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On the Use of Agro-industrial Wastewaters to Promote Mixotrophic Metabolism in *Chlorella vulgaris*: Effect on FAME Profile and Biodiesel Properties

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The increase of greenhouse gases into the atmosphere, mainly due to industrialization, has affected all the ecosystems. Current worldwide living standards are still heavily dependent on non-renewable fuels. The inevitable depletion of fossil fuels and the adverse climate changes push the scientific community to seek renewable and sustainable sources of fuel. In this scenario microalgae can be potentially exploited as renewable and environmentally friendly fuel resources. Wastewaters (WW) can be used as culture media minimizing the costs associated to their cultivation. Hence, the goal of this study was to examine the effect of agro-industrial WWs rich in organic nutrients on algal lipid content and fatty acid methyl esters (FAME) profile. For this purpose, the fresh water green algae *Chlorella vulgaris* was selected. This strain is able to thrive in a wide range of WWs with high biomass productivity and to shift its metabolism from autotrophic to hetero/mixotrophic one. *C. vulgaris* was cultivated in brewery (BWW), dairy (DWW), oil mill WWs and media supplemented with sugarcane molasses. High biomass yields were obtained when *C. vulgaris* was cultivated in BWW and DWW (1.76 g L^{-1} and 1.56 g L^{-1}, respectively) compared to the control and the other WWs. The assessment of FAMEs composition (i.e. level of unsaturation) of algae cultivated under all the investigated conditions demonstrated that the former ones can be viably used as sources for producing biofuels.

1. Introduction

In the last 100 years the exponential use of fossil fuels and the development of industrialization have emitted into the atmosphere tons of carbon dioxide (CO_2) producing a sharp global increase in temperature. Human activities have generated huge amounts of greenhouse gases (GHG) producing disastrous consequences on ecosystems (Elias, 2020). Therefore, there is an urgent call at global level to seek renewable and sustainable sources of fuel (Malins, 2017). In this scenario, the scientific community emphasizes the exploitation of environmentally friendly resources. To this aim, microalgae show high productivities in terms of biomass and lipid content making them suitable for biofuels production (Soru et al., 2019, Concas et al., 2021a).
Wastewaters (WWs) typically contain large amounts of nutrients, such as carbon (C), nitrogen (N), phosphorus (P) and trace elements to sustain algal growth. It is well demonstrated the ability of microalgae to combine their growth with the biological WW treatment and biofuels production (Concas et al. 2021b, Hussain et al., 2021, Lutzu et al., 2020a). The presence inside a WW of inorganic and organic C makes some algae strains able to modulate their metabolism from autotrophic into a mixotrophic one depending on the carbon sources available. The use of food industry WWs, such as dairy (Khalaji et al., 2021), molasses sugarcane (Piaseka et al., 2017), brewery (Ferreira et al., 2019) as a nutrient medium for microalgae cultivation is well established. *Chlorella vulgaris*, one of the well-known single-celled green microalgae, can accumulate lipids and produce biodiesel

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under suitable stress conditions (Ratomski et Hawrot-Paw, 2021). This strain is also able to shift its exclusively autotrophic or heterotrophic metabolism into a mixotrophic one, leading to an increase in biomass production. The influence of mixotrophy on lipid content and FAME composition is well documented for many *Chlorella* strains (Centeno de Rosa et al., 2020). Molasses wastes are particularly rich in glucose which can enhance biomass productivity by microalgae once available in the culture medium (Yan et al., 2011). Hence, by considering the potential use of WWs as media for microalgae cultivation, such as dairy wastewater (DWW), brewery wastewater (BWW), oil wastewater (OWW), and sugarcane molasses effluent (MOL), the effect of different organic wastes from food industry on *C. vulgaris* lipid production is reported in this study. A close analysis of FAMEs profile is also assessed in order to compare its compliance to standard directives for biodiesel.

