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Semi-batch heterotrophic cultivation of *Chlorella sorokiniana* and *Chlorella kessleri*: lipid and protein content, fatty acid distribution and FAME properties

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ABSTRACT

In the present study the heterotrophic growth of *Chlorella sorokiniana* and *Chlorella kessleri* cultivated in a fed-batch mode, using crude glycerol as the sole carbon source, was investigated. The effect of the mode of the organic carbon addition on the lipid and protein content of the biomass, the fatty acid (FAs) distribution of the bio-oil and the fatty acid methyl ester (FAME) properties was studied. The FAME properties namely, the saponification number, the iodine value, the cetane number and the higher heating value, were estimated from empirical equations. The duration all cultivations was 26 d and until the 20th day 800 mL glycerol were added in the cultivation medium (2.3% v/v). For *C. sorokiniana*, during the cultivation period, 400, 200 and 80 mL glycerol were added in the cultivation medium at 2, 4 and 10 equal intervals respectively. For *C. kessleri* 400, 200, 100 and 40 mL glycerol were added at 2, 4, 8 and 20 equal intervals, respectively. For *C. sorokiniana* lipid and protein content ranged from 35.1% to 47.5% and 16.4% to 21.8%, respectively. The percentage of short chain FAs (C10-C14), medium chain FAs (C16-C18) and long chain FAs (>C18) in the total FAs ranged from 6.6% to 19.8%, 80.2% to 91.3% and 0% to 13.1%, respectively. The percentage of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) of the total FAs ranged from 25.9% to 37.2%, 38.4% to 60.0% and 14.1% to 24.4%, respectively. For *C. kessleri* lipid and protein content ranged from 27.7% to 35.1% and 21.5% to 29.8%, respectively. The percentage of short chain FAs, medium chain FAs and long chain FAs of the total FAs ranged from 15.8% to 30.6%, 66.2% to 76.8% and 1.9% to 14.7%, respectively. The percentage of SFAs, MUFAs and PUFAs of the total FAs ranged from 38.7% to 54.1%, 40.7% to 53.1% and 5.2% to 16.3%, respectively. Although the treatment 2 × 400 mL of glycerol shows very good FAME properties for both species, the lipid productivities of *C. sorokiniana* are higher compared to those of *C. kessleri* and thus, *C. sorokiniana* is a better option compared to *C. kessleri* for potential use in biodiesel production.

Keywords: Chlorella sorokiniana; *Chlorella kessleri*; Semi-batch; Lipids; Fatty acid distribution

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1. Introduction

Microalgae are unicellular photosynthetic organisms that use light and carbon dioxide, with higher photosynthetic efficiency than plants, for the production of biomass. Some microalgae species can also grow and multiply heterotrophically in the absence of light if an organic carbon source becomes available [1]. The main advantage of heterotrophic growth is higher biomass growth rates and biomass production because, unlike autotrophic growth, heterotrophic growth is not limited by light transmission through the growth medium [2]. Another advantage of heterotrophic growth is the potential of achieving higher lipid content and, as a result, higher lipid productivities [3]. This is needed if microalgal cultivation is to be useful for biodiesel production. Disadvantages of heterotrophic growth are the susceptibility to contamination which requires that all parts of the bioreactors as well as all growth media must be carefully sterilized and the cost of organic carbon which must be provided to the growth medium [4].

The microalgae growth rate as well as the protein and lipid content are influenced by many parameters, such as the cultivated species, the temperature, the concentration of carbon and nitrogen, the medium pH, the concentration of potassium, phosphorus and micronutrients, dissolved oxygen and the mixing rate [5]. For biodiesel production, except for a high lipid productivity, a favorable fatty acid profile of the lipids is needed as well so that its properties are within acceptable limits [6]. Currently, biodiesel is mainly produced from used oils and various seed oils such as sunflower and cotton seed oils. Vegetable oils contain fatty acid mostly from about C14 to about C22. Used oils contain a small amount of animal fats which have a lower fatty acid chain length.

Depending on the cultivated species and the cultivation mode and parameters the microalgal biomass can be directed towards specific uses, since various components such as proteins, lipids, antioxidants and other compounds can be maximized [7]. Therefore, the microalgal biomass composition can be altered by altering the growth conditions and furthermore microalgal cultivation can be carried out on an industrial scale using basic biochemical engineering principles. A number of review papers focus on the heterotrophic growth of several microalgal species and the trend is that heterotrophic growth enhances both the biomass and lipid productivity [8,9]. Different sources of carbon have been used for microalgae growth, such as glucose, sucrose, fructose, mannose, acetate, lactose or galactose [10].

In heterotrophic cultivation mode, microalgae use organic carbon and oxygen under dark conditions for growth and biomass production [11]. A number of review papers focus on the heterotrophic growth of several microalgal species and the trend is that heterotrophic growth enhances both the biomass and lipid productivity [8,9]. Different sources of carbon can be used for microalgae growth, such as glucose, sucrose, fructose, mannose, glycerol, lactose or galactose [10]. Studies on the heterotrophic cultivation of different microalgal species using glucose as carbon source have been published [12,13].

