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Extraction Behavior of Different Conditioned S. Rubescens

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Abstract: Microalgae utilized for experiments are often not produced by the researchers that are doing experiments with them. The microalgae are made storable through thermal or freeze-drying by the producer. In an industrial scaled process, because of efficiency reasons, microalgae would not be dried, but processed directly. With that, the question is, if drying already could change the composition or structure that much, that a process scaled up from laboratory to productive scale with fresh microalgae would be less efficient or even would not work at all. The effect of freeze drying on the extraction behavior for the species *Scenedesmus rubescens* was investigated. It was obtained in freeze-dried condition and again was delivered in fresh state. The utilized microalgae were extracted with n-hexane, without and with different pretreatments (acidic hydrolysis and hydrothermal carbonization) to examine the differences in the yields. In conclusion, it was demonstrated that freeze drying harms the cell wall and therefore this process already influences the quantity of extracted lipids. Depending on the harshness of the treatment process for cell wall disruption this might influence the extracted yield when the algae are not freeze-dried. The quality of the extracted lipids does not change when freeze-dried.

Keywords: microalgal oils; microalgae extraction; lipids; biofuel; cell wall disruption; hydrothermal disintegration; freeze-dried; fresh harvested; *Scenedesmus rubescens*

1. Introduction

Very often, the microalgae utilized for experiments are not produced by the researchers that are doing the experiments. The microalgae are made storable by the producer, which means they are harvested, centrifuged and heat or freeze-dried. This makes handling and timing for the consumer much easier. But these pretreatment steps are usually very energy intensive and especially the drying would not be conducted when working in an industrial process where microalgae would be produced as a bulk material and afterwards processed further, for example in an extraction process. For saving costs, in such an industrialized process the freshly harvested microalgae would be fed directly into the process. As it is likely, that beforehand in the R&D stage, freeze-dried microalgae were obtained and utilized, the question is, if the pretreatment (freeze drying) already did changes to the structure that makes the industrial process with fresh microalgae not that efficient or even does not work at all?

In the literature, several papers can be found that deal with the question of cell wall disruption because of an inevitable treatment like harvesting or some kind of dewatering or drying.

Falco et al. [1] analyzed spray dried *Spirulina platensis* with scanning electron microscopy (SEM) and showed that their morphology comprised of globular particles with an average size of several

micrometers. The form of an intact *Spirulina (Arthrospira*) cell is helical/spiral. Therefore, the cell is already destroyed by the drying process. This is likely to happen with any process which applies mechanical pressure on a *Spirulina (Arthrospira*) cell.

Although a high disintegration grade for *Arthrospira* is already supposed to be reached by freeze drying and direct extraction, (i.e., a high fraction of lipids can be extracted without the need for a disintegration step), for other species this might be different [2,3]. In [4] *Chlorella* and *Scenedesmus* species needed a harsher method such as acidic hydrolysis and hydrothermal treatment to break up the cell wall structure and enhance the extraction yield.

Bohutskyi et al. [5] investigated the influence of harvesting techniques and storage conditions of several microalgae species and states that microalgae with a polysaccharide cell wall are less sensitive to rupture compared to those without it. In [6] a cyanobacterium *Aphanothece microscopica Nägeli* was dried at temperatures ranging from 40 °C to 60 °C at different thickness layers. The highest protein and lipid yields were achieved with 60 °C and a layer thickness of 7 mm (in comparison to 5 mm).

In [7] two diatoms (*Chaetoceros* sp. and *Phaeodactylum tricornutum*) were investigated towards their chemical composition after different preservation techniques. Direct freezing of the concentrated medium seemed the most reliable technique for the preservation of these microalgae. The proximate composition concerning proteins and lipids did only change slightly, even after a longer period (2 month). For air (30 °C) and freeze-dried algae these preservation techniques resulted in high percentage, around 70%, losses of lipids directly after treatment and also lost further organic components due to oxidation or bacterial activity after 2 months of storage. Protein content and profile did not change significantly. In contrast, Morist et al. [8] investigated treatment methods for *Spirulina platensis* pasteurization, spray-drying, and freeze-drying. Again, the protein content and profile did not change significantly, but the content of five different fatty acids was also measured. In this case the fatty acid content did not change to this high degree between the different treatment processes. The paper concluded that the freeze-drying method is the most recommended method because of almost no product degradation and higher biomass quality.

