Effect of Mixotrophy on Lipid Content and Fatty Acids Methyl Esters Profile by Chromochloris zofingiensis Grown in Media Containing Sugarcane Molasses

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Corresponding Author:	Gianni Lutzu, PhD Teregroup Srl Modena, MO ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Teregroup Srl
Corresponding Author's Secondary Institution:	
First Author:	Lorenzo Vitali, Ms.
First Author Secondary Information:	
Order of Authors:	Lorenzo Vitali, Ms.
	Veronica Lolli, PhD
	Francesco Sansone, Professor
	Alessandro Concas, Professor
	Giovanni Antonio Lutzu, PhD
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Abstract:	The effect of of sugarcane molasses on lipid content and fatty acids methyl ester (FAME) profile by Chromochloris zofingiensis is investigated in this work. For this purpose, the strain has been cultivated under mixotrophic conditions in a medium amended with specific concentrations of molasses (0.5 g/L, 1 g/L, and 2 g/L) able to sustain microalgae growth. Better biomass concentration, lipid content and lipid productivity (1.6 g/L, 38%wt and 286 mg/L/day) than the control (1.32 g/L, 16%wt, and 139 mg/L/day) were obtained with 2 g/L of molasses. The highest value of the total lipid content (42%wt) was reached with 1 g/L of molasses. FAME profile revealed a 97% composition in C16-C18 with no statistically meaningful differences among the three concentrations of molasses tested. The most represented fatty acids were C18:1 oleic (> 38%wt), C18:2 linoleic (> 21%wt), and C16:0 palmitic (> 14%wt). When using 2 g/L of molasses unsaturated fatty acids reached the highest portion (77%) than the control (61%). Specifically, all FAMEs have been quantitatively and qualitatively analyzed in order to improve biodiesel properties. Main characteristics of biodiesel obtainable from the algal lipids appeared in compliance with ASTM standards for unblended biodiesel and fulfilled European regulation (EN 14214 and EN 590) for the quality of biodiesel. Owing the compliance of the corresponding biodiesel characteristics with international standards, a profitable biodiesel can be obtained under mixotrophic conditions triggered by the use of a food industry waste

Effect of mixotrophy on lipid content and fatty acids methyl esters profile by *Chromochloris zofingiensis* grown in media containing sugarcane molasses

Lorenzo Vitali[1], Veronica Lolli[2], Francesco Sansone[1], Alessandro Concas[3,4], Giovanni Antonio Lutzu[5*]

[1]Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 17/A, 43124 Parma (PR), Italy.

[2]Department of Food and Drug, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma (PR), Italy

[3]Department of Mechanical, Chemical and Materials Engineering, University of Cagliari, Piazza d'Armi, 09123 Cagliari (CA), Italy

[4]Interdepartmental Center of Environmental Science and Engineering (CINSA), University of Cagliari, via San Giorgio 12, 09124 Cagliari, Italy

[5]Teregroup Srl, via David Livingstone 37, 41122, Modena (MO), Italy

* Corresponding author: Giovanni Antonio Lutzu

E-mail: gianni.lutzu@teregroup.net

Tel: +39 0594823699

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Abstract

The effect of ~~mixotrophy~~ of sugarcane molasses on lipid content and fatty acids methyl ester (FAME) profile by ~~*Chlorella*~~ *Chromochloris zofingiensis* is investigated in this work. For this purpose, ~~growth media containing~~ the strain has been cultivated under mixotrophic conditions in a medium amended with specific concentrations of ~~sugar cane~~ molasses (0.5 g/L, 1 g/L, and 2 g/L) able~~, are used~~ to sustain microalgae growth. ~~Lipids have been extracted from microalgae and then characterized~~. Better biomass concentration, lipid content and lipid productivity (1.6 g/L, 38%wt and 286 mg/L/day) than the control (1.32 g/L, 16%wt, and 139 mg/L/day) were obtained with 2 g/L of molasses. The highest value of the total lipid content (42%wt) was reached with 1 g/L of molasses. FAME profile revealed a 97% composition in C16-C18 with no statistically meaningful differences among the three concentrations of molasses tested. The most represented fatty acids were C18:1 oleic (> 38%wt), C18:2 linoleic (> 21%wt), and C16:0 palmitic (> 14%wt). When using 2 g/L of molasses unsaturated fatty acids reached the highest portion (77%) than the control (61%). Specifically, all FAMEs have been quantitatively and qualitatively analyzed in order to improve biodiesel properties. ~~Results showed that using specific concentrations of molasses the total lipid content increased up to 20% compared to the control.~~ Main characteristics of biodiesel obtainable from the algal lipids appeared in compliance with ASTM standards for unblended biodiesel and fulfilled European regulation (EN 14214 and EN 590) for the quality of biodiesel ~~are also evaluated~~. ~~Based on the biomass and lipid productivity achieved during the experiments as well as on the compliance of the~~

~~corresponding biodiesel characteristics with its international standards, it can be stated that molasses waste can be seen as is a viable and cheap option as a cheap medium for the cultivation of *C. zofingiensis*.~~ ~~Furthermore,~~ Owing the compliance of the corresponding biodiesel characteristics with international standards, a profitable biodiesel can be obtained under mixotrophic conditions triggered by the use of a food industry waste

Keywords: *Chromochloris zofingiensis*, sugarcane molasses, mixotrophy, lipids ~~content~~, fatty acids, biodiesel properties

1. Introduction

In the last 250 years, the emissions of carbon dioxide (CO_2) driven by the development of industrialization and the consequent intensive use of fossil fuels have been ~~increasing~~ exponentially raising, producing a sharp global temperature increase ~~in temperature~~ [1]. The above premises pushed the world scientific community to seek renewable and sustainable sources of fuel that could substitute the fossil ones [2]. An environmental friendly option that has been employed to solve the soaring demand of fuels is the biodiesel obtainable from plant biomass. Nowadays most of the worldwide biodiesel production relies on food crops (1st generation biofuels) and lignocellulosic biomass (2nd generation biofuels). Both are today considered not sustainable for ethical and economic reasons due to the 'food *vs* fuel' debate and because of the high costs for both pretreatment methods and conversion of biomass into biofuels [3]. Owing to high

productivities in terms of biomass and lipids ~~content make~~ microalgae [4] and cyanobacteria [5] are recognized as suitable feedstock for 3rd generation biofuels production [6, 7]. ~~In this scenario, liquid biofuels can be potentially produced by the exploitation of microalgae as renewable and environmentally friendly resources.~~ However, the full development of microalgal mass-production cultivation systems is still constrained by economic and technical issues [8].

Nutrients and water supply for microalgal cultivation represents one of the main cost-contributory factors for the full exploitation of mass cultivation systems [9, 10]. This last drawback could be tackled through the exploitation of inexpensive and abundant wastewater (WW) resources, which typically contain large amounts of nutrients, such as carbon (C), nitrogen (N), phosphorus (P) and trace elements that are capable to sustain the algal growth. An extensive literature review demonstrates how microalgae can combine the~~ir~~ growth with the biological WW treatment and biofuels production [11, 12]. In addition, some algae strains can modulate the~~ir~~ cell metabolism depending on the culture medium ~~in which they~~ used for the growth and the available C sources ~~available~~.