In the heterotrophic cultivation of *Chlorella sorokiniana*, the growth rate was higher using glucose and sodium acetate as the carbon source compared to fructose [14]. In *Chlorella vulgaris*, optimal cell growth and lipid productivity were attained using glucose at 1% (w/v). Growth of *C. vulgaris* on glycerol had a similar dose effects as those from glucose [15]. In *Chlorella protothecoides*, corn powder hydrolysate instead of glucose was used as organic carbon source in heterotrophic culture medium in fermenters in order to increase the biomass and reduce the cost of cultivation [16]. O'Grady and Morgan [17] studied the heterotrophic growth of *C. protothecoides* with different carbon sources and specifically glycerol, glucose and a glucose/glycerol mixture. They found that the specific growth rate and lipid yields when using glycerol and a glucose/glycerol mixture were higher in comparison when using only glucose. Similarly, Kong et al. [18] found that the growth rate of *C. vulgaris* as well as the biomass production of the species was enhanced when cultivated with a mixture of glycerol and glucose.

As mentioned, studies on the heterotrophic growth of microalgae using glycerol as the carbon source to the growth medium have published, while there are no studies on the heterotrophic growth of *C. sorokiniana* and *Chlorella kessleri* using crude glycerol as the sole carbon source. In the present study the heterotrophic growth of *C. sorokiniana* and *C. kessleri* using crude glycerol as the sole carbon source in a fed-batch mode was investigated. The duration of the microalgae growth period in all cultivations was 26 d. From the beginning of the growing period until the 20th day and in all treatments, 800 mL crude glycerol were gradually added in the medium of growth. Specifically, for the three cultivations of *C. sorokiniana* during the cultivation period, 400, 200 and 80 mL glycerol were added to the growth medium at 2, 4 and 10 equal intervals, respectively. For the four cultivations of *C. kessleri* during the cultivation period, 400, 200, 100 and 40 mL glycerol were added to the growth medium at 2, 4, 8 and 20 equal intervals, respectively. This mode of glycerol addition was determined in order to avoid possible negative effects of initial excess of the organic carbon in microalgae growth or to avoid possible deficiency of the organic carbon during the growing period with adverse effects on microalgal growth. The purpose of the present study was to determine how the mode of the organic carbon addition (different sub-quantities and different intervals) in the growth medium of the heterotrophic cultivation *C. sorokiniana* and *C. kessleri*, using the same total amount of glycerol as the sole carbon source during the growing period, affects the lipid and protein concentration in the biomass of the two species, the fatty acid distribution and the basic biodiesel properties. Additionally, the purpose was to compare the two species, as well as to compare our results with the corresponding results reported in literature using glucose as a carbon source.

2. Materials and methods

2.1. Bioreactors and inoculum preparation

The cultivations of both species were carried out in glass cylindrical bioreactors of 42 L capacity each that were filled to 80% of their volume. The duration of cultivation was 26 d, until the 20th day in the cultivation medium 2.3% (v/v), that is, 800 mL glycerol were added. Air was continuously

provided to each bioreactor though a perforated network of piping placed at the bottom of the bioreactor tank. The volume/rate of aeration was 45 L of air per liter of culture medium per hour. This corresponds to 9.9 L of O_2 per liter of culture medium per hour.

The temperature of the cultures was kept at 30°C ± 1°C with the use of temperature thermostats (Aquael 250 W heaters, Suwalki, Poland) and the pH was held constant at 7 ± 0.3. The pH was adjusted manually as needed with the use of HCl or NaOH solutions. The bioreactors, the glass tubing and the culture medium were sterilized before use.

The microalgae species *C. sorokiniana* (SAG strain 211-31) and *C. kessleri* (SAG strain 211-11h) were obtained from Culture Collection of Algae from the University of Göttingen in Germany (EPSAG). The inoculums were prepared autotrophically using the standard inoculum growth Basal Medium (=ES "Erddekokt + Salze") and each liter of it contained: 0.2 g KNO_3 L^{-1}, 0.02 g K_2HPO_4 L^{-1}, 0.02 g $MgSO_4{\cdot}7H_2O$ L^{-1}, 30 mL of soil extract L^{-1} and 5 mL L^{-1}, of a solution containing the following micronutrients: (1 mg $ZnSO_4{\cdot}7H_2O$, 2 mg $MnSO_4{\cdot}4H_2O$, 10 mg H_3BO_3, 1 mg $Co(NO_3)_2{\cdot}6H_2O$, 1 mg $MoO_4{\cdot}2H_2O$, 0.005 mg $CuSO_4{\cdot}5H_2O$, 700 mg $FeSO_4{\cdot}7H_2O$ and 800 mg EDTA) L^{-1} [19]. The culture medium was inoculated with a standard quantity of 250 mL of 0.5 optical density.

2.2. Estimation of basic fatty acid methyl ester properties

The saponification number (SN) and the iodine value (IV) were calculated theoretically from the fatty acid (FA) distribution using the equations suggested by Kalayasiri et al. [20] and by Azam et al. [21]. Similarly, the cetane number (CN) was evaluated from the theoretical equation suggested by Krisnangkura [22] and the higher heating value (HHV) from the equation suggested by Demirbaş [23].

$$\mathrm{SN} = \sum\left[\frac{\left(560\times\left(\%W_i\right)\right)}{\mathrm{MW}_i}\right] \tag{1}$$

$$\mathrm{IV} = \sum\left[\frac{\left(254\times N_{\mathrm{db}}\times\left(\%W_i\right)\right)}{\mathrm{MW}_i}\right] \tag{2}$$

$$\mathrm{CN} = 46.3 + \left(\frac{5{,}458}{\mathrm{SN}}\right) - \left(0.225\mathrm{IV}\right) \tag{3}$$

$$\mathrm{HHV} = 49.43 - \left(0.041\mathrm{SN}\right) - \left(0.015\mathrm{IV}\right) \tag{4}$$

where % W_i is the % weight of each FA, N_{db} is the number of double bonds and MW_i is the molecular weight of the respective fatty acid methyl ester (FAME).

