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# Development of an *in situ* extraction process of fatty acids from microalgae cultures

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# Abstract

In this work micellar extraction was investigated in order to develop an alternative process for the *in situ* extraction of hydrophobic substances from microalgae cultures. The main requirements for an *in situ* extraction process such as biocompatibility, phase separation behavior and partitioning of the hydrophobic target substance between micellar- and aqueous phases were studied for a number of surfactants. The cloud point temperatures (CPT) as well as the biocompatibility of the nonionic surfactants with the microalgae *C. reinhardtii, C. vulgaris,* and *S. obliquus* was determined as a function of time. It was shown that algae cells are concentrated in the aqueous phase, whereas the hydrophobic compounds (fatty acids) are enriched in the micellar phase. Significant differences in growth and the photosynthetic activity of different algae strains after exposure to surfactants were determined. Thus, besides extraction, surfactants might be applied to control the growth of certain algae enabling the monoalgal culturing in outdoor cultivation of microalgae since the growth of the less surfactant-tolerant microalgae could be suppressed. For the most biocompatible surfactant under study, Triton X-114 with a biocompatibility of 98%, the kinetics of phase separation in the temperature range between 30 and 40°C have been investigated. The fastest phase separation took place at a Triton X-114 concentration of 3%wt at 40°C. The partitioning of representative hydrophobic substances between the both phases was predicted using the model COSMO-RS, the results were compared to experimental data with satisfying agreement ($LogP_{calc.}$ palmitic acid: 0.82; $LogP_{exp.}$ palmitic acid: 0.75). Based on these results the extraction of valuable compounds from the microalgae *Scenedesmus obliquus* was realized on a pilot plant and compared to the lab scale experiments. Overall, the results indicate that a continuous micellar extraction with nonionic surfactants offers the opportunity for an *in situ* extraction of hydrophobic substances directly from the culture medium and is a promising alternative to a classic solvent extraction of dried algal biomass.

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# 1 Introduction and objectives

## 1.1 Introduction

The photobiotechnology is working on solutions for the current problem of shortage of petroleum based fuel and is exploring renewable energy sources for the substitution of fossil fuels. Nowadays, the cultivation of microalgae is attracting more and more attention due to their ability to produce biomass additionally to their carbon dioxide sequestration accomplishment in large scale applications. The cultivation of microalgae using the industrial flue gas with high $CO_2$ content may be an important change in industrial large scale processes [Morweiser, 2010]. In contrast to chemical synthesis methods the production of valuable substances with microalgae has the advantage that the metabolic reactions run with high selectivity and specificity under mild conditions, usually in an aqueous medium. This contributes to the sustainability of technical processes. Algae are photoautotrophic organisms. Therefore, they need compared to heterotrophic organisms no organic substrates, but only light, carbon dioxide ($CO_2$) and inorganic salts dissolved in water. Different species of microalgae are cultivated in order to produce valuable compounds like β-carotene (*Dunaliella salina*), astaxanthine (*Haematococcus pluvialis*) and fatty acids [Borowitzka 1999; Piorreck, 1984]. In particular the fatty acids are interesting because here there is no competition for agricultural land for food production.

Special attention is given to the extraction of these valuable components from algae cultures. State of the art processing of microalgae currently includes energy consuming separation of the cells, dewatering, cell disruption and lipid extraction from the algae biomass with classical extraction methods [Grima, 2003].

Commonly applied extraction methods use supercritical fluids like carbon dioxide or organic solvents (mainly n–alkanes like hexane), as well as ultrasonic-assisted extraction procedures [Mercer, 2011]. All these techniques have in common that the algae cells are disrupted, followed by a time and cost consuming re-growing step of the algae biomass. None of the described methods allow a simultaneous cultivation of algae cells and an extraction of the hydrophobic target substances (*in situ* extraction approach). Such extractive approach is possible in case of a biocompatible two-phase media application. Dodecane/water *in situ* extraction is so far the only extraction system which is extensively investigated. Major drawback of this method is the formation of stable emulsions which prevents proper phase separation. However, this process was never applied to algae cultures before, so that its potential is unclear. Beside organic solvents, also nonionic surfactant based systems can be used for extraction processes. Following section explains the basics of micellar extraction systems.

The amphiphilic molecules of nonionic surfactants form micellar aggregates in aqueous solutions, when their concentration reaches the surfactants critical micelle concentration (CMC). The hydrophobic head of the molecule is directed to the bulk water and the hydrophobic tails form the hydrophobic micelle core. These solutions often form temperature induced two-phase systems. After a temperature increase above the cloud point temperature (CPT), the homogeneous micellar system divides into two phases: a surfactant-rich phase and an aqueous phase [Hinze, 1993]. These micellar structures possess the ability to solubilize hydrophobic molecules like oleic acid, polycyclic hydrocarbons and other fatty acids within their hydrophobic cores [Chen, 1998; Bai, 2001; Lim, 2005]. A comparatively low clouding temperature, allows the application of a cloud point system as a two phase extraction media in whole cell bioprocesses [Wang, 2007; Wang, 2005]. The extraction mechanism, which uses the separation of a surfactant-rich phase and an aqueous phase by the systems temperature above the cloud point, is called cloud point extraction (CPE).

After carrying out the CPE by increasing, microbial cells predominantly accumulate in the aqueous phase [Wang, 2005; Zhang 2012; Hu, 2012]. It was also shown that the CPE process based on Triton X-114 is implementable in continuous counter current extraction columns which are alternatives to batch extraction systems [Ingram, 2012 a].

## 1.2 Objectives

The aim of this work is to develop a feasible separation process to separate valuable substances, especially the hydrophobic fatty acids from microalgae cultures, *in situ*. Therefore, an alternative to the classic extraction of dried algae biomass should be developed, because the separation of the microalgae biomass is a time and cost consuming process step. Therefore, an *in situ* extraction approach is suggested, whereby the hydrophobic substances are extracted directly from the microalgae culture. In this work it is suggest to use cloud point extraction (CPE) for the *in situ* extraction. Since it was never applied for algae cultures before, several requirements such as biocompatibility of the used solvent/ surfactant have to be studied, in addition to the investigations on phase separation behavior. Also the partitioning of the target substances between both phases has to be studied.

Finally with all the gained knowledge, *in situ* extraction of fatty acids from microalgae cultures should be realized in lab and pilot scale.

# 2 Fundamentals and state of the Art

## 2.1 Microalgae

An advantage of microalgae in contrast to rooted plants is the higher productivity, due to the fact that microalgae do not need a trunk or roots (which have no photosynthetic active cells), because they are free suspended in the surrounding water. Hence, every microalgae cell has the ability to perform photosynthesis and therefore reproduce itself. This leads to higher biomass productivity in contrast to rooted plants [Dismukes et al., 2008]. Another advantage is that microalgae cultivation systems can be built up in arable regions where they do not compete with agricultural land for food production [Lehr, 2009].

Various macroscopic marine plants, like seaweeds and tangle are cumulated named with the latin word *alga*. However, today this term (*alga*) is extended on aquatic phototrophic microorganisms and simultaneously excludes higher plants explicitly from this group. `*Algae*` is nowadays a term for the life-form and not for a unique biological group. Currently approximately 40.000 different microalgae species were described, whereas it has been estimated that 200.000 up to 800.000 species exist which differ extremely in their color and shape and the substances they produce [Cardozo et al., 2006]. Most of them have a single cell size of 3 to 10 µm. Microalgae are characterized by the fact they consume carbon dioxide ($CO_2$) and produce oxygen ($O_2$) and biomass through photosynthesis [Campbell, 2003]. The carbon content of dried microalgae biomass is round about 50 wt%, where at the stoichiometric demand of carbon dioxide is 1.83 kg $CO_2$ per 1 Kilogram of dried algae biomass [Sastre, 2010].

Most microalgae species like green algae (*Chlorophyta*), brown algae, red algae and diatoms belong to the biological group of eukaryotes. Blue-green algae or cyanobacteria are representing an exception, since they belong to the prokaryote group. Additionally these species are distinguished between unicellular and multicellular microalgae [Hoeck, 1993; Berg, 2005].

Microalgae have the ability to produce various valuable substances like fatty acids, colorants, vitamins, antioxidants and enzymes which gain commercial importance [Benemann, 1982; Cohen, 1999; Pulz, 2004; Skulberg, 2000]. Fatty acids are basic cellular constituents that can serve both as structural components of the cell and as a storage product for the cells [Behrens, 1996]. Basically, algae can produce three different groups of fatty acids: saturated fatty acids, monounsaturated and polyunsaturated fatty acids [Mandal, 2009]. (If a fatty acid contains a double bound in its molecule, it is termed as unsaturated and if not – as saturated fatty acid). Long-chain fatty acids are well soluble in organic solvents and almost insoluble in water [Oberle, 1996]. For the food and feed industry, the polyunsaturated fatty acids are mainly of interest. Especially ω-3 fatty acids are gaining more and more importance. Nowadays, the ω-3 fatty acids are usually derived from fish oil, but these have a fishy aftertaste [Bhosale, 2010]. The fatty acids used for biodiesel production are mainly 16:1, 18:1 and 14:0 [Schenk, 2008; Scott, 2010].

Both the content of fatty acids in the microalgae (intracellular fatty acids per g of dried algae), and its relative composition (e.g. myristic, palmitic, stearic acid) may vary due to external influences. Especially nitrogen has been found to be an important factor for the production of fatty acids. It is known that limitation of nitrogen leads to an increase of the fatty acid concentration in the cells. Whereas the fatty acid-production is not linked to the production of biomass [Makulla, 2000], optimal fatty acid productivity usually leads to a poor growth rate [Rodolfi, 2009]. One of the most important natural sources of the carotenoid pigment astaxanthin is the microalgae *Haematococcus pluvialis.*

If these microalgae are cultivated under nitrogen depletion, the intracellular pigment astaxanthin can accumulate in oil droplets up to 8% of the dry weight [Mendes-Pinto, 2001; Harker, 1996]. This is an example for the accumulation of e.g. oils as intracellular microalgae products. A further extraction of these valuable substances from wet biomass is difficult due to the fact that the intracellular components are inaccessible for the used organic solvents like hexane, chloroform, dietyl ether or ethanol [Belarbi, 2000]. Therefore, in the majority of recovery processes of intracellular microalgae products a cell disruption step is included [Mendes-Pinto, 2001].

Table 1: Intracellular valuable substances produced by microalgae.

| **Product** | **Strain** | **Amount (mg/g dry wt)** | **Ref.** |
|---|---|---|---|
| γ-Linolic acid | Chlorella sp. NKG042401 | 9 | Hirano,1990 |
| Phycocyanin | Synechhococcus sp. NKBG042902 | 150 | Takano, 1995 |
| Palmitoleic acid | Phormidum sp. NKBG041105 | 47 | Matsunaga,1995 |
| Docosahexanoic acid | Isochrysis galbana TEXLB2307 | 15 | Burgess,1993 |
| Glutamate | Synechhococcus sp. NKBG040607 | 15 | Matsunaga,1991 |
| Coccolith | Emiliania huxeleyi | 740 | Takano, 1995 |

Since the 1970s microalgae were also investigated with focus on the production of lipids needed for the production of biodiesel [Benemann, 1982; Regan, 1983]. Until today the challenge is to find a suitable algal strain which combines a high growth rate with high fatty acid content.

The microalga *S. obliquus* was found to be a promising strain [Gouveia, 2009]. In order to find other commercially suitable microalgae strains beyond the large number of described strains, Nascimento (2013) have explored the volumetric productivity of fatty acids by some microalgae.

Here, *C. vulgaris* had the highest volumetric fatty acid production exhibited with 204 $mg*l^{-1}*day^{-1}$. Other microalgae had a significantly lower productivity (22-112 mg $l^{-1}$ $day^{-1}$).

Also Ördög (2013) found that *S. obliquus* had the highest productivity of all strains tested, which is consistent with the previous results of other research groups [Nascimento, 2013].

The predominant fatty acid in almost all microalgae is palmitic acid (C16: 0). *S. obliquus* has the highest concentrations of up to about 52% Σ FA. Furthermore, also C18: 0 (about 7% Σ FA) and C18: 1 fatty acids (about 21% Σ FA.) are components of the total mass of fatty acids in *S. obliquus* [Mandal, 2009; Nascimento, 2013]. Long-chain fatty acids are interesting because they are essential for the production of biodiesel [Carmo, 2009]. In order to produce such valuable substances, microalgae biomass has to be cultivated. Generally, two different approaches are used for this. Open systems (open ponds) and closed systems (photobioreactors).

In the review article of Morweiser (2010) the developments and perspectives of photobioreactors for biofuel production are systematically discussed. There are different types of closed photobioreactors designs based on flat plate, tubular or annular modules. An air-lift mechanism is used for mixing and gas dispersion. All of reactor designs deal with the most important factors for an optimal the production of valuable substances (e.g. fatty acids) the distribution of light energy to the cells . This can be realized by flat panel airlift reactors [Münkel, 2013].

Nitrogen can be fixed by some microalgae directly from the air in the form of $NO_x$, however the most effective way is to dissolve urea in the culture medium [Brenan, 2010]. Additionally to solar energy, mainly phosphate and carbon dioxide are needed for optimum cultivation. For a sustainable cultivation of microalgae in industrial scale flue gases which contain $CO_2$ are of particular interest.

Also industrial waste streams like $NaHCO_3$, which is used while removal of $SO_2$ from flue gas, can be used to prepare algae cultivation media [Singh, 2013].

The relative composition of fatty acids varies between the different microalgae strains and the circumstances of their cultivation. The microalgae *Scenedesmus obliquus* mainly produces the fatty acids mentioned in the following table.

Table 2: Relative intracellular fatty acid composition in *S. obliquus* culture [Podojil, 1978].

| Fatty acid | name | content in rel. % |
|---|---|---|
| $C_{14:0}$ | Myristic acid | 2 |
| $C_{16:0}$ | Palmitic acid | 18 |
| $C_{18:0}$ | Stearic acid | 33 |
| $C_{18:1}$ | Vaccenic acid | 10 |
| $C_{20:3}$ | Eicosatrienoic acid | 13 |
| | rest | 24 |

Many of these valuable substances have a hydrophobic character which leads to a relatively low water solubility. This may be explained with the microalgae surrounding conditions. Low water solubility minimizes the loss of important intermediate catabolic products through diffusion into the surrounding bulk water.

Table 3: Selected fatty acids produced by microalgae and their water solubility.

| Fatty acid | name | Water solubility* [mg/l] |
|---|---|---|
| $C_{14:0}$ | Myristic acid | 1,070 |
| $C_{16:0}$ | Palmitic acid | 0,040 |
| $C_{18:0}$ | Stearic acid | 0,597 |

*calc. water solubility at 25°C (Wishart, 2013)

Figure 1: Microalgae *Scenedesmus* [www.microscopy-uk.org.uk/.../mmdesmid.html]

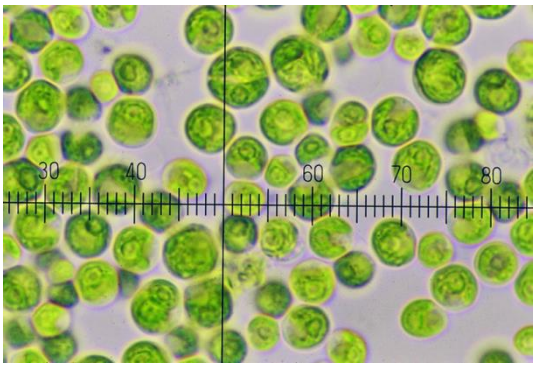



Figure 2: Microalgae *Chlorella vulgaris* [www.chlorella-wholesale.com/Chlorella]

### 2.1.1 Photosynthesis

Many individual reaction steps of the photosynthesis can be divided into two major subtasks. These are the light reactions and the Calvin cycle (dark reaction). While the light reaction the photons are converted into chemical energy (ATP and NADPH). Thereby, oxygen is released by the cleavage of the water. The chemical energy in the form of ATP and NADPH is used for the fixation of $CO_2$. This carbon sources than are converted into higher carbohydrates like sugar and starch [Campbell, 2003].

The resulting, highly simplified net photosynthesis equation is:

$$6\,CO_2 + 6\,H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad \text{(eq. 1)}$$

### 2.1.2 Chlorophyll fluorescence

The light re-emitted by chlorophyll molecules during return from excited to non-excited states is called chlorophyll fluorescence. It is an indicator for photosynthetic energy conversion in higher plants as well as algae. An advantage is that this parameter can be determined in short time. Thus, it is an important tool in plant research, especially to determine the photosynthetic activity.

The light energy which is absorbed of the photo systems, may dissipate in three other forms of energy. This can be the absorption of the light in the photo system II in order to generate energy through photosynthesis; it can be dissipated as heat in non-photochemical quenching or can be emitted as fluorescence radiation. Generally it can be conducted that the higher one dissipation form is, the lower the others are [Baker, 2008].

The fluorescence emission rate, $F$ is proportional to the absorbed amount of light, $l_a$, and the fluorescence factor, $k_F$, divided by the sum of the constants of all competing phenomena like photochemical reactions ($k_P$), thermal radiation ($k_D$) and energy transport in the non- fluorescence- compatible pigments ($k_T$). The main equation that describes the amount of fluorescence is the following, were the fluorescence yield is ΦF [Krause, 1984]:

$$F = l_a \cdot \frac{k_F}{\sum k} \qquad \text{(eq. 1)}$$

$$\Phi F = \frac{F}{l_a} = \frac{k_F}{k_F + k_P + k_D + k_T} \qquad \text{(eq. 2)}$$

An additional electron acceptor is necessary, when the PS II has already absorbed light. Thus, electrons are released and the reaction center is regarded as `closed` [Maxwell, 2000].

This effect of increased fluorescence is known as Kautsky effect. If the electron path between the photosystems is no longer disabled, the transport velocity of the electrons is higher and the fluorescence becomes smaller. This is referred to as `Photochemical Fluorescence Quenching´ Maxwell, 2000].

If the fluorescence is measured prior to irradiation ($F_0$) and after irradiation ($F_m$), the maximum photosynthetic activity of photosystem II (YII) can be calculated.

$$Y(II) = \frac{F_m - F_0}{F_m} \qquad \text{(eq. 3)}$$

In dark-adapted plants or microalgae cultures, the yield of the photosystem II (YII) can be a good `plant stress indicator` which is fast to determine [Baker, 2008].

### 2.1.3 Cultivation of microalgae

The cultivation of phototrophic organisms, in contrast to the cultivation of heterotrophic organisms has the great advantage that light can be used as a cheap source of energy. However, the development of closed photobioreactors is not established on the large scale yet, such as reactors for the cultivation of heterotrophic organisms like *e. coli*.

The advantage to use light as an energy source for phototrophic bioprocesses, also leads to specific problems. The energy source for phototrophic organisms like microalgae is light. In contrast to substrates of heterotrophic organisms (e.g. glucose), light is immiscible with the culture medium and may influence in larger layer thicknesses, mutual shading of the cells and thus to the formation of strong light gradient. The light input, as one of the key factors is currently still under study in order to optimize the design of photobioreactors concerning the highest biomass productivities.

For large scale cultivation of algae there are currently two approaches. One is the cultivation of microalgae in so called `open ponds` which allow a cheap production of algal biomass [Clarens, 2011].

In both systems the supply of each microalgae cell with the energy source light becomes the limiting factor (when all required nutrients are available) for cell growth. Even at low biomass concentrations the mutual shading of the cells results in a light gradient. The light intensity decreases exponentially to the interior of the reactor. Therefore, the flow in the reactor, which is generated by pumps, static mixers or by gassing causes the cells move through zones of different light intensity as mentioned in figure 3.

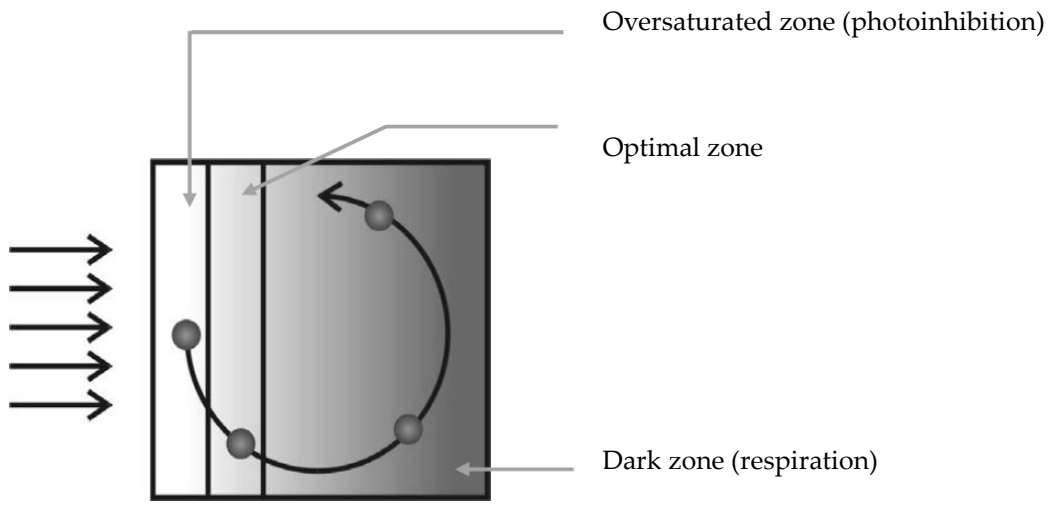



Figure 3: Different light zones in the reactor, which results from the mutual shading of the cells [Degen, 2001].

During the migration through the different zones, each cell passes through a certain light-dark cycle, the frequency of this cycle is flow dependent.

The effects of these light-dark cycles can lead to an increased growth rate when the right frequency is applied.

Therefore, the proper mixing of a microalgae culture is an important parameter for every microalgae cultivation plant. One example of such algae production plant is Cellana´s Kona Demonstration Facility at the big Island of Hawaii. These are used since the 1950s. Both natural lakes, as well as man-made ponds can be used for the cultivation of algae. The most commonly used systems are called ´raceway ponds´ which consist of a 0.2 to 0.5 m deep artificial basin, which have the form of a closed loop.

However, the culture conditions are not adequately controlled, so that no monoseptic conditions can be guaranteed. Contaminations with another algae strain can lead to impurities in products. In addition, the productivity of such systems is very low and the high water evaporation is a serious challenge (water consumption in arid areas) [Lehr, 2009; Bley, 2009; Chisti, 2007].

Figure 4: left: Raceaway-Pond's at Kona Demonstration Facility, Cellana on Hawaii's Big Island., right: Flat Panel Airlift Reactors of SSC Ltd Hamburg [Hindersin, 2013 a].

In closed photobioreactors, the culture conditions can be controlled very well and the risk of contamination by other organisms can be minimized.

Such reactor systems also exhibit a higher productivity per volume and per surface area, than open pond systems [Münkel, 2013].

Main designs used so far are tube or plate reactors in which an optimal light input is realized through high surfaces to volume ratios. However, the investment costs for closed reactor systems are significantly increased by the use of transparent materials and elaborate design in comparison to open pond systems [Posten, 2009; Brenan, 2010].

Due to the increasing request of valuable substances based on sustainable sources, many companies were founded in the last decade. All of them are selling products based on the cultivation and further processing of microalgae biomass [Cuellar-Bermudez, 2015].

Table 4: Global companies' activity in developing process and commercialization of high-value compounds from algae**.** Summarized by [Cuellar-Bermudez, 2015].

| Company name | Location | Company name | Location |
|---|---|---|---|
| Algae. Tec | Australia | AlgaFuel, S.A (A4F) | Portugal |
| Solarvest BioEnergy | Canada | Necton | Portugal |
| Canadian Pacific Algae | Canada | Green Sea Bio Systems s.l. | Spain |
| Solarium Biotechnology S.A. | Chile | AlgaEnergy | Spain |
| BlueBio | China | Fitoplancton Marino | Spain |
| EcoFuel Laboratories | Czech Republic | Simris | Sweden |
| Aleor | France | Taiwan Chlorella Manufacturing Company | Taiwan |
| Fermentalg | France | Vedan | Taiwan |
| Roquette | France | AlgaeLink N.V | The Netherlands |
| Alpa Biotech | France | AlgaeBiotech | The Netherlands |
| IBV Biotech IGV GmbH | Germany | LGem | The Netherlands |
| Subitec | Germany | Solazyme, Inc. | USA |
| Algomed | Germany | Aurora Algae | USA |
| BlueBioTech | Germany | Solix Biosystems | USA |
| Phytolutions | Germany | Synthetic Genomics | USA |
| Algae Health | Ireland | Cellena | USA |
| Seambiotic | Israel | Cyanotech | USA |
| Algatechnologies | Israel | Algaeon | USA |
| UniVerve Biofuel | Israel | Alltech Algae | USA |
| Parry Nutraceuticals | India | Green Star Products, Inc | USA |
| Sunchlorella | Japan | Bionavitas | USA |
| Fuji Chemicals | Japan | Heliae | USA |
| DAESANG | Korea | Kuehnle Agro Systems | USA |
| Algaetech International | Malaysia | Photon8 | USA |
| June Pharmaceutical | Malaysia | Ternion BioIndustries | USA |
| Tecnología Ambiental BIOMEX | Mexico | Algae to Omega Holdings | USA |
| Algae Technology Solutions | Mexico | Sapphire Energy | USA |
| Aquaflow Binomics | New Zealand | Algenol | USA |
| Photonz | New Zealand | | |

Mentioned companies focus on specialty chemical based on intracellular compounds produced by microalgae, because these have higher revenues than bulk chemicals like algae oil for biofuels [Borowitzka, 2013].

### 2.1.4 Extraction of valuable substances from microalgae

Different valuable substances (e.g. fatty acids, pigments, vitamins) are produced by microalgae depending on the species and the surrounding conditions, like nutrient depletion, high light intensities or salt concentrations. The majority of these substances accumulate intracellular. In order to process any valuable product, these substances have to be released from the cells. Commonly, this is done via extraction steps. Due to the fact that 50 to 80% of the total production costs refer to the downstream processing (post treatment after growing the biomass) energy- intensive harvesting and effective extraction of the target substances are important [Grima, 2003; Kumar, 2015]. Following, different extraction methods for valuable substances from microalgae are discussed.

**Solvent extraction**

For the extraction of lipids from biomass the `Folch method` is one of the first, mentioned in 1957. Thereby, a mixture of chloroform and methanol of 2:1 (by volume) is used to extract lipids from endogenous cells. Also the `Bligh and Dyer method` uses the mixture of chloroform and methanol in the same ratio [Bligh, 1959]. This method is widely used for the estimation of the lipid content of microalgae. To both methods, many modifications were made (e.g. addition of 1 M NaCl instead of water) to enhance the extraction efficiency. The `Matyash Method` uses methyl-tert-butyl ether as a solvent for extraction of lipids [Matyash, 2008]. Above mentioned methods are not suitable for large scale application, due to the usage of chloroform which provides environmental and health risks when used in large scale. Therefore, other solvents like ethanol, isopropanol, butanol, acetic acid esters or hexane were used which are less toxic, but also less effective in extraction of lipids [Kumar, 2015]. All these methods do not use an explicit cell disruption step. In addition to the above mentioned classical solvent extraction methods, the extraction of lipids with supercritical carbon dioxide (sc $CO_2$) is an alternative.

Here, the extraction step is carried out at temperatures between 40 and 80°C and pressures between 200 and 350 bars. In general, the extraction ability of sc $CO_2$ can be compared with hexane, but has the advantages of a nontoxic and non-flammable solvent [Rajvanshi, 2012].

**Mechanical extraction**

In contrast to the above mentioned solvent based extraction, also mechanical methods are applied in order to extract lipids from microalgae. Therefore, dried microalgae biomass is pressed in expeller presses.

The mechanism releasing the oil from the cells based on the rupture of the cells due to the applied high pressure. Afterwards the oil is pressed out of the biomass. Typical oil recovery with this method is 70 to 75%. Additionally, an energy consuming dewatering-step of the biomass has to be applied before, because the method requires a very low moisture of the biomass [Kumar, 2015].

**Ultrasonic and microwave assisted extraction**

For liquid cultures, ultrasonic assisted extraction disrupts the cells. The mechanisms of this method are cycles of rapid compression and decompression which forms micro bubbles inside the cells (cavitation). The collapse of a microbubble (cavitation implosion) provides extremely localized heat shock waves which finally disrupt the cells by destroying the cell wall and cell membrane [Brujan, 2001]. Microwave assisted extraction uses the effect of intracellular heating of the cells. Thereby, the electromagnetic oscillating field generates heat which vaporizes the water inside the cells. Major advantage of this method is the short operation time until cell rupture and low operating costs. However the high maintenance cost prevents a large scale application [Refaat, 2008].

**Enzyme assisted extraction**

The addition of enzymes like trypsin and cellulases destroy the cell wall and release the intracellular lipids. This method is very specific and is relatively cost intensive due to high enzyme costs, but ensures rapid cell rupture without mechanical methods or increased temperatures. This makes the method especially applicable for temperature labile products [Taher, 2014]. In general, above mentioned methods for the effective extraction of lipids can be combined with each other. For example the enzymatic treatment can be combined with a classical solvent extraction to achieve a cost effective production of microalgae lipids.

## 2.2 Expanded bed adsorption

The expanded bad adsorption (EBA) is mainly used for the purification of proteins and peptides from a variety of sources. It is related to the classical packed bed column chromatography, like ion-exchange-, affinity-, hydrophobic interaction chromatography and underlines the same binding principles. In contrast to these methods, the EBA uses fluidized adsorption particles and no packed bad as a solid phase, which allows the processing of viscous and particulate liquids like, yeast and mammalian cell culture fluids as well as bacteria fermentation broths [Wang, 2005]. The EBA combines the hydrodynamic properties of a fluidized particle bed with the chromatographic properties of a packed bad. In general the chromatographic resin is placed in a column, provided with an upwards liquid flow which suspended the particles. Thus, a fluidized bed of chromatographic resin particles is formed. Applying this flow regime (from bottom to top), cells, cell debris and other particles of the treated broth are washed out to the top and do not plug the adsorption bed like in packed bed chromatography.

Nevertheless, this flow regime ensures a good mixing of the (cell containing) broth with the adsorber particles, which enhances the target substances to adsorb to the chromatographic resin. After the adsorption step, the bounded target substances are eluted from the particular adsorbent by using a suitable buffer conditions (pH, salinity dependent on adsorbend and bounded substances) [Mattiasson, 1999].

## 2.3 *In situ* separation approach

The term `*in situ*` comes from Latin and means `in a place`. The `*in situ*` separation approach combines the cultivation of microorganisms and the extraction of their products, such as fatty acids in the same step. The energy-consuming harvesting especially of microalgae biomass can be excluded. In conventional harvesting processes the algae are usually centrifuged first and then dried. Only the dried algae are used for further downstream processing for example the extraction with organic solvent or supercritical $CO_2$ due to the fact that the most valuable substances are produced intracellular [Mendes-Pinto, 2001]. For the `*in situ*` extraction, the algal culture is mixed directly with a solvent, so that two phases are formed and the product-rich phase can be further purified [Kleinegris, 2011 a].

An important requirement is that the microalgae are not affected negatively after the direct contact to the used solvent. Extraction solvents which do not decrease the photosynthetic activity of the microalgae should be found in order to return them to the cultivation.

When this extraction method is used, generally there are three different mechanisms how the target substance is extracted, depending on which character the substance has in microalgae's metabolism.

These mechanisms are:

- 1) Excretion of the intercellular product
- 2) Permeabilization of the cell walls
- 3) Cell death

Especially these mechanisms count for intracellular production. However, on the other hand, it may be that the microalgae products are exported to the environment, these products are named extracellular metabolites as illustrated in fig. 5-7.
Ultimately the *in situ* extraction can be defined as a concurrency of three processes: extraction of excreted products, extraction with the help of cell permeabilization and extraction leading to cell dead.

