Lipolysis in *T-Isochrysis lutea* during wet storage at different temperatures

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Abstract

Previously, it has been observed that lipolysis occurring during (even short term) wet storage of microalgal biomass causes high free fatty acid (FFA) concentrations in the biomass. These FFA have a negative impact for different applications of microalgal lipids, e.g. downstream processing problems in biodiesel production, off-flavors and loss of nutritional value for food applications. However, it is not clear which factors influence lipolysis in microalgal biomass and which lipid class are more susceptible to lipolysis. In this study, wet biomass of *T-Isochrysis lutea* was stored at 20°C, 4°C and -20°C during 3 weeks. The extent of lipolysis was followed by analyzing the lipid classes distribution by ultrahigh-performance liquid chromatography–accurate mass mass spectrometry (UHPLC-amMS) and the FFA content. It was observed that FFA were formed very rapidly during post-harvest storage of wet biomass at 20°C and 4°C, the rate of this process being faster at 20°C than at 4°C, while almost no lipolysis was observed at -20°C. However, the FFA content levelled off after several days of storage because FFA reacted with alcohols to form fatty acyl esters.

Keywords

stability
microalgae
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free fatty acids
lipolysis
lipase

1. Introduction

Microalgae are an extremely diverse group of unicellular, photosynthetic microorganisms, found in marine, brackish or freshwater environments. Microalgal cells consist mainly of carbohydrates (4-50%), lipids (10-50%) and proteins (30-60%), which all can be used for several applications in different markets (Li et al., 2015; Koller et al., 2014; Xu et al., 2011; Spolaore et al., 2006). Relative amounts of these compounds strongly depend on the species and the nutritional and environmental factors during cultivation (Koller et al., 2014; Spolaore et al., 2006).

The lipid part of microalgae can have applications in different industries. First, microalgal lipids can have a role in the supply of biodiesel. Hereto, triacylglycerols are converted to methyl esters by a transesterification reaction catalyzed by acid or alkali catalysts or by lipase enzymes (Navarro López et al., 2015). In addition, some marine species (e.g. *Nannochloropsis*, *T-Isochrysis*) contain substantial amounts of long chain omega-3 polyunsaturated fatty acids (ω -3 LC-PUFA), which have been demonstrated to possess health-promoting effects, particularly eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) (Calder, 2014). These are promising ingredients for use in among others functional foods, nutraceuticals, pharmaceuticals and cosmetics (Borowitzka, 2013; Spolaore et al., 2006).

Production of microalgal biomass and products derived thereof is performed in several sequential steps. For each step different techniques are possible with their corresponding efficiency and quality implications. In a first step, microalgae are cultivated in open ponds or photobioreactors during a few days to weeks, after which

the microalgal cells are harvested. In most cases, this occurs by centrifugation, but also alternative, less energy consuming techniques, e.g. flocculation and filtration have been proposed. Harvesting yields a wet, microalgal paste which typically contains 5 to 25% of dry matter, depending on the number and type of harvesting techniques used (Mata et al., 2010). In a next step, this paste can be dried to obtain a dry microalgal powder.

To obtain stable products of good quality, storage of microalgae as dried microalgal powder ('dry storage') is preferred. However, a (short) period of storage of microalgal paste after harvesting ('wet storage') is often inevitable in practice. Moreover, life cycle assessment studies have shown that the drying process accounts for a high percentage of the total energy consumption in the production process (e.g. Slegers et al., 2014; Xu et al., 2011). Therefore, during the last years, numerous studies have explored the potential of oil extraction from wet biomass, as this would avoid the energy required for drying (Tanzi et al., 2013; Xu et al., 2011). An attractive option for biodiesel production is direct transesterification of the lipids in the wet biomass (Wahlen et al., 2011). However, for both direct extraction and direct transesterification on wet biomass, the yield of these processes has been found to be strongly negatively influenced by the moisture content (Tanzi et al., 2013; Wahlen et al., 2011). Moreover, only comparisons of lipid yields have been made. Investigations focusing on quality implications have hardly been performed.