2. Material and Methods

2.1 Inoculum, culture medium and wastewater preparation

The strain used in this study, *Chlorella vulgaris* SAG 211-12, was obtained from the culture collection of algae at the University of Gottingen, Germany (SAG, 2021). Detailed chemical composition of the culture maintenance media is available on the SAG official website. The strain was maintained in 150 mL glass tubes containing the growth medium recommended by SAG at room temperature. Two 32 W white fluorescent tubes continuously provided a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1}. Inoculum was maintained in cultivation for about one week once it reached the end of exponential growth phase. WW samples were collected from brewery, dairy, oil mill and molasses sugarcane facilities located in Modena, Italy. An average range of the main chemical-physical parameters for both effluents is shown in Table 1. Once collected WWs were stored at 4° C before their use. Later they were filtered using glass filter microfiber disks (GF/C™ 47 mm diameter, Whatman, Incofar Srl, Modena, MO, Italy), deprived of solid materials and then sterilized at 121° C and 0.1 MPa for 20 min before microalgal cultivation.

2.2 Algae Cultivation

500 ml glass flasks, thereafter denominated PBRs, were used for algae cultivation. PBRs were covered with a cotton cup for air diffusion (0.03% CO_2 v v^{-1}) and daily shaken manually at room temperature. They were illuminated with a photoperiod of 12 h light/12 h dark by white fluorescent lamps providing a light intensity of 85 µmol m^{-2} s^{-1}. The initial working volume of the PBRs and cell concentration were 300 ml and 0.1 g L^{-1}, respectively. The culture medium used as control was a modified Doucha whose composition was obtained by adding to 1 L of distilled water 10 ml of five stock solutions, $NaNO_3$ (38.92_g 250_{mL}^{-1} H_2O), KH_2PO_4 (2.96_g 250_{mL}^{-1} H_2O), $MgSO_4 \cdot 7H_2O$ (2.55_g 250_{mL}^{-1} H_2O), $CaCl_2 \cdot 6H_2O$ (2.17_g 250_{mL}^{-1} H_2O), EDTA-FeNa (0.5_g 250_{mL}^{-1} H_2O), 1 ml of microelements solutions I and II, and 0.5 ml of NaOH 1 M. Microelements solution I was prepared in the following manner (mg L^{-1}): H_3BO_3 415, $MnCl_4 \cdot 4H_2O$ 1650, $ZnSO_4 \cdot 7H_2O$ 1350, $CoSO_4 \cdot 7H_2O$ 300, and $CuSO_4 \cdot 5H_2O$. For solution II (mg L^{-1}): $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 85 and NH_4VO_3 7. After two weeks of cell growth, the cultures were centrifuged at 9722 g RCF^{-1} for 10 min. The liquid phase was separated from the pellet and the latter used for fatty acids methyl esters (FAME) analysis.

2.3 Characterization of microalgae growth pattern

Microalgae growth in the culture was monitored by measuring the optical density (OD) at 680 nm. The detailed procedure adopted to monitor algal growth was reported in Lutzu et al. (2020b). The cell concentration (dry weight V^{-1}), X_{dw} (g L^{-1}), specific growth rate (µ), doubling time (t_d) calculations were performed according to the procedures reported in detail elsewhere (Zhou and Dunford, 2017). The average biomass productivity (ΔX) was expressed as:

$$\Delta X_{dw} = \frac{X_{\max} - X_0}{t_{\max} - t_0} \quad (1)$$

where the t_0 represent initial time of the cultivation period. The pH of the cultures was recorded using a pH-meter (HI 2210, Hanna Instruments, Woonsocket, RI, US).

2.4 FAMEs determination

FAMEs were prepared according to a modified protocol reported by Lage and Gentili (2018). Briefly, a toluene solution and a 1% H_2SO_4 solution in anhydrous methanol were used to re-suspend freeze-dried cells to improve the methylation of non-polar lipids and their trans-methylation, respectively. A tricosanoic acid methyl ester (TAME) ($CH_3(CH_2)_{21}COOCH_3$) in hexane was added as an internal standard. The FAMEs were then extracted

