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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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# BioEnergy Research

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

<b>Manuscript Number:</b>	BERE-D-22-00329R1
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<b>Abstract:</b>	<p>The effect of of sugarcane molasses on lipid content and fatty acids methyl ester (FAME) profile by <i>Chromochloris zofingiensis</i> 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 (&gt; 38%wt), C18:2 linoleic (&gt; 21%wt), and C16:0 palmitic (&gt; 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</p>

1       1       **Effect of mixotrophy on lipid content and fatty acids methyl esters**  
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6       3       **sugarcane molasses**

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1 **Abstract**

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

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

7  
8 **Keywords:** *Chromochloris zofingiensis*, sugarcane molasses, mixotrophy, lipids  
9 content, fatty acids, biodiesel properties

## 10 11 **1. Introduction**

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

1 productivities in terms of biomass and lipids ~~content make~~ microalgae [4] and  
2 cyanobacteria [5] are recognized as suitable feedstock for 3<sup>rd</sup> generation biofuels  
3 production [6, 7]. ~~In this scenario, liquid biofuels can be potentially produced by the~~  
4 ~~exploitation of microalgae as renewable and environmentally friendly resources.~~

5 However, the full development of microalgal mass-production cultivation systems is  
6 still constrained by economic and technical issues [8].

7 Nutrients and water supply for microalgal cultivation represents one of the main  
8 cost-contributory factors for the full exploitation of mass cultivation systems [9, 10].

9 This last drawback could be tackled through the exploitation of inexpensive and  
10 abundant wastewater (WW) resources, which typically contain large amounts of  
11 nutrients, such as carbon (C), nitrogen (N), phosphorus (P) and trace elements that are  
12 capable to sustain the algal growth. An extensive literature review demonstrates how  
13 microalgae can combine their growth with the biological WW treatment and biofuels  
14 production [11, 12]. In addition, some algae strains can modulate their cell metabolism  
15 depending on the culture medium ~~in which they used for the~~ growth and the available C  
16 sources available.

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

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

10 This possibility to use organic sources opens up interesting scenarios for ~~their~~ algae  
11 exploitation at industrial level using WWs rich in organic matter as growth medium  
12 [20]. This depends both on the specific industrial sector producing the WWs and the  
13 capability of the specific strain of thriving in such non-ideal growth media. The use of  
14 food industry WWs as a nutrient medium for microalgae cultivation is well established.

15 In the last years, many studies have addressed the exploitation of dairy WW [21],  
16 vinasse and molasses from sugarcane and sugar beet [22, 23], brewery [11] and vinegar  
17 WW [24]. Industrial waste sugars can be used to replace other sugars or alcohols (i.e  
18 glucose, glycerol), which are expensive and whose production is not industrially  
19 scalable. From a nutritional point of view, sugarcane molasses is particularly suitable  
20 for heterotrophic [25] and mixotrophic [26] cultivation of microalgae. Sugarcane  
21 molasses is characterized by dark brown color, extensive odor and a very high content  
22 of organic matter in terms of Biological Oxygen Demand (BOD) and Chemical

1 Oxygen Demand (COD). The presence of total sugars (mainly 29% of sucrose, 12%  
2 of glucose and 13% of fructose), water, crude proteins and fats, heavy metals as well  
3 as vitamins makes molasses wastes suitable to boost microalgae growth [27].  
4 ~~Regarding the strains, one valid candidate for this purpose is *Chlorella Chromochoris*~~  
5 ~~*zofingensis* (formerly known as *Chlorella zofingensis*), is a single-celled green~~  
6 ~~microalgae belonging to the class of Chlorophyceae [28]. Beside the production of~~  
7 ~~astaxanthin [29], in the last years *C. zofingensis* has emerged as a potential producer~~  
8 ~~accumulator of triacylglycerides (TAGs) under multiple trophic conditions of~~  
9 ~~astaxanthin and lipid accumulator to produce biodiesel under suitable stress conditions~~  
10 ~~[18]. TAGs are a class of lipids suitable for biodiesel production [30]. It is reported that~~  
11 ~~in this strain the total lipids can account for 65.8%wt of the total biomass [31] while the~~  
12 ~~quantity of TAGs can significantly increase up to 40%wt [32]. This strain tends to~~  
13 ~~accumulate a high level of TAGs under abiotic stresses such as N deprivation, sulfur~~  
14 ~~deprivation, salinity stress, and high light [33].~~  
15 The choice to cultivate *C. zofingensis* could be of great interest for several reasons: a)  
16 a biochemical aspect that makes this strain particularly appealing is the ability to shift  
17 its exclusively photoautotrophic or heterotrophic metabolism into a mixotrophic one,  
18 with the result of an increased biomass production due to the sum of the two processes  
19 [34]. Since this strain is capable of thriving above the three mentioned trophic modes,  
20 the use of an organic waste as culture medium represents a valid strategy to increase  
21 biomass production [35]; b) experimental advantages due to the easiness of cultivation  
22 and fast growth rate [36]; c) the concurrent accumulation of TAGs and astaxanthin

1 enables this microalga an ideal cell factory for integrated production of the two  
2  
3  
4 2 compounds and has the potential to improve algae-based production economics [37].  
5

6 3 The influence of mixotrophy on lipid content and FAME composition is well  
7  
8  
9 4 documented for many *Chlorella* strains [38]. Beside the abundance of organic C, one  
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11  
12 5 of the features of molasses waste is the relative low content of N and P that makes this  
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14  
15 6 effluent particularly suitable to accumulate TAGs [30]. Many studies deal with the  
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18 7 effect of sugarcane molasses on lipids and FAME profile by different microalgae  
19  
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21 8 strains such as *Chlorella* [39], *Scenedesmus* [40], *Dunaliella* [41], *Monoraphidium*  
22  
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24 9 [27], *Micractinium* [42]. On the other hand, as far as *C. zofingensis* is concerned this  
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27 10 aspect has been assessed only under heterotrophic mode [43].