Water soluble organic compounds and inorganic ions can be exported out of the cell via transmembrane carrier or channel proteins. Bigger organic molecules can be excreted only through exocytose [Kleinegris, 2011 b]. The milking of β-carotene molecules from microalgae *Dunaliellea salina* with dodecane for example, can be realized because the target substance is released to the surrounding media through exocytose [Hejazi, 2004 a].

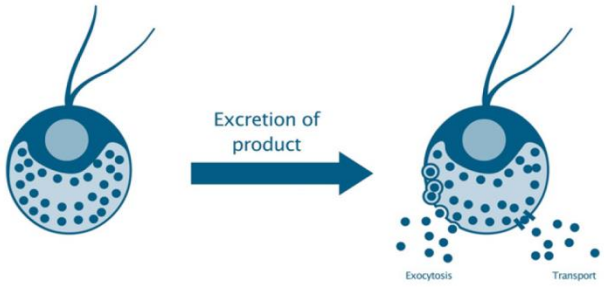



Figure 5: Extraction mechanism: Excretion [Kleinegris, 2011 b].

The uptake of extraction solvent into the cell membrane by active carrier mechanisms or partitioning of the solvent into the membrane can accelerate the extraction of intracellular components without a lethal damage of the living cells [Hejazi, 2004 a]. This extraction mechanism is termed as extraction through permeabilization.

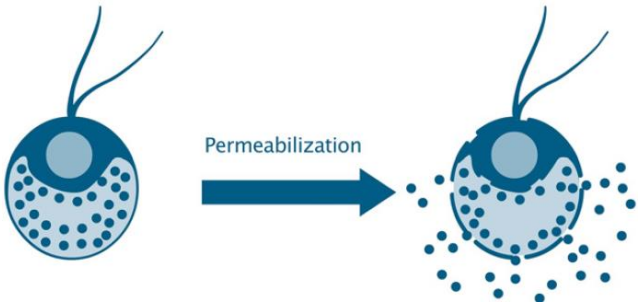



Figure 6: Extraction mechanism: Permeabilization [Kleinegris, 2011 b].

If the cell membranes are destroyed irreversibly by ultrasound, microwaves, mechanically or by cell toxic chemicals, the cell is disrupted into cell fragments [Guedes, 2011].

Accordingly to this cell lyses all cell components including the target substances are released to the surrounding medium and therefore well accessible for the extraction, as shown on figure 7 [Kleinegris, 2011 b].

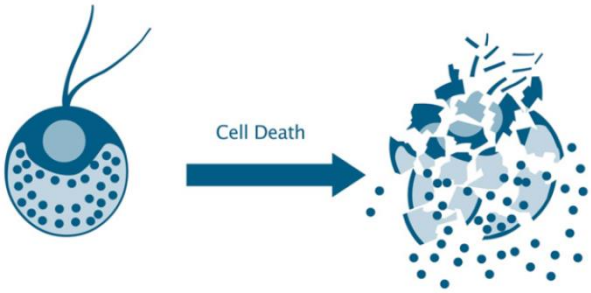



Figure 7: Extraction mechanism: Cell death [Kleinegris, 2011 b]

The key of realizing a milking process is the choice of an appropriate solvent in order to prevent the irreversible destruction of the cells. In this work, surfactant solutions are firstly suggested for this purpose. Their properties are discussed below.

# 2.4 Surfactants and micellar systems

## 2.4.1 Surfactants

Surfactants are surface active molecules which have been used in various fields and applications for centuries. In general, surfactants consist of a hydrophilic head-group and a hydrophobic tail [Tadros, 2006]. The hydrophilic head consists of strongly polar groups. Anionic and cationic surfactants dissociate in aqueous solution to form negatively charged and positively charged ions head groups, while nonionic remain neutral in an aqueous environment. Hence, surfactants are classified in anionic-, cationic-, zwitterionic- and nonionic surfactants [Myers, 2006]. The hydrophobic tail consists almost exclusively of a carbohydrate chain of e.g. perfluoroalkyl, polysiloxane alkylbenzene or alkylnaphthalene which can be linear or branched [Falbe, 1987; Rosen, 2012].

These surface active molecules are widely used as soaps, cleaning agents or detergents, due to their ability to lower the surface-/ as well as the interfacial tension of two liquids (or between liquids and solids). In following sectors of industry like the chemical- and pharmaceutical industry, the personal care and cosmetics-, as well as food and metal processing industry, surfactants are used as emulsifiers, wetting agents, dispersants and foaming agents [Rosen, 2012].

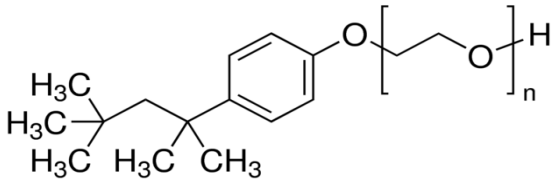



Figure 8: Chemical structure of Triton X-114 with n = 7.5 [www.sigmaaldrich.com]

Due to the amphiphilic character of the surfactant molecules, monomers form micellar aggregates by self-assembly in aqueous solutions, after reaching their critical micelle concentration (CMC).

Is this the case, the hydrophobic heads of the molecules are directed to the bulk water and the hydrophobic tails form the hydrophobic micelle cores [Fabry, 1991]. The critical micelle concentration in water varies with surfactants between 0.1-1.0 mmol/L [Rydberg, 2004] and depends mainly on the tail length and the group of the surfactants. The CMC of the nonionic surfactants is much lower than that of the ionic surfactants [Lindman, 1980]. Basically, the longer the tail, the lower the critical micelle concentration is. Above the critical micelle concentration of the surfactant in aqueous solution, not only the arrangement of the surfactants, but also physical properties (e.g. osmotic pressure, surface tension) of the solution changes [Lindman, 1980].

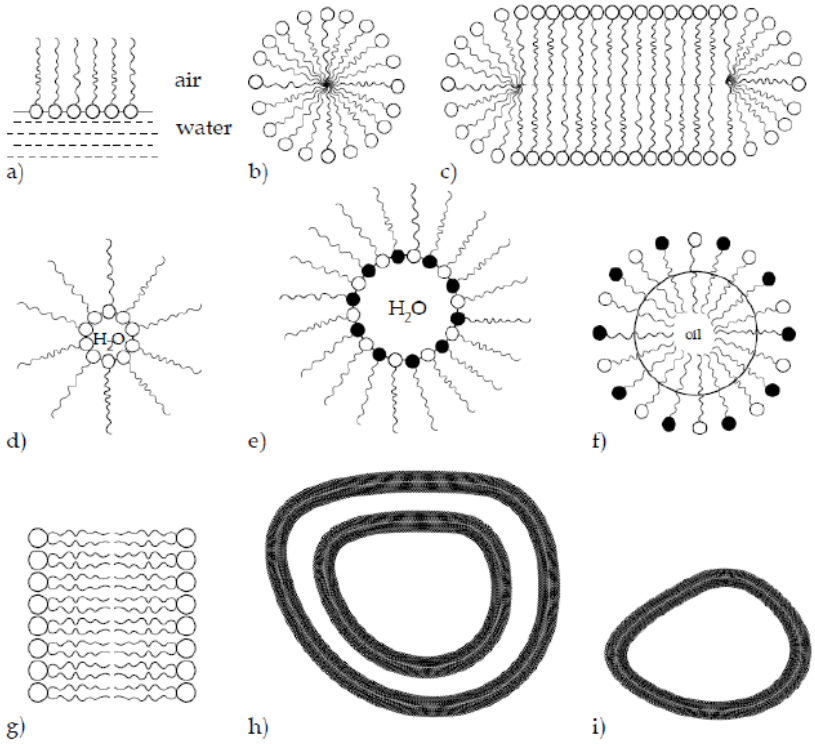



Figure 9: Overview of common micellar structures adopted from [Hinze, 1993] a) monolayer, b) spherical micell, c) rod-like micelle, e) water/oil microemulsion, f) oil/water microamulsion, g) lipid layer, h) multicompartment vesicle, i) single compartment vesicle.

Further increasing of the surfactants concentration leads to the formation of micellar aggregates which can be the shape of cylindrical, worm-like, lamellar shaped. But most common is the spherical form of micelles [Shinoda, 1962].

The form of the micelles results from the fact that the alkyl chains have to fill out the micelle core completely without conformational tensions to minimize the systems interfacial free energy. In addition, the micelle surface is completely covered with hydrophilic heads of surfactant molecules. Additionally, the distance between the hydrophilic groups is as large as possible due to the electrostatic and steric repulsion [Kosswig, 1993; Ingram, 2012 a].

Furthermore, the surfactant monomers at the interface and the micellar aggregates are in a dynamic equilibrium. The aggregation number is an indicator for the average number of surfactant in a single micelle and therefore also a dimension of the micelle size. This size is specific for each surfactant, but it can change with the surrounding conditions, especially the addition of salts [Lucza, 2011; Santos, 2009]. Another parameter for the characterization of surfactants is the hydrophilic-lipophilic balance (HLB). This describes the ratio between the total molecular weight of the surfactant and the molecular weight of the hydrophilic part of the molecule. HLB values between 0 and 9 indicates a stronger oil solubility, values between 11 and 20 greater water solubility. At an HLB value of 10, a hydrophilic - hydrophobic balance is reached [Kosswig, 1993]. This balance was developed empirically to classify surfactants according to their degree of hydrophobicity [Griffin, 1949].

### 2.4.2 Phase behavior of surfactant solutions

Although micellar aqueous solutions are heterogeneous on the molecular scale, they can be assumed as macroscopically homogeneous phases. This leads to the theory that the micelles can be assumed as a separate (pseudo) phase [Shinoda, 1962]. An increasing surfactant concentration above the CMC level leads to the formation of more complex mesophases, which can be lamellar, hexagonal, bicontinous or inverse structured [Kosswig, 1993; Tabazadeh, 2005]. The formation of these mesophases strongly depends on the temperature as well as the surfactants concentration in the solution.

But not only the concentration of the surfactant is important for the micellization, also the so called Kraft temperature should be taken into account. This indicates the surfactant specific temperature above the surfactant forms micelles. Below this temperature the surfactant remains in crystalline form even in aqueous solution [Rosen, 2012].

Especially nonionic surfactant aqueous solutions show a phase behavior which leads to their great potential for extraction of hydrophobic substances from diluted complex mixtures. Above a certain temperature which is surfactant specific, the homogeneous micellar aqueous solution divides into two macroscopic phases: a surfactant-rich phase and an aqueous phase [Hinze, 1993]. This temperature at which the both macroscopic phases coexist is called cloud point temperature (CPT) due to the visible fact that the solution turns turbid (cloudy) at this certain temperature. The surfactant-rich phase or coacervate phase is rich in micelles, the aqueous phase is poor in surfactant micelles, whereas the concentration of surfactant stays around the CMC. This phase separation is reversible and can be converted back to a single phase homogenous micellar solution through decreasing of the temperature below the CPT [Gu, 1995].

Important to mention is that there is an infinite number of clout point temperatures, due to the fact that the transition of the one-phase-region to the two-phase-region (in a phase diagram) is depended on the concentration of the surfactant. Correct would be the term lower critical solution temperature (LCST). But it has been shown that the term `cloud point temperature` is generally accepted to describe the lowest temperature at which the surfactant solution turns turbid.

In the figure10 adopted from [Sandoval, 2012], a typical phase diagram of a nonionic surfactant (here $C_{12}E_5$) is shown. Also anionic- cationic- and zwitterionic surfactants show a specific phase behaviorisms, but these are not discussed in detail here.

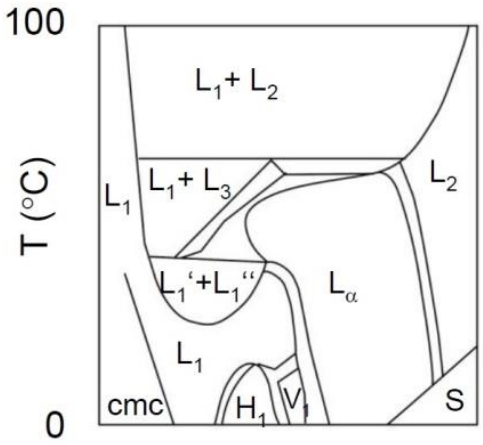



Figure 10: Phase diagram of a nonionic surfactant here $C_{12}E_5$from [Sandoval, 2012] adopted.

$L_1$, $L_2$, and $L_3$ micellar, reverse micellar and an isotropic solution containing bilayers, respectively; $L_1$’ and $L_1$’’ surfactant-lean and surfactant-rich phase; $H_1$ is a hexagonal, $V_1$ a cubic and $L_\alpha$ a lamellar liquid crystalline phase, and S represents the solid surfactant/ hydrated crystals.

For the application of these surfactant systems in separation processes, especially the appearance of the phases $L_1$’ and $L_1$’’ (surfactant-lean and surfactant-rich phase) with different densities is a main requirement.

If only the coexistence of the micellar and the aqueous phase is described, a simplified figure can be drawn. A typical liquid-liquid-equilibrium (LLE) of a nonionic surfactant water mixture which was generated with different methods is shown in the following figure 11 (here Triton X-114).

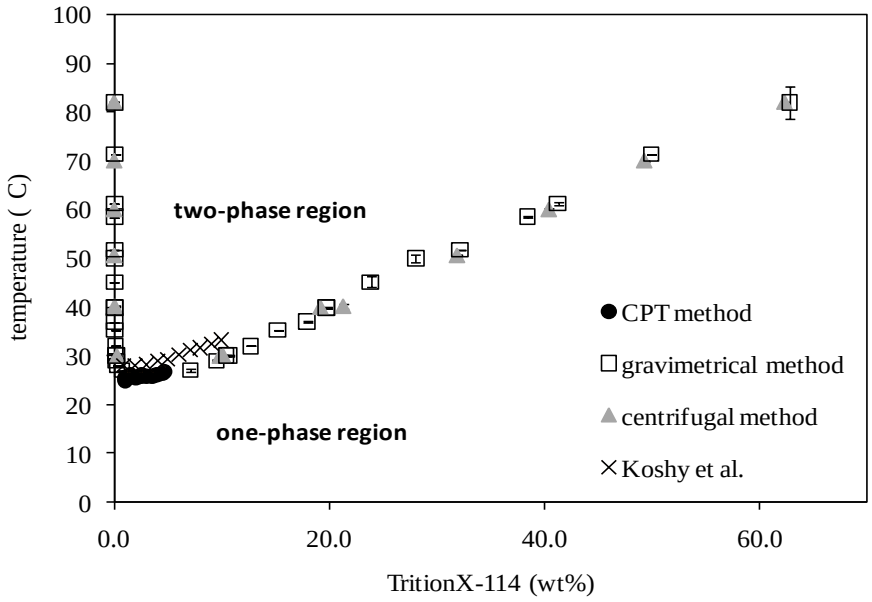



Figure 11: Liquid-liquid-equilibrium for the Triton X-114/water system data generated with different methods [Ingram et al., 2012].

In order to use aqueous surfactant systems for separation of target substances from diluted solutions, the knowledge of the phase behavior of the surfactant system is essential.

### 2.4.3 Solubilization of hydrophobic molecules by micelles

The incorporation of molecules (further named as solutes) at the micelles surface or in the micelles cores is named solubilization which increases the solubility of solutes in aqueous solution. The solubilization of other molecules in the micelles starts at the critical micelle concentration (CMC) and increases linearly with increasing surfactant concentration [Rangel-Yagui, 2004]. Thereby, the exact location of the solubilized molecule depends on the type of the interaction occurring between surfactant and solute that is solubilized including the charge of both molecules (surfactant molecule head as well as the charge of the solute).

Hydrophilic substances are adsorbed at the surface of the micelles, solutes with a low hydrophobicity generally incorporate with the surfactants hydrophilic head groups or between the head group and the first carbon atom of the surfactants tail, whereas strongly hydrophobic solutes are deposited directly into the micelle core [Rosen, 2012; Swe, 2006].

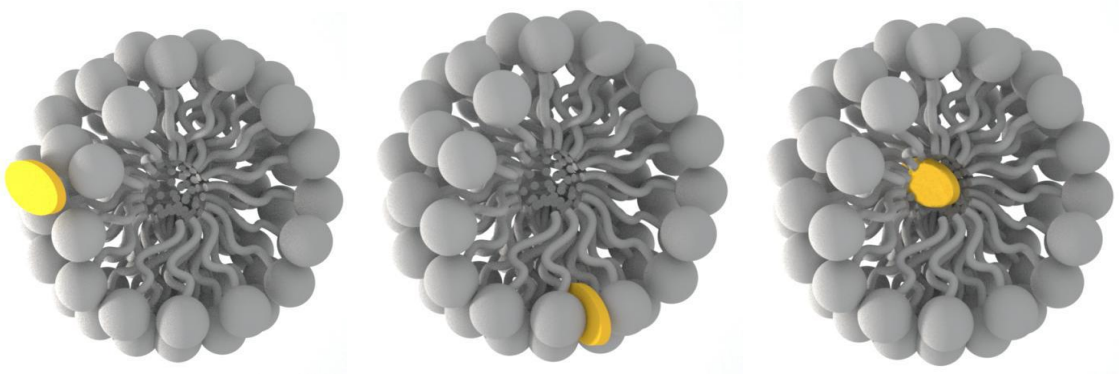


Figure 12: Locations for the solubilization of solutes with increasing hydrophobicity (left to right) within the micelle [Mehling, 2014].

Generally the solubilization and the exact location of the solute in the micelle can be influenced by several parameters like the addition of electrolytes or organic compounds [Maclay, 1956; Holmber, 2002; Javadian, 2008; Myers, 2006; Mehling, 2014].

This refers to changes of intermicellar interactions which lead to different aggregation numbers, CMC`s as well as the micelle size. With the appropriate selection of additives, an aqueous surfactant system can be adapted to the desired properties of separation process. For example an addition of `salting in` additives (as known from the Hofmeister series) increase the CPT, whereas `salting out` additives lower the CPT of a surfactant system [Rocha et al., 2013]. This ability of the specific adjustment of a micellar system, especially the possibility to decrease the CPT plays an important role for the applicability in micelle mediated extraction processes.

The higher the temperature difference between the CPT and the temperature at which the phase separation takes place, the higher is the density difference, which supports faster phase separation.

## 2.5 Liquid-liquid extraction

The liquid-liquid extraction is used when components (solutes) have to be separated from a homogeneous solution (feed solution) and distillation is not possible or expensive. Additionally, the solute can be relatively non-volatile (mineral salts) or heat sensitive like pharmaceutically active substances. Therefore, two immiscible fluids are contacted to enable a mass transfer of the solute between these fluids. The fluid which is enriched in the solute is called extract, the other one is called raffinate. The fundamental mechanism of this unit operation is the partitioning of the solute between the both phases, due to the different solubility of the solute in the liquids. This process can be carried out in many different kinds of extraction apparatus, for example extraction columns or mixer-settler devices. To enhance the mass transfer of the solute, small droplets are favourable which result in a large contact area. Therefore, an additional energy input is often generated with agitators inside the extraction devices [Hanson, 1974].

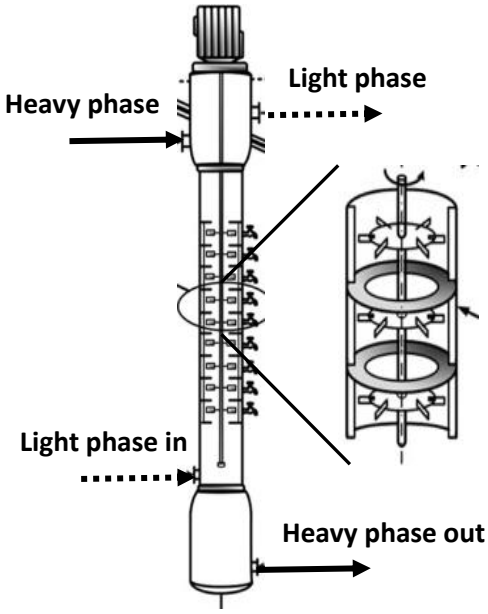



Figure 13: Schematic drawing of an agitated extraction column, adopted from [Asadollahzadeh, 2015].

Generally, continous extraction columns consist of two sections. In the first one, the mixing-section, raffinate and extract are mixed in order to enhance the mass transport. The second one, the settling-section, allows the two disersed phase to settle (settling of the lighter phase on top, settling of the havier phase on the bottom). In most applications a counter current flow of raffinate end extract is realized in order to mantain a high conentration gradient, but also co-current low or cross-flow can be realized. In the countercourrent application, the havier phase is entering the column at the top, whereas the lighter phase is inserted at the bottom. Therefore, the counter-courrent flow by pumping is supported by the gravity force [Wojik, 2005].

**Energy input**

The energy input in extraction colums can be realized by a piston pump or vibrating membranes (pulsed packed columns, pulsed sieve columns, pulsed pervorated plate colums), wich causes shere field at the packing or sieves inside the column, which leads to dispersion of the droplets.

Another possibility in order to enhance the dispersion of droplets, is the use of agitaded colums. In agitated extraction columns, a mixer is placed inside the mixing zone (see scheme of agitated extraction column). The advantage of agitated extraction columns is that the energy input and therefore the turbulence can be adjusted very accurate. The turbulence is generally characterized by the Reynolds number (Re). If (Re) is above 2300 the flow regime is turbulent. The main parameters for adjustment of the turbulence with agitators are the rotation speed of the agitator n (rpm), the mixers diameter $d_M$ (m) and the kinematic viscoity $v$ ($m^2/s$).

$$Re \overset{\text{def}}{=} \frac{n d_M^2}{v} \qquad \text{(eq. 4)}$$

Generally, an increase in the agitator speed, results in higher turbulence and therefore an increase interfacial area, which promotes masstransfer [Haeusler, 1985]. But also the axial backmixing is increased, which leads to a decrease in the exraction efficiency, due to the reduction of the concentartion gradient.

**Separation factor**

The separation factor S is a direct indicator for the quantitative evaluation of a extraction process. For a raffinat (with the target cpmponent a solute), following lodings of the both phases (rafinate α, extract β) leaving the extractor.

$$S_i^{\alpha,\beta} = \frac{X_i^\alpha}{X_i^\beta} \qquad \text{(eq. 5)}$$

with the itroduction of mass fraction $x_i$

$$S_i^{\alpha,\beta} = \frac{X_i^\alpha}{X_i^\beta} = \frac{x_i^\alpha (1-x_i^\beta)}{x_i^\beta (1-x_i^\alpha)} \qquad \text{(eq. 6)}$$

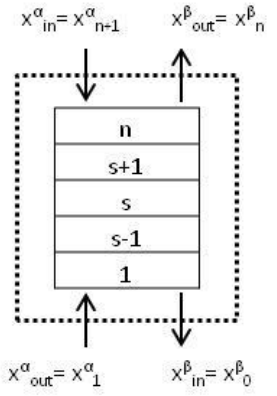



Figure 14: Balancing area of equation.

The number of theoretical stages extraction can be calculated using the Kremser equation by using the given balancing area with the stages s=1,…n-1 [Schönbucher, 2013].

$$N_{theo} = \frac{\ln\left[\left(1-\frac{1}{\varepsilon}\right)\left(\frac{x_{in}^{\beta,i} - x_{in}^{\alpha,i}/\mathrm{K_i^{\alpha,\beta}}}{x_{out}^{\beta,i} - x_{in}^{\alpha,i}/\mathrm{K_i^{\alpha,\beta}}}\right)+\frac{1}{\varepsilon}\right]}{ln\varepsilon} \qquad \text{(eq. 7)}$$

with the extraction factor

$$\varepsilon = \frac{1}{\mathrm{K_i^{\alpha,\beta}}\vartheta} \qquad \text{(eq. 8)}$$

With the number of theoretical stages and the knowledge of the hight $H_c$ of the column, Height Equivalent to one Theoretical Step (HETS) can be calculated.

$$HETS = \frac{H_K}{N_{theo}} \qquad \text{(def.)}$$

## 2.6 Phase separation in two phase systems

For the development of a liquid-liquid extraction process, the knowledge of the settling kinetics of the two phases from their dispersion is essential. For example the geometry of a settling device directly influences the phase separation behavior of the applied system. The faster the phase separation is, the smaller a settler device has to be. A classical used two-phase-system is the one of hexane and water. In this system the density difference of both phases is nearly 0.4 (g/kg) at 25°C. In comparison to this, a micellar two-phase-system containing of micellar and aqueous phases just has a density difference of 0.1 (e.g. 3% TX-114/water system at 30°C). This results in the fact that the micellar system needs minutes for phase separation in contrast to seconds for the hexane/water system. In the following chapter, the batch settling kinetic of two phase systems is described in order to derive the kinetic of nonionic surfactant two phase systems analogous to the classical two phase systems containing organic solvent and water. However, batch-settling of surfactant systems was not investigated in detail yet.

Commonly, for the determination of a batch settling kinetic of a two phase system an organic solvent is mixed with water (or another organic solvent with higher or lower density). The aqueous phase will disperse in the organic phase or vice versa depending on the volume ratio between the two phases.

In following the characteristic zones in a batch settling-experiment are shown schematically as well as the progress of the coalescence and the sedimentation curve as a function of time. It can be seen that different areas within a liquid-liquid extraction system are formed. There are the continuous phase and the dispersed droplets which are moving to the interface. The coalescence of droplets happens within the continuous phase when the contact impulse is high enough or at least the droplet coalescence takes place in the dense packed zone near the interface.

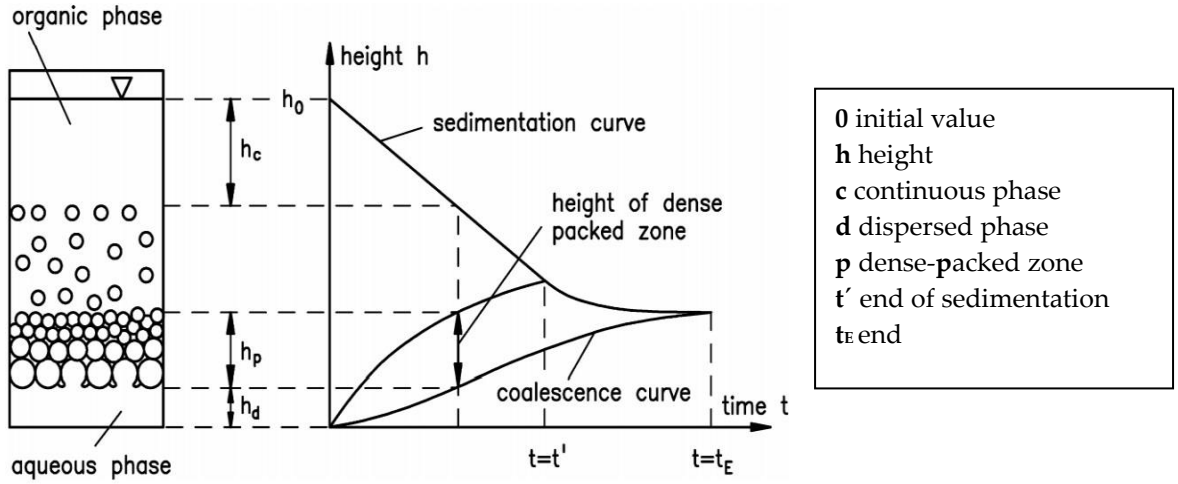



Figure 15: Batch-settling experiment (left-hand side) and respective settling curve (right-hand side) with notation for different heights [Henschke, 2001].

In fig. 16, a typical continuous extraction set up is shown with a dispersed light phase and a heavy continuous phase. Here, special attention is given to the dense-packed layer and the wedge of dispersion, were the coalescence takes place.

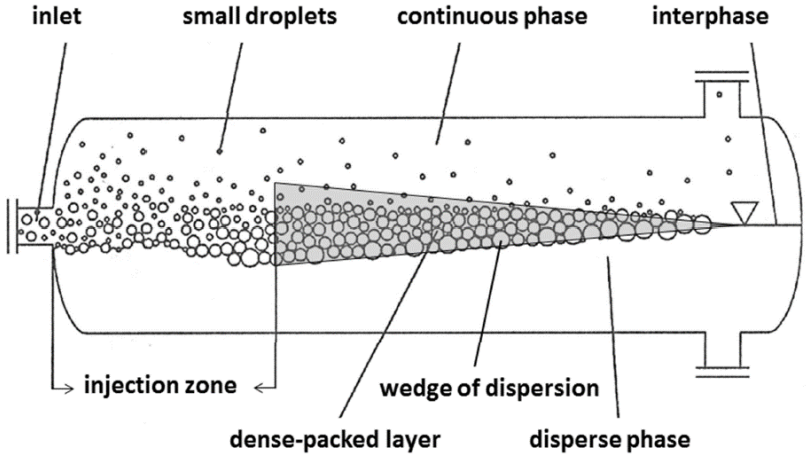



Figure 16: Continuous liquid-liquid extraction modified afterwards [Henschke, 2001].

In general, the following physical parameters are considered to be responsible for the phase separation of a dispersed two phase system. The phase separation itself consists of the following mechanisms: flocculation of droplets, coalescence of flocculated droplets and the sedimentation velocity of single droplets.

Following, the general forces which act on a dispersed droplet in a fluid are shown. These are the gravitational force, which depends on the density of the droplets; and the flotation or frictional force, which depends on the rheological properties of both phases. The balance of these forces influences in which direction the droplet moves [Asenjo and Andrews, 2012], however, it depends which phase (top with lower density, or bottom with higher density) is the continuous one.

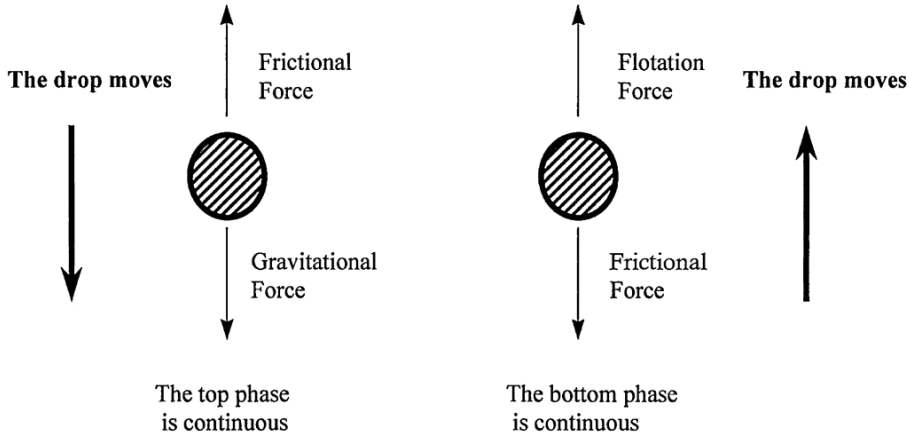



Figure 17: Overview of different forces influencing the phase separation [Salamanca, 1998; Asenjo, 2012].

The Stoke´s law is valid for single solid particles surrounded by a continuous viscous liquid. With the assumption that the droplet underlies no deformation, the velocity of the sedimentation can be described as:

$$v_{St} = \frac{g \cdot \Delta\rho \cdot d^2}{18 \cdot \eta_C} \qquad \text{(eq. 9)}$$

Were ($\upsilon_{st}$) is the Stokes sedimentation velocity, (g) is the gravitation force, ($\Delta\varrho$) is the density difference between the droplet and the surrounding liquid, (d) is the diameter of the droplet and ($\eta$c) the viscosity of the continuous fluid.