2.3. Methods of analyses

The composition of the samples was determined according to AOAC [24] methods. Total nitrogen content in the biomass samples was measured with digestion using the Kjeldahl method [25]. Total protein content of samples was calculated using a conversion factor of 6.25 [26]. For the determination of organic carbon, the method of Ciavatta et al. [27] was used. The samples were first centrifuged and then filtered. According to this method, organic carbon was oxidized by a mixture 5 mL of 2N $K_2Cr_2O_7$ and 20 mL of concentrated H_2SO_4 at 160°C ± 2°C. The excess dichromate was titrated with iron (II) sulphate. The total lipid content was determined with extraction using co-solvents of n-hexane/isopropanol in the microalgal biomass according with the method of Bian et al. [28]. The solvent ratio of n-hexane to isopropanol was 3/2 (v/v), the ratio of co-solvents to

Table 1
The initial nitrogen concentrations and the mode of carbon (glycerol) addition during the fed-batch cultivation of *C. sorokiniana* and *C. kessleri*

Species↓	Mode of glycerol addition				*No (mg L^{-1})	Total Co (g L^{-1})	Co/No
C. sorokiniana	2 × 400 mL	4 × 200 mL	10 × 80 mL	–	80.5	13.2	164
C. kessleri	2 × 400 mL	4 × 200 mL	8 × 100 mL	20 × 40 mL	80.5	13.2	164

*Total N = 80.5 (mg N L^{-1}) = 25.7 mg N L^{-1} from NH_4^+ + 54.8 mg N L^{-1} as NO_3^-; Temperature = 30°C ± 1°C, pH = 7 ± 0.3

Table 2
The content of the biomass of *C. sorokiniana* and *C. kessleri* in lipids and protein for the cultivation treatments shown

*Property	Species						
	C. sorokiniana			*C. kessleri*			
	Treatments			Treatments			
	2 × 400 mL	4 × 200 mL	10 × 80 mL	2 × 400 mL	4 × 200 mL	8 × 100 mL	20 × 40 mL
Biomass (g L^{-1})	2.2 ± 0.1^{b}	2.6 ± 0.2^{a}	2.8 ± 0.2^{a}	1.5 ± 0.1^{c}	1.8 ± 0.1^{b}	2.0 ± 0.1^{b}	2.1 ± 0.1^{b}
Protein (%)	16.4 ± 0.9^{c}	16.7 ± 0.9^{c}	21.6 ± 1.2^{b}	21.5 ± 1.3^{b}	22.1 ± 1.2^{b}	28.2 ± 1.6^{a}	29.8 ± 1.6^{a}
Lipid (%)	47.5 ± 2.8^{a}	42.8 ±2.3^{a}	37.3 ± 1.8^{b}	35.1 ± 1.5^{bc}	32.4 ± 1.3^{c}	28.3 ± 1.2^{d}	27.7 ± 1.1^{d}

*For each chemical property of samples, the values in the rows of the table with the same letter do not differ significantly according to the Tukey's test (P = 0.05), n = 4.

microalgal dry biomass was 10/1 (v/w). Extraction was carried out in horizontal-circular movement ''tehtnica Zelezniki EV-402'' machine (Zeleznica, Slovenia). Stirring speed was 300 rpm, and the extraction time was 48 h. The total lipid extract was determined gravimetrically after filtration and evaporation of the solvents.

The bio-oil was subjected to transesterification and the fatty acid distribution was determined by gas chromatography. Specifically, the bio-oil was first filtered and 100 mg of the bio-oil was reacted with a mixture of heptane and KOH/MeOH (2 N). The anhydrous MeOH was first filtered. Following the separation of the two phases, 1 μL of the FAME (fatty acid methyl ester) phase was injected into an Agilent Gas Chromatographer Model 6890 N. Analysis of the FAME distribution was performed according to the EN 14103 method.

2.4. Materials and initial parameters of the experiments

Crude glycerol was used as the carbon source. It was obtained from a local biodiesel manufacturing plant. Its methanol was removed and its composition was 86% glycerin, 0.5% methanol, 7.5% water, 3.5% MONG (non-glycerin organic matter) and 2.5% ash. Table 1 summarizes the initial nitrogen concentrations and the mode of carbon addition for the cultivation of *C. sorokiniana* and *C. kessleri*.

2.5. Statistical analysis

Comparison of means was performed by subjecting the data to one-way analysis of variance at a significance level of 0.05 using the IBM SPSS Statistics 24 statistical package [29]. The significant differences between treatments were determined using Tukey's multiple comparison test.

3. Results and discussion

3.1. Lipid and protein content of biomass

Table 2 shows the lipid and protein content of the biomass of *C. sorokiniana* and *C. kessleri*. It is noticed that the mode of carbon addition in these semi-batch cultivations affects both the protein and the lipid content of the biomass in both species.

