KN3

Extraction of lipids from wet or dried microalgae

M.Sova[1*], E.Lack[1], J.Hell[1], J.Fernandes[1], S.Pereira[2], A.R. Serra[2], S. Badenes[2], L.Costa[2], S. Reyer[3], M. Stehr[3], V. Verdelho[2]

[1]Natex Prozesstechnologie GesmbH, Ternitz, Austria
[2]A4F-Algafuel, S.A., Lisbon, Portugal
[3]IOI Oleo GmbH, Witten, Germany

* office@natex.at

ABSTRACT:

The extraction of target compounds from algal biomass has become a bottleneck in the algae biorefineries. Algae are becoming a very important raw material for the extraction technologies, opening a variety of new challenges for the recovery of specific fractions from algal biomass.
The extraction using supercritical CO_2 as solvent is a potential technology for the recovery of algal lipids or pigments. We discuss the advantages and requirements of the CO_2 extraction within this topic.
The necessity for a drying step prior to the extraction in the algae biorefinery opened up a discussion about alternative extraction processes. Extraction with liquid propane seems to be a promising technique. During the course of the project PUFAChain (grant agreements n° 613303), we were able to gather first experiences with extraction of the lipid fraction from different algal strains using liquid propane as solvent. The major advantages of this technology are lower extraction pressure compared to the supercritical CO_2 extraction and the fact that the biomass can be extracted without the necessity of drying.

INTRODUCTION

The targets of PUFACHAIN project were highly purified omega-3 fatty acids (EPA and DHA), for nutrition and pharmaceutical applications. Microalgae were chosen as an alternative sustainable source, because a further increase of using fish oil would risk destroying fish populations. The project aimed for the development and pilot-scale implementation of a complete microalga-based process from screening to feedstock production and harvesting to oil extraction and purification, from lab to prototype. Included was also a sustainability assessment based on high quality real data from practical experiences from each step of the production chain. A consortium of 6 companies and 3 research institutes combined research and industrial process development, building the links of the value chain.
The value chain started with the screening and selection of the appropriate strains from the enormous biodiversity of microalgae in two culture collections (SAG at University of Göttingen and Culture Collection of Cryophilic Algae at Fraunhofer, Potsdam). Almost 100 strains were screened for the quantity of EPA and DHA. Finally, 6 strains were selected as relevant for the project.
Previously unexplored and bench-mark strains were further characterised and the most suitable cultivation strategies for both laboratory-scale and industrial-scale applications were developed (A4F, Portugal) using flat panel and tubular photo-bioreactors from 10L to 1.5m³. For harvesting, new integrated membrane-based filtration, including the reuse of process water was successfully tested for algae harvesting (MAHLE, Germany). Various extraction procedures

combined with different cell disruption methods were evaluated to reflect the sensitivity of the unsaturated fatty acids, i.e. for optimally producing high quality oils at lower costs (NATEX). These include extraction of dry biomass using supercritical CO_2 or extraction of concentrated wet algae biomass by propane to provide crude oil and defatted algae pellets. The crude algal oil can be purified to gain highly purified and concentrated fatty acids employing a cascade of purification steps (IOI Oleo, Germany). The integrated value chain was also critically evaluated for its environmental and socio-economic sustainability to support a commercial scale-up with lowest possible impacts (IFEU, Germany, DLO, Netherlands). This way, health benefits of PUFA's can be realized using innovative, optimized and integrated technology while conserving fish populations (Friedl, 2018).

Phycologists regard any organism with chlorophyll *a* and a thallus not differentiated into roots, stem and leaves to be an alga. Cyanobacteria are included in this definition, even though they are prokaryotic organisms. The interest for these two groups of phototrophic organisms lies in their potential utilization, in a similar way heterotrophic microorganisms, to produce biomass for food, feed and fine chemicals, using solar energy. Microalgae are found all over the world. They are mainly distributed in the waters, but are also found on the surface of all type of soils. Although they are generally free-living, a certain number of microalgae live in symbiotic association with a variety of other organisms. (Richmond, 2004)

The biodiversity of microalgae is enormous and they represent an almost untapped resource. It has been estimated that about 200,000-800,000 species in many different genera exist of which about 50,000 species are described. Over 15,000 novel compounds originating from algal biomass have been chemically determined. Most of these microalgae species produce unique products like carotenoids (e.g. astaxanthin, fucoxanthin, phytoene and phytofluene), antioxidants, fatty acids (e.g. EPA, DHA), enzymes, polymers, peptides, toxins and sterols. (Wikipedia, 2018)

The chemical compositions of various microalgae are shown in Table 1. Microalgae contain lipids and fatty acids as membrane components, storage products, metabolites and sources of energy. Algae lipids have a wide range of potential applications. Algal oils possess characteristics similar to those of fish and vegetable oils, and can be considered as potential substitutes for the products of fossil oil.

Table 1 *Chemical composition of algae expressed on dry matter basis (%)* (Baddiley & al., 1994, p. 178)

Strain	**Protein**	**Carbohydrates**	**Lipids**	**Nucleic acid**
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Scenedesmus quadricauda	47	-	1.9	-
Scenedesmus dimorphus	8-18	21-52	16-40	-
Chlamydomonas rheinhardii	48	17	21	-
Chlorella vulgaris	51-58	12-17	14-22	4-5
Chlorella pyrenoidosa	57	26	2	-
Spirogyra sp.	6-20	33-64	11-21	-
Dunaliella bioculata	49	4	8	-
Dunaliella salina	57	32	6	-
Euglena gracilis	39-61	14-18	14-20	-
Prymnesium parvum	28-45	25-33	22-38	1-2
Tetraselmis maculata	52	15	3	-
Porphyridium cruentum	28-39	40-57	9-14	-
Spirulina platensis	46-63	8-14	4--9	2-5

Spirulina maxima	60-71	13-16	6-7	3-4.5
Synechoccus sp.	63	15	11	5
Anabaena cylindrica	43-56	25-30	4-7	-

Microalgae lipids can be divided into neutral lipids consisting of triglycerides and free fatty acids, polar lipids (glycol- and phospholipids) and unsaponifiable matters (phytol, sterols). (Linxing, Gerde, Lee, Wang, & Harrata, 2015)

The success of an extraction process is based on the solubility of target compounds in the solvent and availability of these compounds in the biomass matrix. Considering our target fatty acids to be DHA and EPA, it is crucial to understand the type of microalgae lipids these fatty acids are present in.