Further transformation can be done with the given equations for the Reynolds number (Re) and the Archimedes number (Ar),

$$Re = \frac{d \cdot v \cdot \rho_C}{\eta_C} \qquad \text{(eq. 10)}$$

$$Ar = \frac{\rho_C \cdot \Delta\rho \cdot g \cdot d^3}{\eta_C^2} \qquad \text{(eq. 11)}$$

to dimensionless number:

$$\mathrm{Re}_{St} = \frac{Ar}{18} \qquad \text{(eq. 12)}$$

Due to the fact that Stokes law is only valid for laminar flow (Re > 25), the calculation according to Zimmels [2000] can be expressed for turbulent flow of a droplet during sedimentation.

$$Re_{zi} = \left( \frac{-4.8 + \sqrt{23.04 + 2.91 \cdot \sqrt{Ar}}}{1.26} \right)^2 \qquad \text{(eq. 13)}$$

If the drop sedimentation is faster than drop–interface coalescence (drops combining with their corresponding continuous phase), the droplets will accumulate in a dense-packed zone. Here the drops grow due to drop-drop coalescence, until no drops can be observed [Henschke et al., 2001].

For an emulsion also the interfacial tension ($\sigma$) has to be considered [Berger, 1981; Blaß, 1992; Reissinger, 1981; Stönner, 1975]. In general liquid–liquid phase separation cannot be described by simple correlations of characteristic quantities, because of effects like interfacial mobility, van der Waals attraction or electrostatic repulsion which occur on a microscopic scale at the liquid–liquid interface [Henschke, 2001].

**Coalescence**

Due to the density differences in a liquid-liquid extraction system, the dispersed phase forms droplets which accumulate in the dense packed zone next to the interphase. Coalescence is the interaction of two droplets, or between a droplet and the interface in two phase systems. Following, the principals of droplet interaction and coalescence at the interphase are shown.

I: The droplet approaches the interface.

II: The interfacial tension between the disperse- and the continuous phase leads to the deformation of the droplet. A thin film is formed. The drainage of the film between the droplets is gravity force generated. When film-thickness reaches a critical value, the van der Waals forces cause the film-rupture [Samanta, 2011].

III: The droplets coalesce with the interface, whereby the time of the coalescence is dominated by the film-drainage.

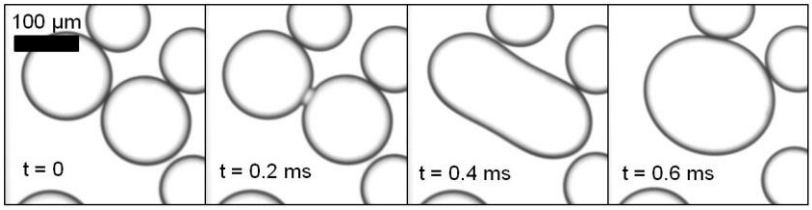



Figure 18: Principle of droplet coalescence (Hexadecane in water) [Berton-Carabin, 2015; Krebs, 2012].

Above mentioned coalescence mechanisms is shown for the coalescence of two pure liquids (Hexadecane in water). If also surface active molecules like surfactants are present, the Marangoni effect plays an important role. The drainage of the film between two droplets leads to a surfactant concentration gradient, which causes a surface tension gradient. This surface tension gradient provokes Marangoni convection, which induces the reflux of the continuous phase between the droplets and the interphase. This implements the droplet coalescence.

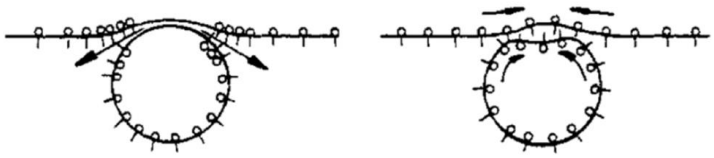


Figure 19: Principle of film drainage when surfactants are present. Marangoni convection [Rautenberg, 1983].

In summary the main parameters which can influence the phase separation are:

- Density differences of the phases;
- Viscosity;
- Interfacial tension.

## 2.7 Partitioning of hydrophobic substances

The liquid-liquid extraction is generally used for processes which focus on target substances which degrade at higher temperatures or form undesirable byproducts. Mostly, these extraction processes take place at ambient or moderate conditions. During the application of a thermal separation process, heat and mass are transferred between the phases of the open thermodynamic system until the equilibrium between both phases is reached [Sattler, 2001]. In order to describe the partitioning of target substances in aqueous surfactant systems (between micellar and aqueous phase) it is convenient to assume the 'pseudo-phase-approach' according to Gmehling (1996). Here, one phase is the ´micellar pseudo-phase` the other one the bulk aqueous phase outside the micelles. On account of this the Gibbs conditions of thermal, mechanical and chemical equilibrium can be applied [Gmehling, 1996].

$$T^M = T^W \qquad \text{(eq. 14)}$$

$$P^M = P^W \qquad \text{(eq. 15)}$$

$$\mu_i^M = \mu_i^W \, , i = 1, \dots, n \qquad \text{(eq. 16)}$$

Here, $M$ represents the micellar phase, $W$ the aqueous phase and $i$ is the certain component.

The partial molar Gibbs free energy, also referred as the chemical potential of the component i, is connected to the thermodynamic potentials namely the internal energy U (S,V,$n_i$) the enthalpy H (S,p,$n_i$), the Helmholz free energy A (T,V,$n_i$) and the Gibbs free energy $G\,(T,p,n_i)$. This can be defined as following:

$$\mu_i = \left(\frac{dU}{dn_i}\right)_{S,V,n_j \neq n_i} = \left(\frac{dH}{dn_i}\right)_{S,p,n_j \neq n_i} = \left(\frac{dA}{dn_i}\right)_{T,V,n_j \neq n_i} = \left(\frac{dG}{dn_i}\right)_{T,p,n_j \neq n_i} \quad \text{(eq. 17)}$$

Therefore, the chemical potential is the partial molar Gibbs free energy. The following equation can be written for an ideal mixture:

$$\mu_i = \mu_i^0 + RTln(x_i) \quad \text{(eq. 18)}$$

Here $\mu_i{}^0$ represents the chemical potential of the component $i$ in standard state and $\Delta\mu_i$ partial molar free energy of mixing of the component $i$.

For the description of real mixtures the use of the mole fraction $x_i$ is insufficient and therefore replaced with the activity $a_i$.

$$a_i^\alpha = a_i^\beta = \cdots = a_i^\pi \quad \text{(eq. 19)}$$

an activity coefficient $\gamma$ is defined as

$$\gamma_i^{(x)} := \frac{a_i}{x_i} \quad \text{(eq. 19)}$$

with $\gamma_i \cdot x_i$ as activity und $\gamma_i$ as activity coefficient of the component $i$. The activity coefficient is thereby defined as:

$$\lim_{x_i \to 1} \gamma_i = 1 \quad \text{(eq. 20)}$$

According equations, for the component i, partitioned at constant pressure and temperature between two immiscible and no ideal liquid phases, is valid:

$$\mu_{0i,M} + R \cdot T \cdot \ln(\gamma_{i,M} \cdot x_{i,M}) = \mu_{0i,W} + R \cdot T \cdot \ln(\gamma_{i,W} \cdot x_{i,W}) \quad \text{(eq. 21)}$$

Whereby the standard state for the two liquid phases is the same with respect to component i.

$$\mu_{0i,M} = \mu_{0i,W} \qquad \text{(eq. 22)}$$

Thus eq. 23 can be simplified to eq. 25:

$$\gamma_{i,M} \cdot x_{i,M} = \gamma_{i,W} \cdot x_{i,W} \qquad \text{(eq. 23)}$$

The partition coefficient is defined as:

$$K_i^{MW} = \frac{x_i^M}{x_i^W} \qquad \text{(eq. 24)}$$

were $K_i$ describes partition coefficient of component i between the micellar *M* and the aqueous phase *W*.

This can also be expressed in terms of activity coefficients [eq. 24].

$$K_i^{MW} = \frac{\gamma_i^W}{\gamma_i^M} \qquad \text{(eq. 25)}$$

In diluted systems where $x_i$ is low, the following form of the Nernst's distribution rule is often applied:

$$K_i^{MW} = \frac{c_i^M}{c_i^W} \qquad \text{(eq. 26)}$$

Here the relation between both partition coefficients is the ratio between phase molar volumes [Sattler, 2001]:

$$K_i^{MW} = K_i \frac{\vartheta_w}{\vartheta_M} \qquad \text{(eq. 27)}$$

In order to apply those theoretic partitioning basics, a stable phase separation in the real liquid-liquid two phase system is a main requirement for the application.
In the following chapter this requirement is studied in detail.

### 2.7.1 Modeling of Partitioning Coefficients using COSMO-RS

Often it is not enough or even no experimental data about $K_i^{MW}$ available. In this case, the prediction of the partition coefficients is of extreme importance.

Generally there are two types of models. First category are models using the activity coefficient derived from excess Gibbs energy ($g^E$). These models are used to describe complex mixtures over a relatively low pressure range, in contrast to the second category of models which are based on equations of state (EOS). EOS are generally less accurate but are appropriate for a wide pressure range [Kontogeorgis, 2010].

Following, the $g^E$ model COSMO-RS is described in general since it was used in this work to predict activity coefficients and therefore partition coefficients $\gamma_i$. Further details can be found at [Klamt, 1995; Klamt, 2011; Mehling, 2014; Ingram et al., 2012]. Important is that the model COSMO-RS has the ability to calculate thermodynamic data based on a limited number of parameters specific for each element [Klamt, 1995]. This is mainly the molecular structure including all information about the bindings including the possible rotations.

Fundamentally for these calculations is the dielectric continuum solvation method, also called `Conductor-like Screening Model` (COSMO). In the model COSMO the solute molecules are put in a virtual conductor environment where polarization charged density $\sigma$ is induced on the interface between each molecule and the conductor, i.e. on the molecule surface. These charges act back on the solute and lead to more polarized electron density than in a vacuum.

The screening charged density is used to apply the COSMO-model for `Real Solvents` (COSMO-RS). A distribution function, referred as σ-profile $p^i(\sigma)$ and describing the polarization charge density distribution on the surface of each solute molecule, presents the relative amount of surface with polarity σ on the molecule's surface. The sum of $p^i(\sigma)$ for each component, multiplied by the mole fraction in the mixture is the σ-profile $p_s(\sigma)$ for the entire mixture of several compounds, S [COSMOlogic GmbH & Co. KG, 2009]. The activity coefficients $\gamma_i$ in both phases can be calculated for infinite dilution of the solute ($\gamma_i \rightarrow \gamma_i^{\infty}$ if $x_i \rightarrow 0$) with the assumption one phase (the micelles) are free of water ($x^M_{iwater} = 0$) [Mehling, 2014].

## 2.8 Biocompatible solvents for extraction

In the design of liquid-liquid extraction processes the selection of a suitable solvent is one of the most challenging tasks. Generally the used solvent should be chemically and thermally stable and should be non-biodegradable and non-hazardous to the microorganism used [León et al., 2001]. Still the main constrains of a suitable solvent is the ability to extract the target substance. Additionally, the living cells should not be damaged lethally, in other words the solvent should be biocompatible. Hence, the ideal solvent of a two-phase system in which living cells are present, should neither impede the organisms' growth, nor interfere with any of its biological and bio catalytic functions [Heipieper, 2007].

The application of the two-phase extraction in so called extractive fermentations allows simultaneously product recovery and separation. This can induce a shift in the reaction equilibrium to higher yields [Wang, 2010; Dhamole, 2012]. Another advantage is that intermediate products that are produced during continuous fermentations by enzymatic processes can be removed continuously from the reactive phase [Wang, 2005]. Therefore, the organic solvents applied in whole cell transformation should have high biocompatibility to the used cells [Heipieper, 2007].

For the microalgae strain *Dunaliella salina,* which accumulates ß-carotene several solvents were investigated in order to determine their biocompatibility. The selection was done according to their hydrophobicity. Therefore, the octanol/water partition coefficient (log $P_{oct}$), was selected. This parameter is often used in the pharmacokinetics and pharmacodynamics were it describes the tendency of a drug in the human body to partition into the lipid bilayer (hydrophobic), or into the blood serum (hydrophilic). It allows characterizing a compound related to the effects in living organisms. The parameter log $P_{oct}$ is defined as the partition coefficient of a given solvent in a mixture of 1-octanol and water [Ogino, 1999]. Generally it can be summarized that the greater the polarity, the lower the log $P_{oct}$ value of the solvent is. Following figure 20 shows the relative activity retention of the microalgae *Dunaliella salina* as function of the solvent´s log $P_{oct}$ and their molecule size. It is concluded that solvents are biocompatible to the algae when they have a log $P_{oct}$> 5, or a molecular weight higher than 150 g/mol. The authors have the thesis that small molecules below a molecular weight of 150 g/mol, can easily penetrate into the organism's membrane in contrast to larger molecules. This critical concentration in the organism`s membrane causes cell death.

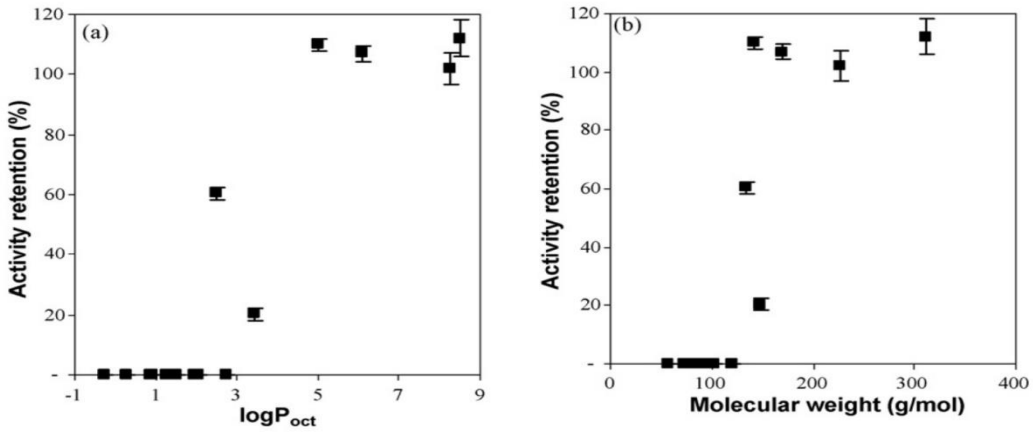



Figure 20: Relationship between activities retention of *Dunaliella salina* cells exposed to an organic solvent and the log $P_{oct}$ value of the solvent (a) and its molecular size (b) adopted from Mojaat 2008.

Essentially two types of toxicity are defined. The so called `molecular toxicity` is caused by dissolved molecules of the solvent in the aqueous phase. These molecules can affect the cell by membrane modification, enzyme inhibition or protein denaturation. In addition to molecular toxicity, phase toxicity can be observed.

This occurs when there is enough solvent to form a separate phase. In this case, the toxicity may be not only attributed to dissolved molecules, but also to the presence of a second phase which can lead to extraction of nutrients, or disruption of the cell wall due to the large difference in surface tension and shear forces [Kleinegris, 2011 b].

The above mentioned facts are important for the application of organic solvents and focus on the toxic effects of solvents to living cells. But also the temperature is affecting the viability of microorganisms, due to the temperature induced denaturation of key enzymes in the metabolism.

Especially when aqueous surfactant systems are applied for the extraction of valuable substances from living cell cultures, the temperature tolerance of the microorganisms have to be taken into account [Hanagata, 1992]. For the special nonionic surfactant extraction systems, which are described in detail later on, the temperature is a key factor. Especially the cloud point temperature is a major parameter, due to the fact that this is the temperature where phase separation starts.

The following figure shows the photosynthetic activity of the microalgae *Scenesdesmus obliquus* as a function of the cultivation temperature.

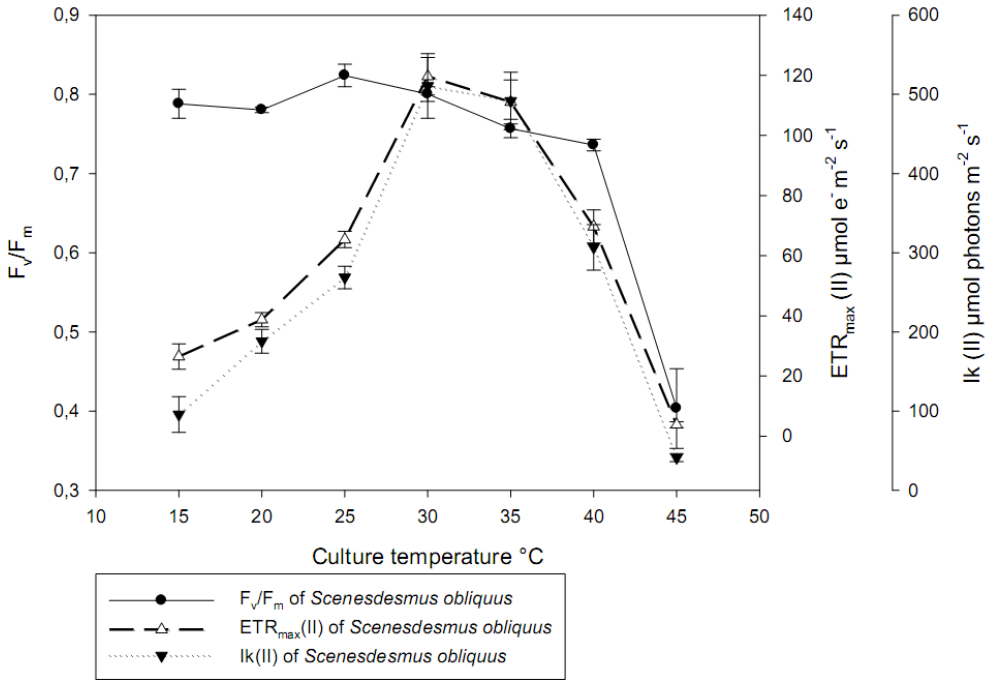



Figure 21: Photosynthetic activity of the microalgae *Scenesdesmus obliquus* as a function of the temperature [Hindersin, 2013 b].

It can be concluded that the maximal tolerable temperature for *Scenesdesmus obliquus* is between 40°C and 45°C. Above 40°C the $F_v/F_m$ parameter, which is an expression for the algae relative photosynthetic activity (RPA), sharply decreases [Hindersin, 2013].

The efficiency of photochemical energy conversion also referred as relative photosynthetic activity (RPA), is a suitable and simple to determine parameter which can be used for the characterization of positive or negative environmental effects (such as toxic medium compounds, temperature induced denaturation of enzymes, of photoinhibition) on the microorganisms.

The working group of Wang was one of the first ones which described the use of surfactant based extraction systems for the separation of target substances from microorganisms. They promote that the cloud-point temperature of a micellar system which should extract target substances *in vivo* (directly from the culture broth), is the first aspect to be taken into account for the surfactant screening process [Wang, 2007].

Applied for a two-phase system including living cells, the selected solvent should have a high critical lethal concentration for the organism. This represents the solvent toxicity [Wang, 2007]. Wang also states the assumtion that nonionic surfactant cloud-point systems provide milder extraction, in contrast to the classic solvent extraction [Wang, 2004].

**Mechanisms of surfactant interactions with mebranes**

Generally, surfactants are expected to bond and cause denaturation of proteins in the cell membrane. Thereby the membrane gets permeable for nutrients and chemicals. Monosaccharide glucosamine is the main constituent of the rigid cell wall of chlorella [Nemcova, 2000]. Thus, cell wall thickness and composition influence the severity of this effect. Charged surfactants (anionic and cationic) have a greater denaturizing effect than nonionic ones [Lewis, 1990]. The toxicity strength of a certain surfactant is always specific for the different cell types. Thus, the surfactant screening must be overviewed in every certain case of observation [Lewis, 1990].

According to [Wang, 2007] a nonionic surfactant is biocompatible from $\log P_{o/w}$ values of 2 upwards. Within a homological series or a series of similarly structured organic compounds, the toxicity increase with the CMC values and the critical aqueous concentration, respectively.

## 2.9 Zeta Potential

The charge distribution on the surface of microorganisms (which are regarded as particles here) is crucial for their interaction with other molecules in their aqueous environment. The electrochemical character of interfaces or surfaces can be measured by the zeta-potential (ζ-potential). It characterizes the electrostatic repulsion or attraction between particles in colloid dispersions and depends on the properties of liquid as well as on properties of the surface [McNaught, 1997].

Particularly at phase boundaries between solid and liquid, charges on both sides are formed mostly by the specific adsorption of ions, or dissociable groups that can be ionized.

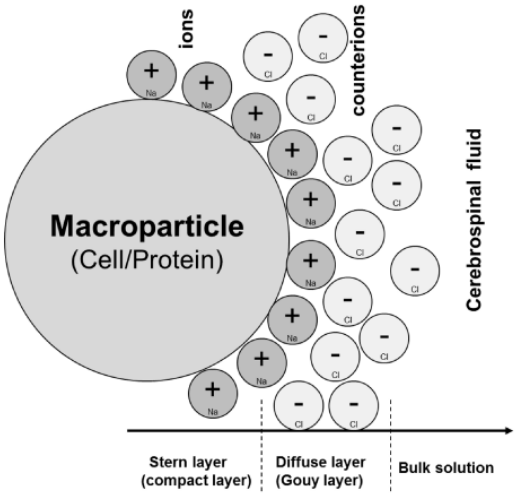



Figure 22: Schematic representation of the electric double layer around the macro particles suspended in an electrolyte containing fluid, adopted from [Herbowski, 2011].

The formed bilayer is composed of the `Stern layer` at the particle´s surface, a rigid layer of charged ions or oriented dipoles on the solid phase and the diffuse double layer, a layer in which the alignment of the ions or dipoles continuously decreases until it reaches the concentration of the surrounding bulk liquid [Wilson, 2001].

## 2.10 State of the Art

After discussing the basic process and phenomena in chapters 2.1 – 2.5, in this chapter the state of the art of the extraction of valuable compounds from microalgae is reviewed.

### 2.10.1 Extraction of valuable substances from microalgae

State of the art technology especially for the extraction of hydrophobic compounds from algae cultures is the batch extraction of dried algal biomass. This approach always includes the energy consuming cell harvesting step by centrifugation or filtration and the following dewatering of the wet microalgae biomass [Norsker, 2011; Grima, 2003]. Important to reveal is that the microalgae cells are disrupted (by pressing, ultrasound or at least by the extraction solvent itself) prior to extraction in order to increase the extraction yield [Dragone, 2010]. The two commonly used extraction methods are the extraction with organic solvents or with supercritical carbon dioxide [Rajvanshi, 2012]. For classical solvent extraction mainly hexane, chloroform and acetone are used to extract the algae biomass [Halim, 2012]. More sustainable concerning the extraction medium is the supercritical fluid extraction (SFE) which uses supercritical $CO_2$ (sc. region reached above 30 °C and 74 bar) which has a nearly the same polarity like hexane, but does not cause the problems such as using organic solvents (recycling, safety). The drawback of supercritical extraction with $CO_2$ is relatively high pressures of at least 100 bars compared to classic solvent extraction at ambient pressure, which reveal to higher equipment costs. Especially the extraction of omega-3 and saturated fatty acids with supercritical $CO_2$ from the algae was successfully shown in technical scale [Andrich et al., 2005; Mercer, 2011]. The working group of the Fraunhofer IGB at Stuttgart shows the extraction of the long-chain omega-3 fatty acid Eicosapentaenoic acid (EPA, 20:5) with sc. carbon dioxide.

To overcome the disadvantages of the commonly used methods and therefore to enhance the realization of an overall economic process the *in situ* extraction approach can be helpful. Thereby, the time and costs for re-growing the biomass in such repeated batch processes (growing, harvesting, extraction, re-growing, etc.) are omitted [Kleinegris, 2011]. Additionally, product inhibition or degradation of organic products by microorganisms can be eliminated by an *in situ* product removal [Xue, 2010].

### 2.10.2 *In situ* extraction with organic solvents

In 2004 a new method for the extraction of β - carotene was described, also called milking (Hejazi, 2004 a). In a two-phase photobioreactor cultivation of microalgae and the extraction of metabolites were combined in one step. It was shown that dodecane seem to be a biocompatible solvent for *Dunaliella salina*. Two possible extraction mechanisms, which involve exocytose or membrane alterations, were suggested [Hejazi, 2004 b]. The process selectivity decreases after several days in aerated flat photobioreactors and also chlorophyll and other pigments were extracted. This was explained with the shear stress which causes cell damage [Kleinegris, 2010 a].

The process consists of a dodecane extraction of astaxanthin from the culture broth (stage 1) and methanol extraction of free astaxanthin from the dodecane extract (stage 2) which can be seen in the following scheme.

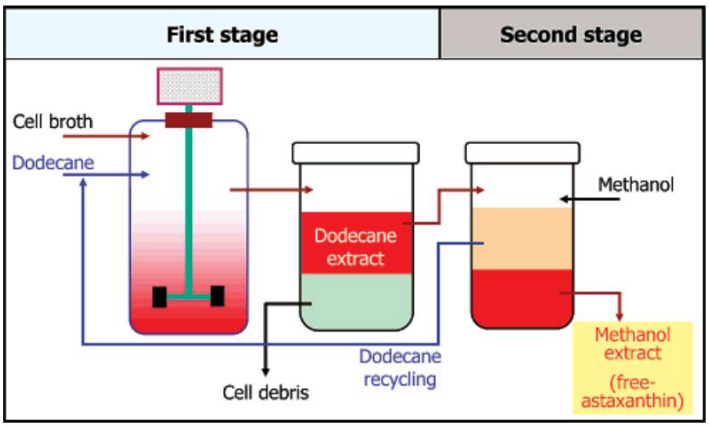



Figure 23: Schematic diagram of a two-stage extraction process for selective separation of free astaxanthin from red encysted *Haematococcus* culture [San, 2007].

Also an extraction of astaxanthin with vegetable oil was successfully demonstrated in lab scale by [Kang, 2008]. Dodecane as biocompatible solvent and potential milking agent for polyunsaturated fatty acids was studied by [Silva, 2006].

### 2.10.3 Biocompatibility of solvents with algae

For the application of this *in situ* extraction approach, direct contact of the used solvent with the living cells is a prior condition. Additionally, it is important that the metabolic activity of the cells remains unaffected by the extraction medium.

Therefore, Leon (1998) studied the biocompatibility of different solvents in two-phase systems for microalgae biocatalysis. Brink (1985) also studied the toxicity of conventional solvents used in two phase bioprocesses.

Several solvents were investigated for the microalgae strain *Dunaliella salina,* which accumulates ß-carotene, in order to determine their biocompatibility to the microalgae. The selection was done according to their hydrophobicity (log $P_{oct}$) and their maximal solubility in water [Ogino, 1999]. Yamane (1984) was one of the first who examined the toxic effect of anionic and nonionic surfactant on marine microalgae. Nonionic surfactants were proven to be less toxic than anionic surfactants. Biological effects on the green microalgae *Chlamydomonas reinhardtii* caused by anionic, nonionic and amphoteric surfactants were investigated by Ernst et al. (1983). It has been proven again that anionic surfactants inhibit cell growth significantly compared with nonionic surfactants.

The toxicity of surfactants to algae has been reported and discussed by, among others, [Hicks, 1966; Roederer, 1987]. Lewis (1990) summarizes all microalgae surfactant toxicity studies. He found out that anionic and nonionic surfactants and detergent builders are relatively non-toxic when compared to various cationic monoalkyl and dialhyl quaternary ammonium salts. Charged surfactants (anionic and cationic) have been reported to have a greater denaturing effect than neutral compounds [Nyberg, 1976]. Furthermore, the dosage of surfactants is inversely proportional to their ability to reduce surface tension [Bock, 1965]. Cell walls of algae species differ in thickness and chemical composition.

Generally the studies show that, surfactants have been observed to denature and bind proteins in the cell wall and therefore alter membrane permeability to nutrients and chemicals. The thicker the cell wall the less likely the impact on the viability of the organism is. High lipid and protein content in the cell wall allows penetration of hydrophobic surfactants. Thereby, the pH value does not influence the sorption of surfactant molecules in the plant cells [Cserháti, 1995].

### 2.10.4 Cloud point extraction for living organisms

The cloud point extraction (CPE) is based on the special phase behavior of aqueous nonionic surfactant solutions, which show a phase separation when the temperature increases above the clod point temperature (CPT) of the system. A major advantage of these systems in contrast to classic organic solvent two phase systems is the ability to change the system from a monophasic- to a biphasic system (or vice versa) just by increasing (or decreasing) the temperature. Especially the extraction of hydrophobic substances from dilute aqueous solutions is a promising field for the application of cloud point extraction systems.

It was shown that the cloud point extraction has a great potential to remove toxic solutes from polluted water [Sakulwongayai, 2000]. In special applications, when biodegradation by microorganisms of non-polar and polluting substances is used the cloud point extraction is an approach for the solubilization of these molecules in the fermentation medium in order to increase the bioavailability [Peng et al., 2008; Pantsyrnaya, 2011]. Nonionic surfactants were described to extract target substances from bacteria cultures as an alternative method which prevents the hydrophobic product from further degradation or product inhibition [Wang, 2007]. Another application is the extractive fermentation of lipase in a cloud point system [Wang, 2010]. The lipase prefers to accumulate in the surfactant rich phase of the nonionic surfactant (Triton X-114; Triton X-45) - water system. Important to mention is that cells remain in the aqueous solution.

Another application of micellar systems in separation processes the combination of functional magnetic nanoparticles and aqueous micellar two-phase systems for the continuous purification of proteins. Thereby, the phase separation of the micellar phase is enhanced by the addition of magnetic particles [Fischer, 2013].
Furthermore, the toxicity of nonionic surfactant on microbial cells is very low so that the microorganism *Monascus purpureus mold* can be cultivated in Triton X-100- water mixture without cell growth inhibition [Hu, 2012].
Cloud-point systems were also used for the microbial transformations of hydrophobic compounds with the nonionic surfactant Triton X-114. It is biocompatible and has a cloud-point temperature below the biotransformation temperature (28°C) [Wang, 2005].

Additionally, from previous studies on the application of micellar extraction with nonionic surfactants in fermentation systems it can be assumed, that the micellar extraction of microalgae cultures can be an alternative to extraction with organic solvents [Pan et al., 2009; Hu et al., 2012].

All the above mentioned works are dealing with the cloud point extraction in batch systems [Malpiedi, 2014]. But there are also applications, where a continuous cloud point extraction is shown. Ingram (2012) showed that a CPE with Triton X-114 in a continuous counter current extraction column is possible and a promising approach to improve the usage of surfactant based extraction systems. The improvement of a continuous cloud point extraction finding the optimal operating point is show in Trakultamupatam et al. (2005).

## Summary of the current state of the art

It has been shown that the state of the art production of valuable substances from microalgae has a main drawback: the low productivity of microalgae biomass (and therefore intracellular compounds) compared to bacteria cultivations. A high density microalgae cultivation results in a biomass concentration of round about 10g dry mass/ l, whereas cultivations of bacteria can reach over 100 g dry mass/l. This fact requires a time and cost consuming harvesting of the microalgae biomass (about 99% of water has to be separated from the microalgae cells).

In contrast to the classic procedure of biomass cultivation, followed by harvest, dewatering and a resulting extraction of valuable substances with classical organic solvents or sc. carbon dioxide, the *in situ* extraction approach offers some advantages. With the use of a biocompatible solvent, the target substance can be extracted directly from the culture, which makes a harvesting of the biomass unnecessary. The *in situ* extraction has the potential to develop a continuous extraction process in contrast to the batch extraction of dried algae biomass.

