Mixotrophic and Heterotrophic Metabolism in Brewery Wastewater by *Chlorella Vulgaris*: Effect on Growth, FAME Profile, and Biodiesel Properties

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Microalgae show potential as renewable and environmentally friendly fuel resources. Wastewaters (WWs) can be utilized as growth media, reducing the associated cultivation costs. Therefore, the aim of this study was to investigate how organic-rich WWs affect the growth and fatty acid methyl esters (FAME) profile of *Chlorella vulgaris*. This particular strain exhibits high biomass productivity and can thrive in a wide range of WWs. It also has the ability to shift its metabolism from autotrophic to hetero/mixotrophic. Glycerol can serve as a means to direct metabolism towards lipids production. Consequently, *C. vulgaris* was cultivated in brewery wastewater (BWW) containing varying concentrations of glycerol under both metabolic conditions. When *C. vulgaris* was cultivated in a mixotrophic environment, it achieved a notably higher biomass yield compared to heterotrophic cultivation. The highest biomass yield, reaching 1.33 g L^{-1}, was achieved utilizing 2 mL of glycerol in BWW, surpassing the control with 1.08 g L^{-1}. However, when a two-phase metabolism was applied, comprising the ten days of mixotrophy followed by the final five days in heterotrophy (MHB), the biomass yield was slightly lower than that obtained under continuous mixotrophic conditions. Nevertheless, it was still double the biomass obtained in a strictly heterotrophic environment. The FAME profile analysis revealed that, within the considered trophic conditions, the highest content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) was observed during mixotrophic cultivation with 10 mL of glycerol, mixotrophic cultivation with 4 mL of glycerol, and MHB with 4 mL of glycerol (35.36%wt, 46.89%wt, and 31.60%wt, respectively). An initial examination of the saturated and unsaturated components of the FAME suggests that lipids extracted from *C. vulgaris* biomass cultivated mixotrophically and heterorophically in BWW could potentially serve as a valuable feedstock for biodiesel production.

1. Introduction

Microalgae are currently recognized as a valuable tool for harnessing environmentally friendly resources, thanks to their high biomass and lipids content, making them well-suited for biofuels production (Concas et al., 2021a). Wastewaters (WWs) typically contain significant quantities of nutrients, including carbon (C), nitrogen (N), phosphorus (P), and trace elements, essential for supporting algal growth (Concas et al., 2021b). The ability of microalgae to effectively integrate their growth with the biological WWs treatment has been extensively demonstrated (Lutzu et al., 2020a). The presence of both inorganic and organic C within WWs allows certain algae strains to adjust their metabolism from autotrophic to mixotrophic, depending on the available carbon

sources. The utilization of WWs from the food industry, such as dairy, brewery and molasses sugarcane, as a nutrient medium for microalgae cultivation, has been firmly established (Lutzu et al., 2016, Miotti et al., 2022, Vitali et al., 2022). Furthermore, the introduction of exogenous organic C sources during heterotrophic/mixotrophic microalgae cultivation stimulates the cell's metabolic machinery, leading to enhanced lipids production (Yun et al., 2021). In this context, glycerol, a by-product of biodiesel industry, can be regarded as a metabolic stress inducer that promotes lipids production (Gaignard et al., 2021).

The microalga *Chlorella vulgaris* has the capability to accumulate lipids and produce biodiesel under appropriate stress conditions (Maltsev et al., 2023). Additionally, this strain can transition from its exclusive photoautotrophic or heterotrophic metabolism to a mixotrophic one, resulting in increased biomass production. The impact of heterotrophy and mixotrophy on lipids content and FAME composition has been extensively studied for various *Chlorella* strains (Miotti et al. 2023; Vitali et al., 2022). BWW is notably rich in sugars, with high BOD and COD values ranging from 0.1 to 100 g L^{-1}, which can significantly boost microalgae biomass productivity when present in the culture medium (Lutzu et al., 2016, Amenorfenyo et al., 2017). Therefore, in examining the potential use of BWW as medium for microalgae cultivation, this study investigates the impact of different concentration of glycerol as additional organic sources capable of inducing stress on lipids metabolism in *C. vulgaris*, affecting biomass accumulation and lipids production. The study further conducts a detailed analysis of the FAME profile, considering the addition of glycerol under heterotrophic, mixotrophic and a two-phase mixo-heterotrophic metabolism.