During storage of wet biomass, endogenous hydrolytic enzymes present in the biomass may cause lipolysis, being the dissociation of free fatty acids (FFA) from the lipids. These FFA are disadvantageous for several reasons. First, during the biodiesel production

process, saponification reactions can take place between FFA and alkali catalysts if the oil contains more than 0.5% FFA, leading to a reduced amount of catalyst present and several downstream separation problems (Navarro Lopez et al., 2015). In addition, the presence of FFA causes rancid flavors, which are not desirable in food applications. Furthermore, FFA are known to have a pro-oxidative effect (e.g. Aubourg, 2001) and are also more easily oxidized by lipoxygenase enzymes than fatty acids in the esterified form (Frankel, 2005). As oxidation products are also known to have several deleterious effects (e.g. off-flavors, decrease of nutritional value, negative impact on biodiesel production process), this once again shows the importance of avoiding lipolysis (Gordon, 2001).

In literature, several recent studies have indicated that lipolysis may be a serious problem during storage of wet microalgal biomass. Firstly, Ryckebosch et al. (2011a) reported that pronounced lipolysis occurred after two days of wet storage at 4°C of centrifuged microalgal paste of *Phaeodactylum tricornutum*, while this was not the case when the microalgae were dried immediately. This led to significant lipid losses in the biomass. Chen et al. (2012) also observed a fast increase in FFA content from trace proportions to 62% FFA in the oil during 4 days of wet storage of *Scenedesmus* sp. paste (75% moisture) at 4°C. At higher storage temperatures, lipolysis reactions occurred even faster, with 70.3% FFA in the oil after 1 day at 25°C and 66.8% after 1 day at 37°C. These studies proved that even short-term storage of wet biomass can possibly induce substantial lipolysis problems. The presence of lipase activity has already been reported in *Dunaliella salina* (Cho and Thompson, 1986), *Phaeodactylum tricornutum* (Barka et al., 2016) and *Spirulina platensis* (Demir and Tükel, 2010). In *Isochrysis galbana*, Godet et al. (2010, 2012) and Kerviel et al. (2014) isolated and characterized genes encoding

for carboxylesterase and/or lipase-like enzymes for potential use in metabolic engineering to enhance lipid yields in microalgae.

Despite the importance of lipolysis for both biodiesel and food applications, the occurrence of this process during wet storage of microalgal biomass has, to the best of our knowledge, not yet been investigated thoroughly. Underlying processes leading to lipolysis, species dependency and enzyme specificity are thus not elucidated yet. The aim of this study was to understand the time and temperature dependence of lipolysis and FFA formation and accumulation during both short and long term storage of wet microalgal biomass. For this study, *T-Isochrysis lutea* was selected, as this species contains high amounts of lipids, including ω -3 LC-PUFA, and is consequently a promising source of lipids for both biodiesel and food applications (Li et al., 2015). As microalgae contain different lipid classes (e.g. triacylglycerols, phospholipids, glycolipids), we also wanted to investigate whether lipolysis affects all lipid classes to the same degree. Therefore, a detailed separation in lipid classes by ultrahigh-performance liquid chromatography– accurate mass mass spectrometry (UHPLC-amMS) was performed at different times during storage of the biomass at different temperatures.

2. Materials and methods

Solvents used for lipid extractions, FFA determinations and UHPLC-amMS analyses (chloroform, methanol, dichloromethane and ethyl acetate) were HPLC grade and were obtained from Carl Roth (Karlsruhe, Germany) or Biosolve (Valkenswaard, The Netherlands). For UHPLC-amMS analyses, ultrapure water with a resistivity of 18.2 M Ω ·cm at 25 °C was generated with a Millipore system.

2.1. Cultivation and harvesting of *T-Isochrysis lutea*

T-Isochrysis lutea, formerly named *Isochrysis* sp. (CCAP 927/14; Culture Collection of Algae and Protozoa, Oban, United Kingdom), was cultured in Wright's Cryptophyte (WC) medium (Guillard and Lorenzen, 1972) to which artificial sea salt (Homarsel, Zoutman Industries, Roeselare, Belgium) was added in a concentration of 30 g/L. The cultivation was carried out in 30 L photobioreactors incubated at controlled temperature (20°C). The reactors were irradiated with daylight fluorescent tubes in a cycle of 16 light hours and 8 dark hours and were aerated with filtered air. The pH of the medium was adjusted to 8-8.1 and an inoculum of microalgae was added. During cultivation, the pH was controlled at 8.5 (± 0.2) by addition of CO₂ using a pH stat system. Growth of the microalgae was monitored by measuring the optical density at 750 nm.