To the genera *Chlorella* belong several strains that have been commercially exploited for the production of human food, animal feed, agro-fertilizers, bioplastics and biofuels [13]. The cultivation under photoautrophic, heterotrophic or mixotrophic growth mode has been reported for *C. vulgaris* [14], *C. sorokiniana* [15], *C. prototechoides* [16], and *C. zofingensis* [17]. Heterotrophic nutrition employs organic substrates as C and energy sources, eliminating the requirement for light provision.

Mixotrophic cultivation allows microalgae to photosynthesize as well as assimilate and metabolize organic C simultaneously reducing the dependency on light penetration, enabling in this way the formation of higher cell densities as compared to photoautotrophy [18]. Heterotrophic cultivation improves biomass productivity compared to photoautotrophic conditions even if scientific reports demonstrate how under mixotrophic cultivation biomass productivity is greatly enhanced compared to heterotrophy [19]. However, it should be pointed out that to make economically favorable a heterotrophic/mixotrophic cultivation the external organic C source should be cheap and easily available.

Th~~is~~ possibility to use organic sources opens up interesting scenarios for ~~their~~ algae exploitation at industrial level using WWs rich in organic matter as growth medium [20]. This depends both on the specific industrial sector producing the WWs and the capability of the specific strain of thriving in such non-ideal growth media. The use of food industry WWs as a nutrient medium for microalgae cultivation is well established. In the last years, many studies have addressed the exploitation of dairy WW [21], vinasse and molasses from sugarcane and sugar beet [22, 23], brewery [11] and vinegar WW [24]. Industrial waste sugars can be used to replace other sugars or alcohols (i.e glucose, glycerol), which are expensive and whose production is not industrially scalable. From a nutritional point of view, sugarcane molasses is particularly suitable for heterotrophic [25] and mixotrophic [26] cultivation of microalgae. Sugarcane molasses is characterized by dark brown color, extensive odor and a very high content of organic matter in terms of Biological Oxygen Demand (BOD) and Chemical

Oxygen Demand (COD). The presence of total sugars (mainly 29% of sucrose, 12% of glucose and 13% of fructose), water, crude proteins and fats, heavy metals as well as vitamins makes molasses wastes suitable to boost microalgae growth [27].

~~Regarding the strains, one valid candidate for this purpose is *Chlorella*~~ *Chromochoris zofingensis* (formerly known as *Chlorella zofingensis*), is a single-celled green microalgae belonging to the class of Chlorophyceae [28]. Beside the production of astaxanthin [29], in the last years *C. zofingiensis* has emerged as a potential ~~producer~~ accumulator of triacylglycerides (TAGs) under multiple trophic conditions ~~of astaxanthin and lipid accumulator to produce biodiesel under suitable stress conditions [18].~~ TAGs are a class of lipids suitable for biodiesel production [30]. It is reported that in this strain the total lipids can account for 65.8%wt of the total biomass [31] while the quantity of TAGs can significantly increase up to 40%wt [32]. This strain tends to accumulate a high level of TAGs under abiotic stresses such as N deprivation, sulfur deprivation, salinity stress, and high light [33].

The choice to cultivate *C. zofingiensis* could be of great interest for several reasons: a) a biochemical aspect that makes this strain particularly appealing is the ability to shift its exclusively photoautotrophic or heterotrophic metabolism into a mixotrophic one, with the result of an increased biomass production due to the sum of the two processes [34]. Since this strain is capable of thriving above the three mentioned trophic modes, the use of an organic waste as culture medium represents a valid strategy to increase biomass production [35]; b) experimental advantages due to the easiness of cultivation and fast growth rate [36]; c) the concurrent accumulation of TAGs and astaxanthin

enables this microalga an ideal cell factory for integrated production of the two compounds and has the potential to improve algae-based production economics [37].

The influence of mixotrophy on lipid content and FAME composition is well documented for many *Chlorella* strains [38]. Beside the abundance of organic C, one of the features of molasses waste is the relative low content of N and P that makes this effluent particularly suitable to accumulate TAGs [30]. Many studies deal with the effect of sugarcane molasses on lipids and FAME profile by different microalgae strains such as *Chlorella* [39], *Scenedesmus* [40], *Dunaliella* [41], *Monoraphidium* [27], *Micractinium* [42]. On the other hand, as far as *C. zofingensis* is concerned this aspect has been assessed only under heterotrophic mode [43].

Hence, by keeping in mind the economic importance of exploring a fast-growing strategy for *C. zofingiensi*s and by considering the potential use of WWs as media for microalgae cultivation, the effect of different concentrations of sugarcane molasses on lipid productivity and FAMEs composition of this strain ~~*C. zofingensis*~~ under mixotrophic cultivaton is investigated for the first time in this work. ~~In fact, literature works so far focused only on the total lipid production while neglecting the effect of sugarcane molasses on FAMEs composition [23].~~ A quantitatively and qualitatively close analysis of FAMEs profile is thus assessed in order to compare its compliance to standard directives for biodiesel ~~for the first time~~ production.

2. Materials and methods

2.1 Inoculum and culture medium preparation

The strain ~~*Chlorella*~~ *Chromochloris zofingiensis* UTEX32 used for this study was obtained from the culture collection of algae at the University of Texas, Austin, USA [~~24~~ 44]. The cell cultures were maintained and cultivated in standard Bold Basal Medium (BBM), whose composition was obtained by adding 10 mL of six stock solutions to 1 L of distilled water as follows: $NaNO_3$ (10_g 400_{mL}^{-1} H_2O), KH_2PO_4 (7_g 400_{mL}^{-1} H_2O), K_2HPO_4 x $3H_2O$ (3_g 400_{mL}^{-1} H_2O), $MgSO_4$ x $7H_2O$ (3_g 400_{mL}^{-1} H_2O), $CaCl_2$ x $2H_2O$ (1_g 400_{mL}^{-1} H_2O), NaCl (1_g 400_{mL}^{-1} H_2O). After autoclaving (Model 760, ASAL, Cernusco s/N, MI, Italy), 1 mL of three different vitamins stock solution and 6 mL of PIV metal solution were added. The vitamin stock solutions were prepared as follows: thiamine 0.1_g 100_{mL}^{-1} H_2O, biotin $25 \times 10^{-6}_g$ 100_{mL}^{-1} H_2O, and vitamin B12 $15 \times 10^{-6}_g$ 100_{mL}^{-1} H_2O. PIV metal solution was prepared in the following manner (mg/L): $EDTANa_2$ 750, $FeCl_3$ x $6H_2O$ 97, $MnCl_4x4H_2O$ 41, $ZnCl_2$ 5, $CoCl_2$ x $6H_2O$ 2, and Na_2MoO_4 x $2H_2O$ 4.

Four 150 mL Erlenmeyer flasks were filled with 50 mL of BBM medium, inoculated with approximately 10 mL of microalgae, closed with a cotton cup and continuously illuminated at room temperature by white fluorescent lamps (Model T8 36 W IP20, CMI, Germany) providing a light intensity of 50 µmol/m^2/s measured with a luxmeter (Model HD 2302.0, Delta OHM, Padua, PD, Italy). Inoculum was maintained in cultivation for about one week once it reached the end of exponential growth phase.