The selection of organic solvents including surfactants for the use of *in situ* extraction from bacterial cultures, was already studied in detail in contrast to the selection of suitable solvents for *in situ* extraction of microalgae.

The *in situ* extraction approach of valuable substances from microalgae cultures with the biocompatible organic solvent n-dodecane was proposed by Hejazi (2004 b). They extracted β-carotene from the marine microalgae *Dunaliella salina* in two-phase bioreactors where the microalgae was cultivated in presents of n-dodecane. As a synonym for *in situ* extraction, he named the process `milking of microalgae`. Thereby, the organic solvent n-dodecane was pumped and cycled continuously through a microalgae culture as can be seen in the following figure.

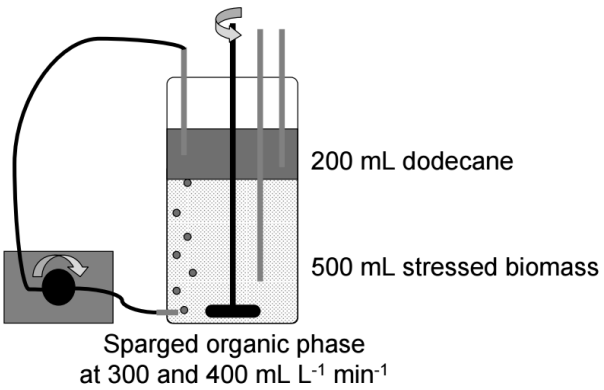



Figure 24: Scheme of *in situ* extraction with use of n-dodecane (organic phase) [Kleinegris, 2010 a].

The application of this `milking of microalgae` over several hours leads to handling problems caused by emulsification. This hinders phase separation and is therefore not applicable. This problem can be principally overcome by using alternative solvent systems.

Concluding, the development of an *in situ* extraction process of valuable substances directly from microalgae cultures, using a surfactant based extraction system was not done yet.

Therefore, the purpose of this work is to prove the feasibility of such an *in situ* extraction system for the extraction of valuable hydrophobic substances from microalgae.

## 2.11 Aim and structure of this work

The process development aimed in this work was structured as follows:

- Selection of a biocompatible micellar extraction system

  In order to use a micellar extraction system, only nonionic surfactants were selected due to their ability to form two phase systems above a certain temperature. Therefore, several surfactants were screened for their biocompatibility to the most commonly used microalgae species *Chlorella vulgaris, Scenedesmus obliquus* and C*hlamydomonas reinhardtii*. The relative photosynthetic activity was used as a parameter for the determination of the surfactants biocompatibility with the microalgae.

- Characterization of phase separation

  The phase separation plays an important role for the application of two-phase extraction systems. If this process step is hindered, due to formation of stable emulsions (see above mentioned dodecane based microalgae extraction approach) the extraction process is not feasible at all. Therefore, the phase separation behavior of a selected surfactant system (Triton X-114) was studied at different temperatures and surfactant concentrations. Furthermore, these studies were also performed for the Triton X-114/ microalgae culture system. Important to mention here is, that the microalgae cells predominantly accumulate in the aqueous phase, whereas the micellar phase remains nearly cell free.

- Partitioning of target substance

  A main requirement for an effective extraction process is the favorable partitioning of the target substance between the extract and the raffinate. In order to evaluate the partitioning behavior of fatty acids in micellar systems, experiments performed and compared with the calculated partition coefficients using the model COSMO-RS.

- Implementation of an extraction process (batch/continuous) in an outdoor plant for cultivation of microalgae

  With the above mentioned requirements for an *in situ* extraction, generated experiences were used to realize an *in situ* extraction process in pilot scale, in order to check the feasibility of such a new developed extraction system. Therefore, the batch extraction process, as well as a continuous extraction process were tested in an outdoor cultivation plant for microalgae, in cooperation with the SSC Ldt.

# 3 Materials and methods

## 3.1 Materials

### 3.1.1 Microalgae

Three different microalgae species *Chlamydomonas reinhardtii, Chlorella vulgaris, and Scenedesmus obliquus* were cultivated in 2000 ml flasks (Schott) at 25° C, illuminated with 200 μmol photons $m^{-2}$ $s^{-1}$, aerated with carbon dioxide-enriched air (4% v/v) in KC cultivation medium [Kessler, 1970]. Prior to the exposure to surfactants, cultures were grown from monoalgal seed cultures (grown from SAG strain collection cells) under mentioned conditions. Algae cultures of every strain were used with a dry mass concentration of 2 g/l.

Investigations on the recovery of the algae culture, in growth and photosynthetic activity, after the phase separation during the CPT was investigated in 1000 ml shake flasks. These were continuously aerated with carbon dioxide-enriched air (4% v/v) on an orbital shaker at 100 rpm. Microalgae cultures (including control culture) were diluted with fresh KC culture medium 1:2 and growth detected in comparison to a control, which had no surfactant contact. This was done by measuring the optical density (OD 750) at 750 nm which is linear correlated with the dry mass concentration.

### 3.1.2 Photobioreactor

For pilot-scale experiments, microalgae were grown in a photobioreactor module (EP 2228432, developed by Strategic Science Consult, SSC Ltd. Hamburg) of a size of 100 x 200 x 2 cm (width x height x depth, according volume of 40 L).

These reactors are located at the outdoor plant for the cultivation of microalgae at Hamburg-Reitbrook. For more detailed description of the cultivation system see Hindersin, (2013).

### 3.1.3 Stirred counter current extraction column

The temperature controlled extraction column used (NORMAG) has a mixing zone of 1000 mm and inner diameter of 30 mm and two storage tanks with a nominal capacity of 10 l. The mixing zone of the column consists of 32 compartments which are separated by symmetrical stators. Stirring speed of the stirrer (Heidolph) can be controlled.

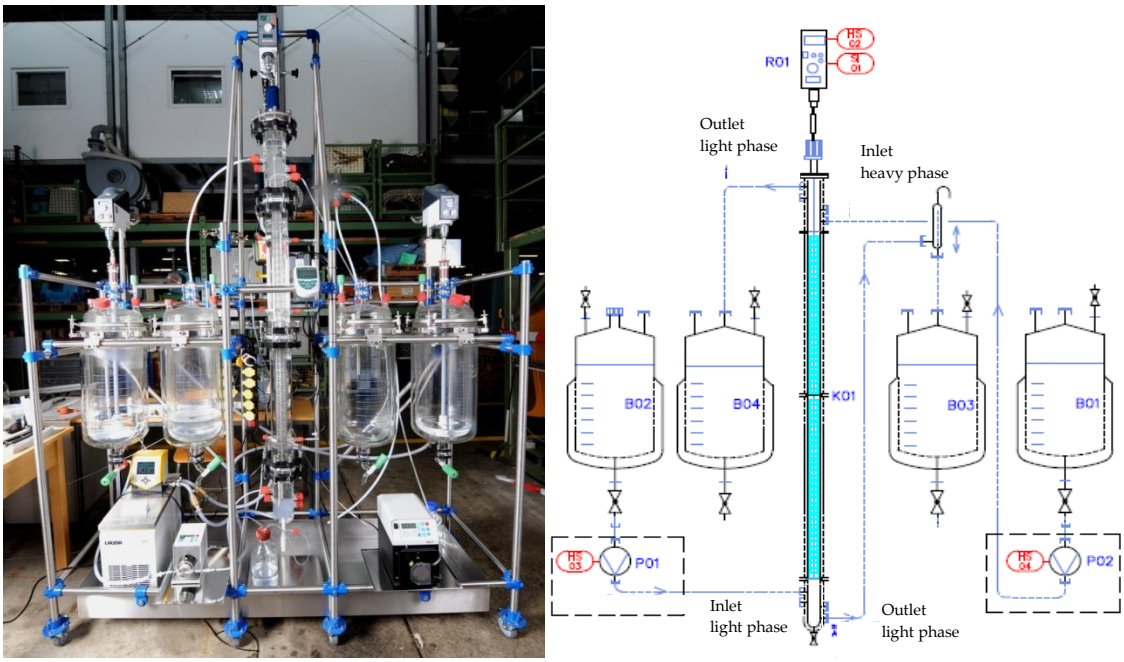



Figure 25: Picture (left) and schematic drawing of the counter current extraction column located at the institute of thermal separation processes of the TUHH.

# 3.2 Chemicals

## 3.2.1 Surfactants

Nonionic surfactants Triton X-114, Tergitol TMN 6, Tergitol 15 s 7,Ecosurf SA-7, Ecosurf EH-6, Triton DF-12, Triton DF-16, Triton CF-32 under study were selected due to their cloud point temperatures of well below 40° C (Tab. 1). Triton X-114, Tergitol 15-S-7, Triton DF-12, Triton DF-16, and Triton CF-32 are alcohol ethers of ethylene oxide. Ecosurf SA-7 and Ecosurf EH-6 are manufactured by reaction of an alcohol with ethylene oxide and propylenoxyde. An aromatic ring is present in the alcohol chain of all Triton surfactants. To investigate the biocompatibility, surfactants were added to 50 ml of *Chlamydomonas reinhardtii, Chlorella vulgaris, and Scenedesmus obliquus* –cultures to final concentrations of 1x cmc to 1 wt% (which is characteristic for each surfactant). The 1 wt% surfactant concentration was chosen as a maximum concentration at which the effects on the different algae strains could be compared. This concentration was chosen due to the fact that the provider's specifications of the cloud point temperature were in this concentration interval. Each solution was prepared directly by mixing of the nonionic surfactant with the microalgae suspension for ca. 5 minutes until getting a homogeneous solution. All experiments on surfactants biocompatibility were done at ambient temperature of about 20° C and thus, well below the cloud point temperature of all tested nonionic surfactants.

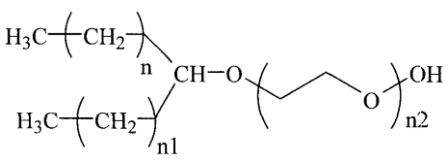
Table 5: Characteristics of surfactants.

| Surfactant | HLB [(a)] | CPT of 1 wt% water solution [°C][(a)] | CPT of 1 wt% water solution [°C][(b)] | Clouding | Stable interfacial border |
|---|---|---|---|---|---|
| Triton X-114 | 12.4 | 25 | 25.4±0.1 | + | + |
| Tergitol 15-s-7 | 12.1 | 37 | 36.1±0.5 | + | + |
| Ecosurf SA-7 | 11.0 | 37 | 41.0±0.2 | + | - |
| Ecosurf EH-6 | 10.8 | 40 | 36.8±0.3 | + | - |
| Triton DF-12 | 10.6 | 17 | 15.4±0.1 | + | - |
| Triton DF-16 | 11.6 | 36 | Proven toxic to *S. obl.*, in own observations | | |
| Triton CF-32 | 11.0 | 25 | Proven toxic to *S. obl.*, in own observations | | |

(a) Manufacturer data; (b) Experimentally determined within this work; HLB - Hydrophilic-lipophilic balance

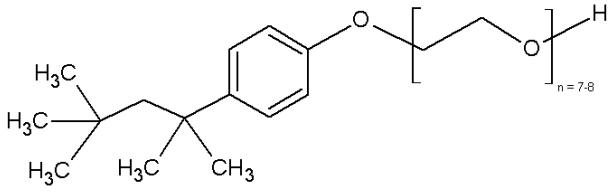
The solvent screening procedure included tests of the toxicity and of the clouding behavior in aqueous solution.

Following the available chemical structures of two surfactants under study were shown. Unfortunately, there are no exact structures of the remaining surfactants due to the corporate secrets of the manufacturers. All mentioned surfactants are belonging to the group of nonionic surfactants which is related to their head group.




Tergitol 15-S-7, n+nl= 12, n2 = 6

[Patent No.: PCT/US2006/061347]




Triton X-114 chemical structure [Sigma Aldrich datasheet]

Table 6: Surfactant properties

| Surfactant | Producer | purity | CPT [°C][(a)] | CMC [g/l] |
|---|---|---|---|---|
| Triton X-114 | SIGMA Aldrich | Lab. grade | 23 | 0.107 |
| Tergitol 15-s-7 | DOW | Lab. grade | 37 | 0.038 |
| Tergitol TMN 6 | DOW | Lab. grade | 36 | 0.800 |

(a) of 1% wt surfactant in water

# 3.3 Analytical and experimentals

## 3.3.1 Fatty acid analysis

The fatty acid concentrations were determined by gas chromatography (Gas chromatograph `7890A Agilent` with flame ionization detector (FID); capillary column: HP5, 30mX320µm X0,1µm, with 80kPa Helium inert mobile phase; temperature program: 150°C (1min), 10°C/min 325°C (20 min); injected sample volume: 1µl, Split 1:8.)

Samples of the aqueous phase and the dodecane were extracted three times with chloroform and evaporated to a volume of 1 ml in a rotary evaporator. This chloroform sample was injected directly to the GC column.

## 3.3.2 Density determination

For the evaluation of the phase separation kinetics the density of a 3 wt% Triton X-114/water solution was determined below (one phase) and above (two phases) the cloud point temperature of the Triton X-114 solution. As already mentioned, the Triton X-114/water solution splits up into a micellar-/ and an aqueous phase when reaching the cloud point temperature of 25°C.

The specific densities were detected with a flexural resonator (DMA-4500 M of `Anton Paar`). The density was determined in triplicates, each with a volume of 1.5 ml. Density measurements were carried out in the temperature range of 20-40°C.

### 3.3.3 Triton X-114 analytics

Triton X-114 concentrations in the surfactant rich and in the aqueous phases were determined using reverse phase HPLC, whereas UV detection (275 nm) was applied, `Agilent 1200` chromatograph was equipped with a `Superspher 125X4 mm RP18e` column. A flow of 0.6 ml/min, mixed isocratic with 0.25% acetic acid/methanol (20/80 vol. %), was applied.

### 3.3.4 Scanning electron microscopy

The microalgae cultures were treated with 1% paraformaldehyde and 0.25% (v/v) glutaraldehyde in phosphate buffer (PBS; pH 7.4) for 20 minutes each. The dehydrogenation was carried out by a graded ethanol series (30-100%) at room temperature. For the final critical point drying of the samples, they were rinsed five times with supercritical $CO_2$. This was done automatically in a critical point dryer located at the bio center of the University of Hamburg. After this step the samples were mounted on stubs and coated with gold. The scanning electron microscope SEM LEO used 1525 was operated at 5 kV.

### 3.3.5 Photosynthetic activity

Changes in the photosynthetic activity (PA) were used to determine possible toxic effects of the surfactants and thus their biocompatibility. The photosynthetic activity of microalgae samples was determined by Puls Amplitude Modulation (PAM) using an imaging-PAM-MAXI chlorophyllfluorometer (Heinz Walz Ltd). This method detects the effective quantum yield of the microalgae photosystem II and thus gives information about the vitality of the microalgae. An effective quantum yield below 0.2 causes irreversible cell damage.

The measurements of the photosynthetic activity were done in 5 ml subsamples in 6 well plates (Greiner Bio-one), every 10 minutes during the first hour of exposition to a surfactant and subsequently at intervals of 60 minutes for four hours. In interim times between each PAM measurement, all cultures were shaken (80 rpm) on an orbital shaker to prevent the cells from sedimentation and to increase the gas transfer rate for a better viability. All analysis were carried out in triplicates, whereas the surfactant concentration was varied from 1 to 5 wt% in steps of 1 wt%. During all experiments, photosynthetic activity was measured after 5 minutes of dark adaptation after 10, 30, 50, 70, 90 120, 150 and 220 minutes. Determined changes in the photosynthetic activity were normalized to the photosynthetic activity (PA) of an algae culture, which was not contacted with surfactant. This relative photosynthetic activity (RPA) was set to 1.

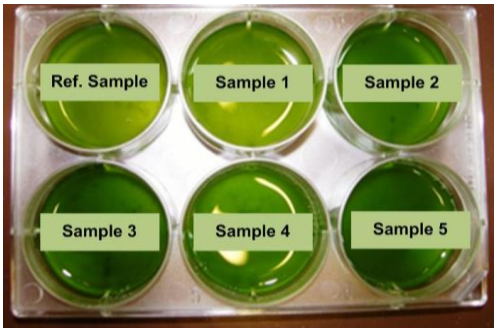



Figure 26: Six-welled plates for multiple measurements.

The maximal fluorescence is captured by the integrated camera. Data are transferred to the Imaging-WIN Software which gives out an image (fig. 27).

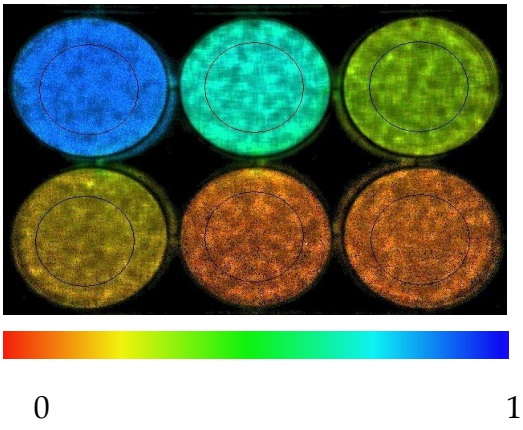



Figure 27: Typically PA of microalgae in presence of Triton X-114 at different concentrations.

Higher PA values are represented in blue and refer to good cell viability. Yellow and orange colors are used for lower values and low cell vitality, respectively. The results can also be obtained in form of an Excel sheet. The results are used by the estimation of the RPA.

### 3.3.6 Cloud point Temperature Determination

For each surfactant under study, the cloud point temperature and the time to form a stable interfacial border were determined. The experimental procedure is based on the one described by [Ingram et al., 2012] which includes cooling of the aqueous solutions of 4 wt% surfactant to 1°C in a water bath (LAUDA, D 20 KP, cooling rate 0.5°C/min). Afterwards the solution was heated with a temperature increase of 1°C $min^{-1}$. According to [Gu, 1995] the CPT was defined as the temperature at which the sample turned turbid.

### 3.3.7 Phase separation

For the investigation of the phase separation behavior in lab scale (20 ml of microalgae culture), mixtures of algae cultures with increasing concentration of surfactant (0; 0.5; 1; 2.5; 5; 7.5; 10 wt%) were prepared by mixing the surfactant (CPT of used surfactant Triton X-114: 23° C) with microalgae culture for 5 minutes. The phase separation was induced by exposing the mixtures for one hour to 30° C in a thermostatic water bath. From former studies, it can be concluded that the obtained two- phase system is nearly in equilibrium (98% of the totally equilibrated two-phase system, according to the volume ratio of micellar and aqueous phase). Experiments in pilot scale, in a 40L outdoor photobioreactor-module described below were performed at a surfactant concentration of 1wt % of Triton X-114 in algae culture. After 15 min mixing by aeration with compressed air, aeration was stopped to induce the phase separation, the culture temperature of 32°C being well above the cloud point temperature of the used surfactant Triton X-114 (23° C). After two clearly separated phases were formed, samples were collected. In order to determine the phase separation kinetic, following investigations were done in lab scale.

The kinetics of the separation of the surfactant rich phase from the aqueous phase (including the microalgae in some cases) was determined at a mass fraction of 3 wt% Triton X-114 at 30, 32, 35, 37 and 40°C. When algae were used, the solution contained algae at a concentration of 0.4 wt%, which corresponds to a cell dry weight of 1.23 g/l. During each experiment changes in the volume of the micellar phase were measured in 5 minute intervals and normalized to the maximal reached micellar phase volume. The results where plotted as phase separation kinetic curves (volume ratios of the micellar phase over time).

$$vol.\%\ of\ micellare\ phase = \frac{phase\ volume\ [mL]}{total\ volume\ [mL]} \cdot 100\ [\%]$$

Samples were examined in triplicates and were heated until T = 50°C was reached.

### 3.3.8 Calculation of partition coefficients

The partitioning coefficients of fatty acids (solutes) between micelles and water were calculated as a ratio of the activity coefficients of the solute in the aqueous bulk phase and in the micelles ($K_i = \gamma_i^{water}/\gamma_i^{micelle}$), were the micelles are considered as a `pseudophase`. The activity coefficients $\gamma_i$ in both phases were calculated for infinite dilution of the solute ($\gamma_i \rightarrow \gamma_i^{\infty}$ if $x_i \rightarrow 0$) and a temperature of 30° C. Additionally, the assumption was made that the micelles are free of water ($x^M_{iwater} = 0$).
These calculations were done with the thermodynamics model COSMO RS [Eckert, 2002; Klamt, 2001]. This model was already validated for the calculation of micelle-water partition coefficients of different organic solutes [Mehling, 2014].

The partitioning coefficient was subsequently calculated by COSMO-RS as follows:

$$P_i^{MW} = \frac{\vartheta_A}{\vartheta_M} \frac{x_i^M}{x_i^A} = K_i^M \qquad \text{(eq. 28)}$$

Where $x_i^{\phi}$ is mole fraction of a component i in an aqueous (A) or micellar (M) phase and $\vartheta$ is the phase molar volume.

For the calculation of the micelle/water partition coefficient, also the linear solvation free energy relationship could be used. The partition coefficient is correlated with solute-dependent parameters expressing its excess molar refraction (E), polarizability/ dipolarity (S), hydrogen bond acidity (A) and basicity (B) and molecular volume (V). The system specific coefficients (c, e, s, a, b, v) could be used to fit following equation [Quina et al., 1995; Abraham, 1997].

$$\log K_i^{MW} = c + eE + sS + aA + bB + vV \qquad \text{(eq. 29)}$$

For the calculations of the partition coefficients in this work COSMO-RS was used.

Experimentally determined partitioning coefficients of myristic (14:0) and palmitic acids (16:0) between the micellar and the aqueous phase of the Triton X-114/water cloud point system were compared with the predicted values using the model COSMO-RS. Within this model the `pseudo phase approach` also given by [Mehling, 2014] was used to calculate mentioned partition coefficients.

### 3.3.9 Cloud point extraction procedure and experimental determination of partition coefficients

Partitioning of fatty acids was studied according to the method described by Frankewich and Hinze [1993]. The aqueous fatty acids stock solution was prepared one week before the experiment by adding 10 mg of myristic, palmitic and stearic acid to 1 liter of deionized water and was shaken at 30°C overnight (water quench shaker, `OLS200`). Subsequently, the solution equilibrated for 6 more days due to the low water solubility of fatty acids.

After the equilibration and filtration of the solid palmitic acid residues, the stock solution was mixed with pure Triton X-114 and homogenized by shaking. In some cases the micellar phase was completely separated from the aqueous phase by centrifugation for 30 minutes at 2500 rpm and 32°C.

### 3.3.10 Cloud point extraction in pilot scale

For the implementation of the cloud point extraction of microalgae culture in pilot scale, the outdoor cultivation system, described by [Hindersin, 2013], was applied. Additionally, a bypass was installed to this system, in order to guarantee a continuous supply of fresh microalgae culture for the extraction experiments. The whole experiments were carried out in the utilities container of the microalgae cultivation plant.

A *S. obl.* culture, with a dry mass content of 3.2 g/l was mixed with Triton X-114 to a final surfactant concentration of 3% wt. Subsequently, the mixture was heated in a thermostatic water bath to 37°C in order to induce the phase separation into an aqueous and micellar phase. After separation time of 30 minutes, samples of both phases were taken for further analytics.

## 3.4 Error calculation

Multiple determinations of the samples have been conducted. The empirical standard deviation and mean values were calculated using equations 31 and 32. For the calculation of the statistical error of dependent factors, the error propagation law was applied (equation 33).

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad \text{(eq. 30)}$$

$$s_x = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \qquad \text{(eq. 31)}$$

$$\Delta y = \sqrt{\sum_{i=1}^{n} \left(\frac{\partial y}{\partial x_i}\right)^2 {\Delta x_i}^2} \qquad \text{(eq. 32)}$$

The standardized estimation function for the mean $\mu$ of a random sample from a normally distributed basic population does not follow a standardized normal distribution but a Student's *t*-distribution. The *t*-distribution depicts the distribution of the differences between the estimated mean from the random sample and the mean value of the basic population. The *t*-value can be determined as a function of a defined level of significance *P* and the sample size *n*.

The latter dictates the degrees of freedom $n$-1 in the equation. The $t$-values in the present study were calculated in Microsoft Excel using the TINV(1-$P$, $n$-1) function.

$$\mu = \bar{x} \pm t_{P,n-1}\frac{s}{\sqrt{n}} \qquad \text{(eq. 33)}$$

For the calculation of a confidence interval for the prediction of a value $y$ from a given value $x$, equation 34 was applied. Besides the $t$-value the sum of squares of deviations $S_{xx}$ and the standard error of the predicted y-value $S_{yx}$ were calculated in Microsoft Excel using the DEVSQ ($x$) and STEYX ($y$, $x$) function respectively (eq. 35). For all statistical calculations, a confidence level of 68.27% was applied, which corresponds to an interval of ± $\sigma$ around the mean.

$$y = \hat{y} \pm t_{P,n-2} \cdot S_{yx} \cdot \sqrt{1 + \frac{1}{n} + \frac{(x-\bar{x})^2}{S_{xx}}} \qquad \text{(eq. 34)}$$

# 4 Results and discussion

In this chapter the development of micellar *in situ* extraction of fatty acid from microalgae cultures is discussed. Therefore, firstly different approaches for the *in situ* recovery of hydrophobic substances from microalgae cultures are presented. This includes the feasibility of the application of fluidized particular adsorber, as well as the usage of organic solvents for *in situ* extraction.

The main results refer to the applicability of micellar systems for the *in situ* extraction of fatty acids directly from microalgae cultures. The main requirements for such an extraction system are discussed. These are: the biocompatibility of surfactants with different microalgae, the characteristics of the phase formation and the partitioning behavior of the target substances between extract and raffinate phase. Furthermore, two *in situ* cloud point extraction approaches are described, a batch and a continuous process.

## 4.1 *In situ* recovery of fatty acids - approaches

The *in situ* recovery of target substances is characterized by the fact that the cultivation of microorganism and extraction of the valuable substances are done simultaneously. This approach disclaims a cost- and energy consuming harvesting of the algae biomass. Not only the extractive process refers to an *in situ* recovery of hydrophobic substances, but also the usage of adsorptive processes is possible.

### 4.1.1 *In situ* adsorption using chromatographic resin

Having its origin in the recovery of proteins from cell containing fermentation broths, the expanded bed adsorption combines the hydrodynamic properties of a fluidized bed with the chromatographic properties of a packed bed. The main advantage in contrast to the packed bed chromatography is that cells and cell debris can pass unhindered through the fluidized adsorber bed. The feasibility of such a system for the recovery of fatty acids from microalgae cultures was tested in lab scale. Additionally, the different size of microalgae cells (10µm) and the chromatographic resin (0.5 to 1 mm) allows good separation of the adsorber particles from the microalgae culture by filtration. Also the counter current classification in an upflowing fluid is possible. Based on a literature review, the particular adsorbent Amberlite XAD 1180 a nonionic, hydrophobic, crosslinked polymer with high surface area (450 $m^2/g$) was selected. This resin adsorbs fatty acids from the culture medium of different algae cultures.

The adsorption was determined by the reduction of DOC (dissolved organic carbon) due to the fact that fatty acids belong to the DOC fraction. In addition, the decrease of the TN content (total nitrogen) was detected. The following figure shows the experimental setup, where the microalgae culture fluidized a resin bed from the bottom.

Figure 28: Tests to determine the suitability of particulate adsorbent in a fluidized bed for the recovery of fatty acids from microalgae culture medium.

The following characteristic trends of the DOC and TN content of a cell free culture medium of a *Ochromonas danica* after the use of adsorbent (Amberlite XAD 1180) for the extraction of a microalgae culture is shown.

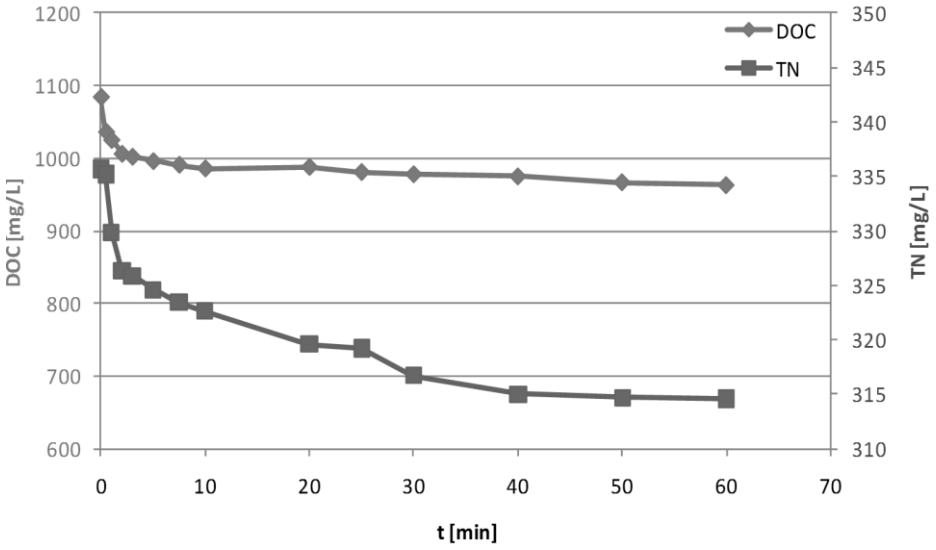



Figure 29: Decrease of dissolved organic carbon (DOC) and total nitrogen (TN) after in cooperation of adsorber resin as with cell free supernatant of *O. danica* culture as function of time.

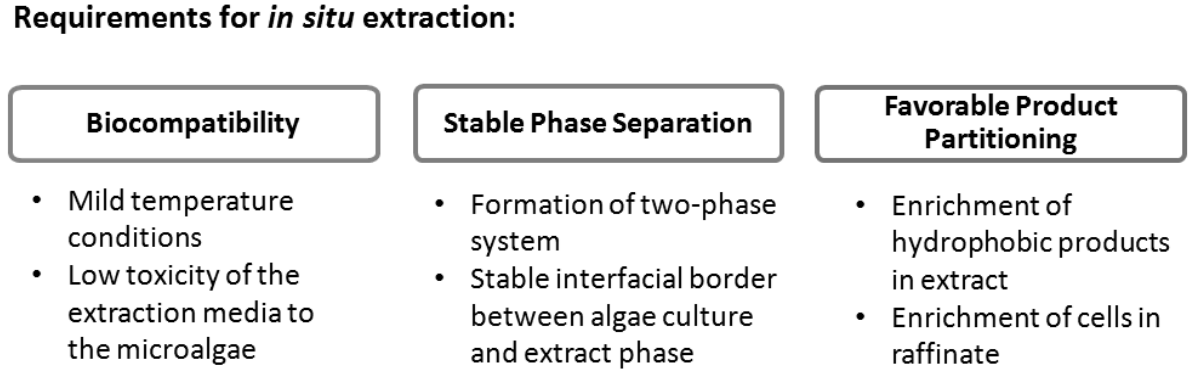
It is visible, that the application of the adsorber resin leads to a significant decrease of the dissolved organic carbon (DOC) as well as the total nitrogen (TN) of the microalgae culture especially in the first 10 minutes of application.

From this it can be concluded that also the fatty acids were adsorbed on the resin due to its (resin) hydrophobic character.

Concluding from the results showed above, the use of chromatographic resin for adsorption of fatty acids from culture medium of microalgae may be generally feasible when cell free microalgae supernatant is used. But for the application as *in situ* recovery of fatty acids from microalgae cultures (especially when applied on an outdoor cultivation plant) this method is not feasible. Furthermore, no selective desorption of fatty acids could be achieved due to unselective binding of target substances. Therefore, this method was not investigated further, due to the problems in handling of the adsorbent. Specifically flocks in the algal culture attached themselves to the adsorber and thus led to blocking or discharge of the adsorber from the particle bed.