Harvesting was carried out in the early stationary phase after 10 days of cultivation using a lamella centrifuge (4000 g), followed by a further concentration by centrifugation (9500 g, Sorvall RC-5B, Du Pont Instruments).

2.2. Wet storage of *T-Isochrysis* paste

The harvested wet microalgal paste (7.8 ± 0.5 % dry matter as determined by drying the wet paste to constant weight in an oven at 100°C) was immediately stored in 50 mL falcon tubes in the dark at 3 different temperatures (20°C, 4°C and -20°C) during a period of 3 weeks. Samples were collected at 8 different time points (0, 0.5, 1, 2, 3, 7, 14 and 21 days) and immediately freeze dried and stored at -80°C until analyses. Preliminary experiments have shown that microalgal biomass remains stable in these conditions (detailed results not shown).

2.3. Analyses

2.3.1. Lipid extraction from microalgal biomass

Total lipids of all collected samples were extracted with chloroform/methanol (1:1 v/v) according to the method described in Ryckebosch et al. (2011b) and determined gravimetrically. Briefly, 4 mL methanol, 2 mL chloroform and 0.4 mL water were added to 100 mg of lyophilized microalgae. In case of extraction for FFA determination (described in 2.3.2), 5 mg of lauric acid (C12:0) (Nu-Check Prep, Elysian, USA), dissolved in chloroform, was added as an internal standard. After homogenizing, 2 mL chloroform and 2 mL water were added. The samples were homogenized again and subsequently centrifuged at 750 g for 10 minutes. The upper (water) layer was removed, while the lower layer was transferred to another tube. The remaining pellet was re-extracted with 4 mL of chloroform/methanol (1:1) and centrifuged again at 750 g for 10 minutes. The supernatant was transferred to the same tube as before while the pellet was extracted again according to the same procedure. The combined solvent layers were filtered through a sodium sulphate layer to remove the remaining water. The lipid content was measured gravimetrically after evaporating the solvents. All extractions were done in triplicate.

2.3.2. Determination of FFA content

The FFA content was determined by derivatization of the FFA in the lipid extract to diethylamide derivatives according to Ryckebosch et al. (2011a), based on Kangani et al. (2008). Therefore, 10 mg of the extracted lipids was dissolved in 1 mL dichloromethane. After addition of 10 µl diisopropylethylamine and 30 µl diethylamine (Sigma-Aldrich,

Bornem, Belgium), the solution was cooled to 0°C. 20 µl of bis(2-methoxyethyl)aminosulfur trifluoride (Sigma-Aldrich, Bornem, Belgium) was added dropwise. After homogenizing, the mixture was kept at 0°C for 5 minutes, subsequently warmed to room temperature and kept there for 15 minutes. Water (2 mL) and hexane (4 mL) were added, after which the solution was homogenized and centrifuged at 750 g for 10 minutes.

The upper layer, containing the amide derivatives, was analyzed by gas chromatography with cold on-column injection and FID detection (Trace GC, Interscience, Louvain-la-Neuve, Belgium). An EC Wax column (length 30 m, ID 0.32 mm, film 0.25 µm; Grace, Lokeren, Belgium) was used with the following time-temperature program: 100 to 160 °C at 10 °C/min, 160 to 240 °C at 2°C/min, 240°C for 54 min. Peak areas were calculated with Chromcard 2.5 for Windows software. The areas of the peaks were summed and compared with the area of the internal standard (C12:0) to calculate the total FFA content. Determination of the FFA content was performed in triplicate on each sample.

2.3.3. Separation in lipid classes by UHPLC-amMS

UHPLC- amMS analysis

A standard stock solution of trans-β-apo-8'-carotenal (Sigma-Aldrich, Bornem, Belgium) was prepared at a concentration of approximately 3000 µg/mL in dichloromethane, to which 0.1% BHT was added (Sigma-Aldrich, Bornem, Belgium) and was used as an internal standard.

