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Fatty Acid Composition and Technological Quality of the Lipids Produced by the Microalga *Scenedesmus dimorphus 1237* as a Function of Culturing Conditions

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Scendesmus dimorphus is an oleaginous eukaryotic microalga, able to produce and accumulate lipids up to a weight fraction of 49%. Aim of the present work is investigating the production of lipids by *S. dimorphus* 1237 and characterize them at a fatty-acid level. The variables accounted for were: the nutrient supply level and the extraction system (Soxhlet and bead beating). Two lipid phases were extracted by sequential, twin-solvent system (hexane and a chloroform:methanol mixture), the quantified gravimetrically and analyzed for their individual fatty acid composition by gas chromatography. The maximal total lipid concentration was measured by Soxhlet extraction in deficient nitrogen conditions (0.88 mg/L) and was found to be 49% (dw). In particular, palmitic acid was increased from 15% to 58% under phosphorous starvation and oleic acid content was increased from 8% to 40% under nitrogen starvation. Finally palmitic, palmitoleic and linolenic acid represented together more than 70% of the extracted lipids.

1. Introduction

Microalgal organisms are primary producers for food chain ecosystems and are considered as a low utility group. However, as land resources are being depleted, microalgae are now treated as substitute resources or new sources for various biological materials (Kim and Lee, 2005). These microorganisms are able to enhance the nutritional content of conventional food preparations and hence, to positively affect the health of humans and animals (Spolalore et al. 2006; Bravi et al. 2012). As the basis of the natural food chain, microalgae play a key role in aqua culture, especially marine culture, being the food source for larvae of many species of mollusks, crustaceans and fish (Pulz and Gross, 2004). Microalgal organisms are also considered one of the most promising feedstocks for biofuels. The productivity of these photosynthetic microorganisms in converting carbon dioxide into carbon-rich lipids, only a step or two away from biodiesel, greatly exceeds that of agricultural oleaginous crops, without competing for arable land. Faced with physiological stresses such as nutrient deprivation, algae store chemical energy in the form of oils such as neutral lipids or triglycerides, by switching from carbohydrates metabolism to lipids metabolism. The algal oil can be extracted from the organisms and converted into biodiesel by transesterification with short-chain alcohols, or by hydrogenation of fatty acids into linear hydrocarbons. The high acidic value of microalgal oil makes them an inconvenient raw material for the traditional biodiesel production. However, by means of a sequential acidic esterification/alkaline transesterification, coupled with a heat integration strategy, the opportunities of accepting microalgae oil as a biodiesel precursor will increase. Biodiesel production from non-edible sources has been extensively studied. Microalgae oil (MAO) can be transformed into biodiesel by several routes. MAO composition have shown high potential for biodiesel production although its free fatty acid (FFA) and poly-unsaturated fatty acid (PUFA, like linolenic acid, C18:3), makes it somewhat technologically troublesome compared to other traditional crops. Recovery of oil from microalgal biomass and conversion of oil to biodiesel are not affected by whether the biomass is produced in raceways or photobioreactors. Hence, the cost of producing the biomass is the only relevant factor for a comparative assessment of photobioreactors and raceways for producing microalgal biodiesel (Chisti, 2007). The possibility to enhance microalgal growth and,