In particular, for *C. sorokiniana* the addition of large amounts (2 × 400 mL and 4 × 200 mL) of glycerol produces biomass with a higher content of lipids compared with the addition of smaller amounts but more frequently (10 × 80 mL). For *C. kessleri* the addition of amounts 2 × 400 mL and 4 × 200 mL of glycerol produces biomass also with a higher content of lipids compared with the addition of smaller amounts of glycerol added more frequently (8 × 100 mL and 20 × 40 mL). Comparing the two species, *C. sorokiniana* is characterized by higher content in lipids compared with *C. kessleri* for the same treatments, reaching up to 47.5% compared to 35.1% in *C. kessleri* for the 2 × 400 mL treatment of glycerol, a difference of 26.1%. *C. sorokiniana* also has a higher content in lipids in the 2nd and 3rd treatments compared to *C. kessleri*. For the *C. sorokiniana* the 2 × 400 mL and 4 × 200 mL treatments produce biomass with a lower content of protein compared with the addition of smaller amounts of glycerol (10 × 80 mL). Comparing the two species, *C. kessleri* is characterized by a higher content in proteins compared with *C. sorokiniana* for the same treatments. The biomass yields of *C. sorokiniana* are also higher compared to the corresponding ones of *C. kessleri* (Table 2). Thus, as lipid productivities are the product of the biomass productivity times the lipid content and cultivation time was the same in all treatments, lipid productivities of *C. sorokiniana* are higher than the corresponding ones of *C. kessleri*.

For *C. sorokiniana* the addition of amounts 4 × 200 mL and 10 × 80 mL of glycerol produce higher biomass yields compared to the addition 2 × 400 mL of glycerol. For *C. kessleri* the addition of amounts (20 × 40 mL, 8 × 100 mL and 4 × 200 mL) of glycerol produce higher biomass yields compared to the addition 2 × 400 mL of glycerol. These results show that the addition of the same total amount glycerol (2.3%, v/v) during of growing period, when added in smaller quantities and more frequently in the growth medium of heterotrophic cultivation *C. sorokiniana* and *C. kessleri*, results in higher biomass yields. Other authors [14] cultivated *C. sorokiniana* with 20 mmol L^{-1} glucose, 20 mmol L^{-1} fructose and 60 mmol L^{-1} Na-Acetate as carbon sources and reported biomass concentrations 1.15, 0.32 and 1.01 g L^{-1} respectively, during the early-stationary phase. These results show a lower biomass concentration compared to our own results (Table 2). The same authors on the heterotrophic cultivation of *C. sorokiniana* with different glucose concentrations (0.2, 2, 20 and 200 mmol L^{-1}) as carbon sources report that the greatest biomass weight (1.60 g L^{-1}) was obtained when the glucose concentration was 20 mmol L^{-1}, during the late-exponential phase. The glucose had inhibitory effects at high concentrations. Liang et al. [15] for the heterotrophic cultivation of *C. vulgaris* with (1%, w/v) glucose as carbon source reported biomass concentration 1.2 g L^{-1} after 12 d of cultivation Other authors [16] in the heterotrophic cultivation of *C. protothecoides* with (1%, w/v) glucose as carbon source in which concentrated glucose solution was batch-fed in a 5-L fermenter containing 3.0 L medium, reported high biomass concentration 3.74 g L^{-1} after 6 d of cultivation.

The results of this study show higher percentages of the lipid content in the biomass of *C. sorokiniana* compared to other carbon sources referenced by other authors for the heterotrophic cultivation of *C. sorokiniana*, who reported a lipid content from 13% to 15% using as carbon sources Na-acetate, molasses and fructose [30]. Qiao and Wang [14] in the heterotrophic cultivation of *C. sorokiniana* with 20 mmol L^{-1} glucose as carbon source reported lipid content 25% in the biomass of *C. sorokiniana* during the early-stationary phase. These results showed a lower lipid content compared to our own results on the heterotrophic cultivation of *C. sorokiniana* (Table 2). Other authors on the heterotrophic cultivation of *C. kessleri* with a carbon source of sucrose under nutrient limitation of N or P have reported a lipid content in biomass of 15% and 16.5%, respectively, these results showed a lower lipid content compared to our own results [31].

3.2. FAs distribution of C. sorokiniana

Table 3 shows the FAs acid distribution of *C. sorokiniana*, while Figs. 1 and 2 show the distribution of FAs with

Table 3
FAs distribution of *C. sorokiniana* for the tree modes of glycerol addition-treatments as shown