2.2 Cultivation conditions and experimental setup

C. zofingiensis was cultivated in 1 L flasks (thereafter named PBRs). The PBRs, with

a working volume of 500 mL, were covered with a cotton cup for air diffusion (0.03% CO_2 v/v) and daily shaken manually at room temperature. The PBRs were illuminated with a photoperiod of 12 h light/12 h dark by white fluorescent lamps providing a light intensity of 50 µmol/m^2/s. A filtered mix of CO_2 and compressed air (2%-98% v/v), provided by a cylinder (Agrifer Srl, Modena, MO, Italy) and air pump (GIS Air Compressor, Carpi, MO, Italy), respectively, was supplied through a perforated rubber stopper to the column.

Molasses was added to the control medium Bold Basal Medium (BBM) as reported in the experimental setup shown in Table 1. Specifically, three different concentrations of molasses were tested as follows: 0.5 g/L (MOL1), 1 g/L (MOL2), and 2 g/L (MOL3), respectively. Two series of experiments were performed to evaluate growth, biomass production, total lipid content as well as FAME of *C. zofingiensis* according to the experiment setup (Table 1). Microalgae growth was monitored for 21 consecutive days through optical density (OD) and biomass concentration (g/L). After the cultivation, the final dry weight, the total lipid content and the FAME profile were obtained. In all the experiments the initial concentration of the inoculum was 0.1 g/L.

2.3 WW collection and characterization

The sugarcane molasses was collected from a sugar factory in Carugate, MI, Italy. An average range of the main chemical-physical parameters for molasses is shown in Table 2. Once collected the molasses was stored at 4 ºC (fridge FKv Liebherr, Incofar Srl, Modena, MO, Italy) before its use. Later it was filtered (filtration unit Sartorius, Incofar Srl, Modena, MO, Italy) 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 ºC for 20 min (Autoclave model 760, Asal Srl, Cernusco sul Naviglio, MI, Italy) prior to microalgal cultivation.

2.4 Cell growth and dry weight determination

C. zofingiensis growth was monitored by measuring the absorbance (ABS) of the culture at 720 nm by a spectrophotometer (model ONDA V30 SCAN – UV VIS, ZetaLab, Padua, PD, Italy) for 21 consecutive days. A regression equation describing the relationship between dried biomass concentration and ABS was also calculated. Dry biomass concentration was evaluated gravimetrically as follows: a) a known quantity (5-10 mL) of culture (V) was drawn from the PBRs, b) the sample was passed through a pre-weighted (W_1) glass microfiber filter (GF/C™ 55 mm diameter, Whatman, Incofar Srl., Modena, MO, Italy), and after filtration the biomass retained on the filter was dried at 105 °C in a forced-air oven (model 30, Memmert Gmbh, Scwabach, Germany) overnight to a constant weight (W_2), c) the filter paper was previously dried ~~in a forced-air oven (model 30, Memmert Gmbh, Scwabach, Germany)~~ at 105 °C for 2 h and then cooled to room temperature in a desiccator (CDL, Incofar Srl, Modena, MO, Italy) and weighed using an analytical scale (model M, Bel Engineering Srl, Monza, MI, Italy).

The cell concentration (dry weight), X_{dw} (g/L), was calculated using the following equation:

$$X_{dw} = \frac{W_2 - W_1}{V} \quad (1)$$

where, W is the "weight" (g) of dried algal biomass, and V is the "volume" (L) of the

algae culture used for the test.

The biomass productivity (ΔX) was expressed as:

$$\Delta X_{dw} = \frac{X_{\max} - X_0}{t_{\max} - t_0} \quad (2)$$

where, max X_{max} is the "maximum biomass" (g/L) obtained at (t_{max}).

The specific growth rate (μ) was calculated according to the following equation:

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (3)$$

where, X_2 and X_1 are the "dry biomass concentration" (g/L) at time t_2 and t_1, respectively.

The pH of culture suspensions was measured by a pH meter (model HI 2210, Hanna Instruments, Woonsocket, RI, USA).

2.5 Total lipid content determination

The total cell lipid content was measured according to a modified version of the classical gravimetric method Bligh and Dyer [~~26~~ 49], tuned by Chen et al. [~~27~~ 50]. After the growth, cell culture was collected and centrifuged (Model 2560 Nakita, Auxilab S.L., Berain, Spain) at 6500 rpm ~~g~~ for about 5 min, the sediments collected and freeze-dried at -80 ºC (Forma Ultra-Low Temperature Chest Freezer MODEL RELEASE – 20, Thermo Fisher Scientific, Marietta, OH, USA) for about 24 h. Before the extraction, the biomass was lyophilized (Lio5P, Cinquepascal Srl, Trezzano s/N, MI, Italy). About 50 mg (m) of lyophilized biomass were placed in a 50 mL falcon tube (FT) where a 7.5 mL of chloroform/methanol mixture (1:2 v/v), called extractive solution I, was added. Th~~is~~ sample was ~~solution was then carefully~~

~~transferred to a 50 mL centrifuge tube and~~ put in a shaking table ~~at 60 ºC for about 1 h~~ (model WB-FM FALC Instruments Srl, Treviglio, BG, Italy) at 60 ºC for about 1 h. Subsequently, it ~~The mixture~~ was centrifuged at ~~65~~9000 rpm ~~g~~ for ~~about 5~~ 10 min and the supernatant was transferred to a new 50 mL centrifuge tube.~~, then t~~The sediment was extracted again with ~~another~~ 7.5 mL of extractive solution I for about 30 min~~utes~~ and subjected to the same treatment. ~~The combined supernatant so obtained was added to a~~ 14 mL of extractive solution II (5 mL of chloroform + 9 mL of aqueous 1% NaCl ~~sodium chloride~~ solution) was added to the combined supernatant to get a final volume ratio of about 2:2:1.8 (chloroform/methanol/water). After vigorous manual shaking, ~~T~~the mixture was ~~subsequently extracted and~~ centrifuged at ~~65~~9000 rpm ~~g~~ for ~~about~~ 5 min allowing a clear separation of the fats containing phase. ~~Then, the chloroform phase~~ This was transferred to a pre-weighted glass tube (m_0, mg), evaporated under vacuum at rotavapor (Büchi rotary evaporator Model R-200, Büchi Labortechnik AG, Switzerland) ~~blow-dried under N_2 flow gas~~ at 60 ºC, completely dried at a high vacuum line ~~(HGC-244-HA, China) for 90 minutes~~ and finally weighted (m_1, mg). The percentage (%) of the lipid content (TL) was calculated by multiplying the obtained value of total lipid content (g/g biomass) by 100 as follows:

$$TL\left[wt\%\right] = \left(\frac{m_1 - m_0}{m}\right)\cdot 100 \quad (4)$$

The lipid productivity (LP) was calculated as follows:

$$LP\left[g\ L^{-1}day^{-1}\right] = TL\cdot\Delta X_{dw} \quad (5)$$

2.6 FAMEs determination

FAMEs were prepared according to a modified protocol reported by Lage and Gentili

[~~28~~ 51]. Briefly, freeze-dried cells were re-suspended in 1 mL of toluene to improve the methylation of non-polar lipids and they were trans-methylated with 1% H_2SO_4 in anhydrous methanol at 80 °C for 2 h under ~~a slow flow of~~ N_2 atmosphere. After cooling, 80 µL of tricosanoic acid methyl ester ($CH_3(CH_2)_{21}COOCH_3$, ≥99.0% (GC)) (Sigma Aldrich, St. Louis, MO, USA) at 2 mg/mL in hexane were added as an internal standard. The FAMEs were then extracted with 12 mL of an extractive solution (5 mL aqueous 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, USA) coupled to a 5977B Mass Spectrometer (Agilent Technologies Palo Alto, CA, USA). The ~~system~~ GC-MS system~~s~~ (split mode 20:1, split flow 19.6 mL/min) 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) 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. It consisted of 50 µL of Supelco 37 Component FAME Mix® (Sigma Aldrich, Saint Louis, MO, USA) at 10 mg/mL added to 40 µL of tricosanoic acid methyl ester internal standard solution at 2 mg/mL and 410 of hexane.