### 4.1.2 *In situ* extraction using organic solvents

In contrast to adsorptive processes wich turned to be not feasible for the current application, extractive processes were chosen. As discussed in chapter 2, the main requirements of an *in situ* extraction with solvents (also micellar phase is considered as `solvent` here) are :



For further development of an *in situ* recovery approach, liquid-liquid extraction systems were investigated in order to prove the suitability for extraction of fatty acids direct from microalgae cultures. As mentioned in the state of the art, organic solvents can be biocompatible with microorganisms.

According to [Kleinegris, 2011], n-alkanes and various natural oils were selected to investigate their influence on the photosynthetic activity of the microalgae *S. obliquus* in order to select the most biocompatible solvent for further investigations.

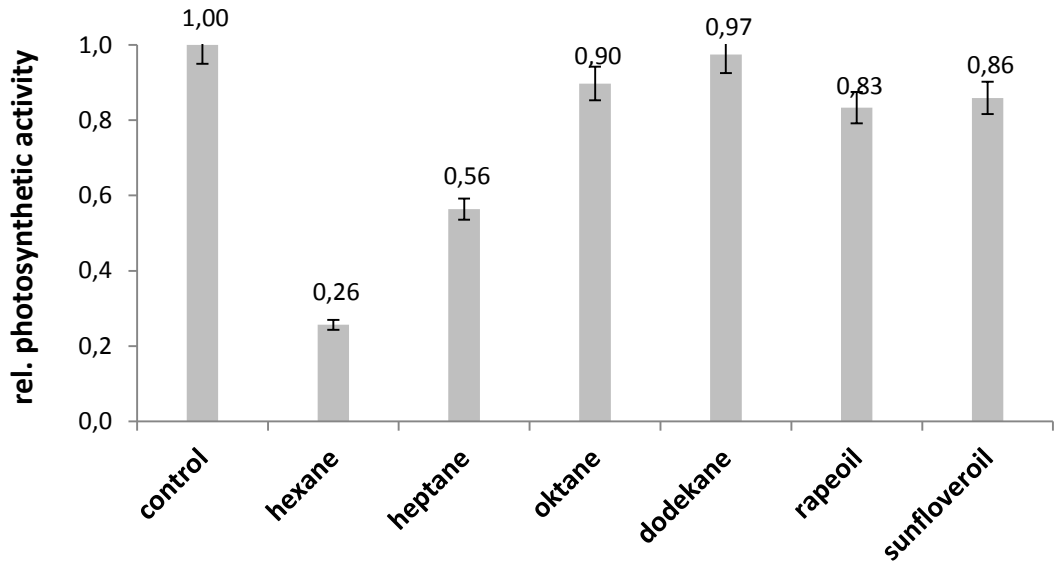



Figure 30: Relative photosynthetic activity (RPA) of *S. obliquus* after 10 minutes of contact with different organic solvents.

The alkane n-dodecane was the most biocompatible organic solvent of those under study (97% of the photosynthetic activity compared to the control). It was shown that with the increasing chain length (hexane, dodecane to C6, C12) also the biocompatibility increases (25 and 97% respectively compared to control).

This is also in accordance to the findings of Hejazi (2004 b) and Kleinegris (2011). Therefore, n-dodecane was used for further investigations.

The investigations showed (fig. 30) that the organic solvent dodecane had the highest biocompatibility with *Scenedesmus obliquus* from all the organic solvents under study. In order to confirm this for long term application, the organic solvent dodecane was added during the cultivation of the microalgae *Scenedesmus obliquus*. Figure 31 represent the growth of two algae cultures with and without the addition of 5% (v/v) dodecane. The optical density [-]of the culture at 750 nm was measured, which correlates with the growth of algae. The addition of dodecane was done after 72h. A further increase of the dodecane content would have reduced the concentration of FS in the dodecane and thus complicated the analysis.

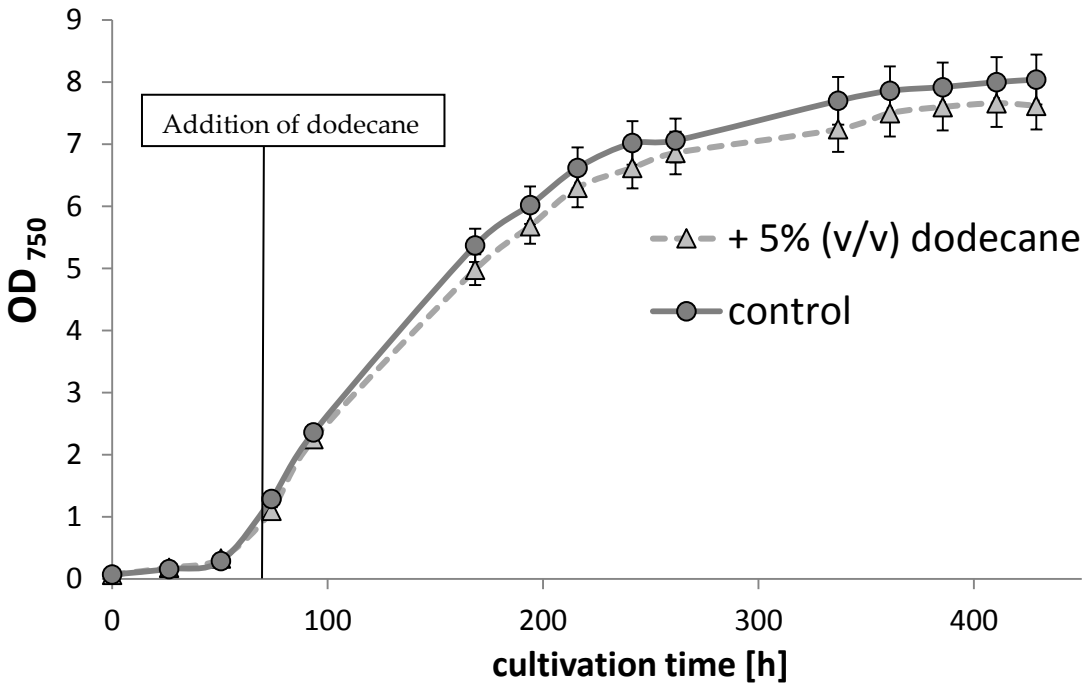



Figure 31: Growth of the microalgae *S. obliquus* with and without addition of 5% (v/v) dodecane.

From the growth curves shown in figure 31 it can be seen, that the microalgae cultivated with dodecane had only slightly lower optical density by about 4% at the end of the cultivation. This confirms again the good biocompatibility of n-dodecane with the algae strain tested.

The findings obtained in determination for biocompatibility were transferred to the pilot plant for the outdoor cultivation of microalgae in order to verify the suitability of this extraction system. For this purpose, an extraction unit with a volume of 3l (fig. 32) was integrated into the pilot plant. With this setup, the continuous extraction of fatty acids directly from microalgae culture could be studied. For these experiments a bypass of 20% (100%flow rate = 5l/min), which results in flow of microalgae culture of 1 l/min, was incorporated the extraction column at the top. The dodecane/ microalgae culture ratio was 50 to 50 (absolute 1.5l dodecane and 1.5l microalgae culture). The microalgae culture disperses upon entering the extraction unit into small droplets. This ensures a high surface area between the algae culture and the dodecane, which is favorable for mass transfer.

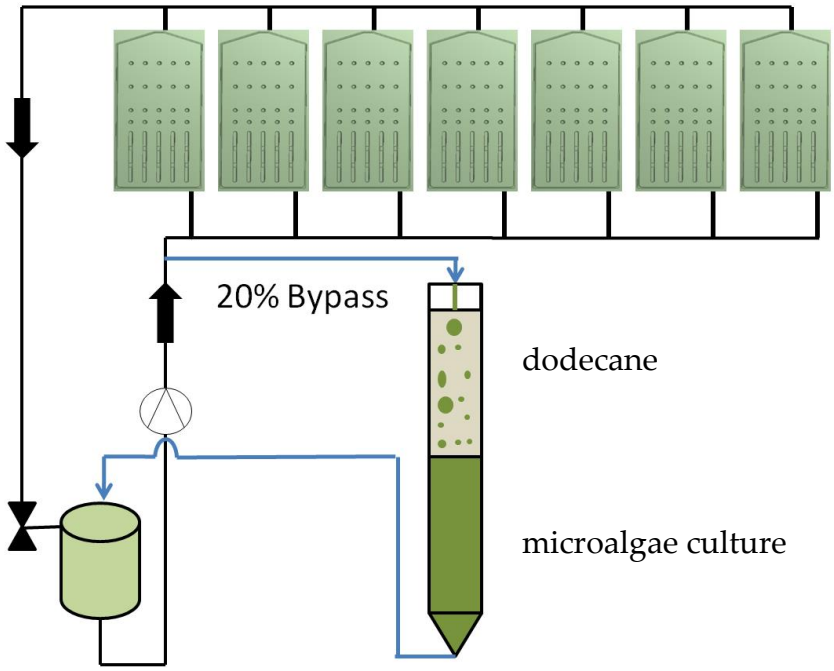



Figure 32: Flow sheet of extraction device integrated in the pilot plant for outdoor cultivation of microalgae circulated by cavity pump with 5l/min flow.

In figure 33, the fatty acid concentrations of the n-dodecane phase are shown. Obviously fatty acids accumulate in dodecane phase. The content of the saturated fatty acids (10:0 to 18:0) is at the level of 30 micrograms/ml dodecane. The polyunsaturated fatty acids like the omega 6 fatty acid 20:2 (n-6), was detected in a much higher concentration of 250 mg/l (fig. 33).

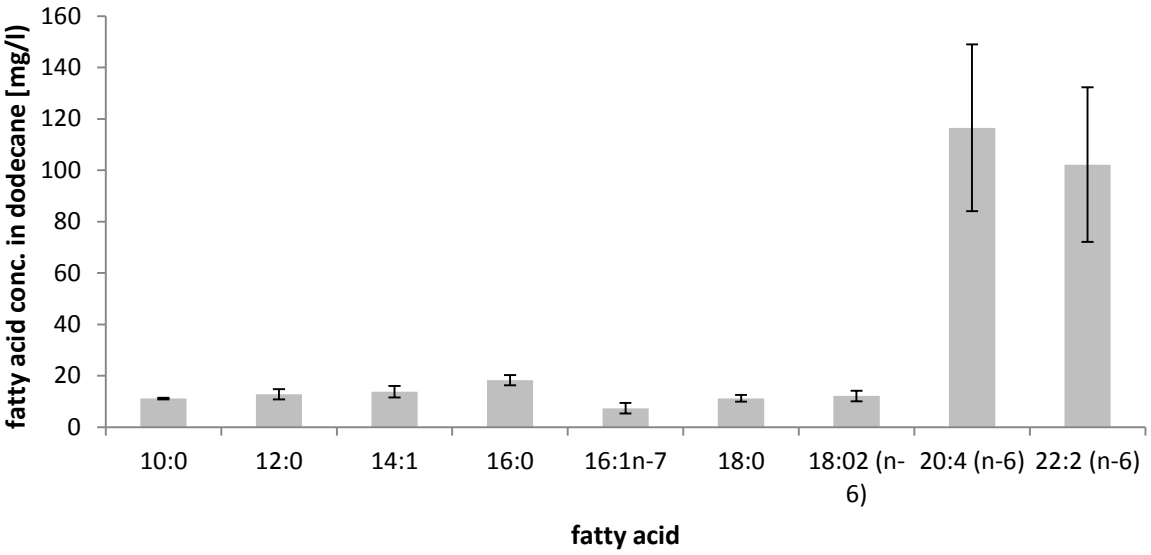



Figure 33: Fatty acid concentration in dodecane after 7 days extraction of *Scenedesmus obliquus* culture in outdoor pilot plant.

To prove if the extraction of fatty acids is feasible, samples were taken at the beginning of the extraction and after 7 days. The DOC (dissolved organic carbon) as an indicator for the extraction of fatty acids from the microalgae culture could be reduced within a week by 35% (fig. 34).

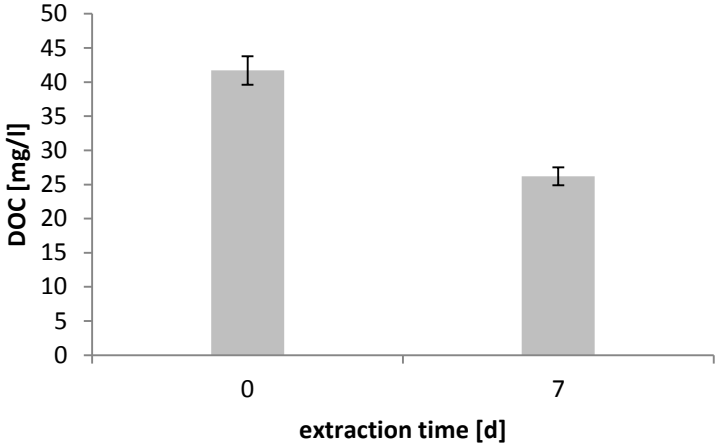



Figure 34: Dissolved organic carbon (DOC) of the microalgae culture before and after the extraction with dodecane.

These results indicate that organic substances (including fatty acids) can be removed from the microalgae culture using the developed extraction unit.

It was observed that the dodecane extraction, depends on the composition of microalgae culture. There was a massive emulsification of dodecane visible, especially when the microalgae *Chlorella vulgaris* dominated the mixed microalgae culture. This phenomenon prevented the two phases (dodecane/ aqueous microalgae culture) from separation in the extraction apparatus. A stable emulsion was formed and thus the dodecane was completely washed out. Particularly when *S. obliquus* dominated the mixed culture, the two phases separated without formation of an emulsion. This different behavior of emulsion formation between the two phases is illustrated in figure 35.

Figure 35: Liquid-liquid extraction of algae culture with dodecane; proper phase separation (left) and emulsification (right).

For further investigations of this phenomenon, phase separation of microalgae cultures of *Scenedesmus obliquus* and *Chlorella vulgaris* with dodecane were studied in lab scale.

Therefore, 20 ml culture of algae in various proportions with water (pure culture; 1:2; 1:5, 1 par microalgae culture to 5 parts water) were added to 10 ml dodecane and mixed for 5 min by vortexing to ensure high dispersion. Thereafter, the samples were stored for 10 minutes to allow phase separation.

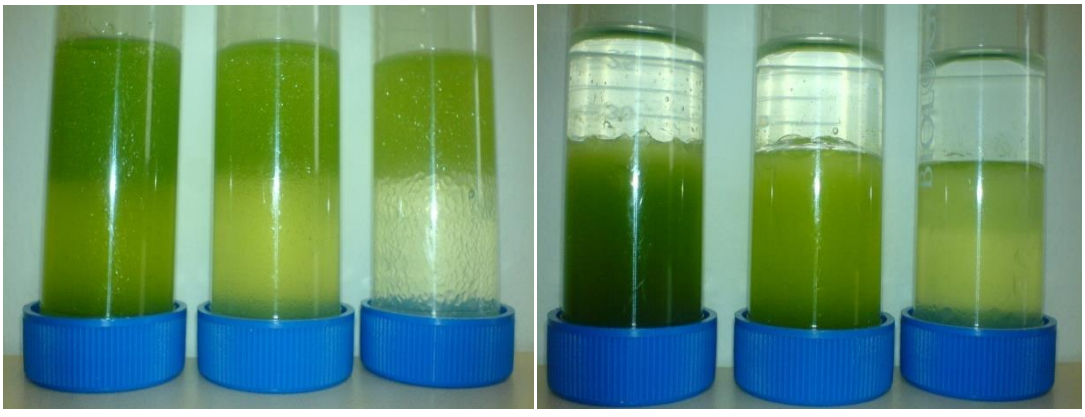


Figure 36: *Chlorella vulgaris* culture (left) and *Scenedesmus obliquus* culture (right) in 3 different dilutions (1:1; 1:2; 1:5) after 5 min shacking and 10 min of phase separation.

A strong emulsification of the sample including dodecane and *Chlorella vulgaris* culture (left) was also obtained, whereas the samples of *Scenedesmus obliquus* culture and dodecane showed a complete phase separation after 10 minutes (upper phase: dodecane, lower phase microalgae culture medium) .

The mechanism of enhanced emulsification in extraction of *Chlorella vulgaris* cultures with dodecane has not yet been clarified. The emulsification during operation of the dodecane extraction on the pilot plant can be explained with the fact that outdoor cultivation of microalgae usually is not under axenic conditions, which leads to the formation of mixed microalgae cultures (esp. *Scenedesmus obliquus* and *Chlorella vulgaris*). Furthermore, the structural differences of the cell wall may lead to different surface charges which can promote emulsification in cases of *C. vulgaris* [Hadjoudja, 2010].

Due to these problems with the formation of stable emulsions of the solvent and the microalgae culture, this particular *in situ* extraction system was no longer studied.

## 4.2 Biocompatibility of nonionic surfactants to microalgae

Due to the fact that both tested *in situ* removal approaches (adsorption on chromatographic resin as well as the extraction with dodecane) turned to be not suitable for the extraction of hydrophobic substances directly from microalgae cultures, a third approach was investigated: surfactant based extraction systems. Wang (2007) have shown that such surfactant based extraction systems are already in application for whole cell extraction of bacteria (see State of the art, 2.10.4). However, the application of surfactant systems for the extraction of hydrophobic substances from microalgae cultures was not reported yet.

The main requirement for such an *in situ* extraction process is the biocompatibility of the used surfactant. In order to select a suitable surfactant for this process the toxicity of commonly used surfactants towards algae has to be tested. The choice of the nonionic surfactants was done based on their cloud point temperature. The microalgae strain *S. obl.,* which was used in this study, does not tolerate temperatures above 40°C as already mentioned in the introduction. If the cells are cultivated at higher temperatures, growth inhibition and cell protein denaturation were observed [Lee, 2008]. Hence, the main criterion for the primary surfactant selection was the cloud point temperature of the corresponding surfactant solution which should be below 40°C.

The influence of nonionic surfactants on three different algae strains were investigated. Generally, all possible combinations of algae and surfactants were investigated, however only the significant results are shown.

Microalgae strains show very different cell viability after the contact with surfactants. It could be observed, that the strain *Chlamydomonas reinhardtii* showed the lowest relative photosynthetic activity (RPA) (no photosynthetic activity (PA) detectable after 120 min, fig. 37) after contact with all tested surfactants, whereas *Scenedesmus obliquus* showed the highest one (RPA > 0.9, after 240 min, Fig 41). When comparing the surfactants among each other under the same concentrations, their biocompatibility decreases in the sequence Tergitol 15 s 7 > Triton X-114 > Tergitol TMN 6.

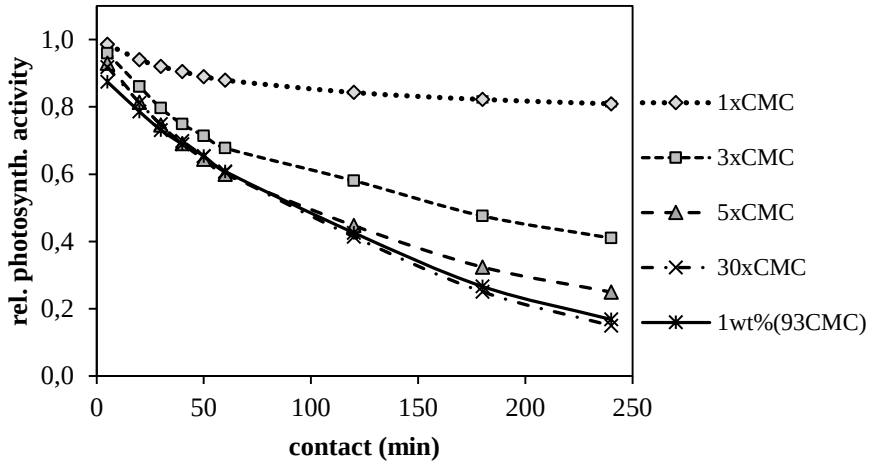



Figure 37: Influence of Triton X-114 on RPA of *C. vulgaris* as a function of time and surfactant concentration.

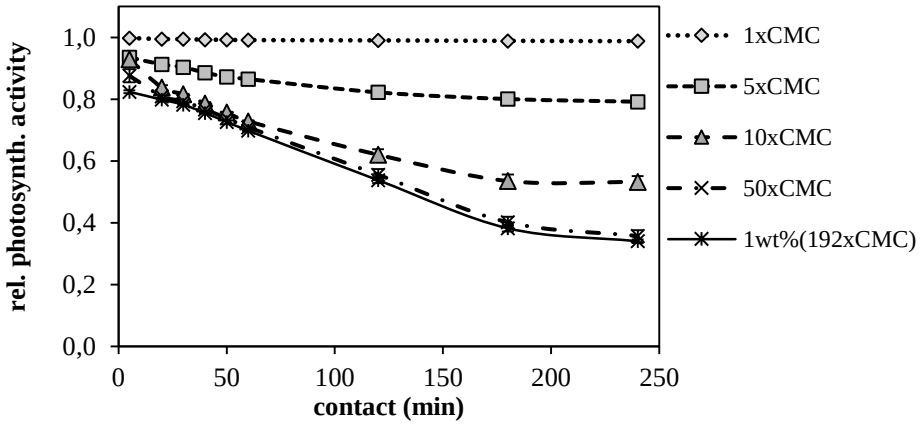



Figure 38: Influence of Tergitol 15 s 7 on RPA of *C. vulgaris* as a function of time and surfactant concentration.

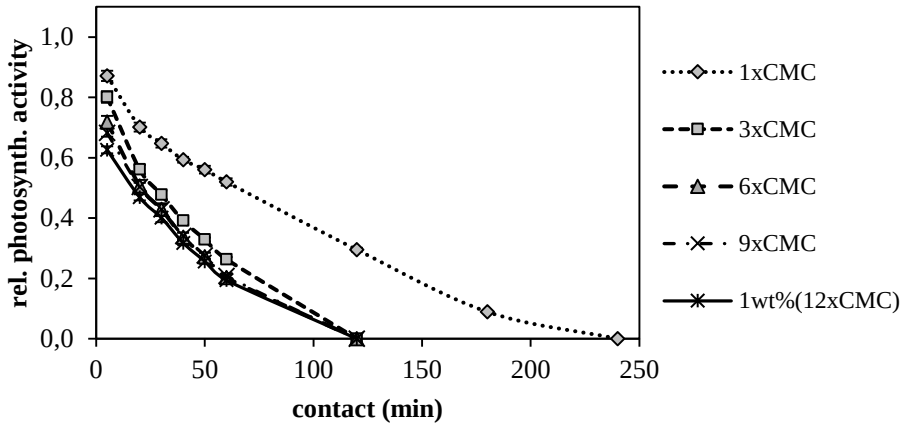



Figure 39: Influence of Tergitol TMN6 on RPA of *C. vulgaris* as a function of time and surfactant concentration.

In figures 37-39 the relative photosynthetic activity of *Chlorella vulgaris* upon contact with the three selected surfactants (Triton X-114, Tergitol 15 s 7, Tergitol TMN 6) is shown. The time shift of 5 minutes in RPA measurements is caused by the time for mixing the microalgae culture with the surfactant as described in the methods chapter. A comparison of the RPA values (1 wt% samples after 180 minutes) shows a RPA of 0.3 for Triton X-114, RPA of 0.4 for Tergitol 15 s 7 and a RPA of 0.1 for Tergitol TMN 6.

The increase of the surfactant concentration leads to a decrease of the RPA values (50% decrease after 120 minutes of contact), at surfactant concentration of 1 wt%, no PA could be detected any more (fig. 39). Overall, it can be concluded that the maximal tolerable concentration of all tested surfactants for *C. vulgaris* is below 1 wt%. The biocompatibility decreases in the order Tergitol 15 s 7 > Triton X-114 > Tergitol TMN 6 (comparison of RPA after 240 min of contact).

The alga *C. reinhardtii* shows even less tolerance towards surfactants (fig. 40). Although Tergitol 15 s 7 showed the best biocompatibility to *C. vulgaris* (compared with other tested surfactants), *c. reinhardtii* culture showed no cell viability after 120 minutes at the latest, even at the lowest surfactant concentration of 1 x cmc.

The contact with Triton X-114 and Tergitol TMN 6 caused cell death of this culture after 10 minutes when contacted with the concentration of 1xcmc (data not shown).

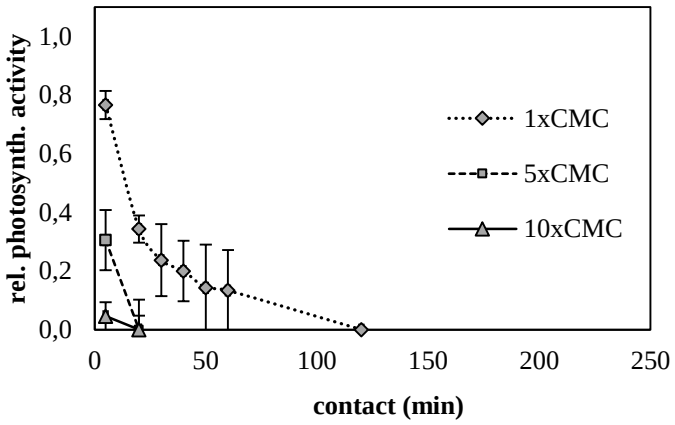



Figure 40: Influence of Tergitol 15 s 7 on RPA of *C. reinhardtii* as a function of time and surfactant concentration.

In contrary, the microalgae strain *S. obliquus* shows a remarkably high tolerance towards all surfactant types. Figure 41 illustrates the effect of all tested surfactants on this strain (at surfactant concentrations of 1 wt%).

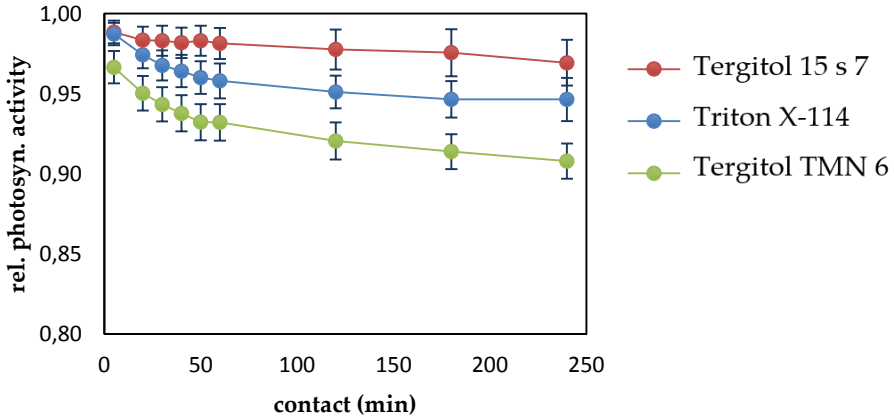



Figure 41: Influence of all tested surfactants on RPA of *S. obliquus* as a function of time. The concentration of each surfactant was 1 wt%.

The biocompatibility of the tested surfactants follows the same trend as observed for the other algae strains: Tergitol 15 s 7 > Triton X-114 > Tergitol TMN 6.

Important to mention is that generally the viability of *S. obliquus* cells after contact with tested surfactants is above 90% (in case of Tergitol TMN 6) compared to the control sample (which had no contact with surfactant).

Thus, the highest biocompatibility to all tested surfactants was observed in case of *S. obliquus*. An explanation for this behavior could be that *S. obliquus* is able to form colonies of 4 to 8 single cells, which stick to each other until further cell division [van Hoeck, 1993]. Hence, the specific surface area of each *S. obliquus* cell exposed to the surrounding medium and therefore the contact area to nonionic surfactant containing growing medium is also reduced. The surfactant might have less effect on the cell growth, in comparison to the single cells of *C. vulgaris* and *C. reinhardtii*.

Besides the nonionic surfactants Triton X-114 and Tergitol 15-s-7, which have shown a high biocompatibility in above mentioned figures, nonionic surfactants from the Ecosurf series (Ecosurf SA-7, Ecosurf EA-6) as well as the low foaming surfactants Triton DF-12, Triton DF-16 and Triton CF-32 were investigated for their biocompatibility.

The influence of the surfactants solutions with different concentrations (1, 2, 3, 4 and 5 wt%) on the RPA (relative photosynthetic activity) of *S. obl.* were studied over a period of 4 hours and illustrate the biocompatibility of the surfactants (fig. 42). Most of the surfactants under study showed a high biocompatibility and RPA remained constant during the whole exposure time. Only Triton DF-16 and CF-32 caused a decrease of the RPA with increasing contact time (fig. 43). Additionally, the interaction intensity increased with raising surfactant concentrations.

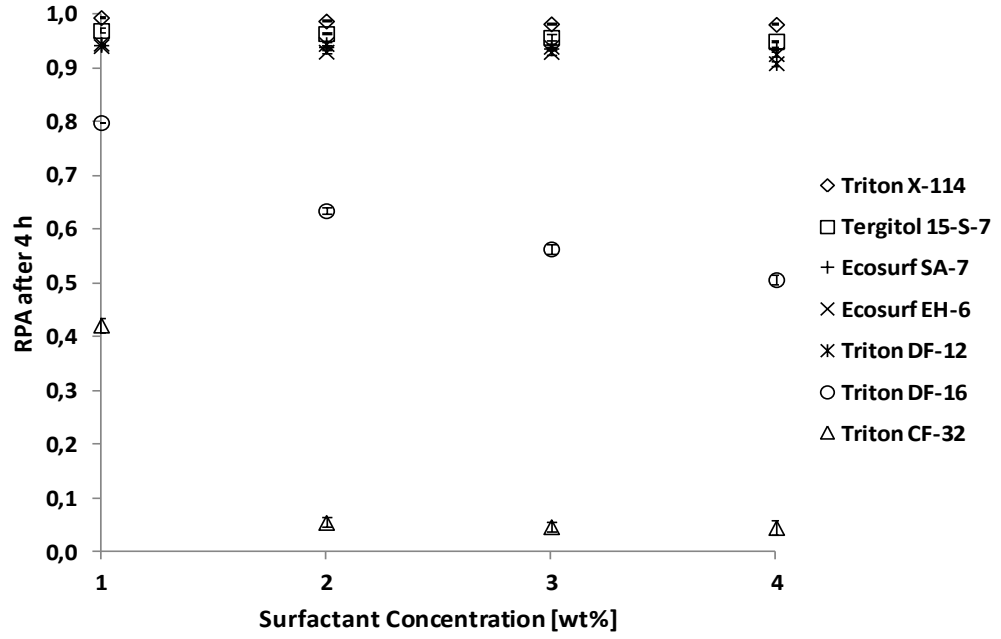



Figure 42: Influence of surfactants on the RPA of microalgae *S. obl.* after 4 hours contact as a function of the surfactant concentration.

The RPA generally decreases with increasing surfactant concentration. Whereas Triton X-114, Tergitol 15-s-7, Ecosurf SA 7, Ecosurf EH-6 and Triton DF-12 show the final RPA (after 4h of exposure) equal to 90% of the reference sample, Triton DF – 16 and Triton CF-32 lead to significant cell vitality decrease (fig. 43).

At higher surfactant concentrations the decrease of RPA with time was even more pronounced (fig. 43). It can be concluded that Triton DF-16 and Triton CF-32 in the observed concentration interval are not biocompatible with the investigated microalgae.