In this publication we investigate the effect of freeze drying for the species *Scenedesmus rubescens.* It was obtained in freeze-dried condition and, while doing experiments for another purpose, we had the chance to obtain algae from the same species from the same producer (IGV GmbH) in fresh state, still in the growth medium. The fresh microalgae were used as is (only centrifuged to lower the water content), but also for comparison, this charge of microalgae was freeze-dried and again processed like the original freeze-dried example. With this, it was possible to directly compare if it made a difference for the pretreatment and extraction if this species was freeze-dried or not. As already conducted in [4], the utilized microalgae were extracted with n-hexane, with and without different pretreatments (acidic hydrolysis and hydrothermal carbonization) to examine the differences in the yield and thereby give an answer on the consequences of making microalgae storable by freeze drying.

2. Materials and Methods

2.1. Material

The species used in this study; *Scenedesmus rubescens (S. rubescens)*, was provided kindly by the IGV GmbH, Germany. *S. rubescens* is a green microalga which grow in freshwater. About 2 kg of it was obtained. The microalgae was freeze-dried after harvesting. It was conducted at IGV GmbH. The freeze-dried algae had a residual water content of approximately 6 wt.%. The microalgae can be stored like this at room temperature for several months. The second (fresh) charge of *S. rubescens* was also obtained from IGV GmbH and was tested directly after centrifugation from the medium. Characteristics resulting from morphology should be the same, but because of seasonal differences and possibly different bioreactor set-ups between the freeze-dried and fresh obtained microalgae the absolute measures of the compounds may vary.

2.2. Freeze Drying

As the fresh obtained *S. rubescenes* from IGV GmbH should be compared to the ones obtained that were already freeze-dried by the producer, freeze drying also was conducted in our own lab. For this work, this was conducted with a special freeze dryer (Alpha 1-2 LDplus, Christ). For this, 100 g of the centrifugate of fresh *S. rubescens* was weighed in a crystallizing dish. The dish was covered by a watch glass and freeze-dried for 72 h until it was a constant mass. The residue was pulverized in a mortar prior to headspace-Karl-Fisher-Titration determining 8.5 wt.% residual water.

2.3. Properties

The raw material was dried in a Laboratory Drying Oven at 105 °C for 24 h to determine the water content [4]. The water content of the hydrothermal carbonization (HTC) product was determined by headspace-Karl-Fisher-Titration (Aqua 40.0, Elektrochemie Halle GmbH) based on DIN EN 14774-1 [4]. Carbon, nitrogen, hydrogen, and sulphur content were measured according to standard methods (DIN EN 15104) using an Elementar Vario Macro Cube (Elementar Analysensysteme GmbH, Hanau, Germany) [4]. C, H, N, and S were reported in weight percentage on a dry basis and higher heating value (HHV) in kJ/kg on dry basis [4]. Determination of the HHV was done by the CEN method (DIN EN 14918) using a Parr oxygen bomb calorimeter 6400 (Parr Instrument (Germany, Frankfurt)) [4].

2.4. Determination of Fatty Acid Profile by GC-MS

Analysis was performed on a 7890A gas chromatograph coupled with a mass-selective detector (5975C; Agilent Technologies, USA) [4]. The injection mode was split 1:15. A deactivated liner was inserted into the injection chamber, which was kept at 300 °C 1 µL of the sample was injected for separation by an ionic liquid phase (1,12-Di(tripropylphosphonium)dodecane bis (trifluoromethylsulfonyl) imide; SPB-IL 60 30 m × 0.25 mm x 0.20 µm, Sigma Aldrich) [4]. The gas chromatography (GC) system was operated in programmed-temperature mode: initial temperature 160 °C, first linear ramp 1 °C min^{-1} until 165 °C, 15 min hold, second linear ramp 5 °C min^{-1} until 250 °C final temperature, 1 min hold. Data acquisition was performed on the mass-selective detector in scan mode (40–450 amu) [4]. Due to changes in laboratory work flow a part of the measurements were done on a Polyethylenglycole phase (HP-INNOWax, 30 m × 0.25 mm × 0.25 µm; Agilent Technologies) The injection mode was split 1:15, the deactivated liner was kept at a temperature of 260 °C [4]. When using the HP-INNOWax-column, the GC system was operated in programmed-temperature mode as well: initial temperature 150 °C, 0.5 min hold, linear ramp 4 °C min^{-1} until 260 °C final temperature, 7 min hold. Data acquisition was performed on the mass-selective detector in scan mode (40–380 amu) [4]. An external calibration was done for both of the capillary columns separately [4].