The content of FAMEs was calculated by manually integrating the~~ir~~ peak areas with respect to the internal standard tricosanoic acid methyl ester, after calculation of the

response factor (RF) using the standard reference solution. Finally, fatty acid (FA) levels were expressed as g/100 g total FAs.

The RF, which expresses the relative content of each FA presented in a percentage of total FA, was derived by the following equation:

$$RF[/] = \frac{m_s A_i}{m_i A_s} \quad (6)$$

where m_s is the "mass of internal standard" (mg), A_i is the "peak area of section i", m_i is the "weight of sample" (mg) and A_s is the "area of standard".

2.7 Estimation of fuel biodiesel properties

The analysis of the fuel properties of the biodiesel obtainable from the extracted lipids was performed based on the FAME composition. Kinematic viscosity (υ), density (ρ), high heating value (HHV), saponification value (SV), iodine value (IV), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF), cold filter plugging point (CFPP), and oxidative stability (OS) were obtained according to the formulas reported in a recent publication [3]. Cloud Point (CP), pour point (PP), allylic position equivalent (APE), and bis-allylic position equivalent (BAPE) were calculated through the software Biodiesel Analyzer© Ver. 2.2. [52]

2.8 Data analysis

Each experimental condition was investigated in triplicate. Statistical analysis on biomass and lipid content, specific growth rate, lipid productivity and FAME profile was performed using MetaboAnalysts 5.0 platform tuned by the McGill University, Montreal, Canada [53]. The difference amongst the groups was statistically analyzed

by using the one-way analysis of variance (ANOVA) followed by Tukey's honestly significance different (HSD) test. Variables were reported as significant at 95% confidence (probability limit of 0.05). ~~Analysis of the fuel properties of the biodiesel obtainable from the extracted lipids was performed based on the FAME composition through the software Biodiesel Analyzer© Ver. 2.2.~~

3. Results and discussion

3.1 Influence of mixotrophy on C. zofingensis growth and lipid content

To verify whether organic source could enhance biomass production and lipid content in *C. zofingensis* a first series of experiments with three sugarcane molasses concentrations (0.5 g/L, 1 g/L and 2 g/L, respectively) were carried out using the BBM medium as control. The inoculums represented 1/3 of the total volume. Molasses water media are easily susceptible of bacteria contamination considering the fact the inoculums seed culture usually is not totally aseptic. Before to run the growth tests with molasses a few tests have been run with different ratios of inoculums and molasses concentrations. It has been noticed that lower inoculums concentration extended the lag phase increasing the risk of bacteria proliferation (based on the molasses concentration investigated in this work). On the other hand, higher inoculums concentration allows microalgae to take over on bacteria when high molasses concentrations are used. After few preliminary growth tests it was found that 1/3 ratio in volume between inoculums and culture medium allowed to run the growth experiments on molasses for three weeks avoiding the risk of bacteria proliferation.

Time evolution of the ~~optical density~~ OD at 720 nm of cultures with different molasses concentrations is shown in Figure 1. It can be clearly observed that the culture MOL3 showed the better growth achieving the highest OD~~720~~ at the end of cultivation (~2.8). A similar trend was also reported for the cultivation of *C. vulgaris* in sugarcane molasses under the same range of concentrations tested in this study (0.5-2 g/L) [26]. In terms of biomass concentration this value corresponded to about 1.6 g/L (cf. Figure 2), that is higher compared to the control (1.32 g/L). On the other hand, when the other two molasses concentrations were used, a slight decrease could be observed. From Figure 2 it can also be observed that, the use of molasses determined almost a doubling of the total lipid content, 42%wt (MOL2), 40%wt (MOL1), and 38%wt (MOL3), respectively, with respect to the control (16-20%wt) meaning that mixotrophy resulted in a boosting of the lipid metabolism. Given the high biomass concentration, the best compromise in terms of total lipid concentration in the PBRs was still represented by MOL3. The increase in sugarcane molasses concentration resulted in an enhanced lipid content for other *Chlorella* strains. In *C. vulgaris* 40.15% of lipid content in the control group achieved a 46.12%, 57.15%, and 62.85% when cultivated with 1 g/L, 2 g/L, and 4 g/L, respectively [26]. *Chlorella sorokiniana* BTA 9031 exhibited a lipid content of 30% when cultivated mixotrophically with molasses which was the double of what obtained when cultivated photoautotrophically [54]. It is known that different organic substrates found in wastes can stimulate distinct metabolic pathways for lipid and FAs synthesis. For instance, it has been demonstrated that when *Scendesmus* sp. was grown in an organic waste rich in glucose under mixotrophic and heterotrophic conditions at

least three metabolic routes, among which the pentose phosphate pathway (PPP), the Embden-Meyerhof-Parnas pathway (EMP) and the Entner-Doudoroff pathway (ED) were involved, with PPP preferred during heterotrophy and EMP employed in mixotrophy [57]. The same effect has been experimentally confirmed using glucose for *C. pyrenoidosa* [56]. Owing to the close phylogenetic relationship between *Scenedesmus* sp. and *Chlorella* sp. (both are Chlorophyceae) and considering that molasses is mainly composed of sucrose and glucose as main sugars it is safe to assume that the also in *C. zofingensis* EMP route could be employed for the synthesis of lipids under mixotrophic cultivation. In addition, it should be highlighted that the enzymes involved in the lipids metabolic pathways become more active in the incidence of light energy and in the presence of glucose, which are two conditions typical of mixotrophy [57].

In Figure 3a the specific growth rates evaluated for the four experiments are shown. It can be observed that, while the addition of 0.5 and 1 g/L of molasses resulted in a slight decrease of the growth rate when compared to the control (0.136 day^{-1}), an increase of growth rate (0.170 day^{-1}) was observed when 2 g/L of molasses was used.

The lipid productivity was greatly enhanced when the strain was cultivated in MOL3 compared to the control~~,~~ (286 mg/L/day and 139 mg/L/day, respectively, ~~(~~Figure 3b). This was due to both the increase in biomass productivity and lipid content achieved when using 2 g/L of molasses. However, MOL1 and MOL2 did not produce an evident increase in biomass concentration compared to the control (Figure 3b).

Microalgae able to use organic sources can shift the~~ir~~ metabolism from

photoautotrophy to mixotrophy. As the discussion so far has confirmed, over a specific threshold of molasses concentration the shifting form photoautotrophic to mixotrophic metabolism can be advantageous in terms of biomass productivity and lipid content resulting in a relevant increase of lipid productivity. This can explain why *C. zofingensis*, cultivated in MOL3 medium, attained a better biomass concentration compared to BBM 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 ratios with respect to the optimal values for algae. This leads to an intracellular excess of C which is stored in the form of neutral lipids such as ~~triacyclglycerols (~~TGA~~s)~~ rather than as proteins which would require N. As depicted in Figure 2, there was an evident increase in lipid content in MOL media ~~which~~ that can be explained by considering that there is a ~~the~~ wide gap in terms of C, N and P concentrations due to the low values of N and P found in ~~between~~ BBM (2.5 g/L and 0.75 g/L, respectively) and the high content of C in molasses (cf. BOD and COD in Table 2) ~~MOL (0.055 g/L and 0.015 g/L, respectively).~~ Similar findings have been reported for *C. vulgaris* grown in dairy ~~wastewater~~WWs [58] and *Scendesmus dimorphus* cultivated in brewery WW [11].