A/A	FAs	*C. sorokiniana*		
		FAs distribution as % of total FAs		
		2 × 400 mL	4 × 200 mL	10 × 80 mL
1	C10:0	1.0 ± 0.06^{b}	2.7 ± 0.13^{a}	0.4 ± 0.02^{c}
2	C10:1	0.9 ± 0.05^{b}	6.0 ± 0.35^{a}	–
3	C12:0	0.6 ± 0.05^{b}	2.8 ± 0.15^{a}	0.3 ± 0.02^{c}
4	C12:1	3.2 ± 0.22^{a}	2.9 ± 0.21^{a}	0.6 ± 0.04^{b}
5	C14:0	1.8 ± 0.14^{a}	2.0 ± 0.15^{a}	1.4 ± 0.11^{b}
6	C14:1	1.2 ±0.08^{b}	3.4 ± 0.27^{a}	3.9 ± 0.30^{a}
7	C16:0	27.1 ± 1.99^{a}	14.9 ± 0.81^{b}	14.8 ± 0.85^{b}
8	C16:1	5.7 ± 0.32^{c}	11.2 ± 0.59^{a}	8.3 ± 0.47^{b}
9	C16:2	1.4 ± 0.11^{b}	1.2 ± 0.09^{b}	7.7 ± 0.59^{a}
10	C18:0	6.7 ±0.42^{a}	3.5 ± 0.25^{c}	5.2 ± 0.31^{b}
11	C18:1	27.4 ± 2.06^{b}	36.5 ± 2.66^{a}	31.7 ± 2.28^{ab}
12	C18:2	20.6 ± 1.48^{a}	8.0 ± 0.58^{b}	9.2 ± 0.70^{b}
13	C18:3	2.4 ± 0.17^{c}	4.9 ± 0.37^{a}	3.4 ± 0.24^{b}
14	C20:0	–	–	4.8 ± 0.35
15	C20:1	–	–	0.6 ± 0.04
16	C20:2	–	–	1.7 ± 0.13
17	C22:0	–	–	1.4 ± 0.10
18	C22:1	–	–	0.9 ± 0.07
19	C22:2	–	–	1.8 ± 0.14
20	C24:0	–	–	1.1 ± 0.09
21	C24:1	–	–	0.8 ± 0.06

*For each chemical property of samples, the values in the rows of the table with the same letter do not differ significantly according to the Tukey's test (P = 0.05), n = 4.

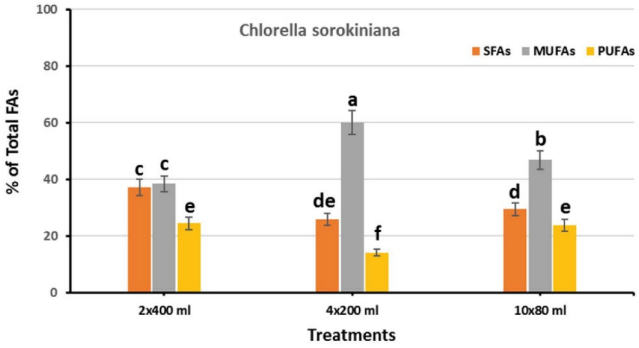

Fig. 1. The distribution of SFAs, MUFAs and PUFAs of the bio-oil of *C. sorokiniana* for the three modes of glycerol addition as shown; The values in the bars of the graph labeled with the same letter on the top do not differ significantly according to the Tukey's test (P > 0.05). Data represent average, (n) = 4.

respect to the degree of saturation and the chain length respectively. FAs from C10 to C24 are produced. From Table 3 it is also noted that the predominant FAs in all treatments is oleic acid (C18:1). Significant amounts of

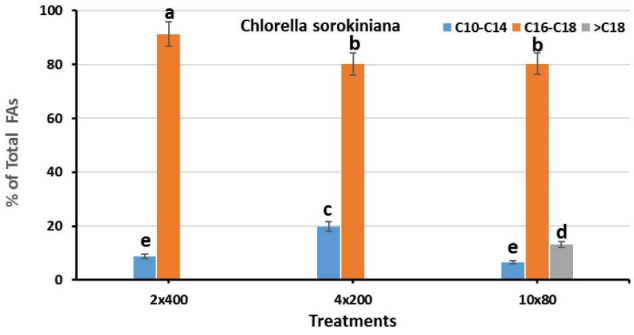

Fig. 2. The distribution of short chain (C10-C14), medium chain (C16-C18) and long chain (>C18) FAs of the bio-oil of *C. sorokiniana* for the three modes of glycerol addition as shown; The values in the bars of the graph labeled with the same letter on the top do not differ significantly according to the Tukey's test (P > 0.05). Data represent the average means ± standard deviation, n = (4).

palmitic acid (C16:0), palmitoleic acid (C16:1) and linoleic acid (C18:2) are also produced. When glycerol is added in large amounts (2 × 400 mL and 4 × 200 mL), FAs up to C18:3 are produced. Adding smaller amounts of glycerol, that is, increasing the frequency of addition, leads to FAs of longer chain length up to C24:1. The addition of 4 × 200 mL of glycerol showed higher percentage of short chain FAs (C10-C14) compared to the other two treatments. The addition of a large amounts of glycerol (2 × 400 mL) showed higher percentage of medium chain FAs compared to the other two treatments (Fig. 2). Medium chain FAs (C16-C18) predominate in all treatments (Fig. 2) and vary from a minimum 80.2% to a maximum of 91.3% (Fig. 2). Short chain FAs (C10-C14) are produced in percentages ranging from 6.6% to 19.8%. However, the mode or frequency of glycerol addition has a more pronounced effect on the saturation of the FAs. Increasing the frequency of addition from 2 × 400 mL to 4 × 200 mL and to 10 × 80 mL decreases the saturated fatty acids (SFAs) from 37.2% to 25.9% and 29.4% of the total FAs respectively. Monounsaturated fatty acids (MUFAs) predominate, attaining their highest value of 60.0% of the total FAs in the 4 × 200 mL treatment.