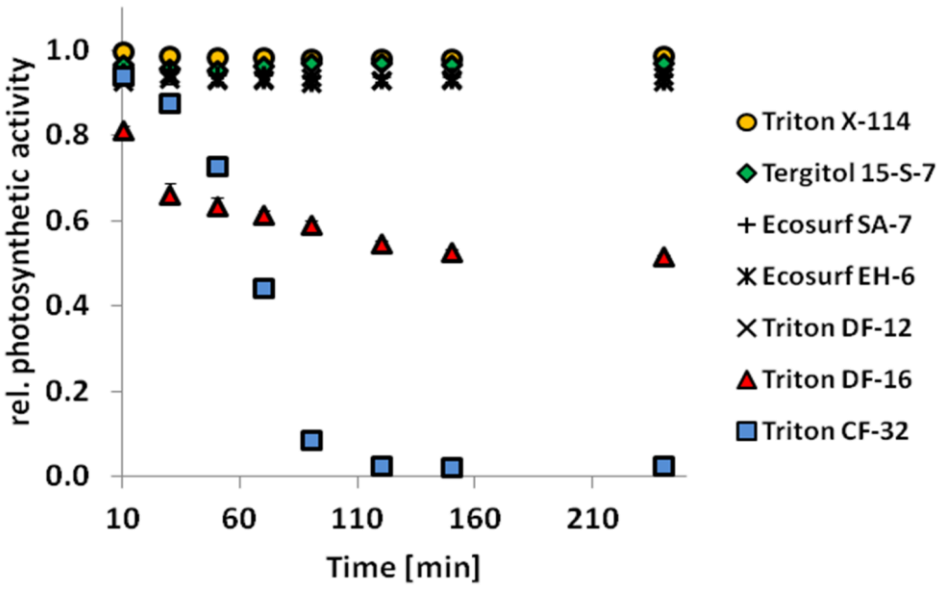



Figure 43: Influence of surfactants on the RPA of microalgae *S. obl*. as a function of contact time. Surfactant concentration in the solution, 5 wt%.

No significant decrease of RPA in case of Triton X-114, Ecosurf SA- 7, Ecosurf EH-6, Triton DF-12 and Tergitol 15-s-7 was observed neither at 5 wt%, nor at any other surfactant concentration studied. Obviously, these surfactants are not toxic to *S. obl.* within the contact time studied.

Data shown on figure 44 were obtained for a constant concentration of the surfactants from the Triton-series (5 wt%) after 4 days of contact with microalgae. Tergitol 15-S-7, Ecosurf SA-7 and Ecosurf EH-6 do not belong to the Triton series and therefore were not added to figure 44.

As discussed in the introduction, $\log P_{ow}$ values might be a criterion for the toxicity of the solvent toward microalgae. Plotting the logarithmic values of each surfactant's CMC and the RPA versus the corresponding $\log P_{ow}$ values (which were selected from manufactures data), the interrelated toxicity effect can be discussed (fig. 44).

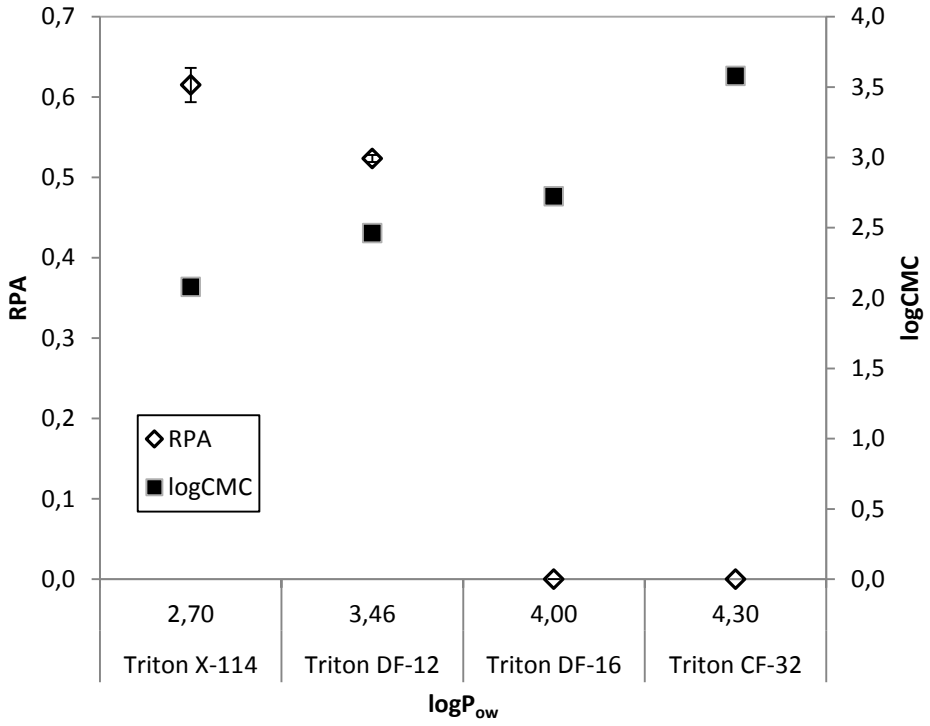



Figure 44: RPA after 4 days at 5%wt surfactant conc. and logCMC as a function of $logP_{ow}$ of Triton surfactants.

Data shown on figure 44 were obtained from the Triton-series surfactants (5 wt%) after 4 days of contact with microalgae. Tergitol 15-S-7, Ecosurf SA-7 and Ecosurf EH-6 do not belong to the Triton series and therefore were not added to the figure 44.

Surfactants with higher CMCs appear to be more toxic to microalgae. This may be induced by the higher ratio of surfactant monomers (in contrast to monomers which have already formed micellar aggregates with a hydrophilic shell) at the same surfactant concentration. On the one hand, more hydrophobic interactions between the surfactant monomers and the cell walls of the microorganisms are induced [Masakorala, 2011]. Thus, cell lyses can take place as shown after contact with Triton DF-16 and Triton CF-32 (RPA after 4 days = 0). On the other hand the HLB values of Triton DF-12, Triton DF-16 and Triton DF-32 are lower than the HLB of Triton X-114. Therefore, micelles of the first three surfactants are more hydrophobic.

It can be assumed that Triton DF-12, Triton DF-16 and Triton DF-32 interact more severely with the outer surface of algae.

Taking into account the $P_{ow}$ value, it has already been confirmed in other studies, that surfactant with $\log P_{ow} \geq 2$ can be considered as nontoxic to bacteria [Wang, 2007].

However, green algae are more sensitive than bacteria [Kleinegris, 2011 b]. *S. obl.* shows constant photosynthetic activities when contacted with surfactants with $\log P_{ow}$ values of 2 and 3. After the contact with solvents having $P_{ow}$ values of 4, the photosynthetic activity of *S. obl.* drops significantly among the studied series. Triton DF-16 and Triton CF-32 showed the highest $\log P_{ow}$ and CMC value. Therefore, the toxicity of the surfactants was intolerably high, thus Triton DF-16 and Triton CF-32 were not used for further studies.

### 4.2.1 Photosynthetic activity at phase separation conditions

The biocompatibility of nonionic surfactants was investigated in the previous chapter, focused on the biocompatibility under cultivation conditions (25°C). For the application of a nonionic surfactant based extraction process, the temperature induced phase separation plays a key role. Important to mention here is the fact that the higher the temperature of the surfactant containing microalgae culture is, in comparison to the surfactants cloud point temperature, the higher the density difference between the micellar and the aqueous phase is. Therefore, the influence of the temperature (25°C up to 37°C) on the photosynthetic activity was investigated.

The relative photosynthetic activity (RPA) during the phase separation of an algae culture containing 3%wt Triton X-114 at 37°C was determined every 5 minutes, in order to prove the effects on the microalgae during a separation process (fig. 45).

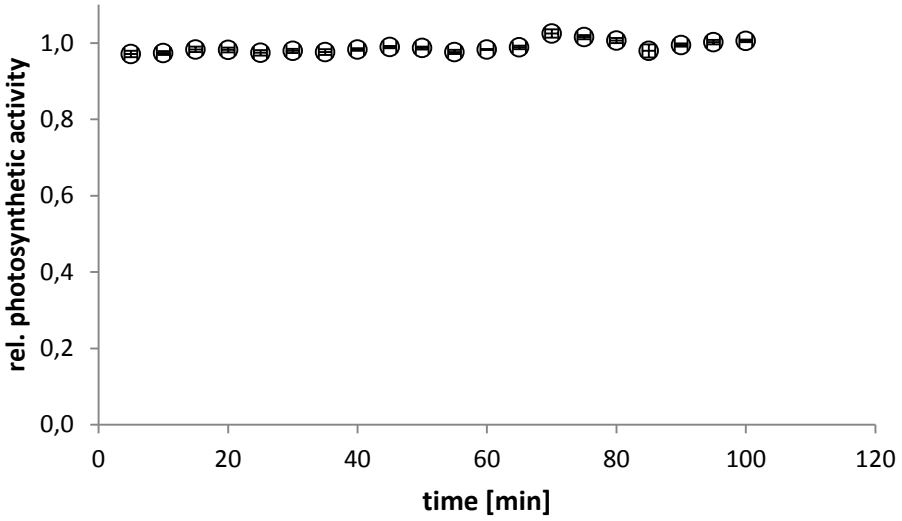



Figure 45: Photosynthetic activity of photosystem II during phase separation of a 3 %wt Triton X-114 algae containing solution (*Scenedesmus obliquus*) during the phase separation process at 37°C.

As shown on figure 45, there is no significant decrease of the relative photosynthetic activity of S. *obl.* during exposure to Triton X-114 of up to 100 minutes. This time was chosen in agreement with the slowest phase separation time of the cloud point system containing 5%wt TX-114, (at 30°C) which is 60 minutes (results presented in chapter 4.3.2.). This result confirms the previous observations, that the surfactant Triton X-114 shows a high biocompatibility with the microalgae *Scenedesmus obliquus* [Glembin, 2013].

**Cloud-point Determination and Phase Stability**

For the application of the *in situ* cloud point extraction, the exact cloud point temperatures as well as the stability of the phases formed in a two phase system are important. Therefore, the cloud point temperatures were measured for all surfactants under study. However, the experimentally determined cloud point temperatures were slightly different from the manufacturers' data. Furthermore, the formation of a stable interfacial border between the two phases did not occur with all surfactants under study.

The surfactant solutions of Ecosurf SA-7, Ecosurf EH-6 and Triton DF-12 remained turbid during the whole experiment until a temperature of 60°C was reached. Due to the fact that a temperature above 40°C is not tolerable for the used microalgae, these surfactants were excluded from further studies. The micellar system containing Tergitol 15-s-7 showed a micellar phase with a lower density than the aqueous phase (resulting in a micellar top-phase). However, the phase separation took extremely long time and formed a clear interfacial phase boundary just above a temperature of 45°C. Hence, this surfactant was not applicable, under tested setup, for a CPE.

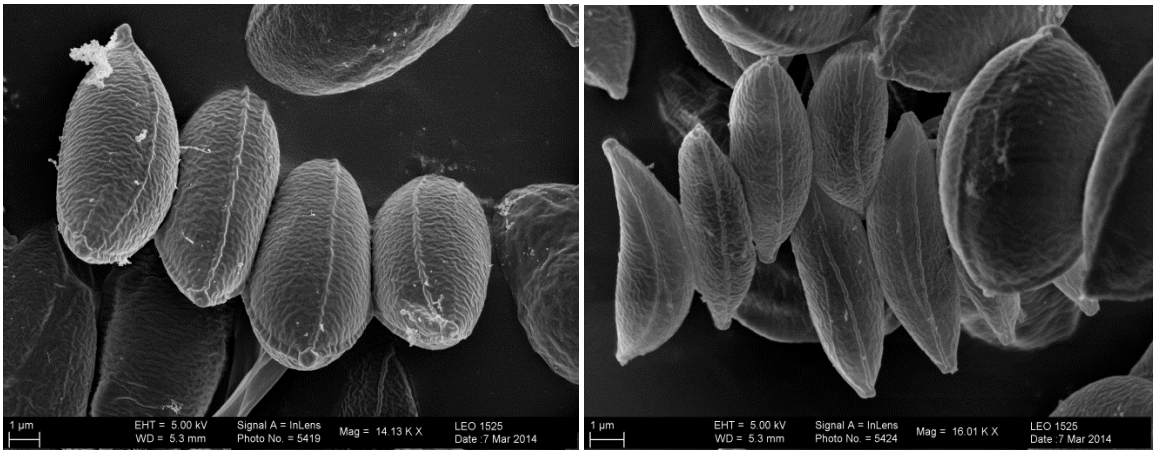
Only Triton X-114 demonstrated stable and rapid phase separation characteristics at temperatures below 40°C. Summarizing all results, it can be concluded that Triton X-114 is the most appropriate surfactant for cloud point extraction of fatty acids from the microalgae *S. obl.*, among the tested surfactants.

### 4.2.2 SEM observation

Previous investigations on the biocompatibility of surfactants to microalgae show, that there are significant differences in the resulting photosynthetic activity.

In order to investigate the interaction of surfactant molecules or micellar aggregates with the microalgae cells of the investigated species *Scenedesmus obliquus*, a scanning electron microscopy was used.

A




B

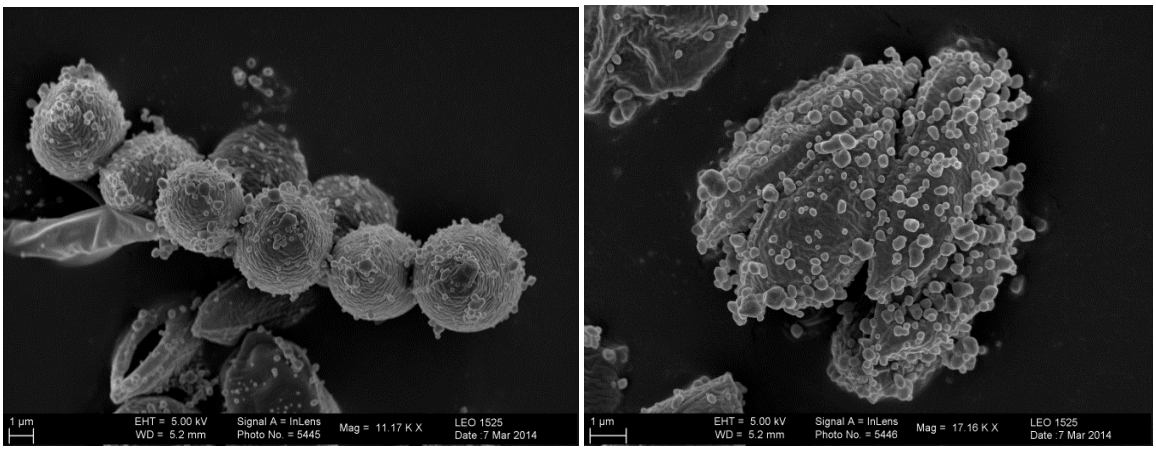



Figure 46: SEM picture of *s. obliquus* control (A), and after cloud point extraction with 3%wt Triton X-114 (B).

Typically for *Scenedesmus* species, cell compounds of four single cells can be identified at a magnification of 10.000. The single cell size of 8 to 10 µm is in accordance with the literature [Linne von Berg, 2005]. It is visible that there are no specific structures on the cell surface of the microalgae in control samples.

After CPE there are obvious changes in the outer appearance of the algae cells (fig 46B. Particle like structures with a size of 0.1 to 0.5 µm are attached to the cells surface. It could be the molecules of the surfactant Triton X-114 or micelles which attached to the surface.

But it may also be substances being released from the algae itself. Unfortunately there are no evidences in literature concerning these findings (It has to be mentioned here that the samples for the SEM are dried with supercritical $CO_2$ in order to maintain the structure of the samples, and prevent shrinking).

In order to investigate this attachment, zeta potential measurements were performed. Generally, the aggregation of cells or particles can be explained with the different zeta potentials. In the following, zeta potential of the microalgae culture *Scenedesmus obliquus* was investigated in order to identify changes after addition of the nonionic surfactant Triton X-114.

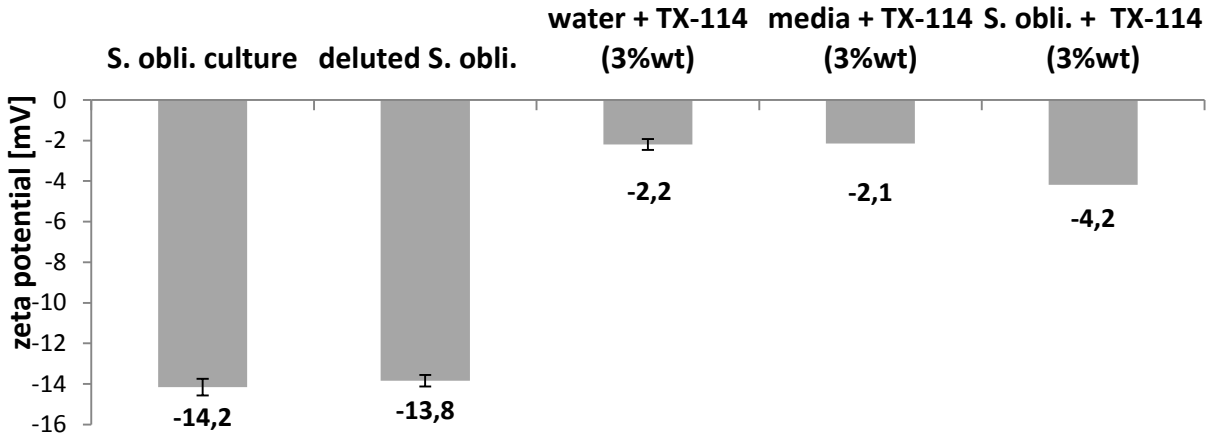



Figure 47: Zeta potential of the algae surface of different microalgae cultures with and without nonionic surfactant Triton X-114.

The zeta potential of the untreated control samples of both microalgae is -14 mV, which is in accordance to the work of [Bhattacharya, 2010].

A 3%wt aqueous surfactant solution had a zeta potential of -2 mV. The addition of Triton X-114 to the microalgae culture causes a sharp increase of the zeta potential from -14 to -4 mV. This increase of the zeta potential leads to promoted flocculation or adhesion of the cells due to the fact that their electrostatic repulsion is decreased [Liu, 2013].This may support the hypothesis that the changes on the surface of the algae cell which is visible in the SEM pictures is caused by the addition of Triton X-114 which forms aggregates at the cells surface.

### 4.2.3 Long term biocompatibility of surfactant to different microalgae

Additionally to the biocompatibility measured directly after and while the phase separation procedure (fig 51), the photosynthetic activity of the surfactant- exposed microalgae (in RPA and cell growth) is important for potential CPE. Furthermore, it is important to mention that the phase separation results in a very high concentrated surfactant phase.

To prove this, the regeneration of *C. vulgaris* and *S. obliquus* after the phase separation (CPE with 1% wt Triton X-114) was investigated. The detection of the microalgae cell growth is an often used parameter to exclude enduring damage of the cells.

As can be seen from figures 48 and 49, both microalgae strains show a completely different regeneration after the cloud point extraction with Triton X-114. RPA of *C. vulgaris* culture drops to 0 (at day 4) and also the optical density (OD 750), which is correlated with the cell number decreases, which indicates the cells death. Both values are shown in comparison to the control (fig. 48).

In contrast, the cell growth of *S. obliquus* (fig. 49) remained unaffected after exposure to Triton X-114 and RPA recovered from 82% (start) to 100% (after 10 days). This finding also confirms that the surfactant Triton X-114 is the most compatible with the microalgae strain *S. obliquus* under study. It can be associated with the particular cell wall of this strain.

In comparison to *C. vulgaris* and *S. obliquus* which both have a thick cell wall consisting of polysaccharides, the cell wall of *C. reinhardtii* is very light and consists mainly of glycoproteins [Lee, 2008].

Additionally *C. reinhardtii* is very sensitive to the loss of its flagella, under osmotic pressure changes caused by contact to surfactant containing growth medium [Hoeck, 1993].

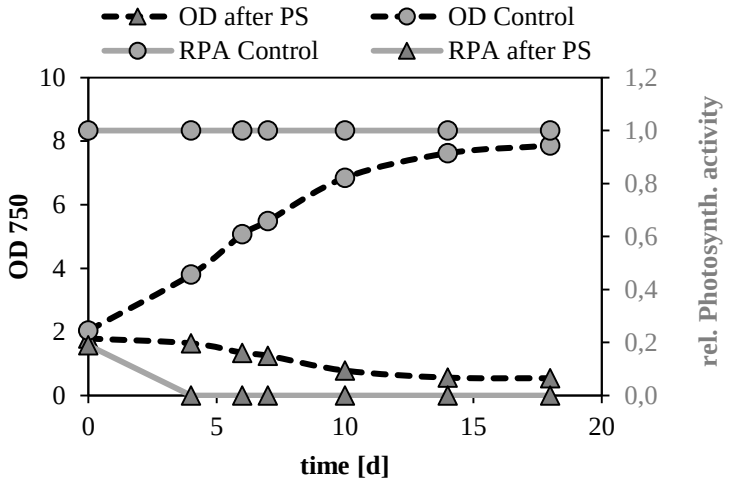



Figure 48: Growth parameter OD 750 (left axis) and RPA (right axis) of *C. vulgaris* culture after accomplished cloud point extraction with Triton X-114 as a function of cultivation time.

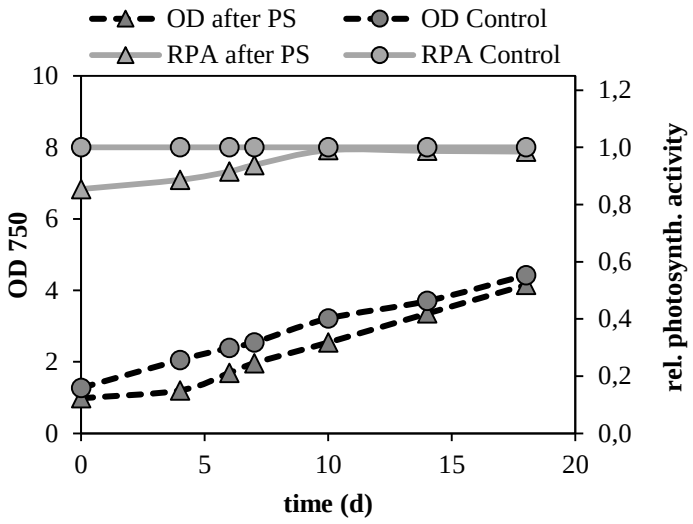



Figure 49: Growth parameter OD 750 (left axis) and RPA (right axis) of *S. obliquus* culture after accomplished phase separation (PS) as a function of cultivation time.

The regeneration and growth behavior after the phase separation of algae culture upon contact with Triton X-114 underlines the important differences in biocompatibility of *C. vulgaris* and *S. obliquus*.

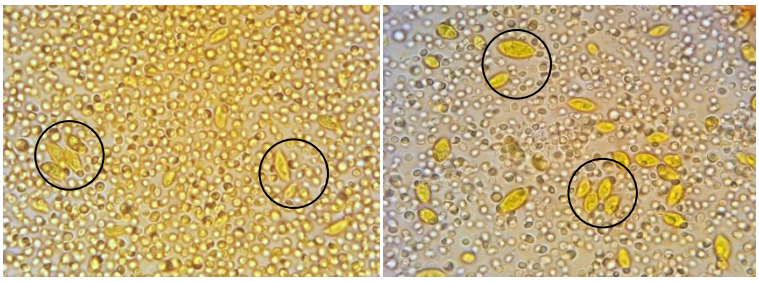


Figure 50: Mixed culture of *Scenedesmus obliquus* (circle) und *Chlorella vulgaris*, before (left) and after (right) cloud point extraction with Triton X-114.

Also light microscopy investigations show that the microalgae strain *S. obliquus* is more robust against Triton X-114 in contrast to *C. vulgaris*. In figure 50 a mixed culture of both microalgae strains are shown before (left) and after (right) the cloud point extraction with Triton X-114. After the extraction the cells of *C. vulgaris* completely lose their chlorophyll which results in bleached cells. These cells cannot carry out photosynthesis any more and therefore are lethally damaged.

It may be a disadvantage which excludes the microalgae strain *Chlorella vulgaris* to be suitable for the Triton X-114 based extraction system. But on the other hand it may be an advantage in order to improve the outdoor cultivation of microalgae. These differences may be used for the control of the growth of a certain algae strain by adding the specific surfactant to the algae culture. This would enable the monoalgal culturing in outdoor cultivation of microalgae since the growth of the less surfactant-tolerant microalgae could be suppressed. Monoalgal culturing is desired, because the target product is produced by only one algae strain. Therefore, a higher productivity (all nutrients are available for just one algae strain) and purity of a product may be achieved. This feature can be another advantage when using surfactants instead of common organic solvents for the *in situ* extraction.

## 4.3 Phase separation behavior

In contrast to two phase solvent extraction systems, in surfactant systems the density differences between micellar and aqueous phase is much lower (0.1, compared to 0.4 for hexane water). Therefore, the separation of the both phases becomes more challenging in the application of those processes. Due to the fact that these investigations have not be done yet for micellar extraction systems, the phase separation (PS) behavior of the used system, especially in terms of its kinetics were discussed in the following chapter. Phase separation was investigated in lab as well as in the pilot scale.

### 4.3.1 Phase separation in lab scale

The phase separation of the micellar and the aqueous phase was investigated for Triton X-114/algae mixtures. Triton X-114 was chosen as the most suitable surfactant under study which was shown in the chapter 4.2.. Additionally, its low cloud point temperature (23° C) prevents the cells from high-temperature-stress (see mentioned max. tolerable temp. of microalgae of 40° C). The temperature difference between the cloud point temperature and the temperature where the phase separation takes place is a key parameter for the phase separation of micellar an aqueous phase. The higher the temperature difference is, the faster the phase separation is due to the greater density differences and lower viscosity. The results presented in figure 51 reveal, that the microalgae culture being mixed with the nonionic surfactant Triton X-114 showed the expected temperature-induced phase separation after being exposed to 30° C, already after 10 minutes (visibly by formation of a clear bottom phase).

After being exposed to 30° C (temperature difference to CPT: 7K) for one hour (immersed in a water bath) two clearly separated phases were observed. Interestingly, the microalgae cells accumulate in the aqueous phase (which has a lower density than the micellar phase) although the microalgae cells themselves have a higher density than the micellar phase.

This is a very important finding, because it enables the simultaneous separation of the extract and the raffinate, as well as the separation of the microalgae cells (which stay in the aqueous phase). One possible explanation for this behavior can be the hydrophilic nature of the algae surface due to the polar functional groups of the cell wall [Hoeck, 1993]. It was observed by Pan et al. (2009) that the gram negative bacterium *S. marcescens* also retains in the aqueous phase of the used cloud point system. These findings prove the principle feasibility of the cloud point extraction to be applied for the surfactant/algae culture.

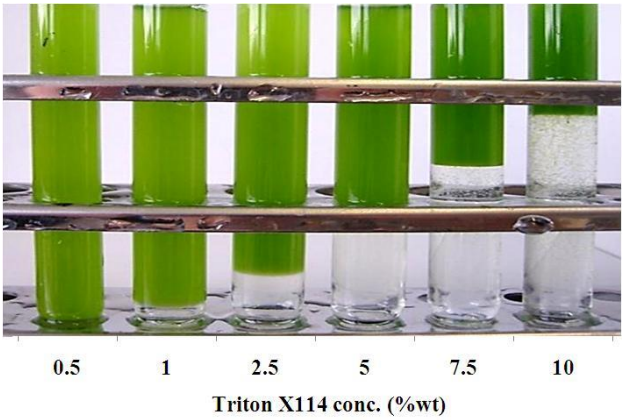



Figure 51: Phase separation of *S. obliquus* culture as a function of Triton X-114 concentration after 1 h, at 30° C.

In order to check the viability of the microalgae culture (*S. obliquus*) after phase separation procedure, samples from the aqueous phase (upper green phase visible in fig. 51) were collected and their RPA values were monitored (fig. 52). All the samples, independent to the surfactant concentration showed RPA values above 90%.

Thus, the phase separation did not have a significant influence on the algae growth in the investigated time period, which is one of the main requirements for the *in situ* extraction.

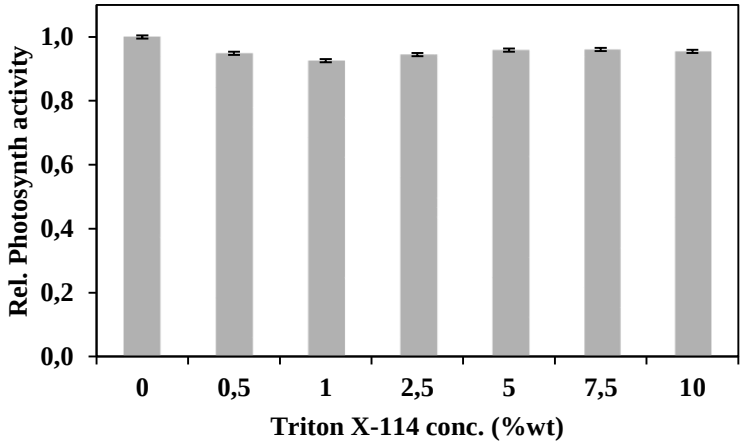



Figure 52: RPA after 1 h Phase separation of *S. obliquus* culture with increasing concentrations of the Triton X-114.

### 4.3.2 Phase separation kinetics

A key parameter for an effective *in situ* cloud point extraction is the duration of the contact between microalgae and relatively concentrated surfactant phase of the micellar phase (see Triton X-114 conc. of micellar- and aq. Phase, at 35°C, fig. 53) in order to minimize negative effects of the surfactants to the microalgae. The faster the phase separation is, the shorter the contact of the microalgae to high surfactant concentrations (as found in the micellar phase) can be. Therefore, the highest temperature at which the microalgae cells are not damaged irreversible, should be selected as operating point for such an extraction application.

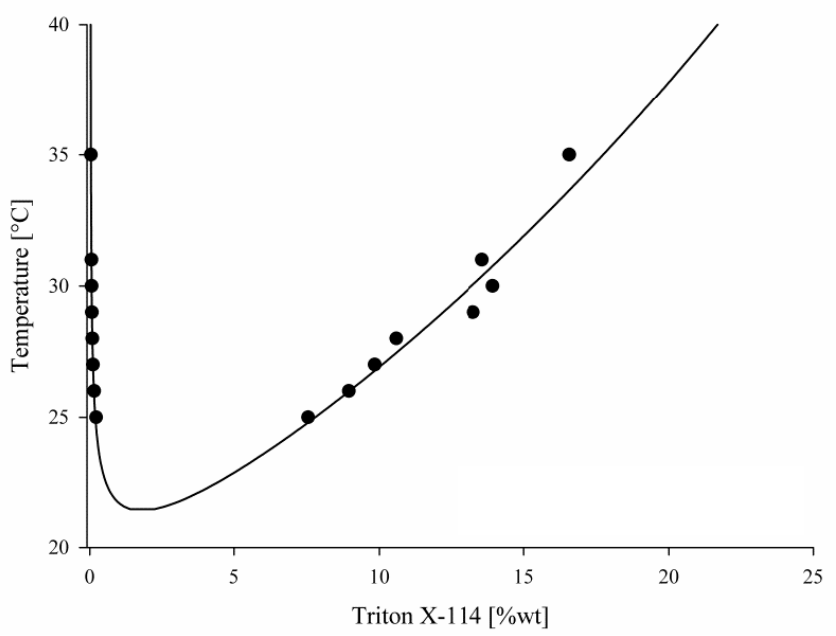



Figure 53: Liquid-liquid-equilibrium for the Triton X-114 system [Fischer, 2013].

In order to investigate the phase separation behaviour of different micellar solutions, two different Triton X-114 concentrations were chosen (3 and 5%wt). Due to the maximum tolerable temperature of microalgae of 40°C and the cloud point temperature of Triton X-114 (25°C), phase separation kinetic in the temperature range between 30 and 40°C was investigated.