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at the other part, to limit process costs have provided in two ways; first, developing new photobioreactor designs (Moroni et al. 2014, Cicci et al. 2015) second, using an alternative media coming from agro-industrial waste waters (Cicci et al.2013, 2014a-b). The high cost of algae lipids is largely due to the lower-than-expected lipid yield (LY) of algae culture. Nitrogen source and concentration in the growth media greatly influence algae lipid yield. In nitrogen-limited situations, algae lipid content usually increases because lipid-synthesizing enzymes are less susceptible to disorganization than carbohydrate synthesizing enzymes due to nitrogen deprivation; thus, most carbon ends up to be bound in lipids. However, biomass growth is often inhibited in nitrogen-lacking situations, so that each algal strain usually exhibits a lipid yield peak at (species-specific) concentrations of nitrogen-carrying molecules (Shen et al. 2009). Moreover one of the main obstacles to fully taking advantage of lipid-producing microalgae, is the ability to successfully and efficiently extract oil from the cell biomass. Additionally, there is the concern of extracting oil in the safest and most environmentally sustainable manner; therefore, solvent extraction may not always be the optimal solution for recovering oil from the microalgal biomass. Algae also synthesize other fuel products, such as hydrogen, ethanol and long-chain hydrocarbons, that resemble crude oil, or the algal biomass can be converted to biogas through anaerobic fermentation (Wijffels and Barbosa, 2010, Di Paola et al. 2015).

Aim of the present work is evaluating the quality of the lipids extracted from *Scenedesmus dimorphus 1237*. The variable accounted for was the nutrient supply level (balanced medium and nitrogen limited, deficient and supplemented media). The lipid fraction was extracted with a twin solvent system (chloroform:methanol 2:1 v/v mixture and hexane only) and the two extracted lipid phases were quantified gravimetrically and analyzed for their fatty acid composition by gas chromatography. A qualitative and quantitative profile was established for every lipid characterized in depends on the its distribution between two solvent systems.

2. Materials and methods

2.1. Microalgal Cultivation

S. dimorphus (UTEX code: 1237) was obtained from the Culture Collection of Algae at the University of Texas at Austin, USA. The strain on agar was inoculated into a 3Modified Basal (3MB) medium (described in Section 2.2) and was carried out for 4－6 d. The inocula were added to the media. Cultures were grown in 400-mL cylindrical glass tube, with di diameter of 6.5 cm, fed with filtered and humidified air or air enriched in CO2 at 5%; final flow rate was 130 NL/h. Light (90－80 μmol photons (m^2·s) -1) was provided by cool white fluorescence lamps (400-700 nm, 32W) with 16:8h light:dark photoperiod; the temperature was 28 ±1 °C.

2.2. Growth media

A liter of Basal Medium contained: $CaCl_2$ 0.17 mM, $NaNO_3$ 2.21mM, $MgSO_4$•$7H_2O$ 0.3 mM, K_2HPO_4 0.43mM, KH_2PO_4 1.29mM, NaCl 0.43mM, Na_2EDTA•$2H_2O$ 2mM, $FeCl_3$•$6H_2O$ 0.36mM, $MnCl_2$•$4H_2O$ 0.21mM, $ZnCl_2$ 0.037mM $CoCl_2$•$6H_2O$ 0.0084mM and Na_2MoO_4•$2H_2O$ 0.017mM. Nitrate and phosphate concentrations have been modified, in one experiment set phosphate concentration was fixed and nitrate concentration was changed; in the other set both concentrations changed (Table 1).

2.4. Biomass measurements

Absorbance was measured in correspondence to the chlorophyll absorption peak and it was correlated to cellular density, determined by Burker chamber count and dry weight measurement. At the end of the experimental run the culture was collected, centrifuged and washed twice with distilled water. Afterwards, the recovered biomass was transferred in a pre-dried glass flask and dried in a vacuum oven for 4 hours. Dry weight was calculated subtracting the glass flask weight from the total weight. Absorbance data were determined with spectrophotometer UV-1800PC (Shanghai Mapada Instruments Co., Ltd). Specific growth rate was calculated from absorbance at 690nm.

2.5. Cell disruption and lipid extraction

Biomass extraction method selected was soxhlet extractor. Biomass was harvested, washed twice with distilled water, centrifuged and oven-dried at 105 °C. Dried biomass was treated in a soxhlet extractor for 24 hours and 4 syphonations per hour. The lipid weight in each sample was measured gravimetrically. Two different solvents were selected for the extraction: a dichloromethane/methanol mixture (ratio 2:1, v/v) and hexane only; the two solvents were used sequentially to separate the two lipid classes, polar and nonpolar. First, a neutral lipid extraction was carried out by adopting hexane as the solvent; thereafter, polar lipids were extracted by a 2:1 mixture of dichloromethane and methanol.