3.3. FA distribution of *C. kessleri*

Table 4 shows the FAs acid distribution of *C. kessleri* for the four modes of glycerol addition while, Figs. 3 and 4 show the distribution of FAs with respect to the degree of saturation and the chain length respectively. FAs from C10 to C24 are produced. The addition of a smaller amounts (20 × 40 mL) of glycerol showed a higher percentage of long chain FAs compared to the other three treatments (Fig. 4). The addition of 8 × 100 mL amounts of glycerol showed a higher percentage of short chain FAs (C10-C14) compared to the other three treatments. The addition of large amounts of glycerol (2 × 400 mL and 4 × 200 mL) showed a higher percentage of medium chain FAs compared with the addition smaller amounts of glycerol (8 × 100 mL and 20 × 40 mL). Medium chain FAs predominate in all treatments (Fig. 4) and vary from a minimum

Table 4
FAs distribution of *C. kessleri* for the four modes of glycerol addition-treatments as shown

A/A	FAs	*C. kessleri*			
		FAs distribution as % of Total FAs			
		2 × 400 mL	4 × 200 mL	8 × 100 mL	20 × 40 mL
1	C10:0	5.27 ± 0.35^{b}	15.07 ± 1.10^{a}	17.05 ± 1.18^{a}	5.85 ± 0.41^{b}
2	C10:1	3.12 ± 0.22^{b}	–	–	4.94 ± 0.37^{a}
3	C12:0	2.82 ± 0.23^{b}	2.32 ± 0.18^{bc}	6.83 ± 0.52^{a}	2.08 ± 0.17^{c}
4	C12:1	0.61 ± 0.05^{c}	1.39 ± 0.11^{b}	2.82 ± 0.23^{a}	–
5	C14:0	2.35 ± 0.18^{a}	1.36 ± 0.11^{b}	1.22 ± 0.09^{b}	1.22 ± 0.10^{b}
6	C14:1	5.19 ± 0.40^{a}	3.48 ± 0.27^{b}	2.67 ± 0.20^{c}	1.68 ± 0.14^{d}
7	C16:0	22.83 ± 1.80^{a}	12.47 ± 0.97^{c}	24.04 ± 1.97^{a}	15.67 ± 1.21^{b}
8	C16:1	10.32 ± 0.82^{bc}	13.05 ± 1.02^{a}	11.86 ± 0.95^{ab}	9.61 ± 0.75^{c}
10	C18:0	4.02 ± 0.33^{ab}	3.40 ± 0.28^{b}	3.39 ± 0.28^{b}	4.87 ± 0.39^{a}
11	C18:1	23.29 ± 1.82^{b}	34.68 ± 2.73^{a}	21.69 ± 1.64^{b}	31.14 ± 2.42^{a}
12	C18:2	12.52 ± 0.97^{a}	6.62 ± 0.53^{b}	1.82 ± 0.15^{c}	6.30 ± 0.46^{b}
13	C18:3	3.82 ± 0.30^{ab}	4.26 ± 0.35^{a}	3.37 ± 0.26^{b}	1.93 ± 0.15^{c}
14	C20:0	0.71 ± 0.06^{c}	1.90 ± 0.16^{a}	1.56 ± 0.14^{ab}	1.11 ± 0.09^{bc}
15	C20:1	0.86 ± 0.07^{b}	–	1.68 ± 0.14^{a}	1.21 ± 0.10^{ab}
17	C22:0	0.76 ± 0.06^{b}	–	–	2.15 ± 0.17^{a}
18	C22:1	1.51 ± 0.12^{b}	–	–	2.25 ± 0.17^{a}
20	C24:0	–	–	–	5.75 ± 0.45
21	C24:1	–	–	–	2.24 ± 0.17

*For each chemical property of samples, the values in the rows of the table with the same letter do not differ significantly according to the Tukey's test (P = 0.05), n = 4.

Table 5
Calculated FAME properties of the biodiesel obtained from the cultivation of *C. sorokiniana* with treatments: 2 × 400 mL, 4 × 200 mL and 10 × 80 mL

*Property	2 × 400 mL	4 × 200 mL	10 × 80 mL
Saponification number (SN)	201.73 ± 7.6^{ab}	210.68 ± 7.8^{a}	195.18 ± 6.2^{b}
Iodine value (IV)	79.66 ± 3.4^{a}	86.05 ± 3.6^{a}	86.19 ± 3.7^{a}
Cetane number (CN)	55.43 ± 2.6^{a}	52.85 ± 2.3^{a}	54.87 ± 2.5^{a}
Higher heating value (HHV)	39.96 ± 1.8^{a}	39.50 ± 1.7^{a}	40.14 ± 1.8^{a}

*Units: SN: mg KOH g^{-1} FA, IV: g I/100 g FAME and HHV: MJ kg^{-1}; For each chemical property of samples, the values in the rows of the table with the same letter do not differ significantly according to the Tukey's test (P = 0.05), n = 4.

66.2% to a maximum of 76.8%. Chain FAs (C10-C14) are produced in substantial percentages ranging from 15.8% to 30.6%. Long chain (>C18) are produced in percentages ranging from 3.84% to 14.71%. Oleic acid (C18:1) predominates in all treatments and substantial amounts of linolenic acid (C18:3) up to 4.3% of the total FAs are produced. The addition of 8 × 100 mL of glycerol showed higher percentage of SFAs compared to the other three treatments. The addition of 4 × 200 mL and of 20 × 40 mL of glycerol showed a higher percentage of MUFAs compared with the addition of 2 × 400 mL and of 8 × 100 mL of glycerol, while the addition of a large amounts (2 × 400 mL) of glycerol showed a higher percentage of polyunsaturated fatty acids (PUFAs) compared to the other three treatments (Fig. 3). What is so remarkable with *C. kessleri* is the high percentage of SFAs that are produced, about 54.1% for the 8 × 100 mL treatment, surpassing that of the MUFAs but, also being quite high in the other three treatments (Fig. 3). MUFAs predominate in the other three treatments (Fig. 3) and vary from a minimum 40.7% to a maximum of 53.1%. However, PUFAs are produced in percentages ranging from 5.2% to 16.3%.