It is assumed that about 13600 tonnes of algae biomass, dry basis, is commercially produced worldwide, almost exclusively in open ponds, mostly of the raceway paddle wheel mixed design. The main microalgal species currently produced (>90% of total) are the cyanobacterium *Arthrospira sp. (Spirulina) (Arthrospira platensis,* about 9100 dry tonnes) and *Chlorella (Chlorella vulgaris,* about 3600 tonnes) cultivated in several dozen plants, ranging in size from several tens to a few thousand tons (dry basis) annual production capacity. China accounts for approximately 2/3 of total world production, which is sold mainly for human food products, with bulk (plant gate) selling prices *Arthrospira sp (Spirulina)* and *Chlorella* dried biomass of typically $10-25/kg and $20-40/kg. (Laurens & al., 2017)

Microalgae are a promising source of nutrients. The main products obtainable are dried algae with high nutrients content and high-value compounds such as fatty acids, pigments and antioxidants. Although the research activities on microalgae based nutrients are very promising, the products currently on the market are still limited. There are two main categories of food market products obtained from microalgae. The first category is dried algae (in particular *Chlorella* and *Spirulina*) with high nutrients content, especially of vitamin B12, C and D2. These products can be directly sold as dietary supplements. The second type is specialty products isolated and extracted from the microalgae that can be added to food and feed to improve their nutritional value. These high-value compounds are pigments (e.g. astaxanthin), antioxidants (e.g. ß-carotene), proteins and fatty acids (e.g. phycocyanin, EPA, DHA). (Vigani, et al., 2015) Many other fractions isolated from microalgae find applications also in the cosmetic industry (e.g. minor carotenoids).

NATEX as specialist for extraction technology has focused its research and developing strategy mainly on the extraction step, which was identified as a bottleneck in the microalgae biorefinery. Compared to the high investigated microalga biomass production technologies and the concentration processes, the extraction step needs still a lot of innovation to reach industrial practice. NATEX is further a competent partner for scale-up and introduced several supercritical processes to industrial applications. Therefore we developed two extraction process lines, one for dried solid raw material and one for wet/liquid algae slurry. The extraction of solid raw material is already done in large quantities especially for astaxanthin from *Haematococcus pluvialis*.

The oil extraction is highly debated topic in the microalgae biorefinery, because this process step is one of the quite costly processes having influence to the economics. In the growth medium the microalgae concentration is just several g/l and the biomass has to be concentrated to a solid matrix to apply a well-known extraction process used to extract oil from oil seeds. There are three main processes:

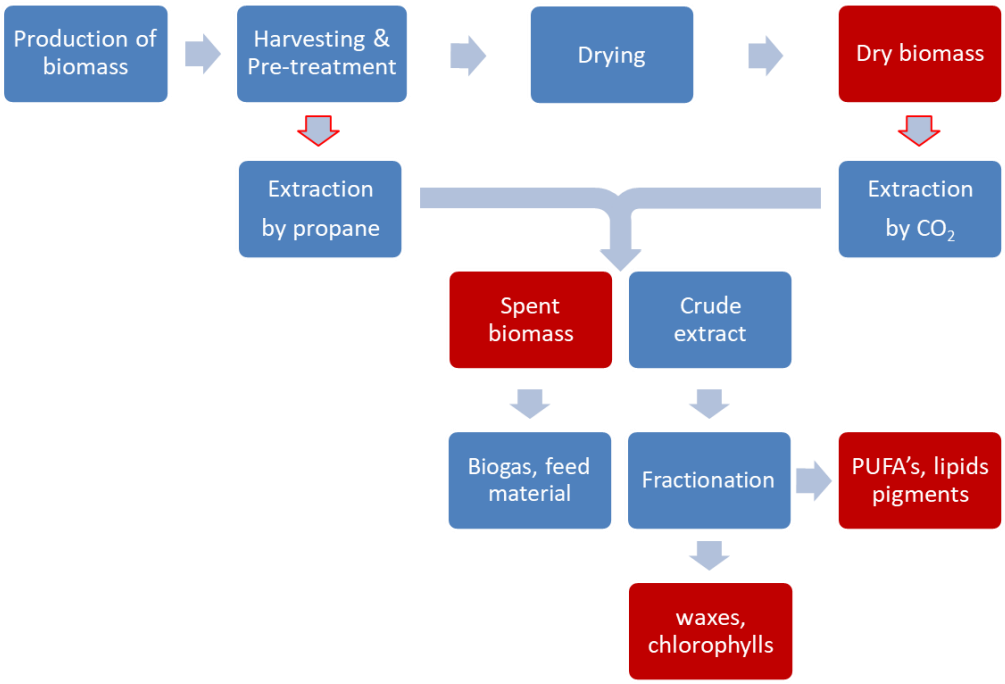
Expeller/Press: For using expeller or press machines the algae material has to be dried. As algae material is so fine a lot of solids go through the strainer and make further separation processes necessary. Further, 6 to 10% of the oil remains in the press cake.

Solvent extraction with hexane: Algal oil can be extracted with chemicals like hexane, this process is relatively inexpensive. The use of hexane brings up same toxic problems in the downstream products of the oil and the extracted residual biomass. Hexane or other organic solvents bring problems with explosion hazards into the biorefinery.
Supercritical fluid extraction with CO_2: The process avoids any toxic problems and explosion hazard and is currently the most important green extraction process to extract triglycerides, carotenoids and sterols out of dried algae biomasses, although it cannot process wet algae material. Water change the equilibrium and reduce the solubility of oil in CO_2. The solubility of polar lipids in CO_2 is very low and extraction not feasible.
Extraction of wet algae biomass by different techniques is also being evaluated. Most of them are in research stage and have not yet reached industrial application.
Enzymatic extraction: This process uses enzymes to degrade the cell wall and the content of the cell is released into the surrounding media. The costs of the process are much higher compared to alternatives.
Osmotic shock: Osmotic shocks can cause cell rupture and release cellular components to the surrounding solution. This process needs additional chemicals and further separation.
Ultrasound-assisted extraction: Ultrasonic waves create cavitation bubbles in the algae medium, after the bubbles collapse the cell wall of the algae can break and release the content of the cell into the surrounding solution. Further ultrasound waves help, that small oil droplet coalescence to larger drops which can be easier separated from the solution. The process is energetically very intensive.
In the project Pufachain, the new whole biorefinery concept of microalgae was evaluated, including following operations:

- production of biomass (with algal crop rotation principle);
- harvesting techniques (based on membrane filtration);
- cell disruption methods specific to microalgae strains (based on osmotic stress, cryo technology or pulsed electric field)
- extraction of dried or wet (concentrated) biomass
- fractionation of crude oils by enzymatic approach

Additionally to the EPA and DHA, any compounds of commercial interest present in the produced biomasses and co-extracted, were at least evaluated in the business models increasing the potential overall feasibility of the biorefinery concepts. The whole biorefinery is shown in Figure 1. The red marked positions are potential final products.