with an extractive solution (5 ml 5% NaCl + 7 ml hexane) and after phase separation, the organic phase was quantitatively analyzed by a 7820A Gas Chromatopraph (Agilent Technologies, Palo alto, CA, US) coupled to a 5977B Mass Spectrometer (Agilent Technologies Palo alto, CA, US). The system GC-MS systems (split mode 20:1, split flow 19.6 ml min^{-1}) was equipped with a low polarity Supelco SLB-5 GC capillary column (30 m x 0.25 mm x 0.25 µm). Helium was used as carrier gas. The injector and detector temperatures were set at 280 °C and 230 °C, respectively. The chromatogram was recorded in the scan mode (40-500 m z^{-1}) with a programmed temperature from 60 °C to 280 °C. The identification and quantification of individual FAMEs were performed by using a standard reference solution obtained by mixing Supelco 37 Component FAME Mix® (Sigma Aldrich, Saint Louis, MO, US), TAME internal standard solution and hexane. The content of FAMEs was calculated by manually integrating their peak areas with respect to the internal standard TAME, after calculation of the response factor (RF) using the standard reference solution. Finally, fatty acid (FA) levels were expressed as g 100 g^{-1} total FAs.

2.5 Data Analysis

All the experiments with algae and analytical tests were carried out at least in duplicate, typically in triplicate, and for all of them the mean values were reported. SAS 9.3 (SAS Institute Inc., Cary, NC, US) was used for the statistical analyses of the data. The regression equations correlating dry biomass concentration to OD, and to µ were calculated using Microsoft Office Excel program (Excel 2016 Ink, Microsoft, US).

3. Results and Discussion

3.1 *C. vulgaris* growth in agro-industrial wastewater

Agro-industrial WWs are characterized by huge amount of organic matter as demonstrated by the high BOD and COD values reported for DWW, BWW, OMW and MOL (Table 1). On the other hands, these waters are poor in N and P. To verify whether the organic load is able to enhance *C. vulgaris* biomass production a series of growth experiments were carried out using three different agro-industrial WWs and regular Doucha medium as control.

Table 1: Range of main chemical-physical parameters reported for wastewater and effluents tested

	BOD_5 (g L^{-1})	COD (g L^{1})	TSS (g L^{-1})	TN (g L^{-1})	TP (g L^{-1})	pH	Ref.
DWW	0.24-5.90	0.50-10.40	0.06-5.80	0.01-0.66	0-0.060	4.0-11.0	Turinayo, 2017
BWW	1.61-3.98	1.09-8.92	0.53-3.73	0.11-0.50	0.075-0.07	4.6-7.3	Erinan et al., 2015
MOL	1.30-4.70	0.80-3.80	1.50-9.10	0.04-0.07	0.01-0.02	3.8-4.3	Brazzale et al., 2019
OMW	35-110	40-220	-	0.60-2.10	0.15-0.30	4.0-6.0	Khdair et al., 2020

Note: DWW = dairy wastewater, BWW = brewery wastewater, MOL = molasses effluent, OMW = oil mill wastewater, BOD = Biological Oxygen Demand, COD = Chemical Oxygen Demand, TSS = Total Suspended Solids, TN = Total Nitrogen, TP = Total Phosphorous.

As it can be seen in Table 2 DWW, BWW and C+MOL greatly increased the biomass concentration (1.76 g L^{-1}, 1.56 g L^{-1}, 1.47 g L^{-1}, respectively) compared to the control (1.37 g L^{-1}), while when OMW medium was used, there was not any growth at all probably due both to the high density and the very dark colour of the solution that prevented light penetration (data not shown). The addition of molasses to the control produced a little increase both in terms of biomass production and biomass productivity. This trend was more evident with BWW and DWW. This aspect can be related to the addition to the culture medium of more C by the organic matter contained in these WWs.

Table 2: Growth characteristics of C. vulgaris cultivated in different media

Growth medium	µ (day^{-1})	t_d (day)	X_{max} (g L^{-1})	ΔX (mg L^{-1} day^{-1})
Doucha	0.195 ± 0.03	3.62 ± 0.53	1.37 ± 0.06	91 ± 0.004
BWW	0.113 ± 0.02	5.63 ± 0.06	1.76 ± 0.25	111 ± 0.02
DWW	0.141 ± 0.03	5.01 ± 0.54	1.56 ± 0.40	119 ± 0.005
C+MOL	0.215 ± 0.21	3.25 ± 0.34	1.47 ± 0.12	94 ± 0.008

Note: µ: specific growth rate, t_d: doubling time, X_{max}: maximum biomass concentration, ΔX: average biomass productivity. Doucha medium: Control, BWW: brewery wastewater, DWW: dairy wastewater, C+MOL: Control + molasses effluent