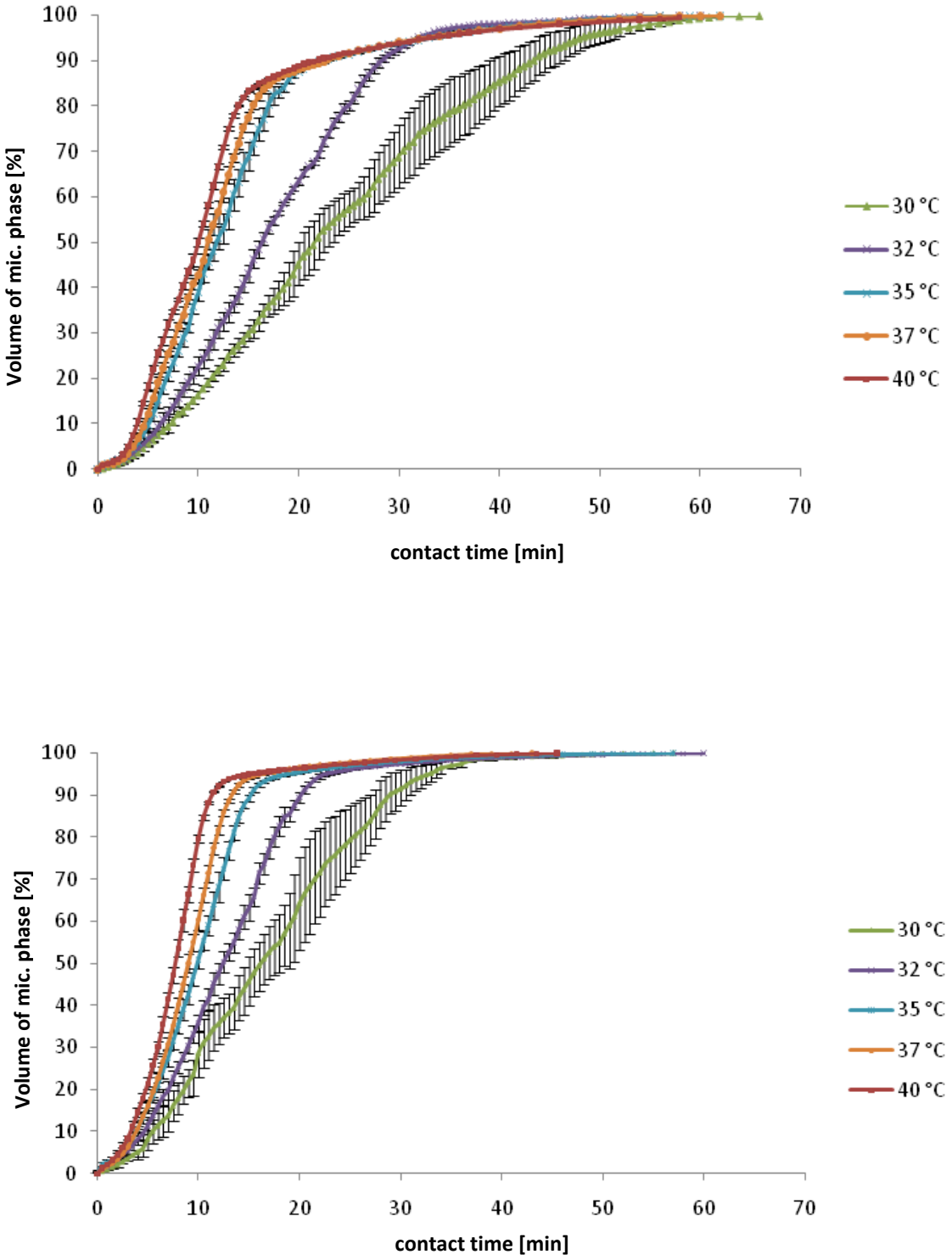



Figure 54: Phase separation kinetics of 1L of a 3wt% (upper) and a 5 wt% (lower) Triton X-114/water solution at different temperatures.

Apparently, the 3% wt Triton X-114 solution shows faster phase separation in comparison to the 5% wt solution at all temperatures under study (fig 54). The higher the temperature, the faster the phase separation is (at both tested concentrations of Triton X-114). Furthermore, the formation of a clear interfacial border caused higher accuracy of the kinetic data. This can be explained by the increasing density difference between the aqueous and the micellar phases (fig. 55). The density of both phases of the micellar system depends on the liquid-liquid equilibrium of the Triton X-114/water system (fig 53). The concentration of the micellar phase strongly increased with increasing temperature, whereas the concentration of Triton X-114 in the aqueous phase remained near the critical micelle forming concentration (CMC).

The different separation times of the biphasic systems (3% and 5%wt TX-114) can be explained with the volume of the micellar and the aqueous phase. The volume of the micellar phase is higher in case of 5%wt Triton X-114 solution compared to 3 wt%, suggesting a longer residence time of the single `droplet` of the aqueous phase, since it has a longer path through the micellar bottom phase, which has a higher density. Furthermore, the viscosity of the micellar phase formed from the 5% wt Triton X-114 solution is higher, which may further decelerate the formation of two clearly bordered phases.

The density difference between both phases is the main driving force (besides the viscosity and the interfacial tension) for the formation of two clearly separated phases. The density of the surfactant solution containing 3%wt Triton X-114 (below its CPT of 25°C) and the densities of the two resulting phases (above the cloud point temperature) are presented in figure 55.

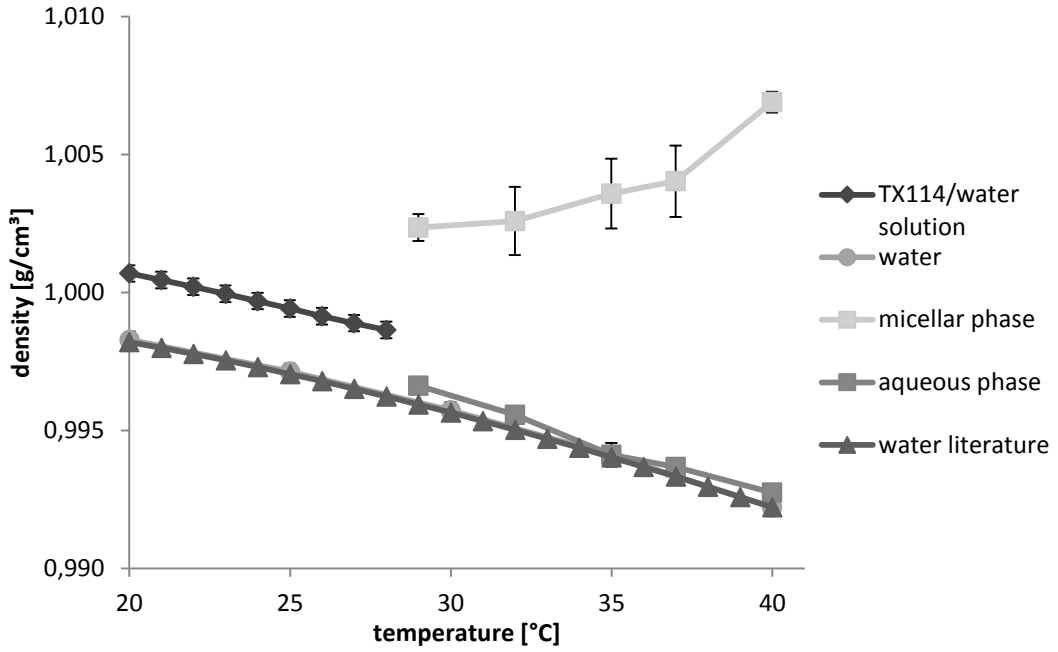



Figure 55: Density of a micellar solution containing 3% wt Triton X-114, below the cloud point temperature and the densities of the resulting aqueous phase and micellar phase, above the cloud point temperature of 28°C, pure water [Bettin, 1990].

The 3% wt Triton X-114 aqueous solution is similar to pure water, whereas the micellar solution has a slighly higher density due to the surfactant content. The density difference between the micellar and the aqueous phases increase with higher temperatures whereas the viscosity decreases. Hence, the time until formation of a stable volume ratio of both phases decrease with increasing temperature.

### 4.3.3 Phase separation in pilot scale

To approve the obtained knowledge about phase separation of a nonionic surfactant-algae suspension system for it's general feasibility, a first scale up approach of the process was tested at a pilot plant for cultivation of microalgae under outdoor conditions.

Therefore, the surfactant Triton X-114 was added to a culture of *S. obliquus* in a photobioreactor at 22 °C (ambient temperature) and mixed gentle (to prevent massive foaming) until getting a homogeneous solution with a surfactant concentration of 1 wt%. One hour after mixing was stopped, phase separation into a micellar and aqueous phase could be observed, since the algae culture was heated up to 32° C (by turning the photobioreactor to direct sunlight it takes about 45 minutes ), which was above the CPT of the used system (CPT of Triton X-114/water: 23° C).

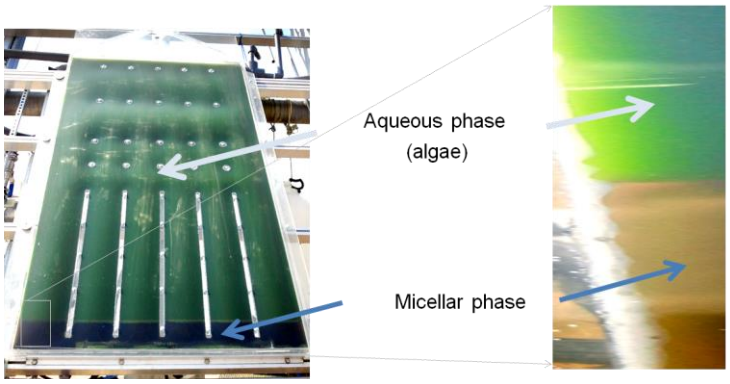



Figure 56: Phase separation of *S. obliquus* culture mixed with 1 wt% Triton X-114 after being exposed to 32° C for 1 hour.

Analogous to the experiments at lab scale, the microalgae cells accumulated in the aqueous phase of the cloud point system, whereas the micellar phase was mostly algae-free what can be seen from the figure 56. Also the light brown color of the micellar phase is visible in the picture. The darker color of the micellar phase in pilot scale may occur due to the fact that the equipment is not that clean like the laboratory equipment, e.g. bacterial biofilms in the piping may release dark pigments.

## 4.4 *In situ* cloud point extraction of fatty acids

Summarizing the previous investigations, the screening of nonionic surfactants with focus on the biocompatibility as well as on the formation of a stable interfacial boundary, was performed. Both main requirements were fulfilled with the selection of Triton X-114 as a suitable surfactant for an *in situ* extraction process for microalgae products. As a last step to transfer the concept to pilot extraction application, the partitioning of the target substance in both phases has to be determined.

To prove the overall concept for the firstly developed *in situ* cloud point extraction, a *S. obliquus* culture was extracted with 3%wt Triton X-114 at a temperature of 37 °C. Samples were taken for analysis of the partitioning of a hydrophobic target substance. In general, it has to be mentioned, that the substances which can be found in microalgae cultures can vary from day to day, due to the changing conditions (light duration + intensity, temperature) while an outdoor cultivation of microalgae.

The content of palmitic acid (16:0) in the micellar phase was 3.3 mg/L and the content of myristic acid (14:0) was 1.7 mg/L. As expected, the concentration of palmitic acid (3.3 mg/L) is much higher than its maximum solubility in water (0.04 mg/L, at 25° C, [Wishart, 2013]. This indicates that due to this enrichment in the micellar phase, it is generally possible to extract fatty acids from microalgae culture by means of *in situ* cloud point extraction.

In order to optimize the parameters of the CPE in the future, a method to predict the partitioning of fatty acids between the micellar and aqueous phase is required. In this work thermodynamic based model, COSMO-RS, was used for this purpose. The calculation of partition coefficients of several fatty acids (Tab. 3) between micelle and water indicate the suitability of CPE for extraction of fatty acids. Log K values from 8 to 13 (in dependence on the fatty acid) indicate that target substances predominantly accumulate in the micellar phase.

Thus, the extraction efficiency is rather dependent on the distribution of micelles between micellar and aqueous phase (see LLE Triton/water; fig. 53) as on the partitioning of fatty acids between micelle and water itself (because the FA are accumulating predominantly in micelles). For comparison, the partition coefficients of FA between water and the organic solvent dodecane are given (Table 7). The Log K values of FA in the surfactant systems are much higher than those in the water-solvent system, allowing for the higher extraction yield.

Table 7: Partition coefficients (Log K) of fatty acid between micelle / water and dodecane /water respectively, calculated with COSMO- RS.

| | Fatty acids | | | | | |
|---|---|---|---|---|---|---|
| | (16:0) | (18:0) | (16:1) | (20:5) | (20:4) | (22:6) |
| Log*K* (micelle/water) | 10.6 | 12.0 | 10.5 | 8.7 | 9.0 | 13.2 |
| Log*K* (dodecane/water) | 4.5 | 5.9 | 4.0 | 2.7 | 3.2 | 7.6 |

Based on these data the partitioning coefficient of each fatty acid between the micellar and the aqueous phase were calculated using the model COSMO-RS with the `pseudo phase approach` also given by Mehling [2014]. Free fatty acids (e.g. palmitic acid) were chosen as model compounds to evaluate the partitioning of hydrophobic substances produced by microalgae, because the palmitic acid shows generally the highest concentration in used microalgae cultures. It can be assumed that also the secretion of fatty acids or lipids by the microalgae is an origin of these hydrophobic substances. The knowledge about the partitioning of different hydrophobic algae products is of great interest for future work.

Due to the low water solubility of myristic acid, analytical difficulties were observed. The detection limit is near the water solubility of myristic acid (1.07 mg/L) which leads to further imprecision of experimental partitioning and extraction efficiency. Determination of the partitioning of stearic acid was not possible.

The stearic acid peak obtained by gas chromatography overlaps with the peaks of Triton X-114 and thus a detection of the 18:0 fatty acid was impossible for samples containing Triton X-114.

Experimental determined partitioning coefficients of myristic (14:0) and palmitic acids (16:0) between the micellar and the aqueous phase of the Triton X-114/water cloud point system were compared with the predicted values (using the model COSMO-RS).

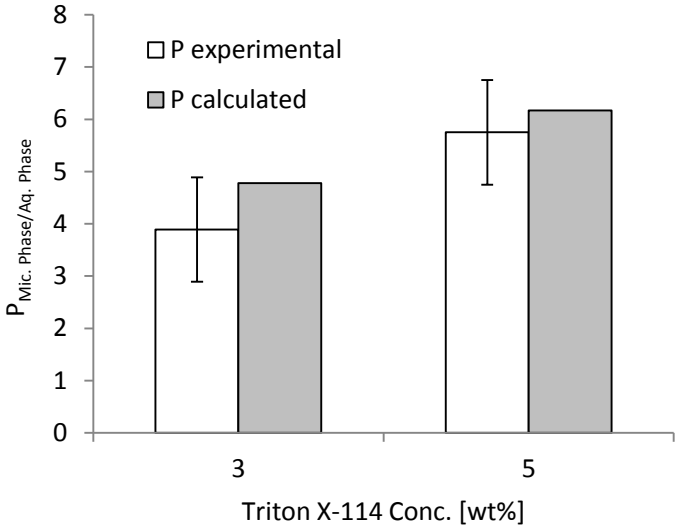



Figure 57: Experimental and predicted partitioning coefficient of palmitic acid (16:0) between micellar and aqueous phases.

These results are satisfying due to the fact that the calculated partition coefficients were in the range of the analytical accuracy. With this knowledge about the partitioning behavior, it is possible to optimize this extraction system, due to the fact that here only one substance (palmitic acid) is taken as a lead substance.

**Extraction yield**

In order to determine the extraction yield, a *S. obliquus* algae culture was extracted with surfactant concentrations of 3 wt% and 5 wt% in a single step cloud point extraction (batch).

Important to mention here is that the substances which can be found in a microalgae culture, can vary from day to day due to different environmental (light, temperature and nutrient depletion) conditions of outdoor cultivated microalgae [Lee, 2008]. This makes it difficult even within one algae species to compare extraction yields of different sampling times. Therefore, a saturated palmitic acid solution (C16:0 stock sol.) as model system for an algae culture was investigated.

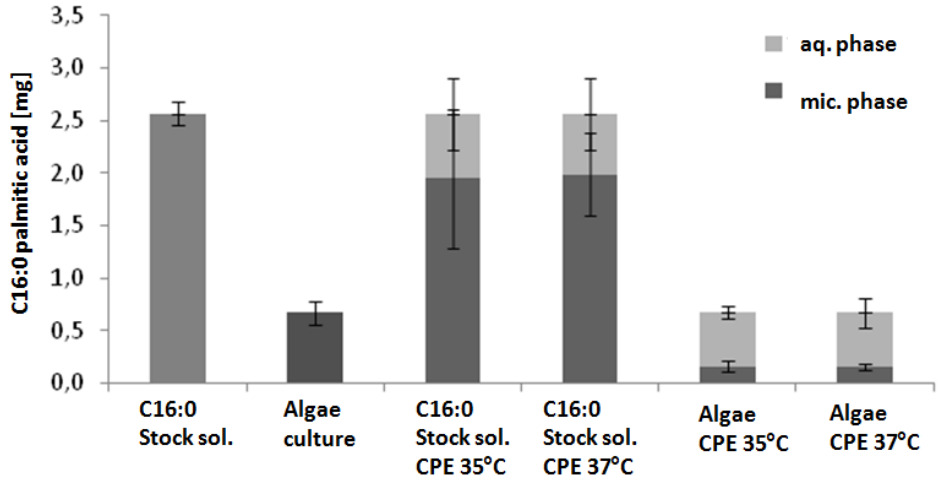



Figure 58: Mass of palmitic acid after a single-stage CPE. A stock solution, a saturated palmitic acid / water solution was used, which was mixed with 3 wt% Triton X-114.

In figure 58 it is visible that the saturated palmitic acid (C16:0 stock sol.) solution has a 4-fold higher amount of palmitic acid than the algae culture. The extraction yields from the algae culture reach 24%. The CPE from the saturated palmitic acid/ water solution shows extraction yields of up to 77%.

In both cases, no influence of the extraction temperature (35°C/ 37°C) on the extraction yield can be detected. Concerning the temperature induced stress to the microalgae the lower temperature is favorable. Nevertheless, faster phase separation takes place at higher temperatures.

The ratio between the fatty acids which are bounded in the biomass (especially the cell walls) and the free fatty acids is approximately 1:1. This may explain why the extraction yield is more efficient in water/palmitic acid solution.

After a single step extraction was carried out at 35°C (37°C), subsequent heating of the micellar phase up to 80°C was done, in order to reduce the water content (of the mic. phase) and therefore increase the palmitic acid concentration of the micellar phase (higher temperature leads to the loss of water according to the LLE of Triton X-114, fig. 53).

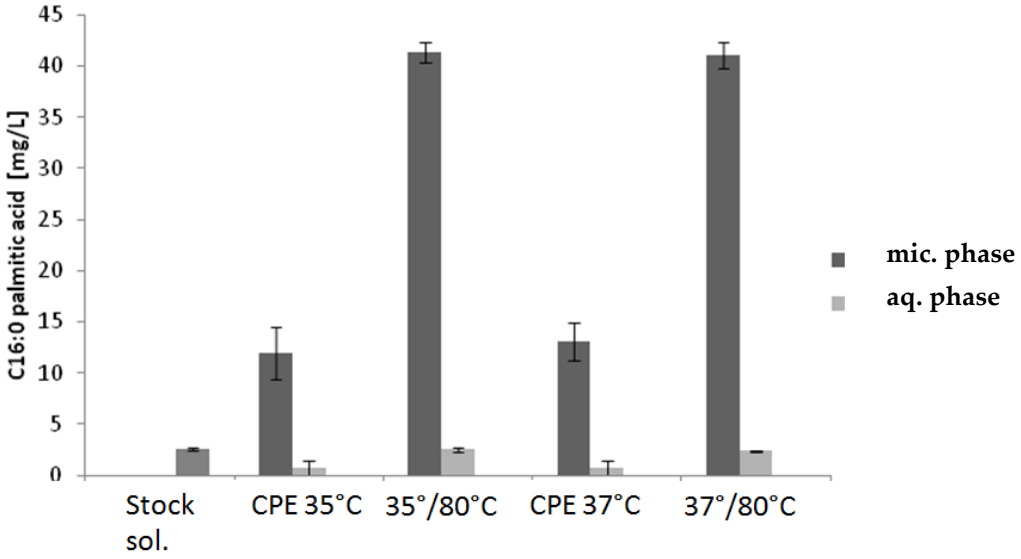



Figure 59: Palmitic acid conc. after a single-stage CPE with concentration of the extract streams. A stock solution, a saturated palmitic acid/water solution was used, which was mixed with 3 wt% Triton X-114.

Increasing the temperature of the cell free micellar phase after the single step extraction, further improves the downstream processing of the target substance. Due to the temperature induced loss of water (see LLE, fig 53), the concentration of the target substance in the micellar phase is increasing.

Summarizing the results, the optimal condition for the cloud point extraction of a *Scenedesmus obl*. culture established in lab scale are: 3%wt Triton X-114 where the phase separation takes place at 37°C.

The previous chapters have shown that it is generally possible to use nonionic surfactant systems to extract hydrophobic substances from microalgae. Therefore, the biocompatibilities of different nonionic surfactants, as well as the phase separation behavior were firstly studied [Glembin et al., 2013].

It was shown that a mixture of the microalgae culture with the nonionic surfactant Triton X-114 forms stable phases after 20 minutes at 37°C, whereby the microalgae cells predominantly accumulate in the aqueous top-phase.

Summarized a surfactant based *in situ* extraction process for the extraction of hydrophobic substances from microalgae cultures is shown in the following figure.

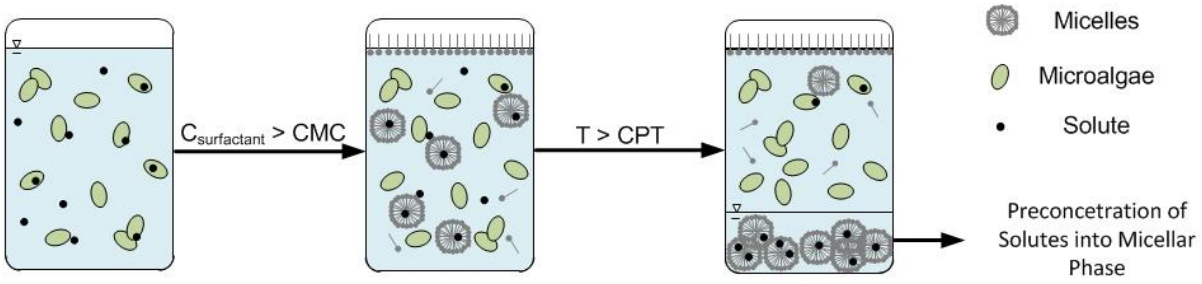



Figure 60: Scheme of the cloud point extraction of hydrophobic target molecules from a green microalgae culture.

These parameters are used to transfer the process from lab to the pilot scale.

### 4.4.1 Cloud point extraction in pilot scale

Since lab scale experiments showed the best results using a 3% wt Triton X-114/ microalgae mixture and temperature induced phase separation at 37°C. These parameters were chosen for the cloud point extraction at a pilot plant for the outdoor cultivation of microalgae. Extraction was done in a bypass, through which 20% (flow rate) of the circulating medium was channeled. The decision for the bypass was made, in order to achieve longer settling times (resulting from lower flow rates) in the extraction device. Triton X-114 was added to this medium until a final concentration of 3% wt was reached and the mixture was temperate to 37°C for phase separation (fig. 61).

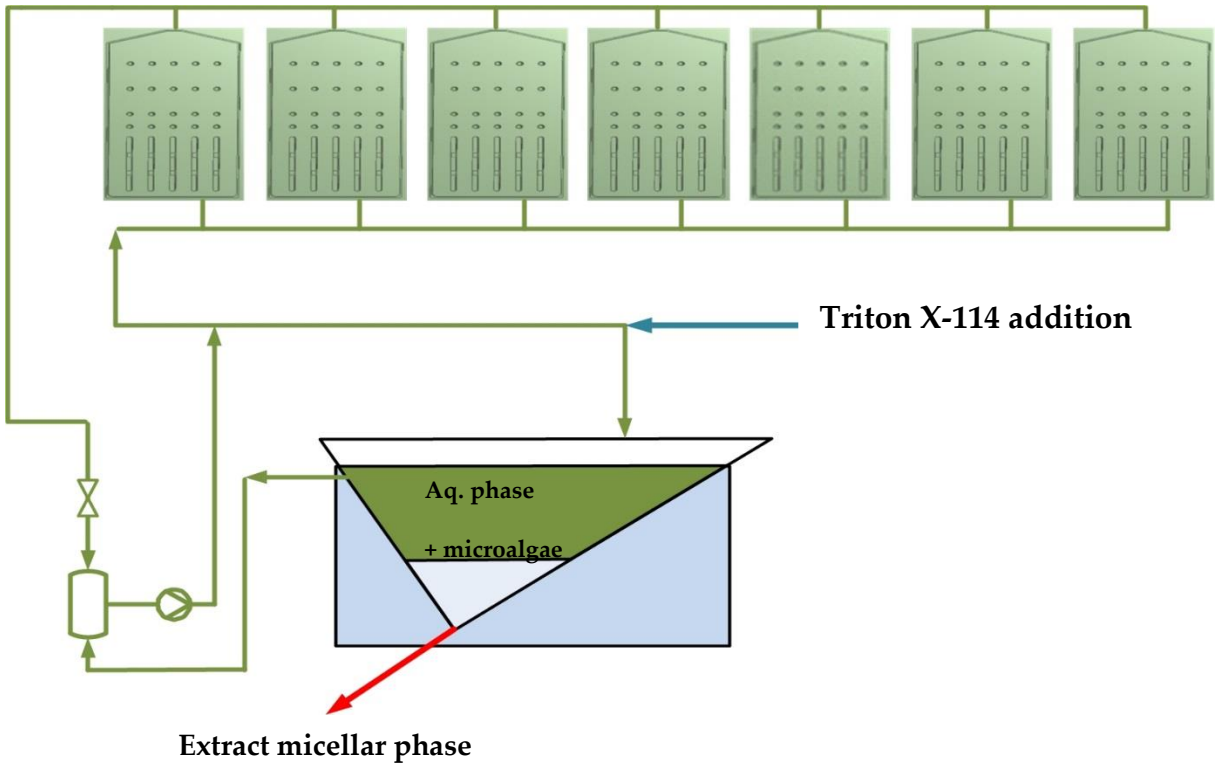



Figure 61: Scheme of the cloud point extraction in pilot scale.

The heating of the microalgae culture mixed with surfactant was realized with a thermostatic water bath, in which the triangular shaped settling device was located.

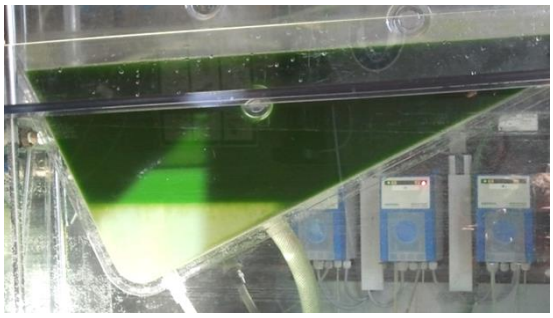


Figure 62: Micellar system (microalgae culture + 3% wt Triton X-114) after being exposed to a 37°C water bath for 30 minutes, with aqueous phase with microalgae (top) and micellar phase (bottom).

Figure 62 shows that the micellar solution splits into an aqueous phase, in which the microalgae accumulated (green top phase) and a surfactant rich phase on the bottom of the phase separation device (white). The triangular form was chosen in order to have a mainly cell free micellar phase at the outlet. The interfacial boundary between the two phases was also proven to be stable in a pilot scale which confirms the lab experiments. Hence, the important requirement to separate the micellar phase from the aqueous phase (with the accumulated microalgae), in order to recycle the cells for further cultivation, was reached with the experimental setup.

In order to determine the partitioning of free fatty acids (myristic acid/ palmitic acid) between both phases, samples from the micellar and aqueous phases were taken for analysis.

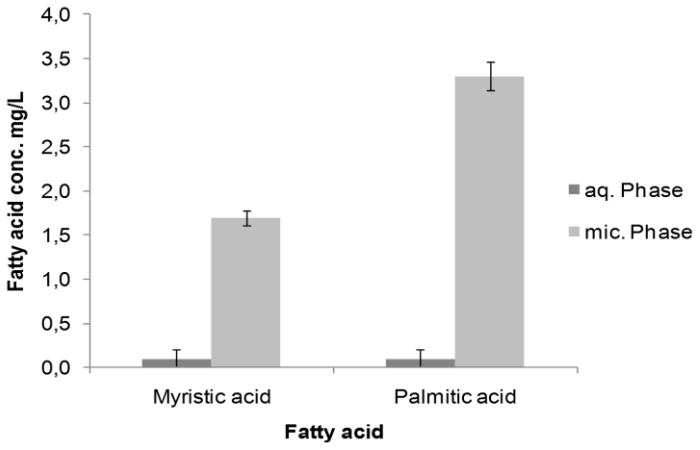



Figure 63: Fatty acid concentration after phase separation in pilot scale.

Enrichment of fatty acid in the micellar phase was detected by the analysis of myristic acid and palmitic acid. The concentration of the fatty acids in the aqueous phase was near the detection limit for the method applied. Important to mention is that the used microalgae strain was cultivated at a nitrogen concentration of about 50 mg nitrogen per liter which does not lead to an optimal production of fatty acids by the algae [Zhao, 2013]. In general, the results confirm the feasibility of the cloud point extraction of microalgae cultures on a pilot scale.

## 4.5 Continous *in situ* extraction

The previously shown results of the development of an *in situ* extraction of microalgae where based on a batch extraction approach, due to the advantages of simple handling and low price of the equipment needed.

However, in industrial applications continuous processes are preferred in general. The investigation of the main requirements for an *in situ* extraction of a micellar based extraction system (presented in the previous chapters) was done for the first time. As successfully shown in previous publications [Ingram, 2012; Mehling, 2014] the application of a surfactant based extraction system in a stirred counter current extraction column is generally possible. These findings should be extended with the gained knowledge about the batch *in situ* extraction of microalgae, in order to develop a continuous *in situ* extraction process for microalgae.

Therefore, the phase separation of micellar and aqueous phases plays an important role, because the extraction column is working in a counter current flow regime. In order to prevent flooding, the energy input by the agitator has to be adjusted. Another constrain is the influence of pumps, pipes, agitators, and the heat jacket of the column on the viability of the used microalgae.

The following chapter deals with the transfer of the batch application of micellar cloud point extraction to a continuous process. First of all, the relative photosynthetic activity (RPA) of the microalgae while passing through the piping and pumps of the heated extraction column was investigated. Therefore, five locations of sampling were chosen as can be seen from following figure 62. The first one was located in the storage tank, where the algae should have the optimal surrounding conditions. The second sampling location was set behind the liquid pump in order to check if the pump leads to the decrease of the RPA of microalgae. Locations 4 and 5 were chosen to evaluate the influence of stirring in the column as well as the temperature increase up to 40°C (due to heating of the column). Location 5 allows to take a sample from the drainage the extraction column.