Figure 1 Biorefinery concept

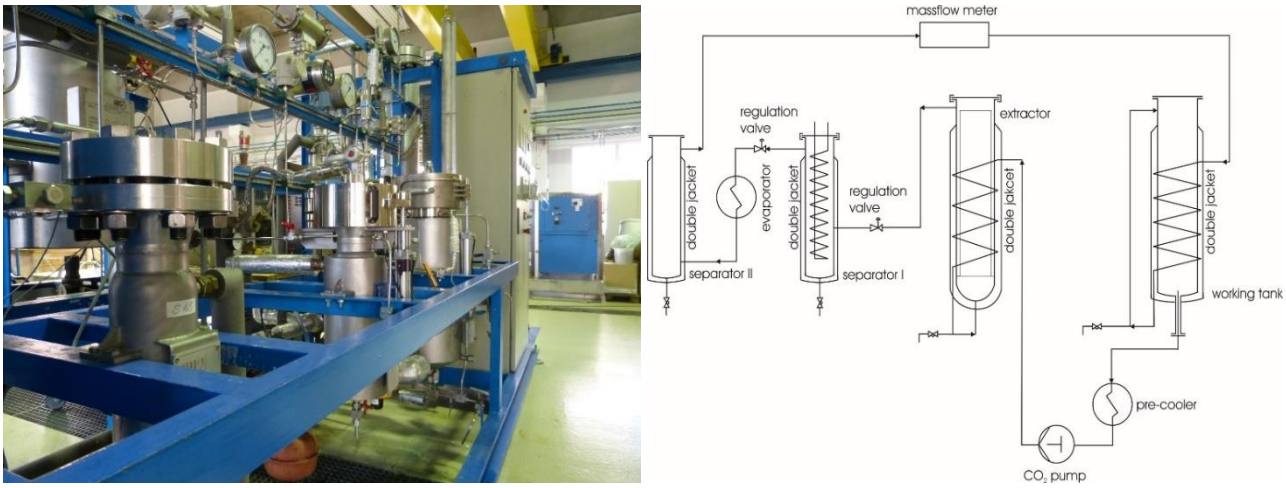


The objectives of the present paper were to provide a general overview of the project PufaChain, especially in the part of the evaluated extraction technologies and to summarize the results. The importance of correct biomass pre-treatment prior to extraction became obvious during the course of the project and will be here highlighted. The limitations of the different extraction techniques are also discussed.

MATERIALS AND METHODS

Extraction by supercritical CO_2. The microalgae biomasses were delivered from partner A4F (Lisbon, Portugal) as spray dried powders. The biomasses were characterized in regards of fatty acid profile, humidity and other potentially relevant parameters (e.g. presence of pigments).
The carbon dioxide of food grade quality was purchased from Messer Austria (Gumpoldskirchen, Austria). Ethanol used as co-solvent in some of the experiments was purchased in 99,9vol.% concentration with 1% methylethylketone from Australco (AustrAlco Österreichische Agrar-Alkohol HandelsgesmbH).
For the extraction experiments with supercritical CO_2 as solvent, the 5L/1000bar R&D unit was used, located in Natex Laboratory. The plant and also the general flow scheme are depicted in Figure ***2***.

Figure 2 *5L/1000bar R&D unit in Natex laboratory*



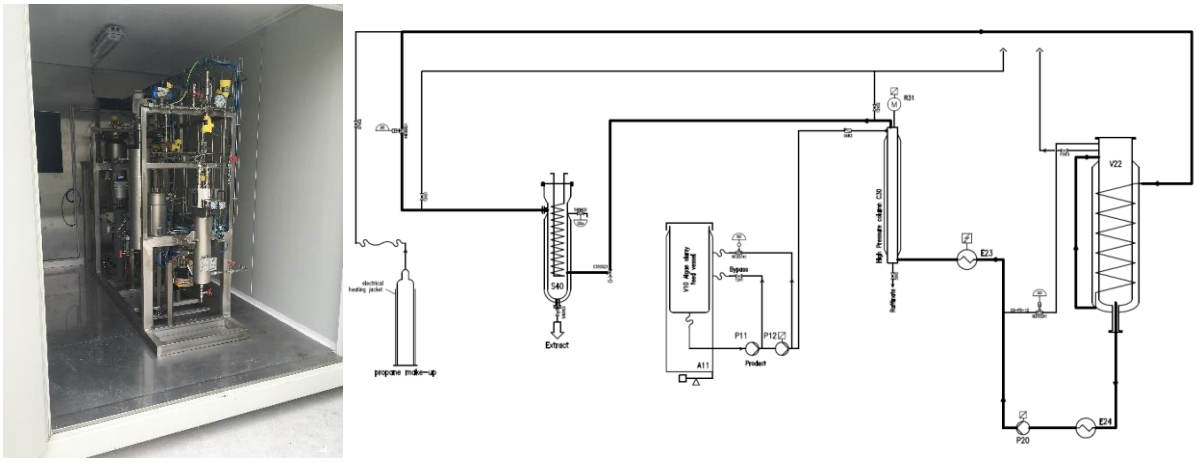
The plant consists of a closed solvent circuit – the CO_2 used for extraction is circulated between the storage (working tank), extraction part (extractor) and separation part (Separator I and II). The liquid CO_2 from the working tank is cooled in the pre-cooler in order to prevent cavitation in the high-pressure CO_2 pump. The pressurized CO_2 is fed over the double jacket of the extractor, where it is heated to the extraction temperature and consequently into the extractor from the bottom. The pressurized and heated solvent comes in contact with the extracted algal biomass. Based on the thermodynamic conditions, the solvent is saturated with all soluble substances as flowing through the bed of the algal biomass. The saturated supercritical CO_2 leaves the extractor towards the regulation valve, where it is depressurized into the Separator I. Due to the change of the pressure and temperature, the solubility of the dissolved substances rapidly decreases and the extract precipitates out of the solvent and accumulates at the bottom of the Separator I. The extract-free CO_2 leaves the Separator I towards the second regulation valve and is further depressurized into the second Separator. The gaseous solvent flows over the mass flow meter towards the working tank and is condensed in the cooled double jacket into a liquid. In this manner the solvent loop is closed. The extracted crude oil can be withdrawn at the bottom of Separator I during the extraction process. The amount of solvent and extract are measured during the experiment, so a mass balance and evaluation of the extraction process can be carried out.