2. Material and Methods

2.1 Experimental setup

Chlorella vulgaris metabolic behaviour was monitored for 17 days under heterotrophy (HB) and mixotrophy (MB) condition using brewery WW (BWW) as culture medium and Doucha medium as control, respectively. A two-phase metabolic condition (MHB) was obtained cultivating *Chlorella* for the first 12 days in mixotrophy and the last 5 days in heterotrophy. For each of the three metabolic conditions (HB, MB and MHB) three replicates were run with four different concentrations of glycerol: 0 mL, 2 mL, 4 mL, and 10 mL.

2.2 Inoculums and wastewater preparation

The strain used in this study, *C. vulgaris* SAG 211-12, was obtained from the culture collection of algae at the University of Gottingen, Germany. Detailed chemical composition of the culture maintenance media is available on the SAG official website. All the details on the inoculums culture maintenance have been provided in our previous work (Miotti et al. 2023). Inoculums was cultured for about one week once it reached the end of exponential growth phase. BWW was collected from a brewery facility "Birrificio Ducale", located in Soragna (PR), Italy. An average range of the main chemical-physical parameters for this effluent is shown in Table 1. Once collected BWW was stored at 4 °C before its use. Later it was filtered using glass filter microfiber disks (GF/C^{TM} 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 microalgae cultivation.

2.3 Culture medium and algae cultivation

1 L glass flasks, referred as 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 500 mL and 0.1 g L^{-1}, respectively. The culture medium used as control was a modified Doucha whose composition is reported in our previous work (Miotti et al. 2023). 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.

2.4 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 for monitoring algal growth was reported in Lutzu et al. (2020b). The cell concentration (dry weight) X_{dw} (g L^{-1}), specific growth rate (μ), and doubling time (t_d) calculations were performed according to Zhou and Dunford (2017). The average biomass productivity (ΔX) was expressed as:

$$\Delta X_{dw} = \frac{X_{max} - X_0}{t_{max} - t_0}$$

(1)

where 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, USA).

2.5 FAMEs determination

FAMEs were prepared according to a modified protocol reported by Lage and Gentili (2018), which has been exhaustively explained in our previous work (Miotti et al. 2023).

2.6 Data Analysis

All the experiments with algae and analytical tests were carried out in triplicate, with mean values for them. MataboAnalyst 5.0, tuned by the McGills University (Montreal, Canada), 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, USA).

3. Results and Discussion

3.1 *C. vulgaris* growth in brewery wastewater supplemented with glycerol

BWWs are characterized by huge amount of organic matter as demonstrated by the high BOD and COD values reported in Table 1. On the other hand, these effluents are poor in N and P.

Table 1: Range of main physical-chemical parameters of brewery wastewaters used for algae growth

BOD_5 (g L^{-1})	COD (g L^{-1})	TSS (g L^{-1})	TN (g L^{-1})	TP (g L^{-1})	pH	Reference
-	8.0-14.0	2.9-3.0	0.08-0.28	0.02-0.09	5.2-6.2	Chen et al., 2015
2.4	6.0	-	0.09	0.005	6.3	Sinbuathong et al., 2015
1.9	2.0	2.5	-	0.025	6.9	Enitan et al., 2016
-	2.1-5.8	-	0.005	0.44	6.9	Lutzu et al., 2016

Note: BOD = Biological Oxygen Demand, COD = Chemical Oxygen Demand, TSS = Total Suspended Solids, TN = Total Nitrogen, TP = Total Phosphorous.