Many studies reported that *C. vulgaris* can live at lower N concentration while it is very difficult that it can survive in absence of P. In this way, P represents the limiting factor for its growth. The optimum N/P ratio for *C. vulgaris*'s growth is set as 16:1 (Wu et al. 2014). In our growth test, N/P ratios of WW are far away from this optimum. In DWW the N/P ratio is 13:1, while in BWW, OMW and C-MOL is 7:1, 7:1, and 3.5:1, respectively. Only the control is close to the optimum, with a N/P ratio of 13:1. It has been also reported that algal specific growth rate can be significantly improved by nutrient supplementation (Lutzu et al., 2020b). In our study μ was lowered in BWW and DWW, while when the control was amended with molasses μ increased. Organic sources can be used by microalgae to shift their metabolism from autotrophy to mixothophy. This can explain why *C. vulgaris*, when cultivated in BWW, DWW and MOL media, attained a better biomass concentration compared to the control alone where there are not at all organic compounds. On the other hand, the scarcity of N and P, typical of wastes rich in organic matter, leads to an imbalance between the ratios C:N:P with respect to the optimal values for algae. This would lead to an excessive intracellular storage of C in the form of neutral lipids such as triacylglycerols rather than as proteins which would require N.

3.2 FAME profile of *C. vulgaris* under agro-industrial wastewaters

The composition of FAs in terms of length and branching of the carbon chain, and degree of unsaturation is a fundamental prerequisite for considering microalgal biomass as a feedstock for biodiesel production. Therefore, the FAME profile of *C. vulgaris*, obtained after the esterification of FAs, is reported in Figure 1. Both the two organic media and the control exhibithed an high perentage of long-chain compounds C16-C18 (91.5%-95.3%). The most represented FAs for the three media were oleic (C18:1), palmitoleic (C16:0) and linoleic (C18:2) ones. In particular C18:1 in BWW and in DWW resulted almsot doubled (42%) and greatly increased (34%) compared to the control (23%), respectively. Interestingly, linolenic acid (C18:3) was the highest (13.6%) only in the control while it was absent in the two organic media. The stearic acid (C18:0) resulted higher in DWW and BWW than the control, high percentages of C16:3 were found in BWW (5.82%). On the other hand, C18:2 was reduced in DWW (13.2%) and increased in BWW (17.1%) compared to the control (15%), respectively. In terms of degree of saturation and unsaturation the unsaturated fatty acids (UFA) represented the main components of FAMEs in BWW (74%), the saturated fatty acids (SFA) in DWW (40%), the monunsaturated fatty acids (MUFA) in BWW (44%), and polyunsaturated fatty acids (PUFA) in the control (38%). The content of total SFA (25.07%), total UFA (73.92%) and C16:0 (14.48%) found in BWW for *C. vulgaris* was in agreement with those reported by Choi (2016) for the same strain cultivated in DWW (22.65%, 77.35%, and 14.32%, respectively). The high UFA/SFA ratio (2.95) was obtained when *C. vulgaris* was cultivated in BWW, being the saturation degree the lowest found for this culture medium. UFA/SFA ratio describes how SFA and UFA are distributed inside the cells. This ratio is strictly linked to the nutritional requirements of microalgae, therefore to the culture medium. Microalgal metabolism can be modulated depending on the conditions in which microalgae are grown. In particular, lipid composition in algal membrane and cytoplasm can be rearranged in terms of SFA and UFA. For example, a redistribution, which lead to an increased SFA portion, can be obtained by increasing the synthesis of neutral triglycerides at the expense of polar membrane lipids (rich in UFA) which can be partially degraded (Xin et al., 2018). This rearrangement of FAMEs can be enhanced under condition of nutrients starvation, such as those that can be found when *C. vulgaris* is grown in DWW and BWW media. One of the FAs suitable for making biodiesel is C16:0. The content of this FA remained high in DWW while decreased in the BWW suggesting that the reduced availability of macronutrients (such as N and P) in organic media compared to the control could influence the accumulation of specific FAs, such as those involved in biodiesel synthesis.