Samples for GC-MS determination was prepared as follows: 100 mg of the extracted algae oil were dissolved in 5 ml tert-butyl methyl ether (MTBE) [4]. 100 µL of the MTBE solution was transferred to a GC-Vial equipped with a 0.2 ml micro-insert [4]. 50 µL trimethylsulfonium hydroxide (TMSH) were added to this solution. The mixture was shaken shortly and measured immediately [4]. The formed fatty acid methyl esters (FAME) were identified by mass spectral data (National Institute of Standards and Technology (NIST) 2008 Mass Spectral Library) and matching of retention times with FAME standard substances [4]. Quantitative determination was carried out by serial dilution of Grain Fatty Acids Methyl Ester Mix (Sigma Aldrich, Taufkirchen Germany) and applying an external linear calibration function [4].

2.5. Acidic Hydrolysis

For comparison and as an example for possible disintegration methods, microalgae were hydrolyzed by hydrochloric acid prior to the solvent extraction. In accordance to DIN 10342:1992-09 approximately 18 g of the homogenized algae sample, 135 mL water and 65 mL hydrochloric acid (37%) were stirred thoroughly and heated slowly until boiling [4]. Smooth boiling was maintained for 1 hour,

followed by filtration and repeated rinsing of the residue and filter paper with distilled water [4]. The filter residue was dried at 105 °C for 24 h and weighed, yielding 61% hydrolyzed product.

2.6. Hydrothermal Treatment

The hydrothermal experiments were conducted in a 500 ml batch autoclave (Berghof, Highpreactor BR-500) with data logger, magnetic agitator and BTC 3000 temperature controller [4]. Temperature and pressure were recorded online. The system was stirred with 100 rpm to increase the heat transfer and avoid hot spots. Reaction temperature was typically 200 °C [4]. Heating time was 90 min (2 K/min rate) Holding time was in general 120 min [4]. Concentration of algae was 20 wt.%, as the algae had a water content of approximately 5.5 to 7 wt.% although being freeze-dried, the corrected dry weight was considered when calculating the yields of HTC char and lipids [4]. The cooling was carried out without external cooling over a period of several hours. After cooling, the resulting solid matter was separated by means of vacuum filtration and dried at 105 °C until a constant weight was attained [4]. The gaseous phase was expanded via a gas sampling valve. The volume of the gaseous phase was measured. As the produced gas mainly consisted of CO_2 and the volume produced is low in general at these process conditions [9] a further examination of the gaseous phase was neglected [4].

2.7. Extraction of Algae Oil

Extraction of the algae oil was done by Soxhlet extraction of the algae sample (freeze-dried or hydrolyzed) or the HTC product [4]. The solid was pulverized and homogenized in a porcelain mortar. The sample was transferred to a fat free extraction thimble and extracted continuously for 7 h [4]. As extraction agent n-hexane was used, as hexane extraction is presently seen as the most economical method for algae extraction [10].

All experiments were conducted only twice because of the limited amount of algae. It was not possible to obtain more microalgae of the same charge [4]. More repetitions of the experiments by using smaller amounts for each run would have led to less products for analyzing and less data. For all educts, intermediates and products, elemental composition, heating value, and, if possible, ash content were analyzed [4]. The extracted lipid yields were measured and put in relation to the input material [4].

3. Results and Discussion

The elemental composition of the microalgae without any treatment (Table 1) is very similar. It shows that there should only be minor differences in the composition of the different charges of *S. rubescens*.

Table 1. Elemental composition of the algae species and products.

Algae Species	C	H	S	N	HHV	H_2O
	wt.% (dw)	wt.% (dw)	wt.% (dw)	wt.% (dw)	kJ/kg (dw)	wt.% (dw)
S. rubescens						
Algae (freeze-dried)	48.40	8.61	0.37	9.30	21,670	5.62
HTC char	54.20	5.39	0.56	11.30	25,100	
Lipid from direct extraction	77.30	11.10	0.32	0.83	39,310	
Lipid HTC pretreated extraction	76.10	9.84	0.34	0.79	38,960	
Fresh *S. rubescens*						
Algae fresh (but freeze-dried)	50.80	8.98	0.44	9.05	22,290	8.49
HTC product from fresh S. rubescens	58.90	6.40	0.79	11.50	26,690	
Lipid from direct extraction of heat dried algae	74.50	10.40	0.37	0.91		
Lipid from direct extraction of freeze-dried (fresh) algae	74.70	10.50	0.33	0.31	37,780	
Lipid HTC pretreated extraction	72.00	10.60	0.34	0.91	38,880	

The HTC char yield of the fresh *S. rubescens* was between 45.5 and 55.9 wt.%. For freeze-dried *S. rubescens* it was between 53.6 and 54.9 wt.%. For the HTC char the carbon and hydrogen content was a bit higher for the fresh algae. For the directly extracted lipids, it was the original freeze-dried algae which had a higher carbon and hydrogen content. As this was only around 3 wt.% and 0.7 wt.% respectively, its questionable if this can be neglected.