To investigate the distribution of the lipid classes, the lipid extracts (obtained in 2.3.1) of all collected samples were analyzed with UHPLC- amMS. After evaporating the

solvent, approximately 4 mg of the dry extract was redissolved in 0.5 mL 1:1 chloroform:methanol + internal standard (*trans*- β -apo-8'-carotenal at a concentration of approximately 15 μ g/mL) and injected with a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) on a Waters Acquity UPLC BEH Shield RP C18 column (2.1 mm x 100 mm, 1.8 μ m; Waters, Milford, MA). Analytes were thermostatically (50°C) eluted at a flow rate of 0.5 mL/min with an Accela™ quaternary solvent manager and a 'Hot Pocket' column oven (Thermo Fisher Scientific, Bremen, Germany). The mobile phase solvents consisted of water + 20 mM ammonium acetate (A), methanol + 20 mM ammonium acetate (B) and ethyl acetate (C). The gradient was set as follows (min/%A/%B): 0.00/50/50, 20.00/0/100, 25.00/0/20, 27.00/0/20, 28.00/50/50, 30.00/50/50. The LC-system was hyphenated to an orbitrap mass spectrometer (Q Exactive; Thermo Fisher Scientific, Bremen, Germany). Full MS scan data were acquired in HESI positive and negative mode within one analysis with a mass-to-charge range (m/z -range) of 90-1350 and resolving power set at 70,000 Full Width at Half Maximum (FWHM). Data were also recorded using selective ion fragmentation (data dependent MS² or ddMS²) in positive and negative mode (one analysis per mode) to obtain additional structural information (resolving power set at 17,500 FWHM, collision energy 30 V, isolation window: 4 m/z). Each sample was analyzed in duplicate.

Data handling

A hybrid quadrupole-orbital trap MS-analyser was used for lipid identification. This enables selective ion fragmentation, a functionality that contributes significantly to compound identification by generating clean product ion spectra. Selective ion

fragmentation is particularly useful for associating product ions with precursor ions during coelution of multiple lipids, as is often the case in complex extracts. The most abundant precursor ions were selected for lipid quantification. The total area per lipid class was obtained by summation of all areas of different components (e.g. different fatty acyl chain) within this lipid class. This area was corrected both for the internal standard (*trans*- β -apo-8'-carotenal) and for the sample weight. Because no response factors were available for all lipid classes, no relative composition of the lipid fraction could be calculated. Instead, arbitrary relative areas were calculated by dividing the corrected areas by the sum of all corrected areas within a sample. Within each lipid class, the highest value was set to 100, thus obtaining relative abundances varying from 0 to 100 in each lipid class. These relative areas do not represent percentages within a lipid sample, but rather the evolution of the amount of each lipid class during the storage period.

2.4. Analysis of volatile compounds in wet biomass by Headspace-Solid Phase

Microextraction – Gas Chromatography – Mass Spectrometry (HS-SPME-GC-MS)

To investigate the presence of alcohols, the wet samples were analyzed by GC-MS. A fully automated sample preparation unit (MultiPurpose Sampler® or MPS®, GERSTEL®, Mülheim an der Rur, Germany), combined with a 6890/5973 GC–MS system (Agilent Technologies®, Palo Alto, CA) was used for compound separation and identification. This method is based on a previous study (Vandamme et al., 2015). Briefly, about 100 mg of the wet biomass was hermetically sealed in 20 mL vials and incubated for 30 minutes at

60°C using agitation. Extraction was done using a 75 µm CAR/PDMS fiber type for 30 minutes. The loaded SPME-fiber was thermally extracted in the GC-injector. Helium was used as a carrier gas (1 mL/min). Injector and transfer lines were maintained at 250°C and 280°C, respectively. The total ion current (70 eV) was recorded in the mass range from 40 to 230 amu (scan mode) and a run time of 52 min. For GC–MS profiling, a ZB-WAXplus column, 30 m x 0.25 mm I.D., 0.25 µm film thickness (Phenomenex®, Utrecht, Netherlands) was used and programmed: 40 °C (5 min) to 250 °C at 5 °C/min, held for 5 min. Identification of volatiles was performed by comparison with the mass spectra of the Wiley® 275 library.