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

## 21 **2. Materials and methods**

### 22 ***2.1 Inoculum and culture medium preparation***

1 The strain *Chlorella Chromochloris zofingiensis* UTEX32 used for this study was  
2 obtained from the culture collection of algae at the University of Texas, Austin, USA  
3 [24 44]. The cell cultures were maintained and cultivated in standard Bold Basal  
4 Medium (BBM), whose composition was obtained by adding 10 mL of six stock  
5 solutions to 1 L of distilled water as follows: NaNO<sub>3</sub> (10g 400<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O), KH<sub>2</sub>PO<sub>4</sub> (7g  
6 400<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O), K<sub>2</sub>HPO<sub>4</sub> x 3H<sub>2</sub>O (3g 400<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O), MgSO<sub>4</sub> x 7H<sub>2</sub>O (3g 400<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O),  
7 CaCl<sub>2</sub> x 2H<sub>2</sub>O (1g 400<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O), NaCl (1g 400<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O). After autoclaving (Model 760,  
8 ASAL, Cernusco s/N, MI, Italy), 1 mL of three different vitamins stock solution and 6  
9 mL of PIV metal solution were added. The vitamin stock solutions were prepared as  
10 follows: thiamine 0.1g 100<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O, biotin 25 x 10<sup>-6</sup>g 100<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O, and vitamin B12  
11 15 x 10<sup>-6</sup>g 100<sub>mL</sub><sup>-1</sup> H<sub>2</sub>O. PIV metal solution was prepared in the following manner  
12 (mg/L): EDTANa<sub>2</sub> 750, FeCl<sub>3</sub> x 6H<sub>2</sub>O 97, MnCl<sub>4</sub>x4H<sub>2</sub>O 41, ZnCl<sub>2</sub> 5, CoCl<sub>2</sub> x 6H<sub>2</sub>O 2,  
13 and Na<sub>2</sub>MoO<sub>4</sub> x 2H<sub>2</sub>O 4.  
14 Four 150 mL Erlenmeyer flasks were filled with 50 mL of BBM medium, inoculated  
15 with approximately 10 mL of microalgae, closed with a cotton cup and continuously  
16 illuminated at room temperature by white fluorescent lamps (Model T8 36 W IP20,  
17 CMI, Germany) providing a light intensity of 50 μmol/m<sup>2</sup>/s measured with a  
18 luxmeter (Model HD 2302.0, Delta OHM, Padua, PD, Italy). Inoculum was  
19 maintained in cultivation for about one week once it reached the end of exponential  
20 growth phase.

## 21 **2.2 Cultivation conditions and experimental setup**

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

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

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

### 17 **2.3 WW collection and characterization**

18 The sugarcane molasses was collected from a sugar factory in Carugate, MI, Italy. An  
19 average range of the main chemical-physical parameters for molasses is shown in  
20 Table 2. Once collected the molasses was stored at 4 °C (fridge FKv Liebherr, Incofar  
21 Srl, Modena, MO, Italy) before its use. Later it was filtered (filtration unit Sartorius,  
22 Incofar Srl, Modena, MO, Italy) using glass filter microfiber disks (GF/C™ 47 mm

1 diameter, Whatman, Incofar Srl, Modena, MO, Italy), deprived of solid materials and  
2 then sterilized at 121 °C for 20 min (Autoclave model 760, Asal Srl, Cernusco sul  
3 Naviglio, MI, Italy) prior to microalgal cultivation.

#### 4 **2.4 Cell growth and dry weight determination**

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

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

$$21 \quad X_{dw} = \frac{W_2 - W_1}{V} \quad (1)$$

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

1 algae culture used for the test.

2 The biomass productivity ( $\Delta X$ ) was expressed as:

$$3 \quad \Delta X_{dw} = \frac{X_{\max} - X_0}{t_{\max} - t_0} \quad (2)$$

4 where,  $X_{\max}$  is the “maximum biomass” (g/L) obtained at ( $t_{\max}$ ).

5 The specific growth rate ( $\mu$ ) was calculated according to the following equation:

$$6 \quad \mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

7 (3)

8 where,  $X_2$  and  $X_1$  are the “dry biomass concentration” (g/L) at time  $t_2$  and  $t_1$ ,  
9 respectively.

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

## 12 **2.5 Total lipid content determination**

13 The total cell lipid content was measured according to a modified version of the  
14 classical gravimetric method Bligh and Dyer [26 49], tuned by Chen et al. [27 50].

15 After the growth, cell culture was collected and centrifuged (Model 2560 Nakita,  
16 Auxilab S.L., Berain, Spain) at 6500 rpm g for about 5 min, the sediments collected  
17 and freeze-dried at -80 °C (Forma Ultra-Low Temperature Chest Freezer MODEL  
18 RELEASE – 20, Thermo Fisher Scientific, Marietta, OH, USA) for about 24 h.