To assess the impact of three trophic conditions - MB, HB and MHB - on *C. vulgaris* biomass production, a series of growth experiments were conducted using BWW as culture medium, with a standard Doucha medium serving as the control. Within each trophic condition, four concentrations of glycerol were examined. Table 2 reveals that MB, especially with 2 and 4 mL of glycerol, significantly increased the biomass concentration (1.33 g L^{-1} and 1.32 g L^{-1}) compared to the control (1.08 g L^{-1}). Conversely, under MHB, only the addition of 4 mL of glycerol showed a notable increase in biomass compared to the control, with the other three concentrations showing no statistically significant differences. In HB, irrespective of glycerol supplementation, biomass accumulation was notably reduced compared to the control. Glycerol supplementation proved effective *C. vulgaris* growth under MB conditions, while its impact was negligible under HB and MHB. The μ in HB were lower than those obtained under MB conditions, approximately 3.5 time higher. Previous studies have indicated that algal μ can be significantly improved through nutrient supplementation (Lutzu et al., 2020b). In our study, μ was lower in the control, but when the brewery medium was amended with glycerol, μ increased, reaching the highest value of 0.547 day with 2 mL of glycerol in MHB. These findings align with prior research, demonstrating that mixotrophy significantly enhances *Chlorella* sp. biomass concentration and productivity in batch systems, compared to autotrophy and heterotrophy (Miotti et al., 2022, Vitali et al., 2022). A plausible explanation could be that mixotrophic cultures exhibit accelerated anabolism, attributed to adenosine triphosphate formed both in photochemical reactions during the photosynthesis and in mixotrophic reactions.

Numerous studies have highlighted the direct impact of the N:P ratio in the culture medium on the growth of *C. vulgaris*, with P identified as the limiting factor for its growth (Miotti et al., 2023). Choi and Lee (2015) investigated the influence of the N:P ratio (ranging from 5 to 70) on *C. vulgaris* cultivation in municipal WWs. They determined that the optimum N/P ratio for biomass productivity and nutrient removal varied between 5 to 20, contingent on the specific ecological conditions of the WWs. In our growth experiment, the N/P ratios of BWW under both MB and HB conditions deviate significantly from this optimum range. In contrast, only the control exhibits proximity to the optimal range with a N/P ratio of 13:1.

Table 2: Growth characteristics of C. vulgaris cultivated with brewery wastewater under different metabolic conditions

Growth medium	μ (day^{-1})	t_d (day)	X_{max} (g L^{-1})	ΔX (mg L^{-1} day^{-1})
CTRL	0.119 ± 0.013	5.81 ± 0.34	1.08 ± 0.06	82 ± 0.004
MB0	0.221 ± 0.012	3.17 ± 0.17	1.13 ± 0.02	66 ± 0.001
MB2	0.194 ± 0.007	3.57 ± 0.12	1.33 ± 0.05	78 ± 0.003
MB4	0.236 ± 0.01	2.94 ± 0.13	1.32 ± 0.01	78 ± 0.001
MB10	0.162 ± 0.039	4.48 ± 1.25	1.11 ± 0.01	65 ± 0.001
HB0	0.166 ± 0.002	4.17 ± 0.04	0.63 ± 0.01	42 ± 0.001
HB2	0.146 ± 0.009	4.75 ± 0.29	0.73 ± 0.01	46 ± 0.001
HB4	0.139 ± 0.008	5.01 ± 0.27	0.79 ± 0.02	49 ± 0.001
HB10	0.144 ± 0.013	4.83 ± 0.43	0.57 ± 0.01	38 ± 0.001
MHB0	0.516 ± 0.027	1.35 ± 0.07	1.04 ± 0.02	61 ± 0.001
MHB2	0.571 ± 0.077	1.23 ± 0.17	1.06 ± 0.03	63 ± 0.002
MHB4	0.547 ± 0.066	1.28 ± 0.15	1.14 ± 0.11	67 ± 0.007
MHB10	0.376 ± 0.019	1.84 ± 0.09	1.05 ± 0.06	66 ± 0.004

Note: μ: specific growth rate, t_d: doubling time, X_{max}: maximum biomass concentration, ΔX: average biomass productivity. CTRL: Doucha medium, MB: mixotrophy in BWW, HB: heterotrophy in BWW, MHB: mixo-hetrotrophy in BWW. 0 mL, 2 mL, 4 mL, and 10 mL represent the amount of glycerol added to the media

Microalgae can utilize organic sources to shift from autotrophy to mixothophy. This phenomenon may elucidate why *C. vulgaris*, when cultivated in BWW, achieved a superior biomass concentration compared to the control, which lacks organic compounds. However, the scarcity of N and P, typical of wastes abundant in organic matter (Table 1), results in an imbalance in the C:N:P ratios compared to the optimal values for algae. This imbalance could 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 (Gao et al., 2016).