The HTC pretreated extracts have differences in the carbon content, but the HHV was again already the same. The elemental composition of the freeze-dried *S. rubescens* and its products char and lipid was comparable to the values for the fresh *S. rubescens* algae and products. Therefore, in terms of the elemental composition the process of freeze drying in this work does not seem to have an influence.

The lipid yields achieved with the freeze-dried and fresh *S. rubescens* under the different pretreatments are given in Figure 1. Direct extraction of the freeze-dried *S. rubescens* gave the lowest yield with 55 g/kg. The acidic treatment gave an approximately 81% higher yield (100 g/kg). The yield after HTC treatment was 63 g/kg. For the fresh *S. rubescens*, again direct extraction gave the lowest yield. Freeze drying gave a yield of around 102 g/kg which was comparable to the yield after HTC treatment with 103 g/kg. The acidic treatment gave an approximately 80% higher yield (180 g/kg).

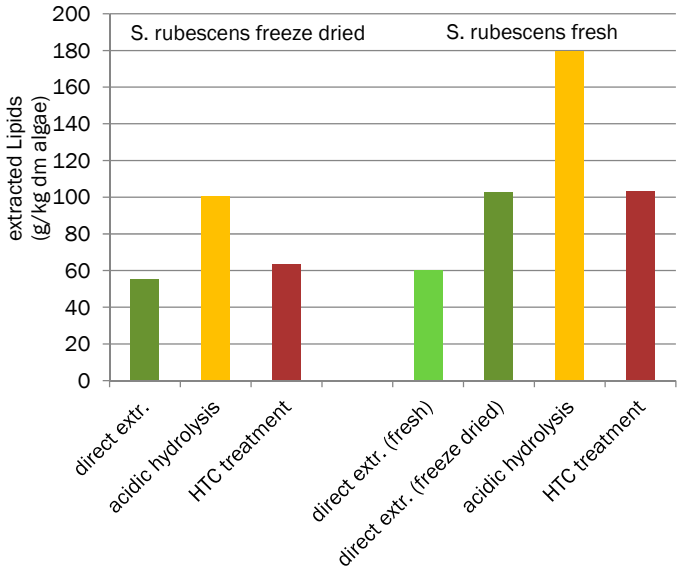

Figure 1. Extraction yields for fresh and freeze-dried *S. rubescens* at different pretreatments.

The lipid yields extracted from the fresh *S. rubescens* did vary in absolute measures from the yields of the freeze-dried *S. rubescens*. The reason for this can be found in seasonal differences and different bioreactor setups. Therefore, the absolute measures of the two charges can not be compared.

Nonetheless, as the species and therefore the morphology of the two charges is the same, the ratio of the different pretreatments is proportional for the two samples. The utilization of the fresh *S. rubescens* shows that mere freeze drying disintegrates the algae and elevates the extracted lipid fraction. For both samples (fresh and originally freeze-dried) the lipid yield was elevated by acidic hydrolysis at a similar ratio (74 wt.% and 81 wt.% respectively). The yield decreases for both fresh and freeze-dried *S. rubescens* when hydrothermally treated.

The GC-MS analysis of the Fatty Acids (FA) (Figure 2) generally showed a good correlation between originally freeze-dried and fresh *S. rubescens*. The sum of the direct extracted lipids was about 10 wt.% lower for the freeze-dried *S. rubescens*, but this is not due to one specific FA.