2.6. Qualitative Lipid Characterization

Fatty acid methyl esthers of each sample were obtained by complete saponification and methylation with boron trifluoride in methyl alcohol, according to ISO 5509:2000. The fatty acid composition was then determined by GC according to ISO 5508:1990. Peak identification was carried out by analogy with retention times of mixtures of methyl esthers with known composition.

2.7. Kinetic and yield parameters

The specific growth rate was calculated by the equation:

$$m\ [\ d^{-1}\ ] = (1/t)\ \ln(X_m/X_0) \quad (1)$$

where X_m and X_0 are the concentrations of biomass at the end and at the beginning of a batch run, respectively, respectively, and t is the duration of the run. The yield in microalgae lipids was calculated by the following equation:

$$L_j\ [\ \%\ ] = 100 \cdot W_{Lj}/W_{Db} \quad (j = \text{neutral, polar}) \quad (2)$$

where W_{Lj} and W_{Db} denote the weights of the extracted lipids of the dried microalgal biomass, respectively.
The lipid productivity in each culturing condition was considered separately for the neutral and for the polar lipid classes (independently from their fatty acid composition) and for the individual fatty acids (independently from their phase distribution during the extraction). The calculation was performed according to the following formulae:

$$\text{j-th lipid class productivity } (Y_j) = \mu * W_L / V_{colture} \quad (j = \text{neutral, polar}) \quad (3$$

$$\text{i-th fatty acid productivity } [\text{ mg } L^{-1}\ d^{-1}] = \Sigma_j \text{ fraction } FA_{ij} * Y_j; \quad (4)$$

3. Results and Discussion

3.1. Lipid Accumulation and extraction yields

Lipid accumulation and microalgal growth were evaluated during balanced (Table 1, run Control) and nutrient limited (Table 1, runs a through g) growth. Table 1 reports the initial Nitrogen and phosphorous ratio of each culture normalized to the initial N:P ratio of the control culture (and therefore it is 1:1 in the control culture).
The nature of solvents and the extraction sequence, first hexane and then dichloromethane - methanol (DM - M), entails that the hexane fraction mostly contains neutral lipids, that is triacylglycerides, while the DM - M fraction mostly contains polar lipids, such as phospholipids, glycolipids and sterols, that compose cell membranes. Only neutral lipids are suitable for transesterification and production of biodiesel (e.g. according to EN 14214 requirements).

Table 1. Nutrients composition of the media and measured growth rate. Relative normalized N:P ratios of the performed cultures. Nn and Pn indicate N and P concentrations normalized to the respective values in the control culture.

Sample	NO_3^-(mM)	PO_4^{3-}(Mm)	$\mu(d^{-1})$	s.d.	Nn	Pn	Nn:Pn
control	8.82	1.76	0.71	0.09	1	1	1
a	4.41	1.76	0.54	0.06	0.5	1	0.5
b	2.2	1.76	0.47	0.02	0.25	1	0.25
c	1.1	1.76	0.46	0.1	0.125	1	0.13
d	0.88	1.76	0.44	0.03	0.1	1	0.1
e	0	1.76	0.07	0.07	0.01	1	0.01
f	8.82	0.44	0.65	0.12	1	0.25	4
g	0.88	0.176	0.35	0.1	0.1	0.1	1

Polar lipids in general, on the other hand, are generally directed to food (nutraceutic) and cosmetic applications. Total lipids were calculated as the sum of neutral and polar lipids. With reference to the control, lipids content tend to increase in reduced-nitrogen cultures, with the apparent exception of moderately reduced-nitrogen cultures which exhibit a minimum of lipid accumulation. This somewhat unexpected phenomenon can be explained by considering that when nitrogen becomes limiting two phenomena can be observed: first, exponential growth ceases and the culture enters the stationary phase; second, the cellular metabolism switches from primary to secondary. Therefore, during this time, a significant portion of the culture time is not benefitting neither of remarkable growth, nor of remarkable lipid synthesis and, as a result, lipid production doesn't allow a highlight accumulation. On the contrary, when a more marked reduction in nutrient

supply is applied, cells enter secondary metabolism right away and lipid synthesis occupies most of the allotted growth time.