3. Results and discussion

3.1. Evolution of lipid content during wet storage

The total **lipid content** (on dry basis) was measured in the start biomass (before storage, i.e. day 0) and in all collected biomass samples that were stored at three storage temperatures (20°C, 4°C and -20°C) for three weeks. The start biomass of *T-Isochrysis* had a total lipid content of 31.3 ± 0.8 %. This is in agreement with values found in literature for *Isochrysis sp.*, varying between 18 and 34% depending on cultivation conditions such as medium, growth phase and light intensity (Huerlimann et al., 2010; Zhu et al., 1997). Although some significant differences could be detected, no clear increase or decrease of the lipid content as function of time was observed for any of the storage temperatures (Table 1).

Table 1: Evolution of the total lipid content on dry basis (g/100 g biomass) during storage of wet microalgal paste at 20°C, 4°C and -20°C.

storage time	Total lipid content (g/100 g biomass)		
	20°C	4°C	-20°C
directly after harvesting	31.3 ± 0.8 ^{ab}	31.3 ± 0.8 ^{ab}	31.3 ± 0.8 ^a
12 hours	29.5 ± 0.4 ^{abcd}	29.8 ± 1.1 ^{abc}	32.0 ± 0.9 ^a
1 day	30.2 ± 0.4 ^{abc}	29.3 ± 1.6 ^{bc}	30.8 ± 0.3 ^{ab}
2 days	27.1 ± 1.7 ^{abc}	29.9 ± 1.3 ^{abc}	31.0 ± 0.6 ^{ab}
3 days	29.2 ± 2.1 ^{bcd}	30.0 ± 1.0 ^{abc}	31.2 ± 1.0 ^a
7 days	28.1 ± 1.0 ^{cd}	27.8 ± 0.9 ^c	29.3 ± 0.4 ^{bc}
2 weeks	29.6 ± 0.6 ^{abcd}	30.2 ± 1.1 ^{ab}	31.5 ± 1.1 ^a
3 weeks	31.9 ± 1.6 ^a	32.0 ± 1.5 ^a	27.7 ± 1.9 ^c

^{abcd} Different superscripts indicate significantly differing values per storage temperature (p≤0.05, Tukey test).

3.2. Evolution of FFA content during wet storage

The **FFA content** in the lipid fraction of all samples was determined and the results are presented in Figure 1.

Figure 1: Evolution of the FFA content (% of total lipids) during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C (●), 4°C (○) and -20°C (▼).

3.2.1. Temperature effects

During storage at -20°C , almost no lipolysis was observed. Although the FFA content was significantly higher after one day storage than in the start biomass, the FFA content remained approximately stable during 3 weeks of storage. When biomass was stored at 4°C , the FFA content increased very rapidly during the first days of storage. In the biomass stored at 20°C , this increase occurred even faster. This demonstrates a strong temperature dependency of lipolysis reactions. These results are also in agreement with the study of Chen et al. (2012), in which the increase in FFA content during 1 day of wet storage of *Scenedesmus* sp. was highest between 25°C and 37°C . When stored at 4°C , a significant increase was also observed, but to a lesser extent, while there were no FFA formed during storage at -20°C and -80°C . Ryckebosch et al. (2011a) also found a

substantial increase in FFA content during 2 days of wet storage of *Phaeodactylum tricornutum* biomass at 4°C.

3.2.2. Origin of hydrolytic enzymes

As the cultivation of *T-Isochrysis lutea* was not conducted axenically, an additional experiment was performed to ensure that the observed lipolysis reactions were caused by endogenous enzymes and not by bacterial lipases. Therefore, a mixture of 5 antibiotics (250 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ gentamycin, 100 µg mL⁻¹ kanamycin, 500 µg mL⁻¹ neomycin, 50 µg mL⁻¹ streptomycin) was added to the wet biomass of *T-Isochrysis lutea* after harvesting and subsequently stored at 4°C during 2 weeks. This method was adopted from Cho et al. (2002), in which it was shown that this antibiotic mixture inhibited bacterial growth in a contaminated culture of *Isochrysis galbana*. The FFA content was followed at different time intervals and compared to the control biomass, to which no antibiotics were added. It was observed that the FFA content was not significantly different between the biomass treated with antibiotics and the control biomass (detailed results not shown). It could thus be concluded that the lipolysis observed in *T-Isochrysis* originated from endogenous hydrolytic enzymes present in microalgal biomass.