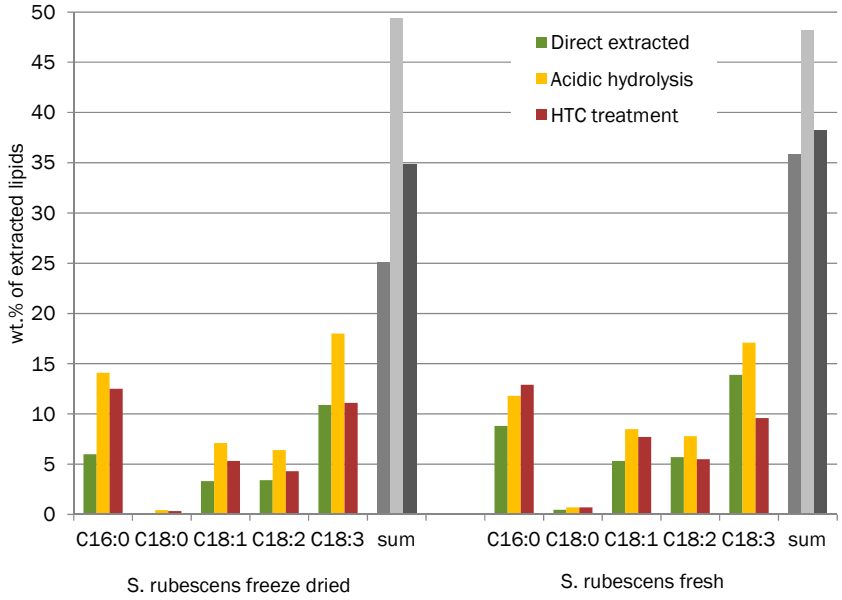

Figure 2. wt.% of main fatty acids and percentage of recognized fraction of *S. rubescens*.

In Figure 3 the percental fraction of recognized FAs was displayed for the fresh and freeze-dried *S. rubescens*. C16:0 increases from direct to acidic hydrolysis to HTC extraction for the freeze-dried example. For the fresh *S. rubescens* only the HTC extracted yield was higher. For C18:0 and C18:1 the increase from direct to acidic hydrolysis to HTC extraction was similar. For C18:2 the differences between the yields of the extraction methods are low, but the decrease from direct to HTC extraction can be seen for both examples. For the C18:3 FA the percental yield decreases for both algal examples. The decrease of the C18:3 FA might be because of a concentration change when breaking the cell wall and diluting other kinds of lipids. On the other hand, one or more of the double bonds could be opened by a hydrolysis or oxygenation reaction. A decrease of C18:3 FA with increase in temperature was also determined in [11]. The increase of the C16:0 FA might be due to a higher content of C16:0 FA in the cell wall.

Thereby, the proportional changes in the FA composition for the different methods show a good correlation between the freeze-dried and fresh *S. rubescens*. The increasing and decreasing of the yields from direct to acidic hydrolysis to HTC extraction was generally quite similar for all fatty acids displayed.

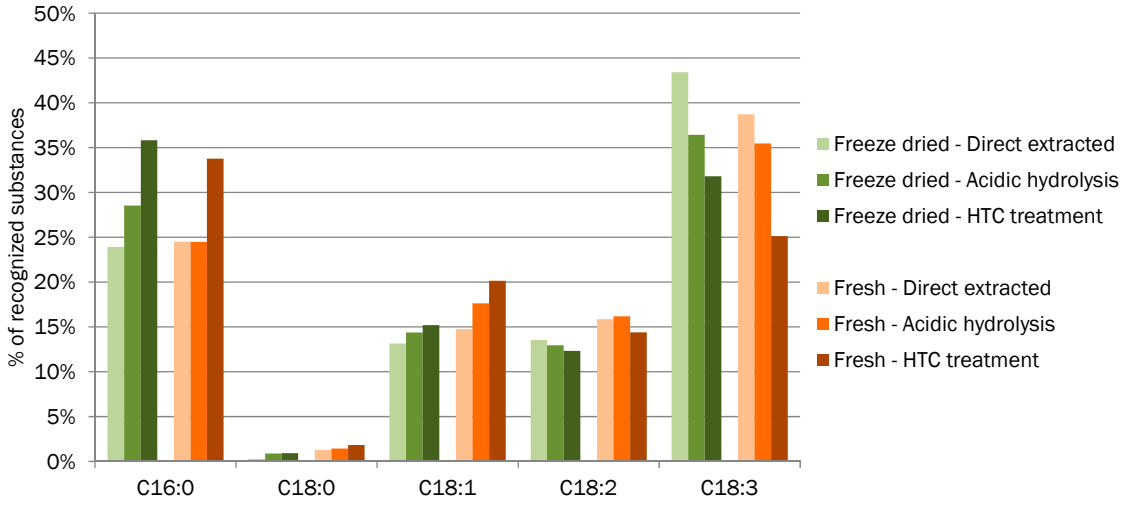

Figure 3. Main fatty acid percentage of the sum of recognized fraction for *S. rubescens*.

4. Conclusions

Concerning the question if using fresh or freeze-dried material for laboratory experiments matters, it has to be stated, that there is a difference in the results found in the conducted experiments for this work. The freeze drying of the fresh *S. rubescens* gave a higher yield of lipids then the direct extraction of the fresh ones. Therefore, there must have been some kind of disruption of the cell wall, which has to be taken into account when scaling up the considered process. For the lipid extracts it can be stated that freeze drying does not thoroughly alter the fatty acid composition and thereby should not lead to problems in process scale up.

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