Table 2. Lipid productivity of the S. dimorphus cultures. Final standard deviation was calculated on total lipids and is ≤0.15.

sample	Polar lipids (mg/(L d)	Neutral lipids (mg/(L d)	Total lipids (mg/(L d)
control	76	46	122
a	19	19	38
b	15	20	35
c	16	34	50
d	35	51	86
e	4	6	10
f	49	28	77
g	40	44	84

Lipid productivity, shown in Table 2, is maximum under balanced growth, as already pointed out by Xin et al. (2010), Becker (1994), and Rodolfi et al. (2009).

Table 3. Extract composition in % by DMM solvent obtained from biomass growth in the different culturing conditions.

Solvent→				DMM				
Common (or chemical) name↓	Control	a	b	c	d	e	f	g
myristic acid	0	0.3	0.5	0.5	0	0.4	0	0
pentadecenoic acid	0	0.3	0	3.2	0	0	0	0
palmitic acid	21.9	23.3	28.7	41.7	23.3	26.5	21.6	26.2
palmitoleic acid	0	3.2	1.6	1.7	0	2.2	0	0
palmitoleic acid (n-9)	22.2	0	0	0	21.3	0	16.6	9.7
palmitoleic acid (n-7)	0.6	0	0	0	0.6	0	0.4	0.4
hexadecatetraenoic acid	2.9	3.6	0.7	0.7	0	1.5	2.4	2
heptadecenoic acid	0	5.5	1.4	2.1	0	4.9	0	0
stearic acid	1.9	2.1	4	3.5	1.8	2.1	0.8	2.8
oleic acid	14.1	27.1	47.5	29.6	19.1	33.1	17.5	34.2
linoleic acid	4.7	12.2	6	6.9	5.2	11.9	6.6	7
linolenic acid (n-6)	0	0.7	0	0	0	0.5	0	0
linolenic acid (n-3)	12.7	16	7.3	6.9	6.1	13.3	11.1	10.9
stearidonic acid	1	2.2	0	0.9	0	2.1	0.9	1
arachic acid	0	0	0.5	0	1	0	0	0
eicosenoic acid	0	0	0.7	0	0	0	0	0
eicosatrienoic acid	0	0	0	0	1.5	0	1.3	0
behenic acid	0	0	0	0	0	0	1.1	0
unknown	18	3.5	1.2	2.3	20.1	1.5	19.7	5.9
Σ Saturated	21.9	23.5	29.6	42.2	24.3	26.9	22.7	26.2
Σ Short chain (4:0--18:0)	21.9	23.5	29.1	42.2	23.3	26.9	21.6	26.2
Σ Long chain (20:0--24:0)	0	0	0.5	0	1	0	1.1	0
Σ Unsaturated	58.2	70.8	65.2	52	53.8	69.5	56.8	65.1
Σ Monodienes	36.8	36.2	51.2	36.6	41	40.2	34.6	44.3
Σ Dienes	4.7	12.2	6	6.9	5.2	11.9	6.6	7
Σ Trienes	12.7	16.7	7.3	6.9	7.6	13.8	12.4	10.9
Σ Tetraenes	4	5.8	0.7	1.7	0	3.6	3.2	3
Σ Others	19.9	5.7	5.2	5.8	21.9	3.6	20.5	8.7
Linolenic								
(ALA)	12.7	16	7.3	6.9	6.1	13.3	11.1	10.9
(GLA)	0	0.7	0	0	0	0.5	0	0
Ratio unsat./sat.	2.7	3	2.2	1.2	2.2	2.6	2.5	2.5

Total lipids more than double (from 21% to 49%) in Soxhlet extractions (Tables 3,4). Total lipids accumulation then decreases, however, in totally nitrogen deficient cultures (from 49% down to 40%). From the point of view of total lipids content, cultures do not benefit from phosphate limitation; rather, phosphate-limitation reduces the potential accumulation which can be obtained owing to nitrogen limitation (from 49% down to 37%).