19 Before the extraction, the biomass was lyophilized (Lio5P, Cinquepascal Srl,  
20 Trezzano s/N, MI, Italy). About 50 mg (m) of lyophilized biomass were placed in a 50  
21 mL falcon tube (FT) where a 7.5 mL of chloroform/methanol mixture (1:2 v/v), called  
22 extractive solution I, was added. ~~This sample was solution was then carefully~~

1 ~~transferred to a 50 mL centrifuge tube and~~ put in a shaking table ~~at 60 °C for about 1 h~~  
2 (model WB-FM FALC Instruments Srl, Treviglio, BG, Italy) ~~at 60 °C for about 1 h.~~  
3 ~~Subsequently, it The mixture~~ was centrifuged at ~~659000 rpm g for about 5~~ 10 min and  
4 the supernatant was transferred to a new 50 mL centrifuge tube., ~~then †~~The sediment  
5 was extracted again with ~~another~~ 7.5 mL of extractive solution I for about 30 minutes  
6 and subjected to the same treatment. ~~The combined supernatant so obtained was~~  
7 ~~added to a~~ 14 mL of extractive solution II (5 mL of chloroform + 9 mL of aqueous 1%  
8 ~~NaCl sodium chloride~~ solution) was added to the combined supernatant to get a final  
9 volume ratio of about 2:2:1.8 (chloroform/methanol/water). ~~After vigorous manual~~  
10 ~~shaking, †~~the mixture ~~was subsequently extracted and~~ centrifuged at ~~659000 rpm g~~  
11 for ~~about~~ 5 min allowing a clear separation of the fats containing phase. ~~Then, the~~  
12 ~~chloroform phase~~ This was transferred to a pre-weighted glass tube ( $m_0$ , mg),  
13 evaporated under vacuum at rotavapor (Büchi rotary evaporator Model R-200, Büchi  
14 Labortechnik AG, Switzerland) ~~blow dried under N<sub>2</sub> flow gas~~ at 60 °C, completely  
15 ~~dried at a high vacuum line (HGC 244 HA, China) for 90 minutes~~ and finally  
16 weighted ( $m_1$ , mg). The percentage (%) of the lipid content (TL) was calculated by  
17 multiplying the obtained value of total lipid content (g/g biomass) by 100 as follows:

$$18 \quad TL[\text{wt}\%] = \left( \frac{m_1 - m_0}{m} \right) \cdot 100 \quad (4)$$

19 The lipid productivity (LP) was calculated as follows:

$$20 \quad LP[g L^{-1} day^{-1}] = TL \cdot \Delta X_{dw} \quad (5)$$

## 22 **2.6 FAMES determination**

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

1 [28-51]. Briefly, freeze-dried cells were re-suspended in 1 mL of toluene to improve  
2 the methylation of non-polar lipids and they were trans-methylated with 1% H<sub>2</sub>SO<sub>4</sub> in  
3 anhydrous methanol at 80 °C for 2 h under ~~a slow flow of~~ N<sub>2</sub> atmosphere. After  
4 cooling, 80 µL of tricosanoic acid methyl ester (CH<sub>3</sub>(CH<sub>2</sub>)<sub>21</sub>COOCH<sub>3</sub>, ≥99.0% (GC))  
5 (Sigma Aldrich, St. Louis, MO, USA) at 2 mg/mL in hexane were added as an  
6 internal standard. The FAMES were then extracted with 12 mL of an extractive  
7 solution (5 mL aqueous 5% NaCl + 7 mL hexane) and after phase separation, the  
8 organic phase was quantitatively analyzed by a 7820A Gas Chromatograph (Agilent  
9 Technologies, Palo Alto, CA, USA) coupled to a 5977B Mass Spectrometer (Agilent  
10 Technologies Palo Alto, CA, USA). The ~~system~~ GC-MS systems (split mode 20:1,  
11 split flow 19.6 mL/min) was equipped with a low polarity Supelco SLB-5 GC capillary  
12 column (30 m x 0.25 mm x 0.25 µm). Helium was used as carrier gas. The injector and  
13 detector temperatures were set at 280 °C and 230 °C, respectively. The chromatogram  
14 was recorded in the scan mode (40-500 m/z) with a programmed temperature from  
15 60 °C to 280 °C.

16 The identification and quantification of individual FAMES were performed by using a  
17 standard reference solution. It consisted of 50 µL of Supelco 37 Component FAME  
18 Mix® (Sigma Aldrich, Saint Louis, MO, USA) at 10 mg/mL added to 40 µL of  
19 tricosanoic acid methyl ester internal standard solution at 2 mg/mL and 410 of  
20 hexane.

21 The content of FAMES was calculated by manually integrating their peak areas with  
22 respect to the internal standard tricosanoic acid methyl ester, after calculation of the

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

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

$$5 \quad RF [/\ ] = \frac{m_s A_i}{m_i A_s} \quad (6)$$

6 where  $m_s$  is the “mass of internal standard” (mg),  $A_i$  is the “peak area of section  $i$ ”,  $m_i$   
7 is the “weight of sample” (mg) and  $A_s$  is the “area of standard”.