During a nutritional unbalance, such as that one triggered by the use of MOL, N depletion appears to be the predominant force which drives the redistribution of C content towards lipid production stimulating the glycolytic metabolism and reducing the expression of enzymatic components involved in the Krebs cycle. This leads to an accumulation of Acetyl-CoA which is conveyed in the lipid synthesis [59]. In this light,

the enhancement of lipid productivity when using MOL (Figure 3b) was probably due to the nutritional deficiency typical of these media, compared to the BBM, which resulted particularly favorable to lipid metabolism leading MOL culture to enter in a state of metabolic stress earlier than the control.

3.2 Influence of mixotrophy ~~and chemical stress~~ on FAME profile

The structure of lipids and the~~ir~~ composition in terms of fatty acids (FAs), such as C chain length, branching of the chain, and degree of unsaturation, should be as much as possible similar to the ones required to determine the quality of biodiesel. This represents a fundamental prerequisite for considering microalgal biomass as a suitable feedstock for biodiesel production [60]. Therefore, FAs were esterified to obtain the FAME profile of *C. zofingensis* as reported in Figure 4.

As it can be noticed, in all the media including the control long-chain compounds with 16 and 18 C atoms are the main FAs with no statistically meaningful differences among ~~them~~ the media ($p > 0.05$). The most represented FAs were oleic (C18:1), linoleic (C18:2), ~~and~~ palmitic (C16:0), and stearic (C18:0) acids. In particular C18:2 in all the three MOL media resulted more than doubled while C16:0 appeared almost halved compared to the control, respectively. It is also interesting to note that oleic acid (C18:1) was the highest in MOL media. The relative high content of C18:2 was almost the same as reported for other *Chlorella* strains when cultivated in dairy WW while C16:0 and C18:1 were lower and higher, respectively [61].

The FAMEs composition depends on the microalgal growth conditions and the predominance of main FAs such as C16:0, C18:0, C18:1 and C18:2 determines the

good quality of a diesel. The trend of main FAs found in this study (C18:1 > C18:2 > C16:0 > C18:0) was in agreement with those reported for the mixotrophic cultivation of *C. vulgaris* and *C. sorokiniana* on dairy effluents [62], and of *Chlorella* sp. and *Micractinium reisseri* cultivated mixotrophycally in sugarcane bagasse [63] and molasses [42], respectively. Changes in the composition of FAs with enhanced production of C16:0, C18:1, and C18:2 by shifting from photoautotrophy to heterotrophy or mixotrophy after exposing microalgae to stress and C enrichment has been reported also for the heterotrophic cultivation of *Chlorella minutissima* [64].

An interesting aspect is the relatively high content of linoleic acid (C18:2) which was almost doubled compared to the control. In the standard medium BBM this FA attained a value of 14%wt, while when cultivated with 0.5-1 g/L of molasses it reached ca 21%wt increasing up to 24%wt with 2 g/L of molasses. Beside the fact that the high content of this FA increases the whole level of unsaturation, C18:2 is also present in most of the vegetable oils suggested in diets rich in omega-3 FAs. Once assumed, C18:2 is converted to gamma-linolenic acid (GLA) in the body which can then break down further to arachidonic acid (ARA). The last one is necessary for the function of all cells, especially in nervous system, skeletal muscle, and immune system [65]. Therefore, *C. zofingensis* biomass grown in molasses can be potentially exploited as feedstock not only for biodiesel production but also for nutraceutical and pharmacological application.

In terms of degree of saturation and unsaturation the unsaturated fatty acids (UFA) represented the main components of FAMEs for all the culture media (Figure 5). The

percentage of total saturated fatty acids (SFA), UFA, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) did not show a significant difference among the three MOL media while they statistically differ ($p > 0.05$) if compared to the control. In particular, it was found out an inversely proportional relationship between total SFA and UFA, especially PUFA, when *C. zofingensis* was cultivated in MOL compared to the control, with a predominance of SFA on PUFA as confirmed also by a decrease in UFA/SFA ratio (Figure 5). The same trend in terms of SFA reduction and UFA increment was reported for the mixotrophic cultivation of *Dunaliella salina* in molasses waste compared to the control F/2 medium [41]. The distribution between saturated and unsaturated FAs inside the cells (represented by the UFA/SFA ratio) is related to the nutritional requirements of microalgae, which depends on the culture medium. In particular conditions microalgae can modulate ~~their~~ cells lipid composition with a rearrangement of saturated and unsaturated compounds. For example, a common way used to increase the SFA portion inside the cell is to increase the synthesis of neutral triglycerides at the expense of polar membrane lipids (rich in UFA) which can be partially degraded [~~34~~ 66]. This metabolic rearrangement of FAMEs can take place when microalgae are grown under condition of nutrients starvation, such as those that can be found in MOL media. C16:0 is suitable for making biodiesel. Therefore, the oil-rich *C. zofingensis* ~~C~~ C would possess high potential as a feedstock for biodiesel synthesis.

3.3 Analysis of biodiesel properties based on FAME profile

The analysis of FAME profile allows determining whether the lipidic fraction of algal

biomass guarantees oxidative stability and ignition quality of the biodiesel, which are two of the main fuel properties able to greatly affect biodiesel quality. These two parameters are positively influenced by the presence of long chain fatty acid (C16–18), high content of C18:1 and the degree of unsaturation [~~35–~~67]. As it can be seen in Figure 5, beside the control, more than 96% of *C. zofingensis* total FAs were represented by C16–C18 groups, C18:1 was 39.46% (MOL2), and UFA were in the range 75~~.34~~% (MOL1) - 77~~.11~~% (MOL3).

The possibility of using the extracted lipids for producing biodiesel was further evaluated on the basis of the FAME profile. In particular, by relying on suitable mathematical relationships, implemented in the software Biodiesel analyzer, the compliance of the resulting biodiesel to the international standards is assessed. This analyzer allows evaluating the relevant characteristics of biodiesel that would be obtained by trans-esterification of the concerned algal lipids. The obtained results are summarized in Table 3.

All the parameter values related to the biodiesel obtainable comply with the range of values prescribed by the ASTM standards for unblended biodiesel. In addition, most of the prescriptions of European regulation for quality biodiesel (EN 14214 and EN 590) are fulfilled by the biodiesel obtained using the culture media investigated.

According to the ASTM standards the cetane number (CN) should be characterized by a minimum value of 40, as it is reported for all the media where *C. zofingensis* was cultivated. Beside CN, other parameters that should be taken into consideration to guarantee biodiesel quality are cloud point (CP), pour point (MP), lubricity (L),

viscosity (υ), and density (ρ). In particular, CP and PP should be the lowest as possible, as found for MOL media. This condition is obtained when in FAME profile the content of UFA is high, as for MOL media (Figure 5). The reduction of PP depends on the high content of PUFA C18:2, as in particular for DWW and MOL3. Viscosity and density (in ~~red~~blue-bold in Table 3) were slightly lower than the prescribed ones. In fact, according to the European standard they should be in the range 3.5 – 5 $mm^2 s^{-1}$ and 0.86 – 0.9 ton m^{-3}, respectively. However, it can be observed that the difference with the prescribed values is very small and might be easily adjusted by mixing the biodiesel with specific additives.