Extraction by liquid propane. The microalgae biomasses were delivered from partner A4F (Lisbon, Portugal). The biomass samples were concentrated by membrane technology to around 20% dry matter and were packed and transported frozen. The biomasses were characterized in regards of fatty acid profile, humidity and other potentially relevant parameters (e.g. presence of pigments).

The propane (mixture of > 85% propane and 5%butane) was purchased from Messer Austria (Gumpoldskirchen, Austria).

The basic idea behind the technology of extraction with propane is the fact, that propane can very effectively dissolve triglycerides even in presence of excess of water. In this manner, the drying step in the biomass processing can be omitted, increasing the energetic efficiency of the whole biorefinery. Furthermore, pressure up to only 25-30bar is needed to run such extraction, which is considerably lower compared to supercritical CO_2 extraction process, finally decreasing the investment cost for such extraction equipment. On the other hand, propane is easily flammable, so the equipment has to be designed in an explosion proof manner, which increases the overall cost of the investment.

***Figure* 3** *Extraction plant for wet algal biomass*



The liquid biomass is injected into the extraction column at the top and due to gravity and higher density than propane, flows downwards. Counter current, liquid propane at 22bar and 50°C is injected into the column from the bottom and flows due to lower density upwards. The liquid biomass is wiped on the inner surface of the column by means of a wiper system, creating a thin film of the biomass. In that manner, the diffusion distances between the oil in the biomass and propane are minimized. The oil dissolved in propane leaves the extraction column towards the separator. Before entering the separator, the propane is depressurized from 22bar to 8bar. The propane and oil enter the separator. The propane is fully evaporated by means of a heating jacket and heating coil in the separator. The oil with other components like water and pigments accumulate at the bottom of the separator and can be collected over a drain valve. The gaseous propane is recirculated back into the condenser and storage tank. The residual – non-extracted part of the biomass, called raffinate, can be collected from the bottom of the column.
The plant is built with its own computer control system, so all process parameters (pressure, temperature, mass flow of propane etc.) can be precicelly controlled and are recorded.
Before each experiment, a frozen sample of wet biomass was thawed in a microwave oven. Further, it was necessary to lower the viscosity of the biomass by diluting it with water, so it was possible to inject it into the extraction column. The biomass was mixed with water and the final concentration was measured by thermogravimetry.

RESULTS

Strain selection. Almost 100 microalgae strains were screened mainly in regards of EPA/DHA content and production rate, suitability for mass culture production in horizontal tubular photobioreactors, preferably suitable for specific culture mediums, suitability for harvesting by cross-flow membrane microfiltration and easy cell disruption. All over, the algae crop rotational principle was also to be implemented into the biorefinery concept, so the selection of the strains was aiming also for their ability to grow during warm or cold season. Based on the objectives of the screening, the following strains were selected as reference for further development:

- *CCAP 1085/18 Thalassiosira weissflogii*
- *SAG 1090-6 Phaeodactylum tricornutum*
- *SAG 2.99 Nannochloropsis gaditana*

Following novel strains were selected:

- *SAG 40.80 Prorocentrum cassubicum*
- *SAG 51.91 Chloridella simplex*
- *CCCryo 381-11 Raphidonema nivale*

Biomass production. The strains were successfully produced at pilot scale at A4F. Information gathered from over 30 pilot scale production trials during a period of 1200 cultivation days provided the necessary data for comparison of biomass and PUFA productivity between strains.
Biomass harvesting. The produced biomasses were harvested by filtration from the culture medium using the MAHLE crossflow- filtration system omitting the centrifugation step as an additional harvesting process, thus lowering the overall energy consumption of the biorefinery. The new developed membranes, integrated into modules and easily scalable, were successfully tested and suit all requirements for algae filtration, like high solids content and salt-water. Beyond that, it is possible to reduce the membrane fouling, particularly by using appropriate cleaning agents.
The concentrated biomasses were either directly spray dried or prior to spray drying, the biomass was treated for cell disruption in order to release the target compounds from the closed cells. For the extraction with liquid propane, the biomasses were only concentrated to around 20w.% solids and transported frozen to the extraction facility.
Biomass extraction. As already mentioned, the success of an extraction process is based on a proper preparation of the raw material. In particular, the compounds to be extracted have to possess possibly high solubilities in the solvent, all diffusion barriers for the solvent to reach the solutes should be removed and the raw material particle's geometry should be in a specific range to ensure a uniform flow of the solvent through the bed of the biomass.
Extraction of dry microalgae with supercritical CO_2. In order to effectively extract lipids with supercritical CO_2, the raw materials should fulfil the following criteria:

- Stabilized microalgae biomass – enzyme inactivation, especially lipases naturally present in the microalgae, can immediately after the harvesting step start with the hydrolysis of the lipids. In opposition, when triglycerides are hydrolysed and free fatty acids are produced, these possess much higher solubilities in supercritical CO_2 and thus can be extracted much easier. After enzyme inactivation, the biomass is stable when it is dried and stored at -18°C.
- Particle size – optimal particle size is in the range of 300 to 800µm. This particle size will ensure no problems with channelling phenomena during the extraction. Since almost all microalgae biomass producers use spray dryers to dewater the biomass, an adequate pelletizing, agglomeration of the dry powder should be considered or an alternative drying procedure applied (e.g. belt drying).
- Water content of the biomass should be in the range of 8 to 12% by weight.
- Availability of the solute in the matrix can be ensured by a proper cell disruption method, which seems to be strain specific. Cryo technology (slow cooling of the cell mass), osmotic stress, sonication, steam jet technology (application of dry steam), pulsed electric field and disruption by high pressure CO_2 were tested during the course of the project Pufachain.