3.2.3. Lipolysis induction mechanism

A very high FFA content (7.5 ± 0.3 % of total lipids) was already observed in the biomass immediately after harvesting. To clarify whether these FFA were already present in the biomass prior to harvesting or were formed during harvesting, this value was compared to the FFA content of a smaller sample of *T-Isochrysis* biomass that could be harvested

more rapidly and subsequently freeze dried immediately. In this sample, the FFA content was 2.50 ± 0.04 % of total lipids, which is significantly lower, but still not negligible. However, it is unlikely that high amounts of FFA are present in intact, living cells. FFA are possibly interfering with enzyme functions and inhibit electron transfer. Moreover, the higher sensitivity of FFA to oxidation causes the production of degradation products that are toxic for cells (Berge et al., 1995). Therefore, it was hypothesized that the FFA observed in freshly harvested biomass were produced during and immediately after harvesting. The shorter harvesting protocol caused a lower FFA content in the start biomass but was not able to completely avoid lipolysis.

The rapid formation of FFA in *T-Isochrysis* biomass may be caused by an innate defense mechanism against grazers. When diatoms are consumed by grazers and the cell integrity is compromised, lipase enzymes are activated and these rapidly form FFA, which are then further converted by lipoxygenase and hydroperoxide lyase enzymes into several oxylipin compounds (e.g. aldehydes), which are toxic for several types of grazers (Cosse et al., 2008; Pohnert, 2002). It is suggested that in *T-Isochrysis* cells, which have a weak cell wall consisting of mannose (Espinosa et al., 2010) or even do not have a cell wall (Zhu and Lee, 1997), this mechanism initiated by lipase enzymes starts very rapidly, activated by the rupture of the cells during harvesting. This could possibly explain the high amounts of FFA in the start biomass and the very rapid increase during the first hours or days of wet storage.

3.2.4. Possible causes for the level off effect

A remarkable observation in Figure 1 is the level off effect that was observed in the FFA content starting after 7 days of wet storage at 20°C and after 2 weeks at 4°C. Several hypotheses can possibly explain this effect.

Firstly, it is possible that conditions in the harvested, microalgal paste are evolving towards unfavorable for enzymatic reactions. The most important factors determining enzymatic reactions are temperature and pH. Temperature was kept constant during storage, and thus could not explain changes in lipolysis rate. The pH was followed during the storage experiment and was found to decrease from 6.0 to 5.2 when storing at 20°C. As in literature rather high pH optima, varying between 6.5 and 9.0, are reported for lipases in microalgae (Demir et al., 2010; Terasaki et al., 2002), this pH decrease could possibly affect the enzymatic reaction. Therefore, an additional experiment was conducted in which the pH was set at 4.0 ± 0.1 immediately after harvest to make the pH even more unfavorable for lipolytic enzymes. The pH was kept at this value during 2 weeks of storage at 4°C. Figure 2 compares the obtained results with a sample in which the pH was not adapted. The FFA content in the start biomass was higher (14.3 ± 0.5 % of total lipids) than in the previous experiment (7.5 ± 0.3 % of total lipids). This can be explained by the fact that different batches always vary in composition. In addition, the rapid increase of the FFA content during the first hours of wet storage causes a high variation in the beginning of the storage period. It can be observed that lipolysis was not inhibited by lowering the pH. On the contrary, the process was found to start faster and reach the same end value as the control sample after 1 week of storage. Probably, the lower pH caused a more rapid rupture of the cell and consequently faster lipolysis reactions. From this additional experiment, it could be concluded that the level off of

the FFA content in the lipid fraction was not caused by unfavorable conditions for hydrolytic enzymes.

Figure 2: Evolution of FFA content (% of total lipids) during storage of wet microalgal paste of *T-Isochrysis lutea* at 4°C, both without adapting the pH (control, ●) and with holding the pH at a lower value (pH 4.0, ○).