Comparing the distributions of the two microalgal species *C. sorokiniana* and *C. kessleri*, we note significant differences in the distribution of the FAs of the two species. While, the mode or frequency of glycerol addition affects the distribution of FAs in both species, *C. kessleri* produces excessive amounts of SFAs compared with *C. sorokiniana*. Also, the percentage of PUFAs is lower in *C. kessleri*. Another substantial difference between the two species is the substantial

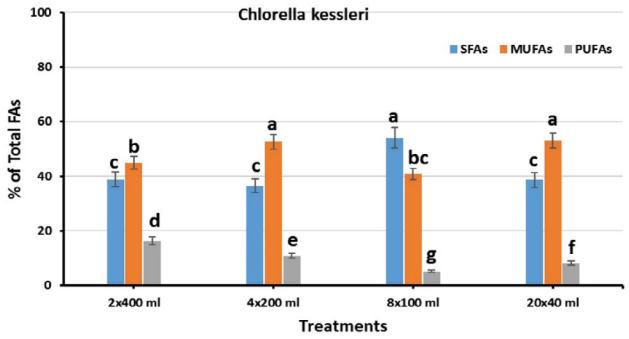

Fig. 3. The distribution of SFAs, MUFAs and PUFAs of the bio-oil of *C. kessleri* for the four modes of glycerol addition as shown; The values in the bars of the graph labeled with the same letter on the top do not differ significantly according to the Tukey's test ($P > 0.05$). Data represent average, (n) = 4.

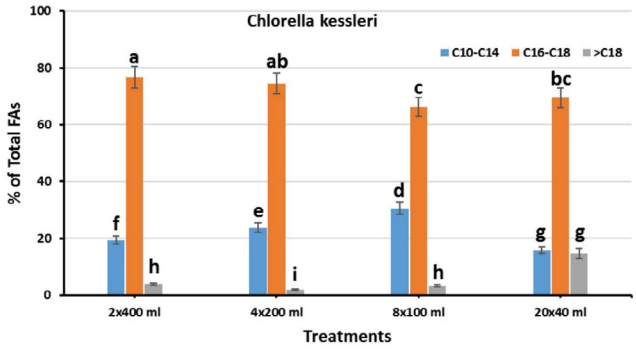

Fig. 4. The distribution of short chain (C10-C14), medium chain (C16-C18) and long chain (>C18) FAs of the bio-oil of *C. kessleri* for the four modes of glycerol addition as shown; The values in the bars of the graph labeled with the same letter on the top do not differ significantly according to the Tukey's test ($P > 0.05$). Data represent average, (n) = 4

percentage of short chain FAs produced by *C. kessleri*, reaching up 30.6% of the total FAs while, the corresponding highest value for *C. sorokiniana* is 19.8%. The distribution of FAs of *C. sorokiniana* and *C. kessleri* are expected to affect FAME properties as is discussed in the next paragraph.

3.4. FAME properties

The FAME properties, SN, IV, CN and HHV are affected, as noted in Eqs. (1)–(4) by the FAs distribution, both with respect to the degree of saturation and also with respect to the chain length. Increasing the degree of saturation leads to a decrease in ignition delay [32]. The ignition delay is related to the CN. As the ignition delay decreases the CN number increases. The CN number is an indication of the quality of the biodiesel produced [21]. The degree of saturation which, affects the IV, is also related to the cold flow properties of the biodiesel. As the degree of saturation increases the IV decreases. Low iodine values lead to biodiesel which is more combustible but it is not suitable for colder climates as it has rather poor flow properties at low temperatures [32]. Also, increasing the average chain length of FAs decreases both the SN and the IV. As the SN and IV decrease the CN increases. However, changes in the value of the SN, according to equation 3, cause more pronounced changes in the value of CN.

Tables 5 and 6 show the values of the four FAME properties, namely the SN, the IV, the CN and the HHV, with respect to the cultivation treatments for the microalgal species *C. sorokiniana* and *C. kessleri* respectively.