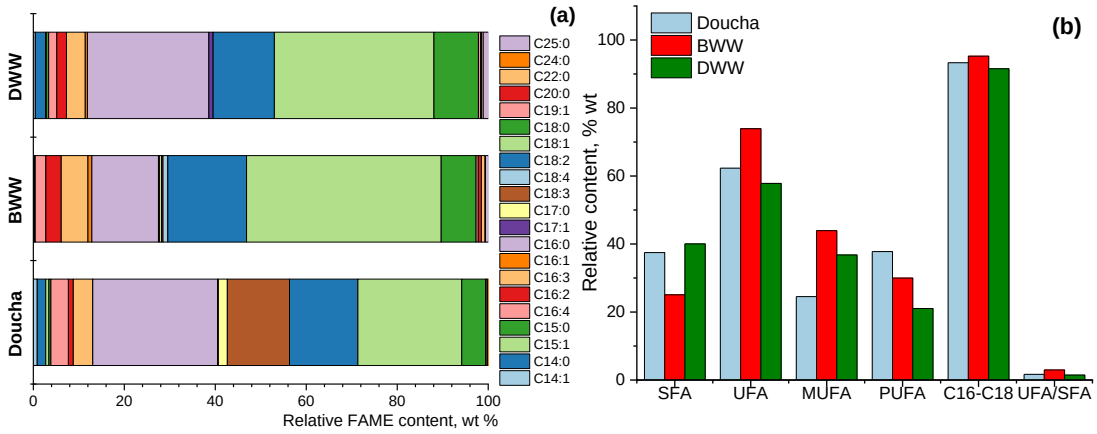

Figure 1. Fatty acids methyl ester profile (a) and general characteristics of fatty acids (b) of C.vulgaris

3.3 Biodiesel properties based on FAME profile

The possibility of using the FAMEs profile for *C. vulgaris* cultivated under organic media to evaluate the feasible production of biodiesel was further investigated by taking advantage of the software Biodiesel Analyzer© Ver. 2.2. The results of such analysis are reported in Table 3 only with reference to the parameters for which specific prescriptions of international standards existed (cf. Table 3).

Table 3. Composition of biodiesels obtainable from C. vulgaris cultivated in Doucha, BWW and, DWW

				ASTM 6751-12		EN 14214	
Parameter	Doucha	BWW	DWW	Min	Max	Min	Max
C18:3 Linolenic Acid (%wt)	13.62	0.33	0.00	-	-	-	12
Iodine Value (/)	85.16	75.48	56.44	-	-	-	120
Cetane number (/)	56.84	60.38	63.46	47	-	51	-
Oxidation Stability (hr)	6.70	9.28	11.35	6	-	8	-
Viscosity ($mm^2\ s^{-1}$)	3.19	3.30	3.38	1.9	6	3.5	5
Density ($kg\ m^{-3}$)	0.78	0.76	0.78	-	-	0.86	0.9

When *C. vulgaris* was cultivated in DWW and BWW most of all the parameter values related to the biodiesel obtainable complied with the range of values prescribed by the ASTM standards (ASTM 6751-12) for unblended biodiesel. In addition, most of the prescriptions of European regulation for quality biodiesel (EN 14214 and EN 590) were fulfilled by the biodiesel obtained using *C. vulgaris*, except for density values which were slightly lower than the prescribed ones. In fact, according to the European standard should be in the range 0.86 – 0.9 ton m^{-3}. Therefore, it can be stated that the biodiesel obtainable from *C. vulgaris* cultivated under organic media would be of particularly good quality even without blending with fossil diesel.

4. Conclusions

Two organic sources of waste have been investigated to improve biomass production and FAME profile by *C. vulgaris*. The results demonstrated that BWW and DWW media could represent a costless resource of organic nutrients able to trigger biomass production (1.76 g L^{-1} and 1.56 g L^{-1}) of this microalgal strain. As far as the FAME profile is concerned, *C. vulgaris* was able to modify its internal metabolism to achieve an improvement in terms of unsaturation based on the two organic media used. In particular, mixotrophy condition reduced the saturation of FAs by increasing the unsaturation level, with the highest MUFA and PUFA contents obtained under BWW. The final microalgae biomass, considering its FAME profile as well as its compliance with the standards for the quality of biodiesel, makes BWW and DWW viable options as priceless media for the cultivation of *C. vulgaris*.

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