Non polar lipids almost quadruply (from 8% to 29%) upon nitrogen limitation (Table 4), while a moderate increase of polar lipids is observed only under severe phosphorus limitation (Table 3). While lipid content strikes the investigator's eye most, lipid productivity is the quantity most related to process cash flow. We therefore calculated this quantity, yet remarking that a low lipid concentration in the raw biomass increases the lipid recovery cost, and may partly or totally defeat the benefit of an overall higher productivity. The runner-up of this experimentation is the maximally nitrogen-limited (but not deficient) culturing condition, for which half of

the obtained biomass was found to be lipids. Other culturing conditions which approach this lipid productivity (culture g) feature a lower total lipids content.

Table 4. Extract composition in % by Hex solvent obtained from biomass growth in the different culturing conditions.

Solvent→				Hex				
Common (or chemical) name↓	Control	a	b	c	d	e	f	g
lauric acid	1.0	0.0	0.0	0.0	0.9	0.0	0.6	1.2
myristic acid	1.6	0.3	0.4	0.5	1.1	0.4	0.9	1.4
pentadecanoic acid	0.0	0.2	0.0	0.2	0.0	0.1	0.0	0.0
pentadecenoic acid	0.0	0.1	0.0	1.2	0.0	0.4	0.0	0.0
pentanoic acid	0.0	0.0	0.0	0.0	1.0	0.0	0.4	0.0
palmitic acid	64.8	29.6	19.6	42.8	33.5	27.5	54.6	56.4
palmitoleic acid	0.0	0.0	1.9	2.0	0.0	2.3	0.0	0.0
palmitoleic acid (n-9)	1.6	4.7	0.0	0.0	8.6	0.0	3.2	1.6
palmitoleic acid (n-7)	0.5	0.0	0.0	0.0	0.6	0.0	0.5	0.4
hexadecatetraenoic acid	0.0	0.8	2.1	0.0	0.0	0.7	0.0	0.0
heptadecanoic acid	0.0	1.3	0.5	0.2	0.0	0.0	0.0	0.3
heptadecenoic acid	0.0	0.0	2.6	0.6	0.0	2.9	0.0	0.0
stearic acid	1.9	3.7	3.3	4.4	2.6	2.8	1.7	3.7
oleic acid	12.6	37.7	45.6	36.2	21.5	40.4	19.2	18.5
linoleic acid	1.9	8.4	8.2	4.0	3.9	9.9	0.9	2.1
linolenic acid (n-6)	0.0	0.4	0.0	0.0	0.0	0.3	1.8	0.0
linolenic acid (n-3)	1.0	7.0	12.0	2.2	2.8	9.2	1.4	0.6
stearidonic acid	0.0	1.4	1.3	0.0	0.0	1.3	0.0	0.0
arachic acid	0.0	0.5	0.0	0.3	0.5	0.2	0.0	0.0
eicosenoic acid	2.3	1.0	0.6	0.6	1.0	0.4	2.2	2.7
eicosatrienoic acid	0.9	0.0	0.0	0.0	0.0	0.0	0.9	0.0
behenic acid	0.9	0.0	0.0	0.0	0.7	0.0	1.2	0.7
unknown	9.1	3.0	2.0	4.6	21.3	1.3	10.6	10.3
Σ Saturated	68.3	31.8	20.5	44.1	37.8	28.2	57.6	60.1
Σ Short chain (4:0--18:0)	67.4	31.3	20.5	43.8	36.5	28.0	56.5	59.4
Σ Long chain (20:0--24:0)	0.9	0.5	0.0	0.3	1.3	0.2	1.2	0.7
Σ Unsaturated	20.7	61.6	74.1	46.9	38.4	67.8	30.1	25.9
Σ Monodienes	16.9	43.5	50.6	40.7	31.8	46.4	25.2	23.2
Σ Dienes	1.9	8.4	8.2	4.0	3.9	9.9	0.9	2.1
Σ Trienes	1.9	7.5	12.0	2.2	2.8	9.5	4.1	0.6
Σ Tetraenes	0.0	2.2	3.4	0.0	0.0	2.0	0.0	0.0
Σ Others	11.0	6.6	5.3	9.0	23.8	4.0	12.2	14.0
Linolenic (ALA)	1.0	7.0	12.0	2.2	2.8	9.2	1.4	0.6
(GLA)	0.0	0.4	0.0	0.0	0.0	0.3	1.8	0.0
Ratio unsat./sat.	0.3	1.9	3.6	1.1	1.0	2.4	0.5	0.4