Supercritical CO_2 is a non-polar, inert and a very mobile solvent. Principal solubilities of substance classes are summarized in **Table 2**.

Table 2 Principal solubilities of substances in supercritical CO_2

EASILY SOLUBLE	REDUCED SOLUBILITY	NOT SOLUBLE
esters, alcohols	triglycerides	sugars
aldehydes, ketones	waxes	organic acids
volatile oils	polyphenols	polysaccharides
free fatty acids	alkaloids	proteins
aromas	pigments	phospholipids
monoterpenes		glycolipids

The target substances to be extracted from microalgae biomass are underlined and it becomes clear that polar lipids, containing reasonable amounts of EPA and DHA are not extractable by supercritical CO_2. Although, a complete hydrolysis of polar lipids induced by either naturally present or added lipases could enhance the extractability of these valuable molecules.

Extraction of Thalassiosira weissflogii. *Thalassiosira weissflogii* is a unicellar seawater diatom naturally found in marine and brackish enviroments and also in inland freshwaters across the world although it grows better at salinities of 28-30g/L. *Thalassiosira* is shaped as a short cylinder sized from few µm to 30µm and has a tendency to have oscillations between enlargement and reduction of cell size which, consequently, affects the growth rate. *Thalassiosira* is reported to naturally contain high amounts of specific interesting PUFA. Currntly the main commercial application is feed for aquaculture, namely, feeding shrimp and shellfish larvae in hatcheries (Pereira, et al., 2014).

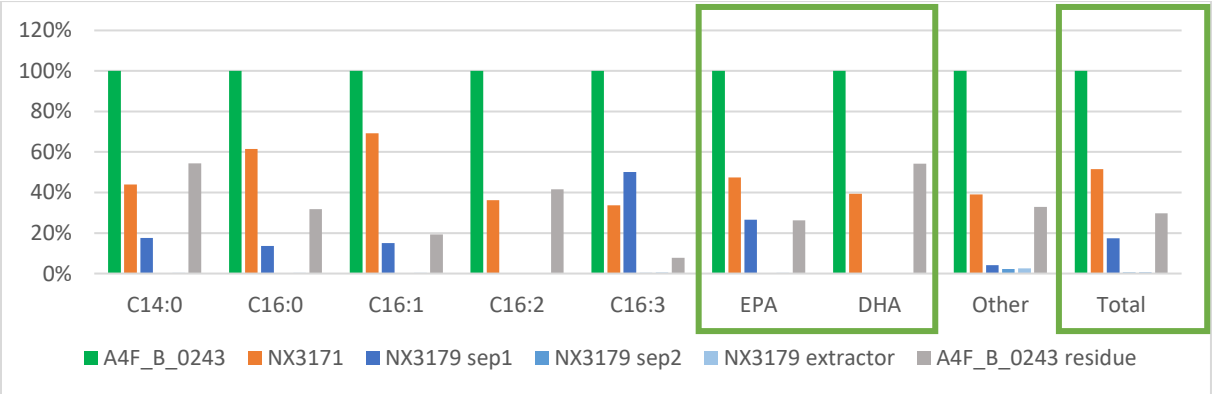
After harvesting, the biomass was treated with osmotic shock for cell disruption and spray dried in a subsequent step. 1002g of the biomass were loaded into the extraction basket and extracted in two steps. In the first extraction step, only CO_2 without modifier was used. The pressure and temperature for the extraction were chosen to reach the maximum possible solubilities of lipids. During the first step of the extraction (NX3171), no channeling was observed. In total, 57g of crude extract were produced. During the following extraction with modifier, the overall yield could be increased. The extraction was, in spite of very small particle size of the biomass, without any channeling problems (NX3179). The exact extraction conditions are listed in Table 3.

Table 3 Overview of the experiments with biomass of Thalassiosira weissflogii

Test	**Raw material**	**Extractor**	**Separator 1**	**CO_2 mass flow**	**Ethanol as modifier**	**Time**	**CO_2 total**
		bar/°C	bar/°C	kg/h	kg/h	h	kg
NX3171	Fresh	460/65	60/50	10	-	1	10
NX3179	Residue NX3171	460/65	60/50	10	2	1	10

The extracts were kindly analyzed by partner A4F. The mass balance of the FA over the course of the two-step extraction is depicted in the following diagram.

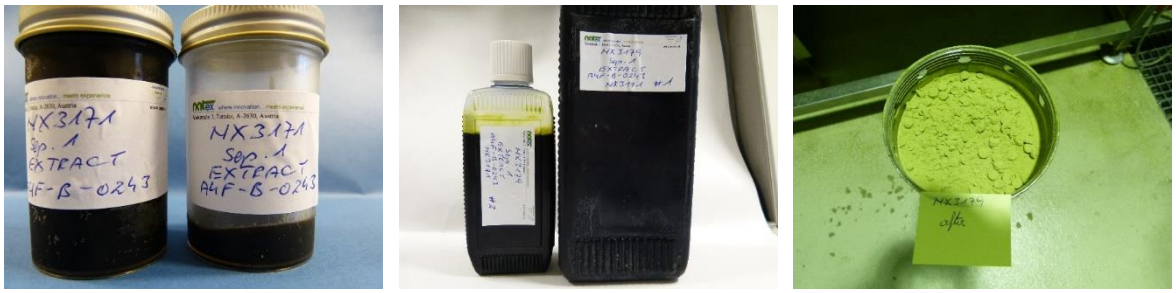
Figure 4 Mass balance of the oil extraction (in weight %)



It can be concluded, that in the first extraction step 52w.% of the total fatty acids were extracted. Regarding EPA and DHA, 47w.% and 39w.% were extracted in the first step. In the second extraction step, with modifier, additional 18w.% of total FA were extracted, whereas additional 27w.% EPA and 0w.% DHA were recovered from the biomass.

The crude extracts were further processed in order to separate the oil from the water fraction. The oil fraction yielded in 69% of the crude extract, the rest was water and cell fragments. The samples are depicted in Figure 5.