It is evident that the cultivation treatment, that is, the mode of glycerol addition, affects these four FAME properties because, as discussed in the previous two paragraphs, it affects the distribution of FAs both with respect to the chain length and with respect to the degree of saturation. FAME properties for both species are within the limits suggested by EN standards. We note that relatively low IV are calculated for *C. kessleri*, especially for the treatment 8 × 100 mL. This IV of 49.2 g I/100 g FAME is caused by the very high degree of saturated FAs in this treatment equal to 54.1% of the total FAs, as shown in Fig. 3. So low iodine values, although they give impart better combustibility properties to the biodiesel, are not acceptable in cold climates as they would cause poor flow properties of the biodiesel at low temperatures. These unusually low IV for *C. kessleri* the treatments 8 × 100 mL and 20 × 40 mL coupled with a higher percentage of long chain FA (>C18) and a lower percentage of short chain FAs (C10-C14) lead to relatively high cetane numbers. However, the bio-oil obtained from the addition of a large amounts (2 × 400 mL) of glycerol for *C. kessleri* compared to the other three treatments contains a higher percentage of medium chain (C16-C18) FAs and of PUFAs leading to a higher iodine value and combined with the highest percentage in bio-oil produced in this treatment (Table 2), is a good option for use in biodiesel production. For *C. sorokiniana* the treatments 2 × 400 mL and 10 × 80 mL lead to CN well above the minimum required value of 51 set by EN standards while, the treatment 4 × 200 mL produces FAME with the lowest cetane number. For *C. sorokiniana* the 2 × 400 mL treatment, compared to the other two treatments, produces the lowest iodine value and the highest cetane number leading to better quality biodiesel.

The continuing dependence on fossil fuels as the prime source of energy raises serious issues for the energy supply in the future and the global warming. The utilization of renewable energy sources is essential both for economic growth and reducing carbon emissions. Microalgal cultivation not only contributes towards atmospheric carbon sequestration but also produces biomass which can be totally valorized. Thus, lipids can be used for renewable energy production and the remaining biomass can be used, for example, as animal feed supplementation. Cultivation of microalgae can therefore contribute, as an alternative renewable energy source, towards reducing dependency on fossil fuels.

4. Conclusions

These results confirm the hypothesis that the mode of glycerol addition affects the lipid and protein content in the biomass of *C. sorokiniana* and *C. kessleri*, the distribution of FAs of the bio-oil obtained and the FAME properties. The addition of 2 × 400 mL and 4 × 200 mL of glycerol in the

Table 6
Calculated FAME properties of the biodiesel obtained from the cultivation of *C. kessleri* with treatments: 2 × 400 mL, 4 × 200 mL, 8 × 100 mL and 20 × 40 mL

*Property	2 × 400 mL	4 × 200 mL	8 × 100 mL	20 × 40 mL
Saponification number (SN)	209.56 ± 7.7^{ac}	215.20 ± 7.9^{ac}	222.90 ± 8.3^{a}	203.55 ± 7.3^{c}
Iodine value (IV)	77.54 ± 3.2^{a}	69.91 ± 2.9^{b}	49.23 ± 2.1^{c}	64.31 ± 2.8^{b}
Cetane number (CN)	54.90 ± 2.4^{a}	55.93 ± 2.5^{a}	59.71 ± 2.7^{a}	58.64 ± 2.6^{a}
Higher heating value (HHV)	39.68 ± 1.8^{a}	39.56 ± 1.8^{a}	39.55 ± 1.9^{a}	40.12 ± 1.9^{a}

*Units: SN: mg KOH g^{-1} FA, IV: g I/100 g FAME and HHV: MJ kg^{-1}; For each chemical property of samples, the values in the rows of the table with the same letter do not differ significantly according to the Tukey's test ($P = 0.05$), $n = 4$.

growth medium of both *C. sorokiniana* and *C. kessleri* produces biomass with a higher content of lipid compared with the other treatments. *C. sorokiniana* is characterized by higher content in lipids, as well as a higher biomass yield compared to *C. kessleri*. The addition of a smaller amounts (10 × 80 mL) of glycerol for *C. sorokiniana* produces biomass with a higher content of protein compared to the other two treatments. For *C. kessleri* the addition of 8 × 100 mL and 20 × 40 mL of glycerol produces biomass with higher content in protein compared to the two other treatments. *C. kessleri* is characterized by a higher protein content compared with *C. sorokiniana* for the same treatments.

For *C. sorokiniana*, medium chain FAs (C16-C18) are predominant in all treatments and vary from a minimum 80.2% to a maximum of 91.3%. The addition of a large amount of glycerol (2 × 400 mL) led to a higher percentage of medium chain FAs and of SFAs compared to the other two treatments while, the addition of a smaller amount of glycerol (10 × 80 mL) led to a higher percentage of long chain FAs compared to the other two treatments. For *C. sorokiniana* the treatments 2 × 400 mL and 10 × 80 mL lead to CN well above the minimum required value of 51 set by EN standards.

For *C. kessleri*, medium chain FAs predominate in all treatments and vary from a minimum 66.2% to a maximum of 76.8%. The treatments 2 × 400 mL and 4 × 200 mL led to a higher percentage of medium chain FAs compared to the other two treatments while, the 2 × 400 mL treatment showed the highest percentage of PUFAs compared to the other three treatments. The unusually low values IV for *C. kessleri* in the treatments 4 × 200 mL, 8 × 100 mL and 20 × 40 mL, although they give better combustibility properties to the biodiesel, are not acceptable in cold climates as they would cause poor flow properties of the biodiesel at low temperatures. However, the addition of 2 × 400 mL of glycerol leads to better FAME properties (IV and CV) and combined with the highest percentage in bio-oil in the biomass of *C. kessleri*, is a good option for use.

Although the addition of a 2 × 400 mL of glycerol for both species shows very good FAME properties, the biomass of *C. sorokiniana* contains bio-oil in higher percentage compared with biomass of *C. kessleri* and also the biomass yield of *C. sorokiniana* is higher. Therefore, *C. sorokiniana* is a good option for biodiesel production and a better candidate than *C. kessleri*.

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