## 9 ***2.7 Estimation of fuel biodiesel properties***

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

## 18 ***2.8 Data analysis***

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

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

### 7 **3. Results and discussion**

#### 8 ***3.1 Influence of mixotrophy on *C. zoofingensis* growth and lipid content***

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

1 Time evolution of the optical density OD at 720 nm of cultures with different molasses  
2 concentrations is shown in Figure 1. It can be clearly observed that the culture MOL3  
3 showed the better growth achieving the highest OD~~720~~ at the end of cultivation (~2.8).  
4 A similar trend was also reported for the cultivation of *C. vulgaris* in sugarcane  
5 molasses under the same range of concentrations tested in this study (0.5-2 g/L) [26].  
6 In terms of biomass concentration this value corresponded to about 1.6 g/L (cf. Figure  
7 2), that is higher compared to the control (1.32 g/L). On the other hand, when the other  
8 two molasses concentrations were used, a slight decrease could be observed. From  
9 Figure 2 it can also be observed that, the use of molasses determined almost a doubling  
10 of the total lipid content, 42%wt (MOL2), 40%wt (MOL1), and 38%wt (MOL3),  
11 respectively, with respect to the control (16-20%wt) meaning that mixotrophy resulted  
12 in a boosting of the lipid metabolism. Given the high biomass concentration, the best  
13 compromise in terms of total lipid concentration in the PBRs was still represented by  
14 MOL3. The increase in sugarcane molasses concentration resulted in an enhanced lipid  
15 content for other *Chlorella* strains. In *C. vulgaris* 40.15% of lipid content in the control  
16 group achieved a 46.12%, 57.15%, and 62.85% when cultivated with 1 g/L, 2 g/L, and  
17 4 g/L, respectively [26]. *Chlorella sorokiniana* BTA 9031 exhibited a lipid content of  
18 30% when cultivated mixotrophically with molasses which was the double of what  
19 obtained when cultivated photoautotrophically [54]. It is known that different organic  
20 substrates found in wastes can stimulate distinct metabolic pathways for lipid and FAs  
21 synthesis. For instance, it has been demonstrated that when *Scenedesmus* sp. was grown  
22 in an organic waste rich in glucose under mixotrophic and heterotrophic conditions at

1 least three metabolic routes, among which the pentose phosphate pathway (PPP), the  
2 Embden-Meyerhof-Parnas pathway (EMP) and the Entner-Doudoroff pathway (ED)  
3 were involved, with PPP preferred during heterotrophy and EMP employed in  
4 mixotrophy [57]. The same effect has been experimentally confirmed using glucose for  
5 *C. pyrenoidosa* [56]. Owing to the close phylogenetic relationship between  
6 *Scenedesmus* sp. and *Chlorella* sp. (both are Chlorophyceae) and considering that  
7 molasses is mainly composed of sucrose and glucose as main sugars it is safe to assume  
8 that the also in *C. zofingensis* EMP route could be employed for the synthesis of lipids  
9 under mixotrophic cultivation. In addition, it should be highlighted that the enzymes  
10 involved in the lipids metabolic pathways become more active in the incidence of light  
11 energy and in the presence of glucose, which are two conditions typical of mixotrophy  
12 [57].

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

17 The lipid productivity was greatly enhanced when the strain was cultivated in MOL3  
18 compared to the control, (286 mg/L/day and 139 mg/L/day, respectively, (Figure 3b).

19 This was due to both the increase in biomass productivity and lipid content achieved  
20 when using 2 g/L of molasses. However, MOL1 and MOL2 did not produce an evident  
21 increase in biomass concentration compared to the control (Figure 3b).

22 Microalgae able to use organic sources can shift their metabolism from

1 ~~photo~~autotrophy to mixotrophy. As the discussion so far has confirmed, over a specific  
2 threshold of molasses concentration the shifting form ~~photo~~autotrophic to mixotrophic  
3 metabolism can be advantageous in terms of biomass productivity and lipid content  
4 resulting in a relevant increase of lipid productivity. This can explain why *C.*  
5 *zofingensis*, cultivated in MOL3 medium, attained a better biomass concentration  
6 compared to BBM where there are not at all organic compounds. On the other hand, the  
7 scarcity of N and P, typical of wastes rich in organic matter, leads to an imbalance  
8 between the ~~ratios~~ C:N:P ~~ratios~~ with respect to the optimal values for algae. This leads  
9 to an intracellular excess of C which is stored in the form of neutral lipids such as  
10 ~~triacetyl~~glycerols (TGAs) rather than as proteins which would require N. As depicted in  
11 Figure 2, there was an evident increase in lipid content in MOL media ~~which that~~ can  
12 be explained by considering that there is ~~a-the~~ wide gap in terms of C, N and P  
13 concentrations ~~due to the low values of N and P found in between~~ BBM (2.5 g/L and  
14 0.75 g/L, respectively) and ~~the high content of C in molasses (cf. BOD and COD in~~  
15 ~~Table 2) MOL (0.055 g/L and 0.015 g/L, respectively).~~ Similar findings have been  
16 reported for *C. vulgaris* grown in dairy ~~wastewater~~ WWs [58] and *Scenedesmus*  
17 *dimorphus* cultivated in brewery WW [11].  
18 During a nutritional unbalance, such as that one triggered by the use of MOL, N  
19 depletion appears to be the predominant force which drives the redistribution of C  
20 content towards lipid production stimulating the glycolytic metabolism and reducing  
21 the expression of enzymatic components involved in the Krebs cycle. This leads to an  
22 accumulation of Acetyl-CoA which is conveyed in the lipid synthesis [59]. In this light,

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

### 5 **3.2 Influence of mixotrophy ~~and chemical stress~~ on FAME profile**

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

12 As it can be noticed, in all the media including the control long-chain compounds with  
13 16 and 18 C atoms are the main FAs with no statistically meaningful differences among  
14 ~~them the media~~ ( $p > 0.05$ ). The most represented FAs were oleic (C18:1), linoleic  
15 (C18:2), ~~and~~ palmitic (C16:0), ~~and stearic~~ (C18:0) acids. In particular C18:2 in all the  
16 three MOL media resulted more than doubled while C16:0 appeared almost halved  
17 compared to the control, respectively. It is also interesting to note that oleic acid (C18:1)  
18 was the highest in MOL media. The relative high content of C18:2 was almost the same  
19 as reported for other *Chlorella* strains when cultivated in dairy WW while C16:0 and  
20 C18:1 were lower and higher, respectively [61].