Figure 5 Crude oil extracted in first step (left); crude oil with ethanol collected in 2nd extraction step and residual biomass after 2nd extraction step in the extraction basket (right)



Extraction of *Phaeodactylum tricornutum*. *Phaeodactylum tricornutum* is a diatom widely studied by the scientific community and commonly used on aquaculture due to its content on EPA and, in some strains, small amounts of DHA. (Pereira, et al., 2014) Unlike other diatoms *P. tricornutum* can exist in different morphotypes (fusiform, triradiate, and oval), and changes in cell shape can be stimulated by environmental conditions. *P. tricornutum* has emerged as a potential microalgae energy source. It grows rapidly and storage lipids constitute about 20-30% of its dry cell weight under standard culture conditions. (Wikipedia, 2018)

After harvesting, the biomass was treated with osmotic stress for cell disruption and spray dried in a subsequent step. The powder contained 66w.% of biomass, 18w.% moisture, 16w.% salt and 12w.% total fatty acids (TFA). The fatty acid profile analysis found 20,8w.% of EPA and 1,4w.% of DHA out of total fatty acids present in the biomass. Arachidonic acid with 9,0w.% of TFA was also present.

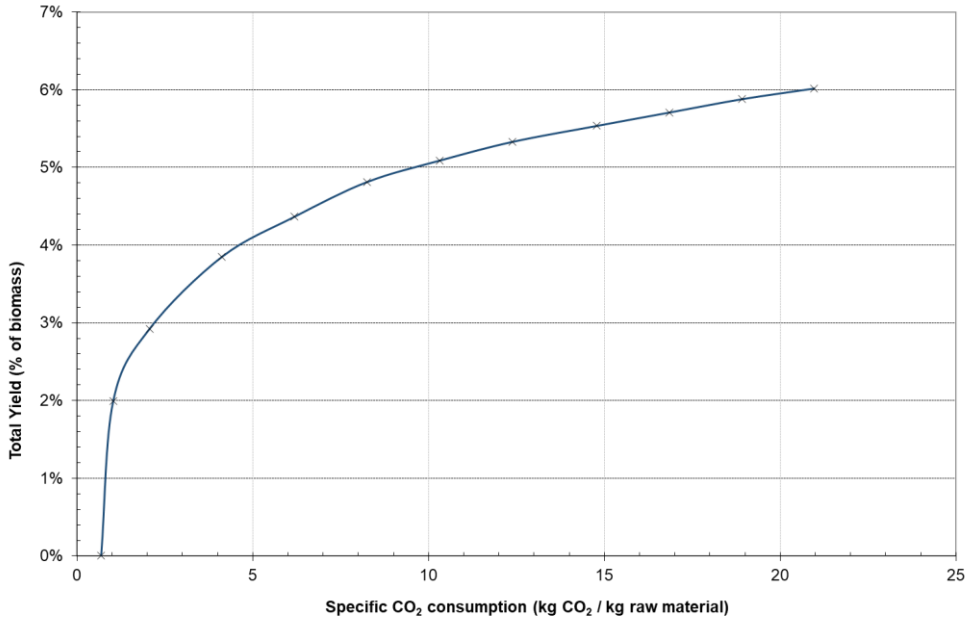
2909g of the biomass were extracted using conditions for the recovery of triglycerides from plant materials. The conditions are listed in Table 4.

Table 4 Experimental conditions for extraction of *Phaeodactylum tricornutum biomass*

Test	Raw material	Extractor	Separator 1	CO_2 mass flow	Ethanol as modifier	Time	CO_2 total
		bar/°C	bar/°C	kg/h	kg/h	h	kg
NX3010	Fresh	460/65	60/50	12	-	5	61

175g (6,0w.% of raw material) of crude extract were collected from Separator 1 during the experiment. The crude extract was further fractionated into an oil and water phase. 74,0±3,7w.% and 6,8±0,1w.% was the content of total free fatty acids and pigments in the extract, respectively.
129,5g of oil were finally recovered from the biomass, which corresponds to 37,1w.% of the total oil available in the biomass. From the evolution of the yield curve it can be concluded, that the extracted oil was easily accessible for the CO_2. The yield versus specific CO_2 consumption indicates very effective extraction of the algae oil (Figure 6).
The acid value of the oil was 146,1mg KOH/g, which indicates a high degree of hydrolysis of the triglycerides. It seems, that native lipases become active immediately after the harvesting of the biomass. This phenomena was observed over the whole course of the project.

Figure 6 Crude oil yield versus specific CO_2 consumption



The lipid profile of the extracted oil is listed in Table 5.

Table 5 Lipid profile of the NX3010 extracted oil

Lipid profile	(mg/g oil phase)
C14:0	81,4 ± 6,4
C16:0	58,5 ± 3,3
C16:1	163,0 ± 4,4
C16:2	39,1 ± 1,4
C16:3	77,5 ± 5,9
C18:0	7,0 ± 0,1
C18:1	33,6 ± 1,5
C18:2	7,6 ± 0,4
C20:4w6 (ARA)	57,7 ± 2,3
C20:5w3 (EPA)	182,7 ± 10,1
Total PUFA	364,6
Total Omega 3	182,7
Total Omega 6	57,7
Omega 3/6 Ratio	3,2

The concentration of total polyunsaturated fatty acids in the oil of 364,6mg/g, especially 182,7±10,1 mg/g of EPA show the potential of this technology for delivery of these compounds.

Extraction of *Prorocentrum cassubicum.* 3187g of the cell ruptured and spray dried biomass were extracted with CO_2 without modifier. The experiments were without any channeling problems. The conditions used in the experiments are listed below in Table 6.

Table 6 Overview of the experiments with *Prorocentrum cassubicum*

Test	**Raw material**	**Extractor**	**Separator 1**	**CO_2 mass flow**	**Time**	**CO_2 total**
		bar/°C	bar/°C	kg/h	h	kg
NX3258	1604g	460/65	60/50	20	2,5	50
NX3259	1583g	460/65	60/50	20	2,5	50

109g (6,8w.% of biomass input) and 111g (7,0w.% of biomass input) extract were recovered from 1604g and 1583g of biomass in experiment NX3258 and NX3259, respectively. The extracts were analyzed by partner IOI for fatty acid profile. The crude extract of NX3258 consisted of 63,0w% of oil phase and 37w.% of cell residues and water. In the case of NX3259, the crude extract contained 66,2w.% oil phase. The results are summarized in Table 7.