Conclusion

~~Organic sources of waste have been investigated to improve lipid productivity and FAME profile by *C. zofingensis*.~~ ~~The results~~ This work demonstrate~~ds~~ that ~~MOL media~~ molasses WW could represent a cheap resource capable to trigger mixotrophic growth ~~lipid productivity~~ of *C. zofingensis* and boost the lipid productivity of this ~~microalgal~~ strain. ~~The use of these WWs would also lead to a further benefit related to their partial treatment. In fact, this WW allowed a relevant increase in lipid content.~~ The increase of lipid content is probably due to the high amount of C internalized by the cells in the presence of molasses and the consequent storage of the excess C in the form of lipids. For specific concentrations of molasses ~~the case of MOL3~~ even a significant increase of biomass productivity was observed, ~~that~~ along with the increase in lipid content ~~resulted in an advantageous augmentation of lipid productivity~~. As far as the FAME

profile ~~is concerned~~, it is affected by the use of molasses since it leads to a statistically significant increase of UFA and in particular of PUFA ones ~~*C. zofingensis* was able to modify its internal metabolism to achieve an improvement in lipid production based on the organic medium used.~~ The assessment of FAMEs composition of algae cultivated under all the investigated conditions demonstrated that the former ones can be viably used as sources for producing biofuels with characteristics very close to the ones required by the relevant standards for the quality of biodiesel.

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Authors contribution

Vitali L, Sansone F, Lutzu GA, and Concas A contributed to the study conception and

design. Material preparation and data collection was performed by Vitali L and Lutzu GA, analysis were performed by Vitali L, Lolli V and Sansone F. The first draft of the manuscript was written by Lutzu GA and Concas A and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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Figure captions

Fig 1 Time evolution of the optical density at 720 nm of cultures with different molasses concentrations. CTRL is the control medium BBM, MOL1 is the medium with the addition of 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2

g/L molasses.

Fig 2 Biomass (green bar) and lipid concentrations (red bars) achieved at the end of cultivation in the reactor. The numbers in white represent the percentage of lipid weight in dry biomass. CTRL is the control medium BBM, MOL1 is the medium with the addition of 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2 g/L molasses. (*) $p < 0.05$ test T.

Fig 3 Specific growth rates (a) and lipid productivities (b) obtained using different concentrations of molasses in BBM (CTRL). MOL1 is the medium with the addition of 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2 g/L molasses. (*) $p < 0.05$ test T.

Fig 4 Fatty acids detected in *C. zofingensis* grown in different concentrations of molasses. CTRL is the control medium BBM, MOL1 is the medium with the addition of 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2 g/L molasses. (*) $p < 0.05$ test T.

Fig 5 Main categories of FAMEs under different concentrations of molasses. CTRL is the control medium BBM, MOL1 is the medium with the addition of 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2 g/L molasses. (*) $p < 0.05$ test T.

Highlights

Sugarcane molasses can be used by *Chromochloris zofingiensis* to produce lipids.

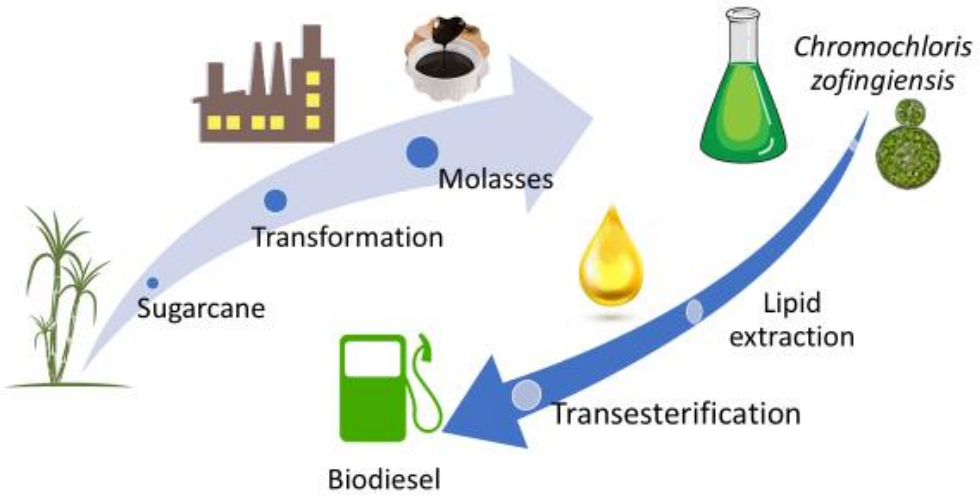
Molasses enhanced better cell growth than control medium.

Molasses triggered biomass and lipid productivity better than control medium.

Internal cell metabolism was modified by molasses to improve lipid production.