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

1 good quality of a diesel. The trend of main FAs found in this study (C18:1 > C18:2 >  
2 C16:0 > C18:0) was in agreement with those reported for the mixotrophic cultivation  
3 of *C. vulgaris* and *C. sorokiniana* on dairy effluents [62], and of *Chlorella* sp. and  
4 *Micractinium reisseri* cultivated mixotrophically in sugarcane bagasse [63] and  
5 molasses [42], respectively. Changes in the composition of FAs with enhanced  
6 production of C16:0, C18:1, and C18:2 by shifting from photoautotrophy to  
7 heterotrophy or mixotrophy after exposing microalgae to stress and C enrichment has  
8 been reported also for the heterotrophic cultivation of *Chlorella minutissima* [64].  
9 An interesting aspect is the relatively high content of linoleic acid (C18:2) which was  
10 almost doubled compared to the control. In the standard medium BBM this FA  
11 attained a value of 14%wt, while when cultivated with 0.5-1 g/L of molasses it  
12 reached ca 21%wt increasing up to 24%wt with 2 g/L of molasses. Beside the fact  
13 that the high content of this FA increases the whole level of unsaturation, C18:2 is  
14 also present in most of the vegetable oils suggested in diets rich in omega-3 FAs.  
15 Once assumed, C18:2 is converted to gamma-linolenic acid (GLA) in the body which  
16 can then break down further to arachidonic acid (ARA). The last one is necessary for  
17 the function of all cells, especially in nervous system, skeletal muscle, and immune  
18 system [65]. Therefore, *C. zofingensis* biomass grown in molasses can be potentially  
19 exploited as feedstock not only for biodiesel production but also for nutraceutical and  
20 pharmacological application.

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

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

### 21 ***3.3 Analysis of biodiesel properties based on FAME profile***

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

1 biomass guarantees oxidative stability and ignition quality of the biodiesel, which are  
2 two of the main fuel properties able to greatly affect biodiesel quality. These two  
3 parameters are positively influenced by the presence of long chain fatty acid (C16–18),  
4 high content of C18:1 and the degree of unsaturation [35–67]. As it can be seen in  
5 Figure 5, beside the control, more than 96% of *C. zofingensis* total FAs were  
6 represented by C16–C18 groups, C18:1 was 39.46% (MOL2), and UFA were in the  
7 range 75.34% (MOL1) - 77.41% (MOL3).

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

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

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

1 viscosity ( $\nu$ ), and density ( $\rho$ ). In particular, CP and PP should be the lowest as possible,  
2 as found for MOL media. This condition is obtained when in FAME profile the content  
3 of UFA is high, as for MOL media (Figure 5). The reduction of PP depends on the high  
4 content of PUFA C18:2, as in particular for DWW and MOL3. Viscosity and density  
5 (in ~~redblue~~-bold in Table 3) were slightly lower than the prescribed ones. In fact,  
6 according to the European standard they should be in the range  $3.5 - 5 \text{ mm}^2 \text{ s}^{-1}$  and  $0.86$   
7  $- 0.9 \text{ ton m}^{-3}$ , respectively. However, it can be observed that the difference with the  
8 prescribed values is very small and might be easily adjusted by mixing the biodiesel  
9 with specific additives.

## 11 **Conclusion**

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

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

8

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14

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20 The authors have no relevant financial or non-financial interests to disclose

### 21 **Authors contribution**

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

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

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## 19 **Figure captions**

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

1 g/L molasses.

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

7 **Fig 3** Specific growth rates (a) and lipid productivities (b) obtained using different  
8 concentrations of molasses in BBM (CTRL). MOL1 is the medium with the addition of  
9 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2 g/L molasses. (\*)  $p <$   
10  $0.05$  test T.

11 **Fig 4** Fatty acids detected in *C. zofingensis* grown in different concentrations of  
12 molasses. CTRL is the control medium BBM, MOL1 is the medium with the addition  
13 of 0.5 g/L molasses, MOL2 with 1 g/L molasses and MOL3 with 2 g/L molasses. (\*)  $p$   
14  $< 0.05$  test T.

