead to the production of biomass, suitable to be use as protein-rich animal feed, biofertilizer and biofuel productions.

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