Table 7 Composition of the extracts from *Prorocentrum cassubicum* biomass

	NX3258		NX3259	
	% TFA	mg FA/g	% TFA	mg FA/g
C14	3,1 ± 0,1	19,8 ± 0,9	2,5 ± 0,0	13,6 ± 0,6
Σ C16	18,4 ± 2,6	115,7 ± 14,4	17,3 ± 1,5	95,2 ± 9,4
Σ C18	31,2 ± 1,0	196,5 ± 9,3	31,9 ± 0,5	175,0 ± 6,5
C18:4	19,3 ± 0,7	121,5 ± 6,6	20,5 ± 0,4	112,5 ± 4,4
Σ C20	26,3 ± 0,9	165,7 ± 8,4	26,3 ± 0,5	144,4 ± 5,4
C20:4 (ARA)	n.d.	n.d.	n.d.	n.d.
C20:5 (EPA)	26,2 ± 0,9	165,0 ± 8,4	26,2 ± 0,5	143,9 ± 5,4
Σ C22	19,8 ± 0,6	124,3 ± 6,0	21,3 ± 0,5	116,6 ± 3,9
C22:6 (DHA)	19,3 ± 0,6	121,7 ± 6,0	21,1 ± 0,5	115,5 ± 3,9
Σ Others	1,1 ± 0,0	7,0 ± 0,2	0,7 ± 0,0	3,7 ± 0,3
Total	100,0 ± 0,0	629,1 ± 12,0	100,0 ± 0,0	548,5 ± 19,4

It can be concluded that from the total fatty acids 45,5±1,1w.% and 47,3±0,7w.% in NX3258 and NX3259 respectively, are the target omega fatty acids. Further, the content of the target molecules in the oil phase of the extract is as high as 286,7±10,3mg/g and 259,4±6,7mg/g.

Extraction of *Raphidonema nivale.* The biomass was after harvesting treated with sonication for cell disruption. Finally, before extraction, the biomass was spray dried. Following table summarizes the experiments.

Table 8 Extraction conditions for *Raphidonema nivale*

Test	**Raw material**	**Extractor**	**Separator 1**	**CO_2 mass flow**	**Time**	**CO_2 total**
		bar/°C	bar/°C	kg/h	h	kg
NX3284	1426g	460/65	60/50	20	2,5	50
NX3285	1433g	460/65	60/50	20	2	40

Figure 7 Extraction basket with biomass after extraction

In experiment NX3284, 87g of crude extract were produced. During the experiment, 37g of extract were collected after 15min (3,5kg CO_2/kg biomass). The residual extract produced during the rest of the experiment was collected separately and analyzed. The total yield was 6,1w.% of the biomass input. In experiment NX3285, 105g (7,3w%) of crude extract were produced. The fatty acid profile of the NX3285 extract was very similar to that achieved in NX3284.

The detailed analysis results of the extract NX3284 are summarized in Table 9.

Table 9 Composition of the extracts from *Raphidonema nivale* biomass

	NX3284 – 0 - 15Min		NX3284 – 15Min – 150Min	
	% TFA	mg FA/g	% TFA	mg FA/g
C14	1,1 ±0,0	8,9 ± 0,3	0,9 ± 0,0	7,1 ± 0,3
Σ C16	26,9 ± 0,0	224,9 ± 8,7	25,0 ± 0,1	193,2 ± 6,5
Σ C18	45,6 ± 0,7	381,3 ± 21,1	45,0 ± 0,1	347,4 ± 8,8
C18:4	3,6 ± 0,0	29,9 ± 0,9	3,5 ± 0,0	27,1 ± 0,8
Σ C20	25,5 ± 0,6	212,9 ± 3,1	27,9 ± 0,0	215,8 ± 5,7
C20:4 (ARA)	4,0 ± 0,1	33,4 ± 0,3	4,4 ± 0,0	33,8 ± 0,9
C20:5 (EPA)	19,8 ± 0,4	165,3 ± 3,0	21,7 ± 0,1	168,2 ± 4,0
Σ C22	0,1 ± 0,0	1,1 ± 0,1	0,1 ± 0,0	1,1 ± 0,1
C22:6 (DHA)	n.d.	n.d.	n.d.	n.d.
Σ Others	0,9 ± 0,0	7,5 ± 0,0	1,1 ± 0,0	8,1 ± 0,2
Total	100,0 ± 0,0	836,6 ± 33,2	100,0 ± 0,0	772,8 ± 21,6

The crude oil after 15 minutes of extraction had an acid value of 90,3mg KOH/g and the residual crude oil 82,9mg KOH/g. It can be concluded, that both oil samples are slightly different in fatty acids profile. The small differences indicate faster extraction of fatty acids with lower molecular weight in the beginning of the extraction, which can be explained by higher solubilities of these molecules in supercritical CO_2. The target omega fatty acids are distributed evenly over the two extract samples.

Extraction of wet biomass by liquid propane. Before each experiment, a frozen sample of wet biomass was thawed in a microwave oven at 800W for 10 minutes. Further, it was necessary to lower the viscosity of the biomass by diluting it with distilled water, so it was possible to inject it into the extraction column. The biomass was mixed with water and the final concentration was measured by thermogravimetry. The goal of the experiments was to gather first experience with the new extraction technology and to produce crude algae oil for analysis and further downstream processing. The extraction conditions used in all experiments were targeting maximum oil yield.

Extraction of *Chloridella simplex.* The biomass was delivered frozen, concentrated and without any cell rupture. Prior to extraction it was thawed and diluted to approx. 10% dry mass and put into the feed tank in the plant. The injection pump was started in bypass mode in order to avoid sedimentation of the biomass in the feed system.

The plant was filled with fresh propane from a bottle. The propane is stored in the plant in liquid form in a working tank at 8bar and 20°C. From the working tank, the propane is fed through pre-cooler, where it is cooled to around 11°C to prevent cavitation in the high pressure propane pump. The liquid propane is pressurized to 22bar in the propane pump and is further fed through a preheater to increase the temperature to extraction point of 50°C. The propane at 22bar and 50°C enters the extraction column at the bottom and flows upwards, passing by the thin film of the algae biomass. The propane has to diffuse through the thin layer of water surrounding the ruptured cells, dissolve the oil and diffuse back out of the biomass. The propane with oil leave the extraction column at the top towards the expansion valve. In the expansion valve, the mixture is depressurized to 8bar and 18°C. The 2 phase mixture, consisting of liquid propane with oil and gaseous propane enters the separator. The liquid propane is here evaporated by means of heating coil and jacket – the temperature of propane leaving the separator is around 45°C. The extracted oil remains at the bottom of the separator and can be collected over the drain valve. The gaseous propane leaves the separator, passes through a Coriolis mass flow meter and is finally condensed back into the working tank. In this way, the propane is circulated in the plant with a mass flow of approx. 6kg/h until the extraction experiment is finished.