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

18

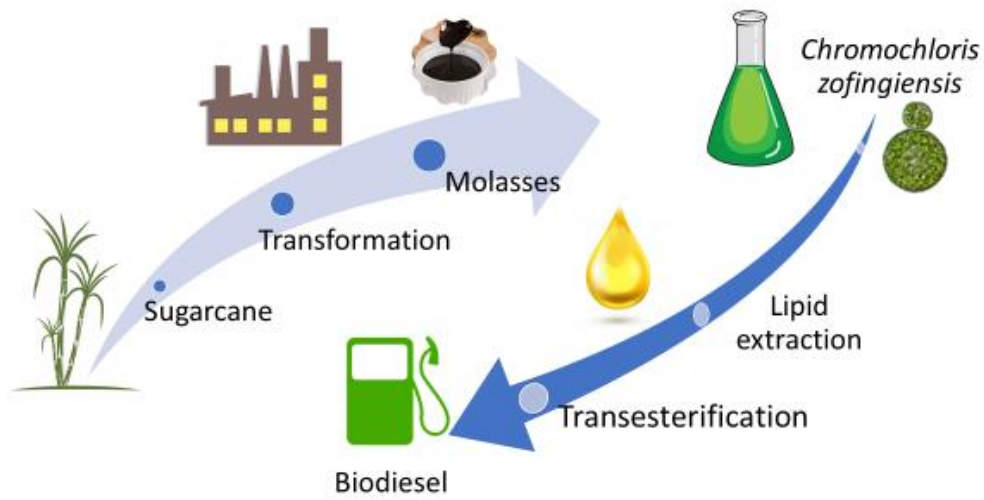
## 19 Highlights

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

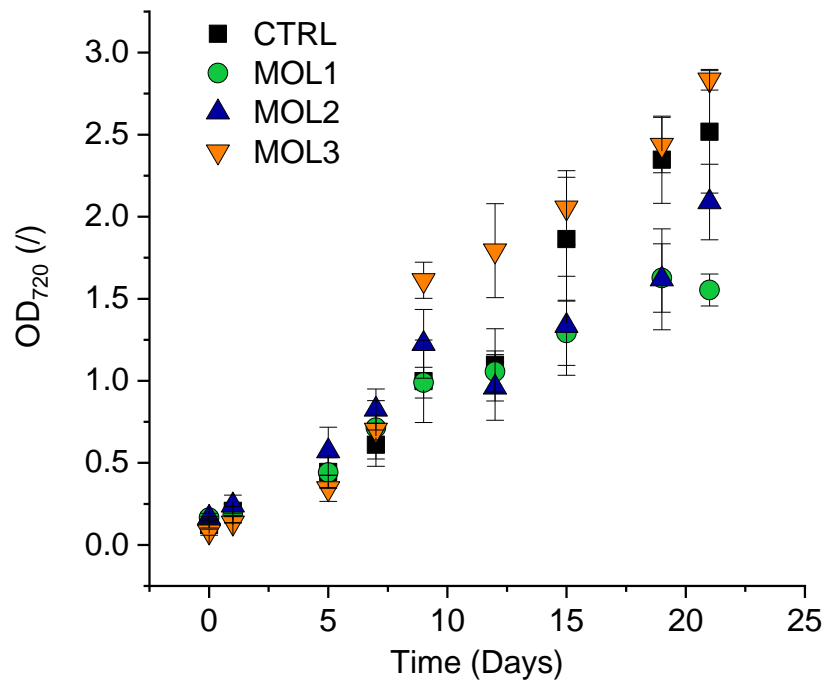
21 Molasses enhanced better cell growth than control medium.

22 Molasses triggered biomass and lipid productivity better than control medium.

- 1 Internal cell metabolism was modified by molasses to improve lipid production.
- 2 Saturation and unsaturation levels in FAMES were directly influenced by molasses
- 3
- 4 Graphical abstract