Figure 1 show that severely nitrogen-starved (but not deficient) culture conditions boost the productivity of the neutral lipid fraction, corresponding to a 5% increase, while they strongly depress the productivity of neutral lipids to a much larger extent than they promote polar lipids; thus, not only does nitrogen starvation promote an overall increased lipid accumulation making recovery more efficient, but also increase the fraction which is suitable for automotive application. Phosphorous-starved cultures, instead, exhibit a lipid class productivity balance leaning toward the polar group. Further technology-related properties are related to the fatty acid profile. Biodiesel production and food and or nutraceutical use have opposite requirements with regard to PUFAs because these latter impair oxidative stability of a diesel fuel (Ballat and Ballat, 2010) while they are an essential (or conditionally essential) nutritional supply. Especially important are, in this latter respect, linolenic and eicosatrienoic acids, with 3 double bonds (Barrow and Shaidi, 2007), and stearidonic acid, with 4 double bonds (Whelan, 2009); while questionable fatty acids such as behenic acid or erucic acid are low or totally absent. Our maximal lipid recovery (from a nitrogen-starved culture subjected to sequential extraction reached 29% of neutral lipids and 20% of polar lipids of the dry biomass weight, for a combined extraction yield of 49% (Figure 1).

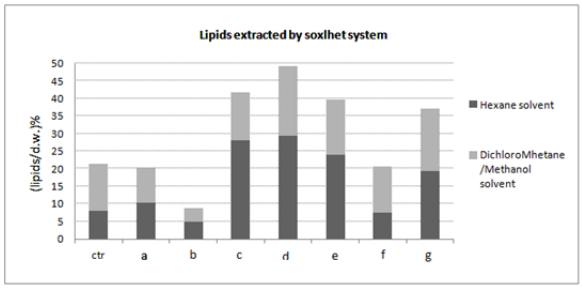

Figure 1 Total lipids sequentially extracted with two solvents by the Soxhlet system.

4. Conclusions

This work showed that *S. dimorphus 1237* under balanced culturing conditions has a total lipid content of 21% by weight. The concentration increase is mainly justified by palmitic, palmitoleic and linolenic acid, representing together more than 70% of the extracted lipids. Nitrogen starvation increases the biomass concentration in total lipids, and especially that of neutral lipids (reaching 49% and 29%, respectively); a significant increase was observed for the productivity of oleic acid (up to +46.4%), linoleic acid (up to +8.8%) and linolenic (n-3) acid (up to +7.2%). Phosphorous starvation increases the biomass concentration in total lipids with respect to the control, but less than nitrogen starvation does, and especially that of polar lipids (reaching 37% and 29%, respectively); a remarkable increase was observed for oleic acid, stearic acid and linoleic acid. Insaturation generally decreases with starvation, which is favorable for biodiesel stability. Starvation decreases or leaves unaltered the productivity of most fatty acids, but somawhat promotes production of heptadecenoic acid, pentadecenoic acid and pentanoic acid.

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