The non-extracted part of the feed biomass – raffinate, is collected at the bottom of the extraction column and can be withdrawn from its bottom over a drain valve. In Table 10, all experiments with *Chloridella* biomass are listed.

Table 10 Extraction experiments for *Chloridella simplex*

Batch Nr.	Input (g)	Dry mass content (w%)	Dry mass (g)	Extraction time (min)	Propane total (kg)	Crude Extract (g)	Oil in extract (g)	Raffinate (g)	Yield (w.%)
NXP31	70,8	8,6	6,1	250	26,64	13,2	0,45	57,6	7,4%
NXP32	68,5	8,6	5,9	250	26,95	21,1	1	47,4	17,0%
NXP33	55,6	10,3	5,7	250	23,5	6,2	0,41	49,4	7,2%
NXP34	69,7	8,6	6,0	286	32,1	1,7	0,1	68	1,7%
NXP35	46,3	8,6	4,0	260	22,9	4,3	0,16	42	4,0%
NXP36	72,4	8,6	6,2	256	23,6	5,8	0,16	66,6	2,6%
NXP37	53,1	8,6	4,6	303	20,1	5,3	0,96	43,5	21,0%
NXP38	failed								
NXP39	failed								
NXP40	79,8	8,6	6,9	276	22,3	7,8	?	72	0,0%
NXP41	16,8	8,6	1,4	200	15,1	3,6	0,3	12,9	20,8%
NXP42	31,6	8,6	2,7	328	28	0,6	0,1	31	3,7%
NXP43	211,8	8,6	18,2	290	23,2	15,5	0,82	163,4	4,5%

In total 776g of biomass were extracted within this series of experiments. The crude extracts collected from separator are mixtures of oil, water and cellular parts. The major fraction of the extracts was water. As far as possible, the oil was either decanted or consequently extracted from the extract solution by hexane and measured. The yield was calculated based on the dry biomass in the extractor and oil in extract. The big discrepancies in the yield were caused by the collection method of the extract. When the extract with depressurized propane enter the separator after the expansion valve, the sample is sprayed through the narrow piping with high velocities into the separator. The water will easily accumulate at the bottom of the separator, the oil sticks on the coil and cannot be drained. So after certain amount of batches, the separator was washed with hexane and all oil collected.

Since all experiments were carried out with almost same extraction conditions, an overall oil yield could be calculated. From 776,4g of wet biomass, containing 67,7g of dry algal biomass, 4,46g of oil were extracted, which corresponds to 6,6w.% yield.

The extract from NXP31 was kindly analysed by partner IOI. From the total extract of 13,2g, 0,45g of oil were separated and analysed on acid value and fatty acid composition. The results are summarized in Table 11.

Table 11 Analysis of NXP31 extract

	% total fatty acids	mg FA / g oil
C14	8,55	52,64
sum C16	40,31	248,11
sum C18	13,21	81,45
C18:4 (stearidonic)	n.d.	n.d.
sum C20	35,01	215,72
C20:4 (eicosatetraenoic)	3,66	22,35
C20:5 (EPA)	31,35	193,37
sum C22	1,19	7,36
C22:6 (DHA)	n.d.	n.d.
sum Others	1,72	10,58
Sum	100,00	615,86

The propane extracted oil sample contained a reasonable amount of EPA.The analysis of wet biomass is not available. The acid value of the oil was 158,1mg KOH/g, which indicates high degree of triglyceride hydrolysis. Enzymatic activity of lipases during harvesting can be one of the reasons and will be investigated.

CONCLUSION

Extraction of lipids from microalgae biomass is still a bottleneck in the microalgae biorefinery. The preparation of the biomass prior to extraction is critical for the success of the technology. Effective cell disruption techniques, tested for the specific biomass strains, need to be developed, especially in regards of industrial processing. Spray drying of the biomass produces very fine powders, causing problems in the subsequent extraction step. Drying techniques, delivering biomass with bigger particle sizes are needed. Unpolar lipids from dried microalgae biomass can be extracted by supercritical carbon dioxide. Extraction of polar lipids, containing part of the polyunsaturated fatty acids present mainly in the membranes of the microalgae is limited by the low solubility of these compounds in the supercritical solvent. First experiments using liquid propane showed positive results, although further improvements regarding distribution of the biomass in the extraction column are needed.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the European Commission's Seventh Framework Programme (www.pufachain.eu, GA Nr.613303). The authors want to thank all partners of the project for good cooperation.

REFERENCES

Baddiley, J., & al., e. (1994). *Microalgae: Biotechnology and Microbiology.* Cambridge, NY: Cambridge University Press.

Friedl, T. (2018). *Publishable final report Pufachain.*

Laurens, L., & al., e. (2017). *State of Technology Review - Algae Bioenergy.* Golden, CO, USA: IEA Bioenergy Task 39.

Linxing, Y., Gerde, J., Lee, S., Wang, T., & Harrata, K. (2015). Microalgae Lipid Characterisation. *Journal of Agricultural and Food Chemistry*(63-6), 1773-1787.

Pereira, S., Costa, L., Santos, E., Verdelho, V., Wörz, K., & Baum, T. (2014). *Pufachain, Deliverable 4.1 - Data from laboratory trials - cultivation parameters.*

Richmond, A. (2004). *Handbook of microalgal culture; biotechnology and applied phycology.* Blackwell Science Ltd.

Vigani, M., Parisi, C., Rodriguez-Cerezo, E., Barosa, M. J., Sijtsma, L., Ploeg, M., & Enzing, C. (2015). Food and feed products from microalgae: Market opportunities and challenges for the EU. *Trends in Food Science & Technology, 42*, pp. 81-92. doi:j.tifs.2014.12.004

Wikipedia. (2018, February). Retrieved from https://en.wikipedia.org/wiki/Thalassiosira_weissflogii

Wikipedia. (2018, March). Retrieved from https://en.wikipedia.org/wiki/Phaeodactylum_tricornutum

Wikipedia. (2018, January). Retrieved from https://en.wikipedia.org/wiki/Microalgae