Saturation and unsaturation levels in FAMEs were directly influenced by molasses

Graphical abstract



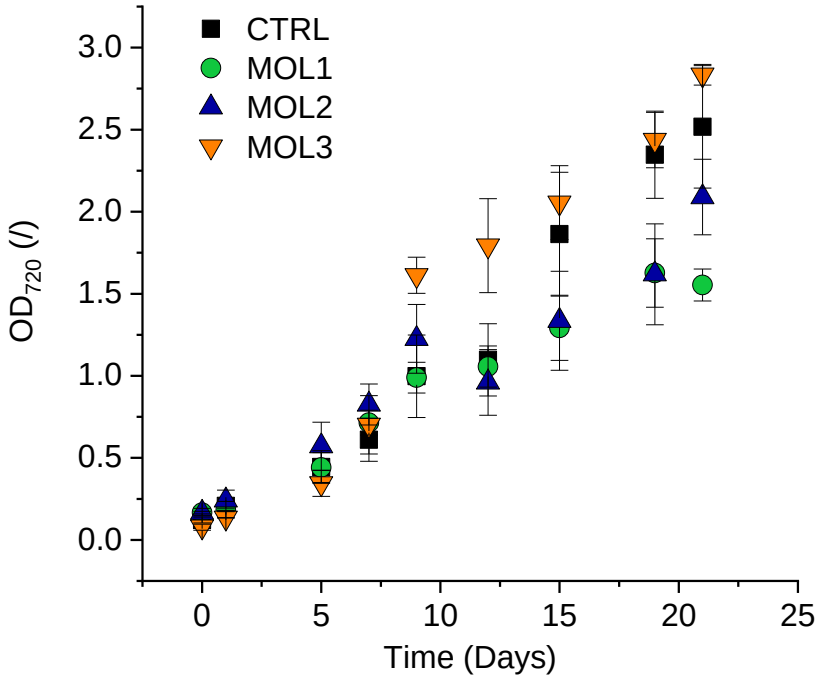


Figure 1

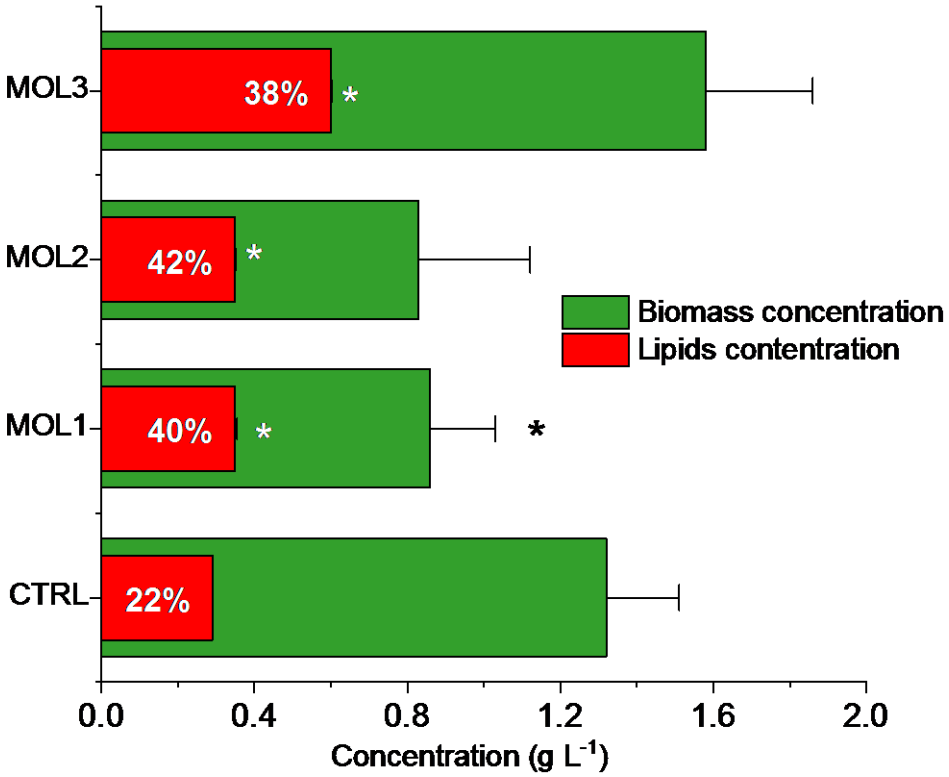

MOL3
38%
*
MOL2
42%
*
MOL1
40%
*
*
CTRL
22%
Biomass concentration
Lipids contentration
0.0
0.4
0.8
1.2
1.6
2.0
Concentration (g L-1)

Figure 2

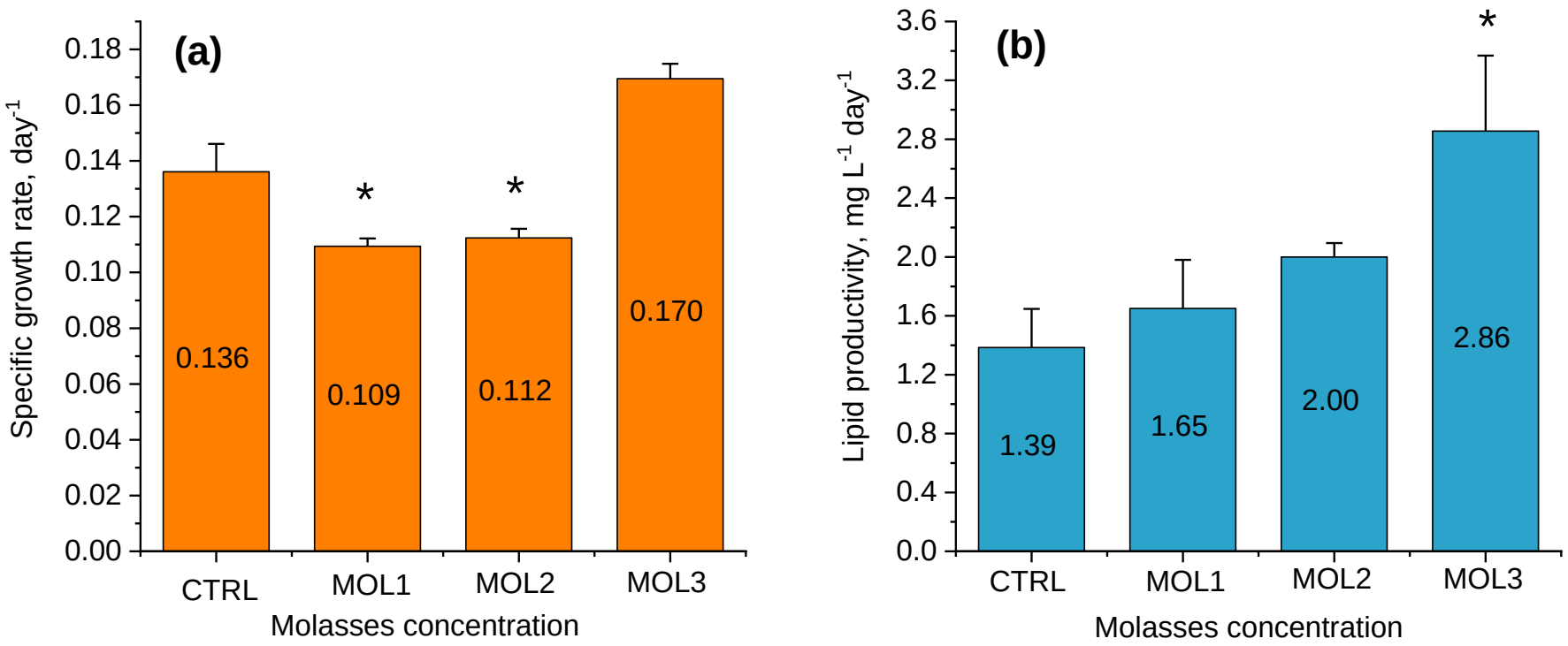

(a)
Specific growth rate, day-1
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.136
0.109
0.112
0.170
*
*
CTRL
MOL1
MOL2
MOL3
Molasses concentration
(b)
Lipid productivity, mg L-1 day-1
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
1.39
1.65
2.00
2.86
*
CTRL
MOL1
MOL2
MOL3
Molasses concentration