A second possible explanation for the stabilization of the FFA content is the lack of substrate. Possibly, the lipase enzymes present in *T-Isochrysis* act specifically on some lipid classes, causing termination of the process when these specific lipid classes are exhausted. To investigate this hypothesis, a separation in lipid classes was conducted on all collected samples. Figure 3 presents the evolution of all lipid classes during wet storage of *T-Isochrysis* at 20°C. During storage at 4°C, almost the same trends were observed, although occurring at a lower rate, while at -20°C, no substantial changes were observed. This confirms the (from Figure 1) expected temperature dependency of the degradation reactions.

Figure 3: Evolution of different lipid classes during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C. Amounts are represented as relative abundances (arbitrary units).

The **neutral lipid** class consisted mainly of FFA, TAG (triacylglycerols), DAG (diacylglycerols) and MAG (monoacylglycerols), for which the evolution during storage

at 20°C is presented in Figure 3A. The evolution of the FFA showed the same trend as was obtained previously by the method described in 2.3.2 (Figure 1). All other neutral lipids showed a decreasing trend during wet storage. TAG were thus converted to DAG, followed by conversion to MAG and eventually to glycerol, in each step accompanied by the release of FFA. This process started immediately after harvest. TAG, DAG as well as MAG followed the same evolution, decreasing to a value that is not equal to zero. However, because no response factors were available, the remaining percentage of these lipid classes in the lipid fraction after 3 weeks of storage could not be calculated.

In the **glycolipid** class (Figure 3B), the following compounds were found: MGMG (monogalactosyl monoacylglycerol), MGDG (monogalactosyl diacylglycerol), DGMG (digalactosyl monoacylglycerol) and DGDG (digalactosyl diacylglycerol). MGDG and DGDG can be converted to respectively MGMG and DGMG by release of FFA and can be further degraded to galactosylglycerols, also accompanied by the release of FFA. It can be observed from Figure 3B that all glycolipids were degraded very rapidly during storage at 20°C and disappeared after only three days of wet storage. **Phospholipids** (mainly phosphatidylglycerol and phosphatidylinositol) were only found in very small amounts in the lipid samples, as peak areas in LC-MS analyses were very small. Nevertheless, a clear evolution could be observed during storage (Figure 3C), again being a very fast decrease during storage. The same observation could be made for the other polar lipids in the samples, which were DGTS (diacylglycerol-O-(N,N,N-trimethyl)-homoserine), MGTS (monoacylglycerol-O-(N,N,N-trimethyl)-homoserine), SQDG

(sulfoquinovosyl diacylglycerol) and SQMG (sulfoquinovosyl monoacylglycerol). DGTS and SQDG are degraded to respectively MGTS and SQMG, which can then be further degraded by loss of the last fatty acyl chain, in each step accompanied by the release of a FFA. It could thus be concluded that all polar lipids were broken down very rapidly by a degradation process that started immediately after harvesting and which resulted in the absence of polar lipids after 3 days of wet storage at 20°C. This is in contrast with the study of Chen et al. (2012) on wet storage of *Scenedesmus* sp., in which the phospholipid content remained constant during 4 days of storage at 4°C.

A remarkable observation is the increasing amount of **fatty acyl esters**, mainly ethyl-, propyl-, butyl- and pentylesters present in the lipid fraction, as presented in Figure 3E. Fatty acyl esters were probably formed by an esterification reaction between free fatty acids, released during degradation of lipids, and alcohols. As storage was conducted in the dark and no oxygen supply was present, alcohols were possibly formed during dark fermentation reactions (Atteia et al., 2013). To confirm the presence of these alcohols, the volatile components of all wet biomass samples (before freeze drying) were analyzed by headspace-solid phase microextraction – gas chromatography – mass spectrometry (HS-SPME-GC-MS). Via this method, the presence of ethanol, propanol and pentanol could be confirmed and the amounts were found to increase during storage at 20°C and 4°C. During storage at -20°C, none of these alcohols were found. The increasing amounts of ethanol even completely matched with the increasing amounts of the corresponding ester (Figure 4). These findings confirm the hypothesis that the formation of fatty acyl esters is associated with the presence of alcohols. Possibly, the reaction between alcohols and free fatty acids is an enzymatic reaction catalyzed by the

lipase enzymes, as external lipases are already used in several applications to convert free fatty acids to fatty acyl esters, for example in enzymatic transesterification during biodiesel production (Navarro Lopez et al., 2015).