- 5
- 6

**Figure 1**

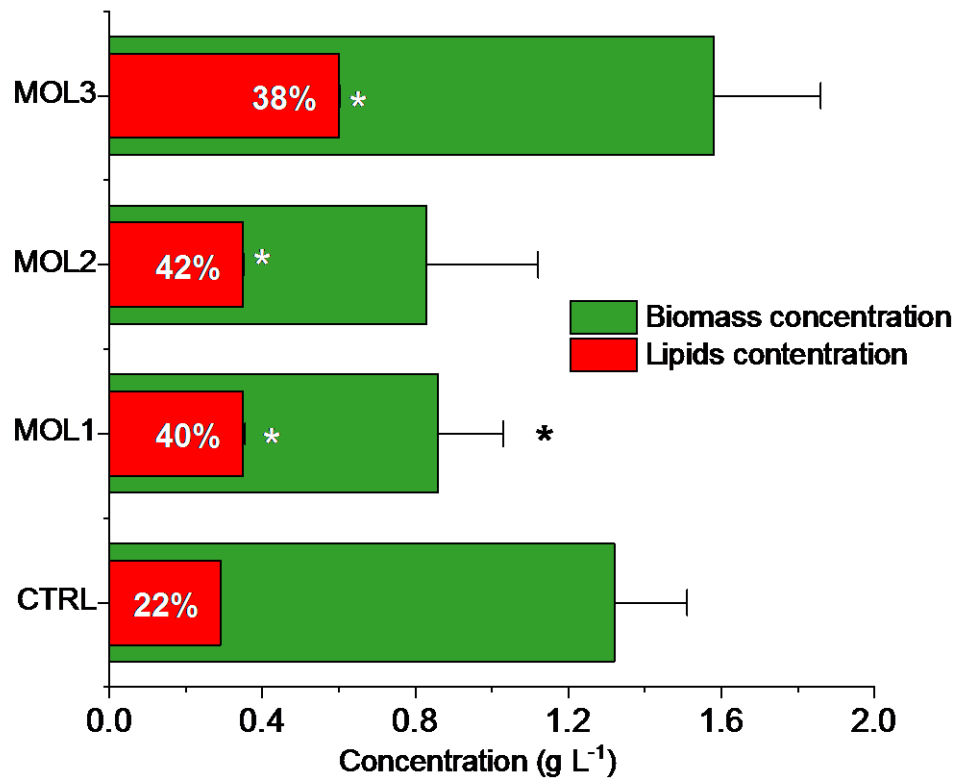
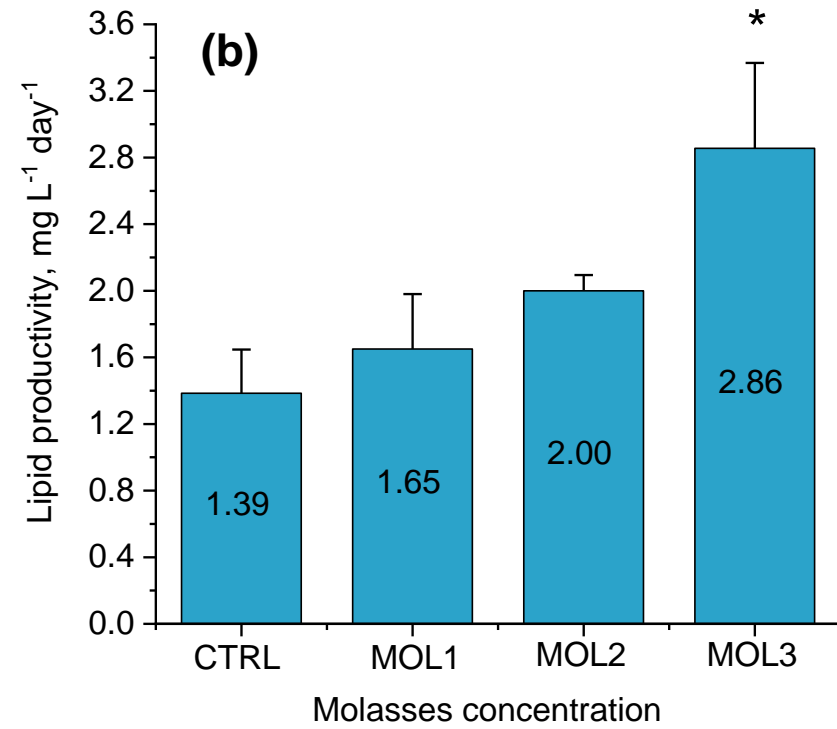
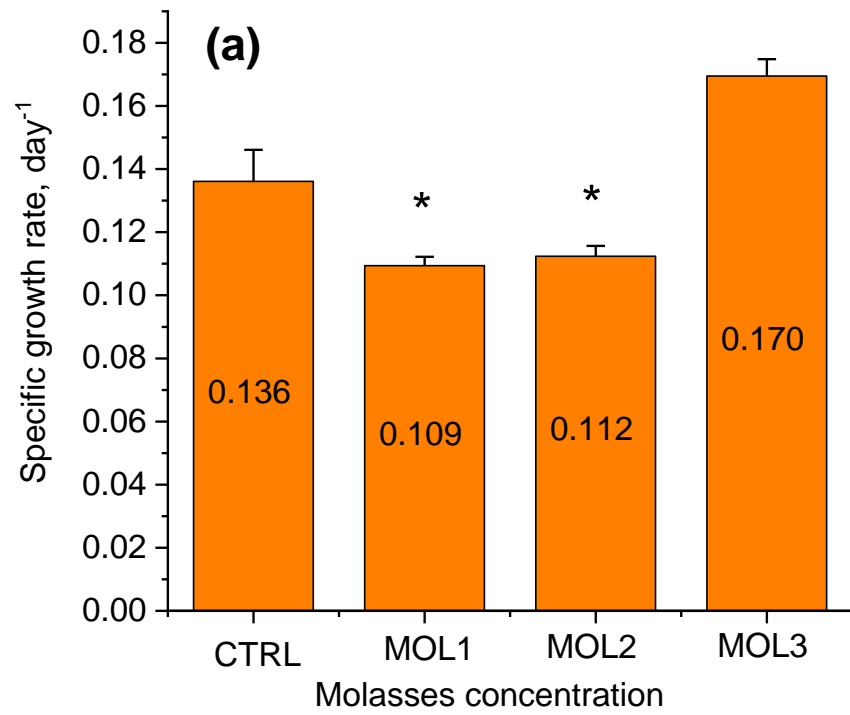