3.2 FAME profile of *C. vulgaris* under mixotrophy and heterotrophy mode

The composition of FAs, including the length, branching of the carbon chain, and degree of unsaturation, is a crucial factor to consider when evaluating microalgal biomass as a potential feedstock for biodiesel production. Thus, the FAME profile of C. vulgaris, obtained through the esterification of FAs, is presented in Fig 1. FAME derived from MHB and HB exhibited higher percentages of long-chain compounds C16-C18 (94-97%wt and 96%wt, respectively) compared to those obtained under the control (93%wt) and MB (86-93%wt) (Fig 1b). The predominant FAs in MB were oleic (C18:1cis) > palmitic (C16:0) > linoleic (C18:2) > stearic (C18:0); in HB, they were C16:0 > C18:1cis > C18:1trans (elaidic acid) > C18:0 > C18:2; and in MHB, they were C18:1 > C16:0 > C18:2 > C16:3 (ω-3 palmitolinolenic) FA (Fig. 1a). Examining the impact of glycerol addition across the three trophic systems, it is observed that in MB, there were no statistically significant differences in the range 0-10 mL of glycerol, except for 4 mL of glycerol, which resulted in a % increase of C18:1. In HB, the addition of glycerol incrased the % of C18:4 (stearidonic FA) by more than 10 times and gradually increased the % of C18:1cis, while reducing that of C18:1trans. On the other hand, the presence of glycerol in MHB did not produce significant changes in the % of FAs. Remarkably, the addition of glycerol led to a nearly 50% reuction in the % of C18:0 in MB, HB and MHB, while substantially increasing the % of C16:0 in all the three trophic systems. When considering the degree of saturation and unsaturation (Fig 1b), glycerol addition resuted in an increase in unsaturated fatty acids (UFA) in all the systems - MB (64.63-67.42), HB (68.63-72.70%), and MHB (67.79-72.77%) - compared to the control (63.06%wt). Coversely, saturated fatty acids (SFA) decreased in MB (32.58-35.36%wt), HB (27.30-31.36%wt), and MHB (27.23-32.20%wt) compared to the control (36.94%wt). The highest % of UFA were observed in MHB with 10 mL of glycerol (72.77%wt), while the highest % of SFA, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were found in MB with 10 mL of glycerol (35.36%wt), MB with 4 mL of glycerol (46.89%wt), and HB with 10 mL of glycerol (36.22%wt), respectively. When C. vulgaris was cultivated in MB and MHB with 2 and 10 mL of glycerol, the PUFA/SFA ratio exceeded 1. The highest PUFA/SFA ratio (1.28) was achieved when C. vulgaris was cultivated in HB with 10 mL the of glycerol, compared to the control (0.97). The PUFA/SFA ratio characterizes the distribution of SFA and UFA within cells. This ratio is closely tied to the nutritional requirements of microalgae, thus reflecting the composition of the culture medium. Microalgal metabolism can be adjusted based on the conditions in which microalgae are cultivated.

Specifically, the lipids composition in algal membranes and cytoplasm can be reorganized in terms of SFA and UFA. For instance, a shift leading to an increased SFA portion can be achieved by upregulating the synthesis of neutral triglycerides at the expense of polar membrane lipids (rich in UFA), which may undergo partial degradation (Xin et al., 2018). This rearrangement of FAMEs can be accentuated under conditions of nutrients starvation, such as those encountered when *C. vulgaris* is cultured in BWW. C16:0 is one of the FAs suitable for biodiesel production. The content of this specific FA increased by 6-7 times in all trophic systems, both with and without glycerol. This suggests that the accumulation of C16:0 could be influenced by the reduced availability of macronutrients (such as N and P) in organic media compared to the control.