Figure 3

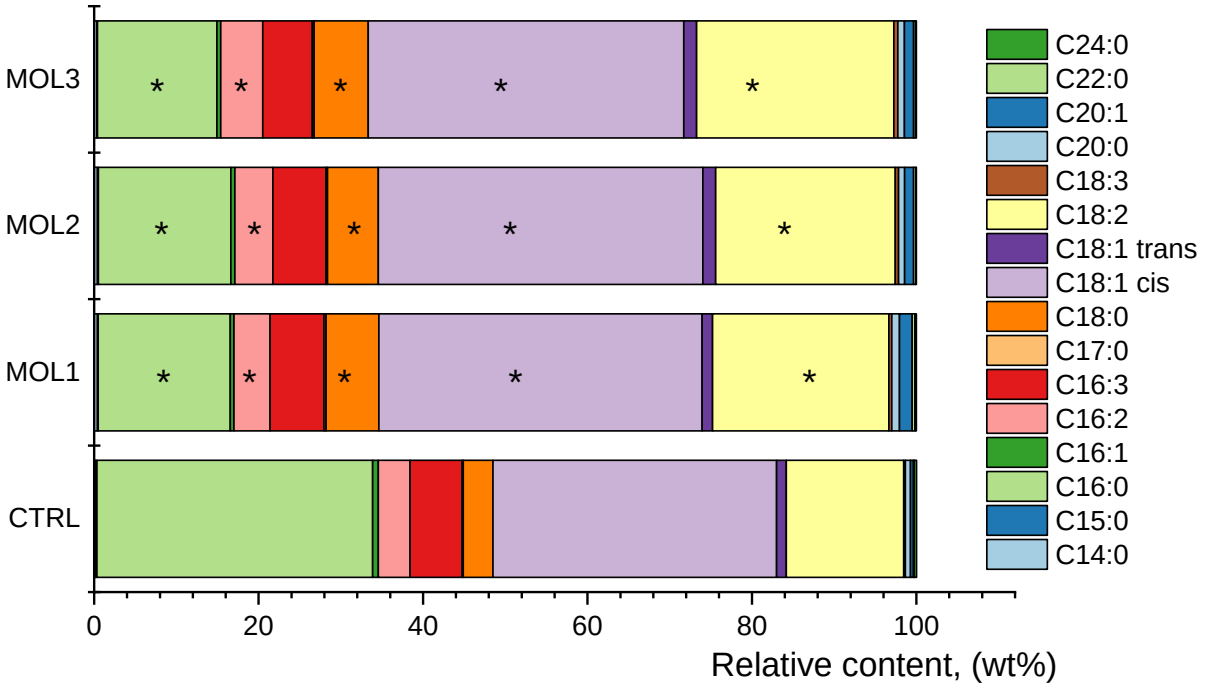

MOL3
MOL2
MOL1
CTRL
0
20
40
60
80
100
Relative content, (wt%)
C24:0
C22:0
C20:1
C20:0
C18:3
C18:2
C18:1 trans
C18:1 cis
C18:0
C17:0
C16:3
C16:2
C16:1
C16:0
C15:0
C14:0

Figure 4

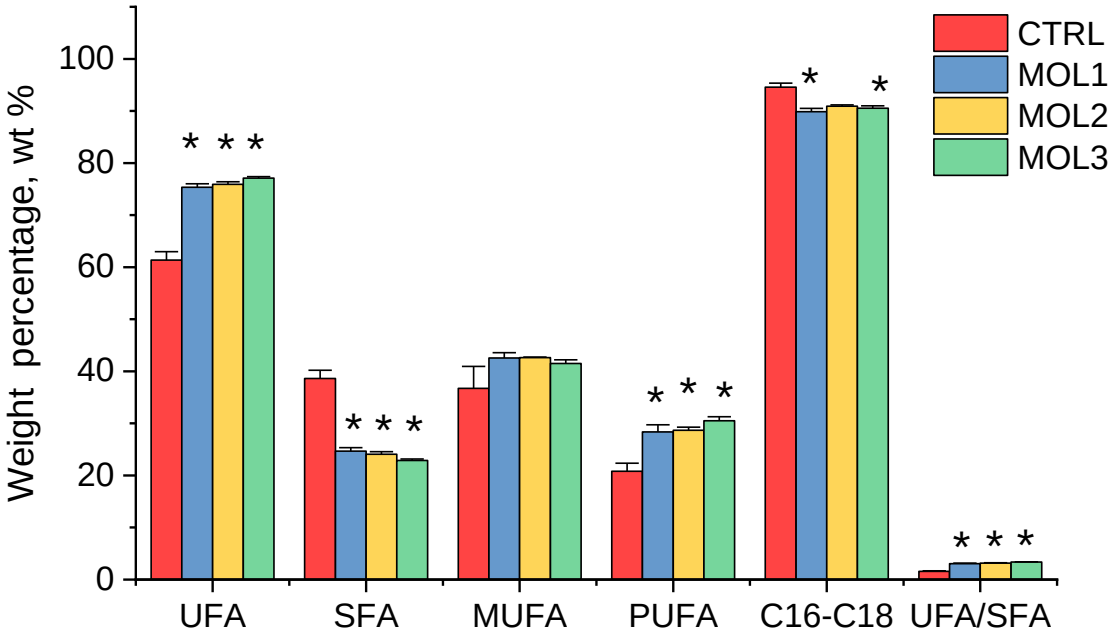

Weight percentage, wt %
100
80
60
40
20
0
CTRL
MOL1
MOL2
MOL3
UFA
SFA
MUFA
PUFA
C16-C18
UFA/SFA

Figure 5

Table 1. Experimental setup

Experiment ID	**CTRL**	**MOL1**	**MOL2**	**MOL3**
Molasses concentration (g/L)	0	0.5	1	2
Cultivation time (days)	21	21	21	21
Reactor	1 L flask	1 L flask	1 L flask	1 L flask
Working volume (L)	0.500	0.500	0.500	0.500
Volume medium (L)	0.333	0.333	0.333	0.333
Volume inoculum (L)	0.167	0.167	0.167	0.167

Note: CTRL = Bold Basal Medium (BBM), MOL1, MOL2 and MOL3 = CTRL + 3 concentrations of molasses (0.5, 1 and 2 g/L, respectively)

Table 2. Range of main chemical-physical parameters reported for molasses effluent

BOD_5 (g/L)	**COD (g/L)**	**TSS (g/L)**	**TN (mg/L)**	**TP (mg/L)**	**pH**	**Ref.**
1.30 - 4.70	3.80 - 16.15	1.50 - 9.10	~~0.0~~40 - ~~0.0~~70	~~0.0~~16 - ~~0.0~~20	3.8-4.3	[~~25~~45]
	0.636		176	2		[46]
		24	458	67		[47]
65	145	13.6	10		3.5	[48]

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

Table 3. Evaluated composition of the biodiesel obtainable using FAMEs from *C. zofingiensis* cultivated under mixotrophic condition ~~and metabolic stress~~

Parameter	Units	CTRL	MOL1	MOL2	MOL3
LA	%wt	0.2	0.36	0.39	0.48
DU	/	65.68	86.18	87.11	90.46
SV	mg/g	184.98	179.70	179.74	179.24
IV	/	59.48	78.03	78.94	82.06
CN	/	62.42	59.12	58.90	58.29
LCSF	/	6.50	6.59	5.94	6.11
CFPP	°C	3.94	4.23	2.19	2.73
CP	°C	12.64	3.46	3.48	2.65
PP	°C	6.90	-3.07	-3.04	-3.94
APE	/	64.63	84.18	85.50	88.89
BAPE	/	15.94	24.04	24.09	26.58
OS	hr	10.74	8.00	7.89	7.41
HHV	MJ/kg	35.42	35.18	35.14	35.12
υ	mm/s	**3.40**	**3.38**	**3.36**	**3.35**
ρ	ton/m^3	**0.78**	**0.78**	**0.78**	**0.78**

Notes: CTRL = Bold Basal Medium (BBM), MOL1, MOL2 and MOL3 = CTRL + 3 concentrations of molasses (0.5, 1 and 2 g/L, respectively), LA = Linolenic Acid, DU = Degree of Unsaturation, SV = Saponification Value, IV = Iodine Value, CN = Cetane number, LCSF = Long Chain Saturated Factor, CFPP = Cold Filter Plugging Point, CP = Cloud Point, PP = Pour Point, APE = Allylic Position Equivalent, BAPE = Bis-Allylic Position Equivalent, OS = Oxidation Stability, HHV = Higher Heating Value, υ = Kinematic Viscosity; ρ = density. Values in blue-bold are not compliant with the international standards for quality biodiesel ASTM 6751-12, EN-14214 and EN-590.