Figure 2



**Figure 3**

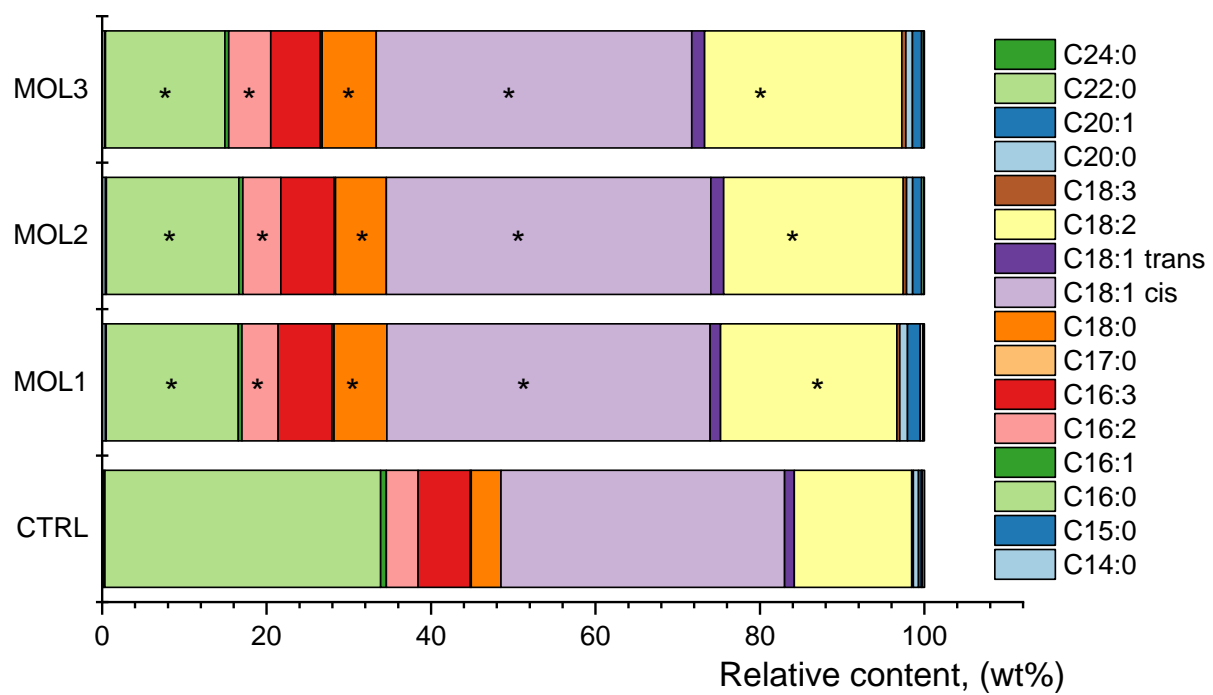
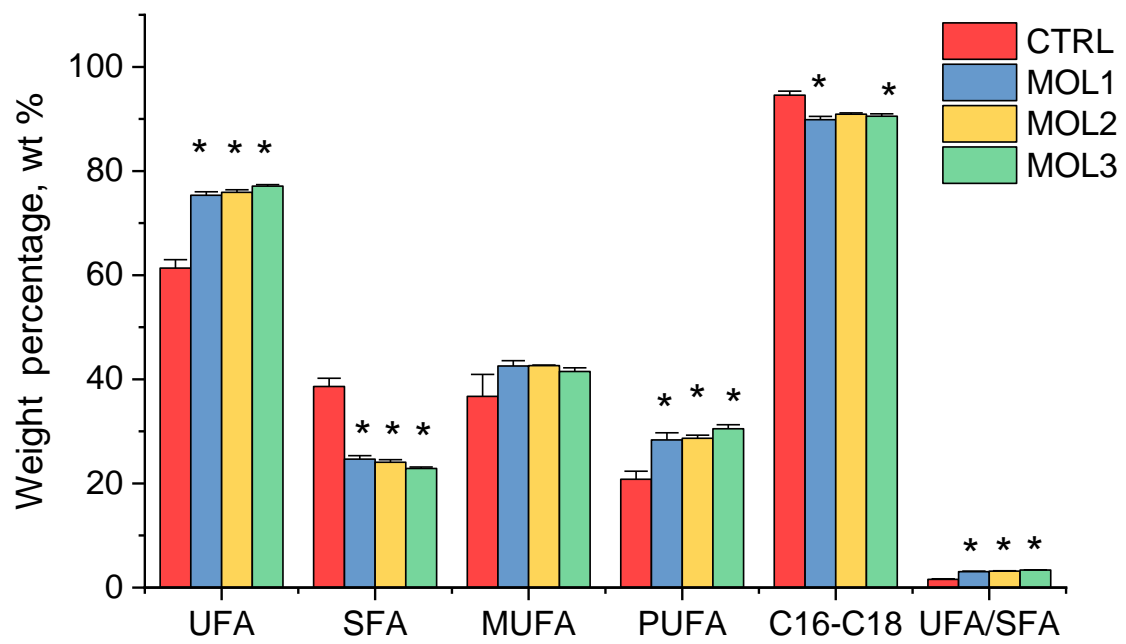


Figure 4



**Figure 5**

**Table 1.** Experimental setup

<b>Experiment ID</b>	<b>CTRL</b>	<b>MOL1</b>	<b>MOL2</b>	<b>MOL3</b>
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

<b>BOD<sub>5</sub> (g/L)</b>	<b>COD (g/L)</b>	<b>TSS (g/L)</b>	<b>TN (mg/L)</b>	<b>TP (mg/L)</b>	<b>pH</b>	<b>Ref.</b>
1.30 - 4.70	3.80 - 16.15	1.50 - 9.10	<del>0.040</del> - <del>0.070</del>	<del>0.016</del> - <del>0.020</del>	3.8-4.3	[2545]
	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
$\nu$	mm/s	<b>3.40</b>	<b>3.38</b>	<b>3.36</b>	<b>3.35</b>
$\rho$	ton/m <sup>3</sup>	<b>0.78</b>	<b>0.78</b>	<b>0.78</b>	<b>0.78</b>

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,  $\nu$  = Kinematic Viscosity;  $\rho$  = density. Values in blue-bold are not compliant with the international standards for quality biodiesel ASTM 6751-12, EN-14214 and EN-590.