Figure 4: Evolution of fatty acyl ethyl esters (FAEE), determined by UHPLC- amMS) compared to the evolution of the corresponding ethanol, determined by HS-SPME-GC-MS, during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C (●), 4°C (○) and -20°C (▼).

Figure 3F presents an overview of the trends in the amounts of the different lipid classes. For this figure, the different lipid compounds within one class are taken together, because they followed the same evolution, as could be seen in Figure 3A-E. In general, it can be seen that all lipid classes, and not one lipid class specifically, were degraded from the beginning of the storage experiment, immediately after harvest. This implies the presence of non-specific hydrolyzing enzymes, possibly rather esterases than lipases. The polar lipids seem to disappear faster than the neutral lipids, but this may be explained by the lower amounts of polar lipids present in the beginning of the storage experiment. After 7 days of storage at 20°C, the FFA content started to level off, while the neutral lipids were not yet at the minimum value. It could thus be concluded that substrate limitation could not be the reason of the level off effect. It is hypothesized that the decline in the rate of FFA formation over time is due to a dynamic balance between hydrolysis of esterified fatty acids and re-esterification of free fatty acids with alcohols.

3.2.5. Implications for lipid applications

The results of this study will have important implications for the different applications of microalgal lipids. The fast increase in FFA during storage at 20°C and 4°C makes storage of wet microalgal paste of *T-Isochrysis* at 20°C or 4°C undesirable. Even short term storage at these temperatures will result in high amounts of FFA (7 to 30% of total lipids). To completely avoid lipolysis problems, wet biomass should be stored at -20°C or further processed (dried) immediately. But even then, a high amount of FFA (7.5% of the lipids) is already present in *T-Isochrysis* due to hydrolysis processes going on during harvesting. This high percentage already exceeds the maximum value for biodiesel

production (0.5% FFA) (Navarro Lopez et al., 2015) and also the provided value for edible oils (0.1% FFA) (Belitz et al., 2009) and is consequently problematic for these applications. In current applications with high FFA oils, refining is conducted either by soap formation with alkali materials, or by a pretreatment with acid or alkali catalyzed transesterification processes, in order to strongly reduce the FFA percentage. However, this results in yield losses and much higher production costs (Chai et al., 2014) and should thus better be avoided. Further research should reveal the microalgal species dependency of lipolysis processes and possible remediating actions to diminish lipolysis during wet storage.

4. Conclusions

It was observed that FFA were formed by endogenous enzymes during and/or immediately after harvesting of *T-Isochrysis lutea*. This FFA content increased rapidly during post-harvest wet storage at 20°C and 4°C, while almost no lipolysis was observed at -20°C. However, the FFA content levelled off after several days of storage, probably because FFA reacted with alcohols to form fatty acyl esters. It could be concluded that even short term wet storage of *T-Isochrysis lutea* biomass at 20°C or 4°C is detrimental for lipid quality and should be avoided to have biomass of good quality for food and fuel applications.

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Figure captions

Figure 1: Evolution of the FFA content (% of total lipids) during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C (●), 4°C (○) and -20°C (▼).

Figure 2: Evolution of FFA content (% of total lipids) during storage of wet microalgal paste of *T-Isochrysis lutea* at 4°C, both without adapting the pH (control, ●) and with holding the pH at a lower value (pH 4, ○).

Figure 3: Evolution of different lipid classes during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 4°C. Amounts are represented as relative abundances (arbitrary units).

Figure 4: Evolution of fatty acyl ethyl esters (FAEE), determined by UHPLC- amMS) compared to the evolution of the corresponding ethanol, determined by HS-SPME-GC-MS, during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C (●), 4°C (○) and -20°C (▼).

Caption of supplemental material

Appendix 1: Spectral and chromatographic data used for tentative identification of lipids in the *T-Isochrysis lutea* samples.