Oxidizability (OX), allylic position equivalent (APE), and bis-allylic position equivalent (BAPE) indices collectively contribute to the calculation of oxidation stability index (OSI). Following the methodology outlined by Pinto et al. (2021), an OSI can be generated through the FAMEs profile of microalgae. A high OSI value for an oil indicates stability, signifying its potential use for biodiesel production without the need for additional antioxidants during the OSI period. This period denotes the time until the quality of the oil/biodiesel remains unchanged, and biodiesel must be fully utilized for engine operation. According to the ASTM standard, oils can be classified as best, moderate, and poor. The best oils are characterized with an OSI ≥ 3 h, indicating stability and negating the need for added antioxidants. Interestingly, in all trophic conditions investigated in this study (MB, HB, and MHB), both with and without glycerol, the OSI values obtained for *C. vulgaris* through these indexes were > 3.90, except for the control (3.89) (data not shown). These values surpass 3.87, which is the OSI reported by Kumar and Sharma (2015) for the best-performing microalgae investigated (*Scenedesmus obliquus*). The heightened level of unsaturation, the high % of C16-C18 and C16:0 FAs, along with the elevated OSI index, suggest that biodiesel derived from *C. vulgaris* cultivated in BWW with the addition of glycerol could be well-suited for biodiesel production.
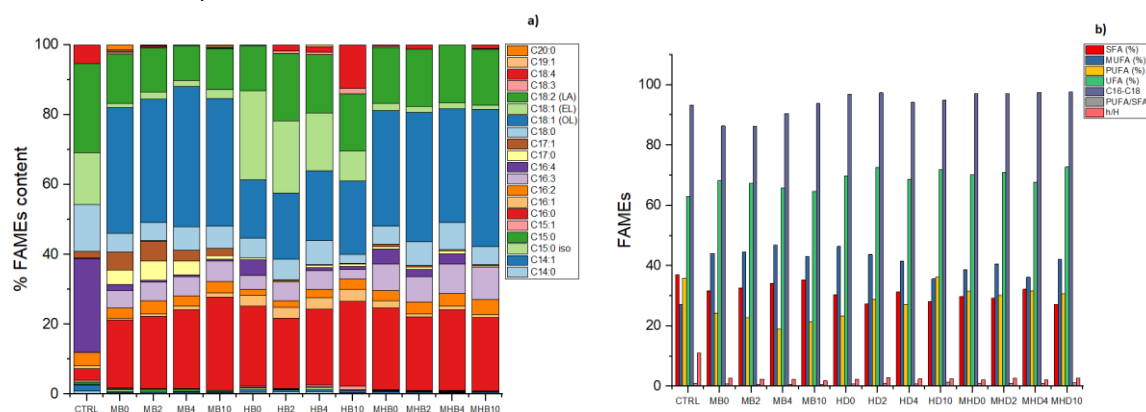

Figure 1. Fatty acids methyl ester profile (a) and total fatty acids (b) of C. vulgaris when cultivated under three different trophic conditions (MB, HB, MHB) in the presence of glycerol and in the control (CTRL)

4. Conclusions

The study investigated BWW as an organic waste source to enhance biomass production and FAME profiles in *C. vulgaris* with the addition of glycerol under three different trophic modes. The findings underscored that BWW could serve as a cost-effective source of organic nutrients, effectively stimulating microalgal growth. Furthermore, biomass production saw improvements with increasing glycerol content, particularly under mixotrophic conditions. Regarding the FAME profile, *C. vulgaris* showcased its capacity to adjust internal metabolism, achieving enhanced unsaturation levels based on the trophic conditions employed. Specifically, the addition of glycerol under MHB conditions reduced FAs saturation while elevating unsaturation levels, resulting in higher MUFA and PUFA compared to the control. The obtained microalgal biomass from BWW cultivation thus emerges as a viable and cost-efficient option. Considering the FAME profile, particularly in terms of unsaturation levels, microalgal biomass obtained through mixotrophic cultivation of *C. vulgaris* could potentially serve as promising feedstock for biodiesel production.

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