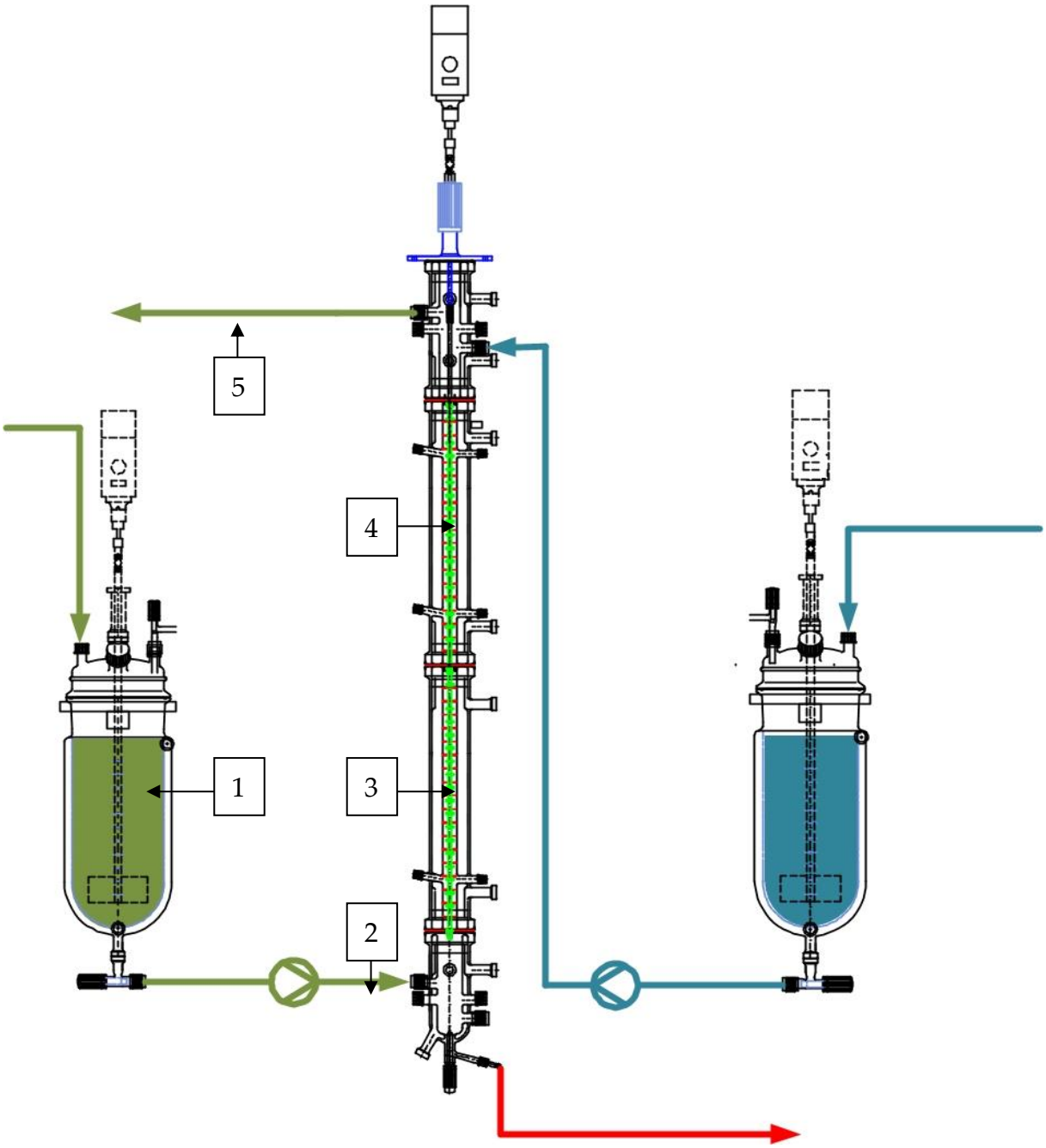



Figure 64: Locations for sampling 1: storage tank, 2: outlet of pump, 3: inlet in extraction column, 4: center of extraction column, 5: drainage of the column.

Figure 65 shows the RPA of the microalgae cultures samples taken at the described location, in the extraction process. Neither the pumping of the culture, nor the temperature increase, cause a significant decrease in the microalgae's RPA. This finding allows the transfer of the batch extraction to a continuous process.

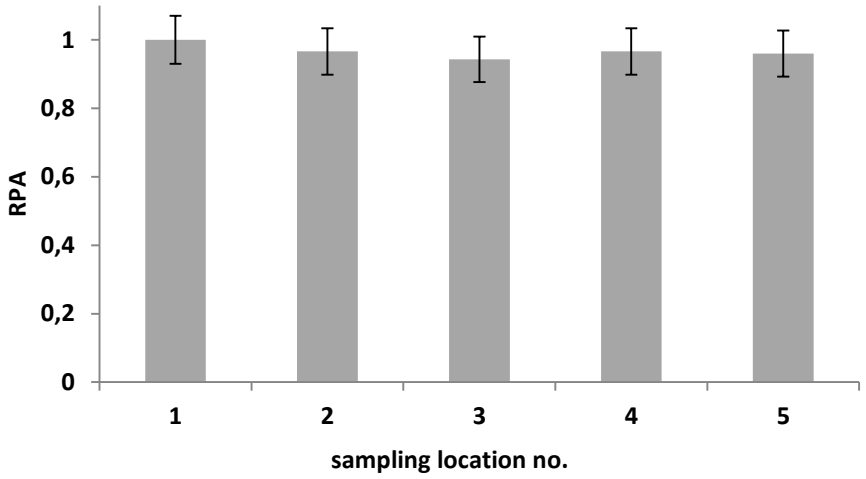



Figure 65: RPA of the microalgae culture at the different locations of sampling.

In order to investigate how fast the steady state is reached under given parameters (RPM: 60; TX-114 conc. in feed: 22%wt) the concentration of Triton X-114 was measured in the aqueous phase (containing the microalgae cells) leaving the extraction column on top.

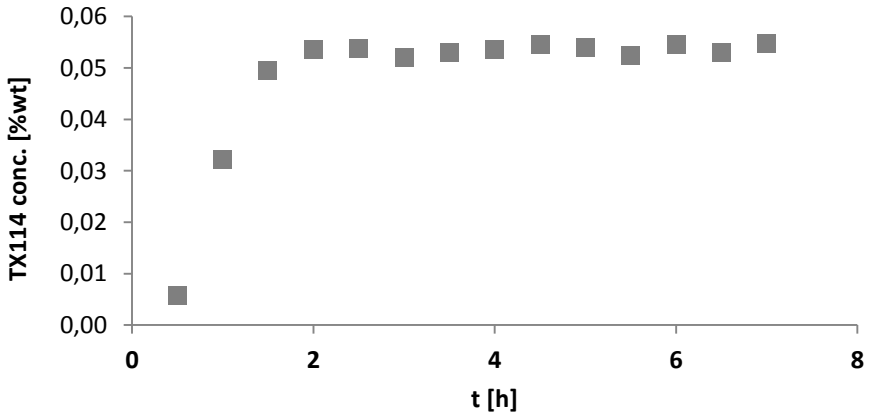



Figure 66: Concentration of Triton X-114 in the drainage of the extraction column as a function of time.

The figure 66 illustrates the start-up process of the continuous micellar extraction of microalgae. The steady state is reached after about 2 hours of operating.

In the following figures 67, 68, the schematic process sheet with the main flows of the microalgae culture (bottom to top) and the micellar solution (top to bottom) is shown. The composition of the surfactant solution was chosen with 22%wt due to the fact that this is the equilibrium composition of the micellar phase at a temperature of 40°C. At this temperature the experiments were carried out.

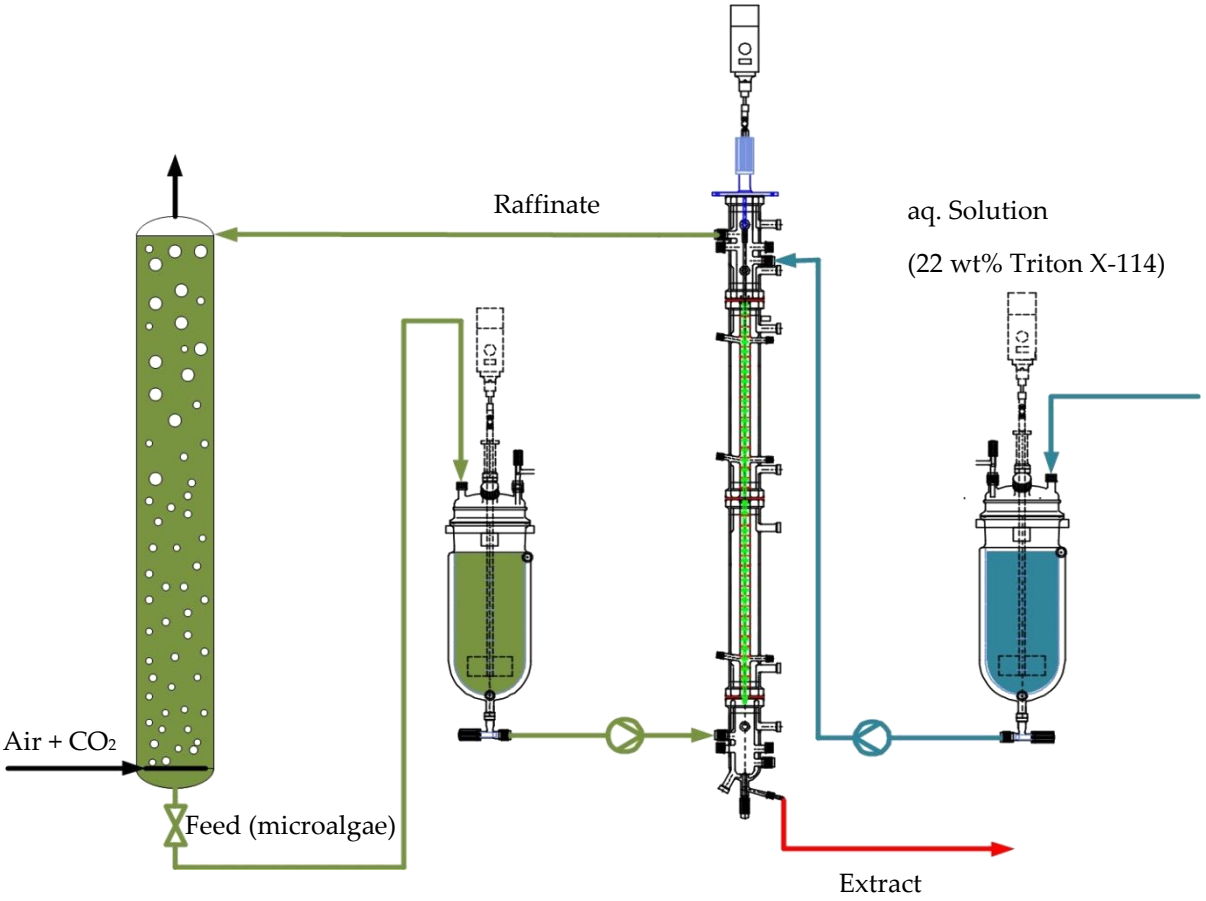



Figure 67: Scheme of the continuous counter current extraction of microalgae [adopted from Racheva, 2012].

The following illustrations (fig. 68) show different locations in the counter current extraction process. The first picture shows an overview of the agitated extraction column with the peripheries like solute and raffinate tanks. The second one on the upper right corner represents one compartment of the mixing zone, were the micellar phase is dispersed in the aqueous phase via agitators to increase the mass transfer surface. The settling zone, were the micellar phase with the higher density separates from the aqueous phase at the bottom of the extraction column, is shown in the lower right corner of the figure 68.

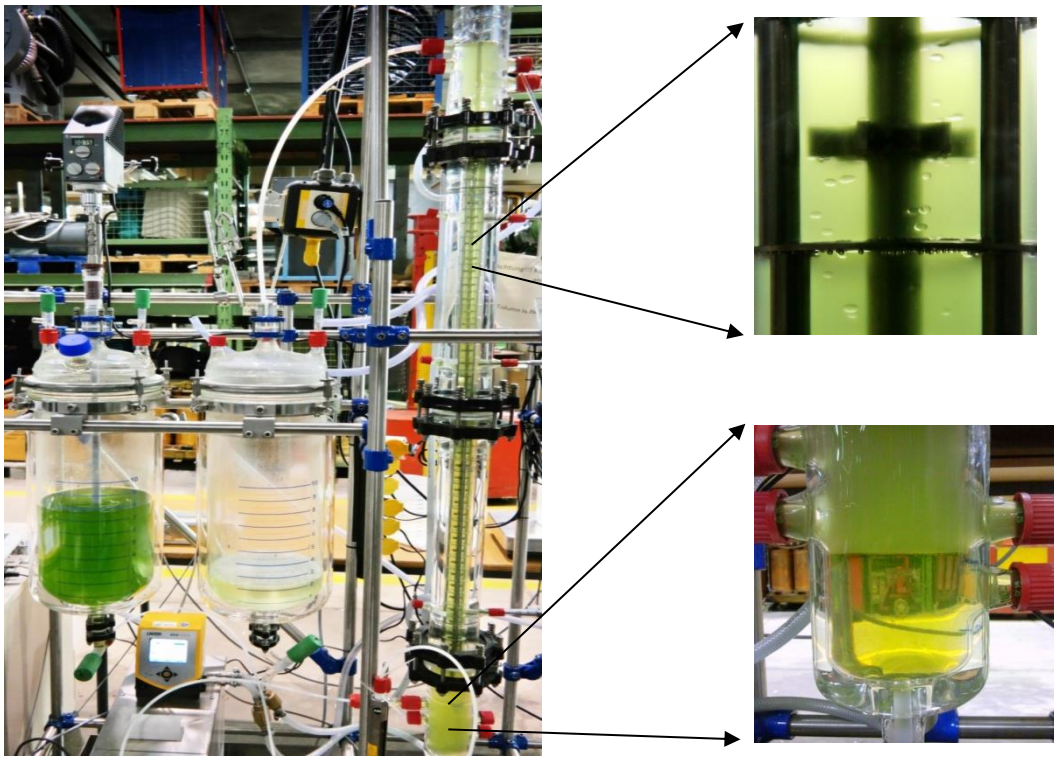


Figure 68: Counter current extraction column during first continuous extraction of microalgae culture with detailed views of the mixing zone and the settling zone.

The results shown in the figures 65 to 68 prove the feasibility of the *in situ* extraction approach in a continuous counter current extraction column. The results shown in figure 69 were derived from a continuous CPE carried out at following parameters: agitator 60 rpm, at 40 °C, feed concentration of 22%wt, resulting in a concentration in the mixing zone of 3%wt Triton-X-114. The palmitic acid analysis of both phases show that fatty acids accumulate in the micellar phase (figure 69). This is a main requirement for the industrial application and principally allows the *in situ* extraction of microalgae cultures.

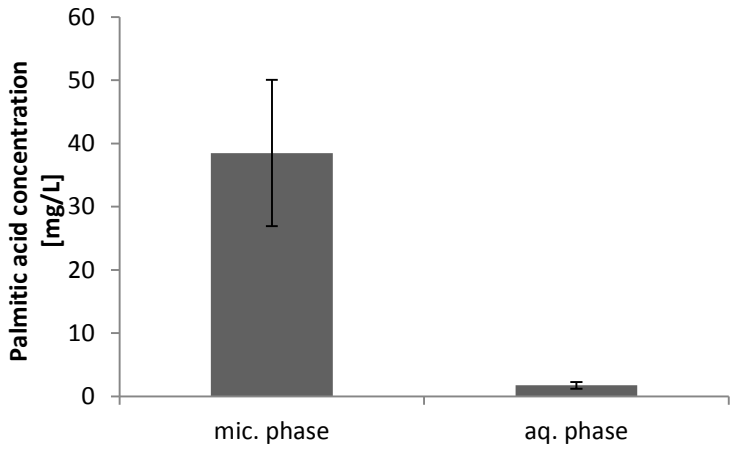



Figure 69: Fatty acid analysis after continuous cloud point extraction.

Compared to classical solvent based extraction processes the very small density difference between micellar and aqueous phase is one reason for the low volume flows in the used extraction column.

Compared to the batch extraction of microalgae culture with Triton X-114 (fig. 62), the concentration of the palmitic acid in the micellar phase is significantly higher. This can be explained with the more effective mass transfer between the both phases due to active agitation which results in higher surface area of the contacted phases. Different algae cultures (but the same strain) were used for these experiments which could lead to slightly different biomass concentrations.

**Some aspects of cost estimation for continuous extraction**

For the estimation of costs for the application of a micellar *in situ* extraction of fatty acids from microalgae cultures, first it has to be discussed which processes are compared. Therefore, different scenarios can be taken into consideration.

Comparing the micellar *in situ* extraction to the dodecane based *in situ* extraction, the main difference are the costs of the extractive phase.

1) **Extraction with Triton X-114 compared to the extraction with dodecane**

   At current market prices 1 l of the surfactant Triton X-114 has the same price as 1 l of the organic solvent dodecane (80 €/l). This results in costs of 80 €/l dodecane (as extractive phase). The assumptions for the Triton X-114 extraction system are that a 3%wt Triton X-114 containing solution disperses in aqueous phase with a surfactant concentration near the CMC and micellar phase above the cloud point temperature. At 37°C the micellar phase of this system has a surfactant concentration of 15% (v/v). Hence, one liter of the micellar phase generates costs of 16€. Concluding, the application of the mentioned surfactant system saves 80% of the costs for the extractive phase. Since the application of a dodecane based *in situ* extraction is anyhow not feasible in pilot scale (formation of stable emulsions, which hinders phase separation) the micellar *in situ* extraction has to be compared to classical microalgae downstream processes.

2) **Extraction with Triton X-114 compared to classical microalgae harvesting and downstream**

   Classical solvent extraction always include the separation of the microalgae biomass followed by further steps of drying, cell disruption and refining of the product. The company `EnAlgae` made a comparison of the economics of downstream processes for different substances from microalgae [Spruijt, 2015].

This includes a scenario for the production of biodiesel. The fact that this process based on the extraction of lipids from microalgae biomass, micellar *in situ* extraction can be compared with this process. In the following table the investment costs for the biodiesel downstream route are given.

Table 8: Investment costs for the processing of 10 tons dry algae biomass [Spruijt et al., 2015].

| | Biodiesel route | |
|---|---|---|
| | **investment** | **life span (y)** |
| Dryer | € 5,000 | 20 |
| Ball Mill | € 10,000 | 15 |
| $SCO_2$ extractor | € 120,000 | 15 |
| Refining | € 15,000 | 20 |
| Transesterification equipment | € 22,520 | 20 |
| Process control | € 16,000 | 5 |
| Infrastructure | € 30,000 | 20 |
| **Total** | **€ 218,520** | |

For the comparison of the micellar *in situ* extraction with the above mentioned downstream processing of biodiesel, following assumptions are made.

The cultivation of the microalgae biomass is the same in both cases. Therefore, the drying, milking and the extraction of lipids with supercritical carbon dioxide are not necessary when an *in situ* extraction is applied. Additional assumptions are that an extraction column for the micellar *in situ* extraction would generate estimated investment costs of 15.000€ (Column volume of 5L, 2 pumps). The costs for the refining transesterification equipment, process control and infrastructure remain the same.

Roughly estimated 120.000€ of invest cost can be saved in this scenario when a micellar *in situ* extraction is applied.

# 5 Conclusions

In this work *in situ* cloud point extraction of fatty acids from microalgae was firstly investigated in the lab and the pilot scale. In order to realize this new extraction process, the biocompatibility of surfactants toward the microalgae *Scenedesmus obliquus*, phase separation kinetics and the partitioning of a hydrophobic target substance (palmitic acid) were studied. Generally, the results indicate that the development of a suitable cloud point extraction system with nonionic surfactants can be realized in both batch and continuous extraction devices. The implementation depends especially on the knowledge of the biocompatibility of used surfactant with the microalgae, the phase separation behavior and a favorable partitioning of the target substance. Based on the results obtained for the different surfactant/algae combinations so far, *S. obliquus* was the most suitable microalgae candidate for the development and application of a cloud point extraction system. The nonionic surfactant Triton X-114 is the most suitable surfactant (among the investigated ones) for an *in situ* extraction process of *S. obl*. microalgae cultures, due to its high biocompatibility with the used microalgae, combined with the rapid formation of the stable phase boundary. Finally, the extraction process with Triton X-114 was tested successfully in both, batch- and the continuous mode in pilot scale. Therefore, an agitated counter current extraction column was firstly tested for the *in situ* extraction of hydrophobic substances. These results prove that micellar extraction is a suitable *in situ* extraction process for microalgae cultures. The separation of the extracted fatty acids from the micellar solution can be done e.g. by re-extraction, rectification or pervaporation processes whereby the constrains (e.g. temperature and pressure) can be chosen much higher due to absence of living microalgae cells. Further, an extraction process using a food grade surfactant would be of great interest.

In this case a further processing of the target substances can be avoided. Under mentioned circumstances the *in situ* CPE of microalgae cultures can be a promising technique to improve the overall efficiency of valuable algae products. Considering the requirements mentioned above, the application of CPE-systems can replace the classic extraction with organic solvents or carbon dioxide.

# 6 Future prospects

In this work the aqueous micellar system based on the nonionic surfactant Triton X-114 was investigated. This system results in an aqueous phase, which has a lower density than the micellar phase. This hinders an acceleration of the phase separation process for example through centrifugation, because the microalgae cells would mitigate into the micellar phase due to their density. Therefore, an improvement for other surfactant systems could be to change the density of the micellar phase. The favorable densities order would be:

density of mic. phase < density of aq. phase < density of microalgae cells

Surfactants which form a micellar phase with a lower density than the aqueous phase of the system may simplify the phase separation in a cloud point extraction process. Such systems can enhance the formation of a stable micellar phase (on top) and enable a separation of the phases using centrifugation. This separation method cannot be recommended to the investigated Triton X-114 system, due to the fact that the microalgae cells have a higher density than the micellar phase, which leads to the contamination of the micellar phase. For instance the system of Tergitol 15 s 7 can be proposed for optimization of the phase separation step.

First experiments with the surfactant Tergitol 15 s 7 underline the suitability of this system as can be seen from following pictures (fig 70, 71). The micellar top phase is in accordance with the expectations.

Figure 70: CPE with 3 wt% Tergitol 15 s 7/water with 0. 1 M C (left); CPE 3 wt% Triton X 114/ water, each at 37 C.

Figure 71: CPE with 3 wt% Tergitol 15 s 7/water at 37°C and addition of: 0. 3 M, 0. 2 M und 0.1 M potassium sulfate.

Furthermore, the surfactant used in an *in situ* extraction process of hydrophobic substances which were processed in the food and feed industry should have a food grade classification in order to avoid the purification of the micellar phase.

Generally, diverse applications where hydrophobic target substances should be purified from a microorganism containing broth (e.g. algae or bacteria), may be covered with a micellar *in situ* extraction.

Another improvement of the developed extraction process may be reached, when this process is applied to production systems in which thermophilic microorganisms produce the target substances. In this case, the maximum tolerable temperature can be increased compared to the studied microalgae system, (max 40°C). Therefore, the temperature induced phase separation could take place at higher temperatures (e.g. 60°C), which would accelerate the phase separation and decrease the volume fraction of the micellar phase.

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# List of figures

# List of tables

# Symbols

| Symbol | Signification |
|---|---|
| c | Molar concentration, [mol/L] |
| $\bar{c}$ | Mass concentration, [g/L] |
| E | Extraction efficiency,[%] |
| K | Molar partitioning coefficient |
| m | Weight,[g] |
| M | Molar weight, [g/mol] |
| n | Molar amount, [mol] |
| p | Pressure, [Pa] |
| P | Partitioning coefficient in the means of concentrations |
| S | Standard deviation |
| t | St. coefficient |
| T | Temperature, [°C] |
| V | Volume, [L] |
| W | Mass fraction, [g/g] |
| x | Mole fraction,[mol/mol] |
| $\gamma$ | Activity coefficient |
| $\mu$ | |

| | |
|---|---|
| ν | Chemical potential, [J/mol] |
| ϱ | Molar volume, [$cm^3$/mol] |
| σ | Density, [g/ $cm^3$] |
| | Charged density, [$e/m^2$] |

| **Greek symbol** | **Unit** | **Meaning** |
|---|---|---|
| *A* | - | Power-law transformation factor |
| *B* | - | Polynomial regression coefficient |
| *E* | - | Statistical error |
| *μ* | - | *t*-distributed mean of a random sample |
| *Σ* | - | Standard deviation |

| **Indices** | **Meaning** |
|---|---|
| N | Sample size |
| P | Level of significance |

# Abbreviations

| Abbreviation | Signification |
|---|---|
| CMC | Critical micelle concentration |
| CP | Cloud point |
| CPE | Cloud-point extraction |
| CPT | Cloud-point temperature |
| CT | Clouding temperature |
| FA | Fatty acids |
| GC | Gas chromatography |
| HPLC | High performance liquid chromatography |
| LLE | Liquid-liquid equilibrium |
| PA | Photosynthetic Activity |
| RPA | Relative Photosynthetic Activity |
| *S.obl.* | *Scenedesmus obliquus* |
| *c. vul.* | *Chlorella vulgaris* |
| *c. reinh.* | *Chlamydomonas reinhardtii* |

**Subscripts and Superscripts**

| | |
|---|---|
| i | Component |
| M | Micellar phase |
| O | Octanol |
| s | Solute |
| w | Water |
| W | Aqueous phase |
| Φ | Phase, =W or M |

# 8 Appendix

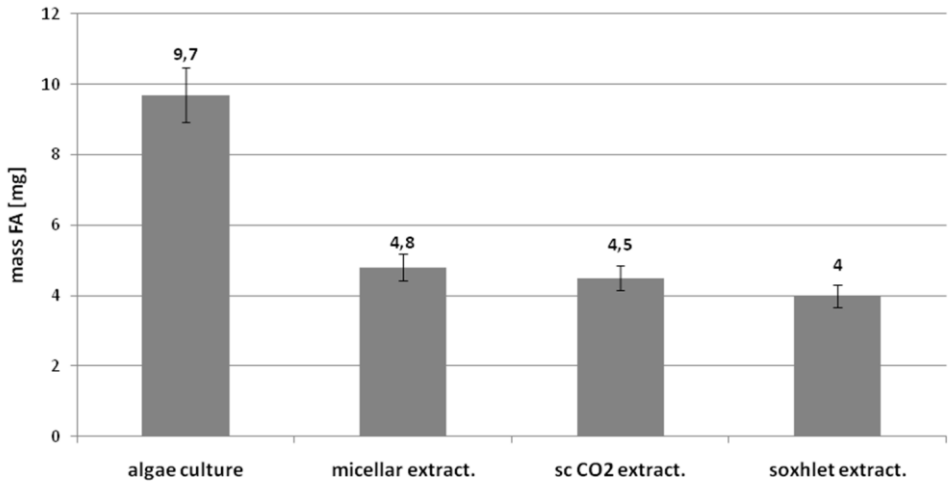



Figure 72: Comparison of different extraction methods. 1l of algae culture was extracted in each case

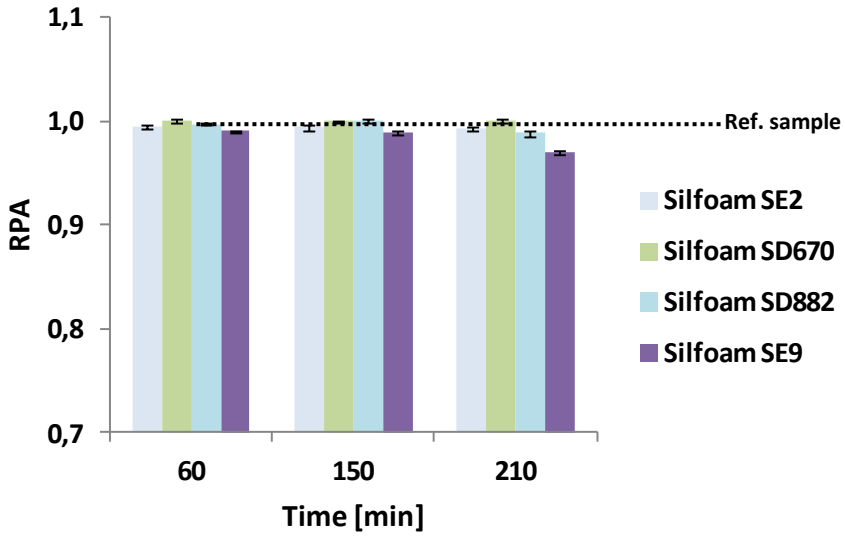



Figure 73: *S.obl* RPA within 210 min contact with different defoamers

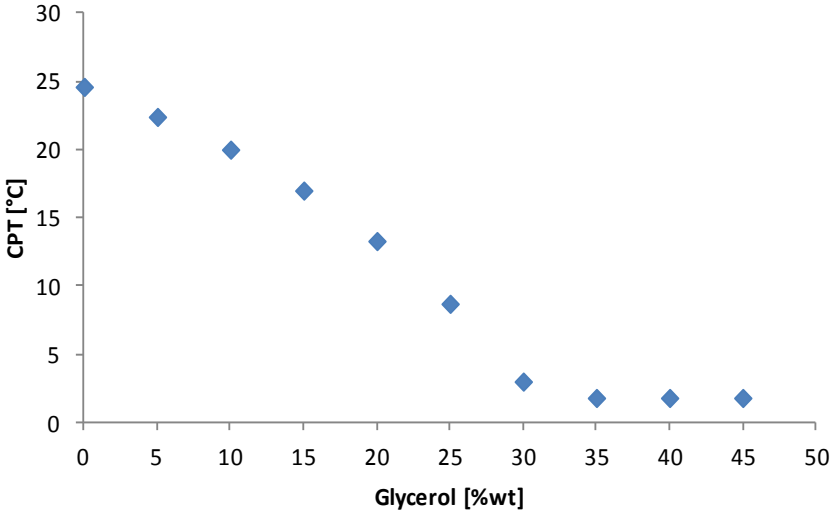



Figure 74: Cloud point temperature in Triton x-114 system (3%wt) as a function of the glycerol concentration

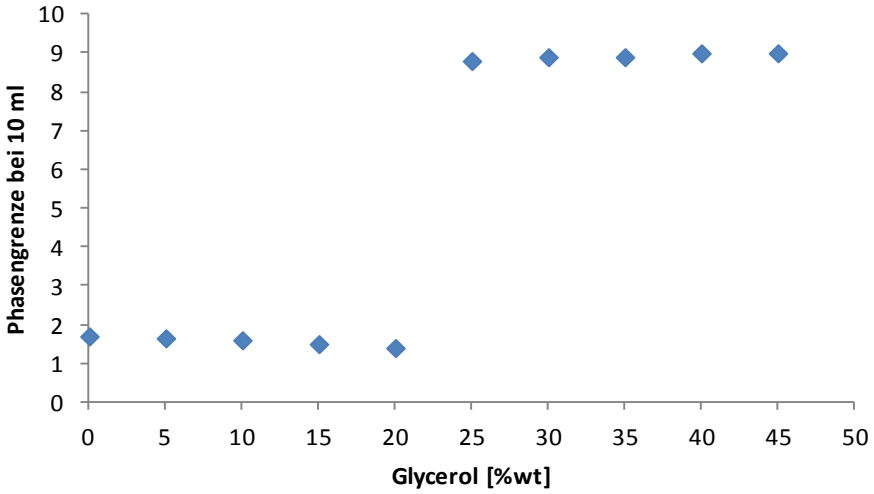



Figure 75: Phase boundary of an aqueous Triton X-114 solution above its cloud point as a function of glycerol concentration

DLS measurtements:


Figure 76: DLS measurement of an aqueous solution containing 3%wt Triton X-114

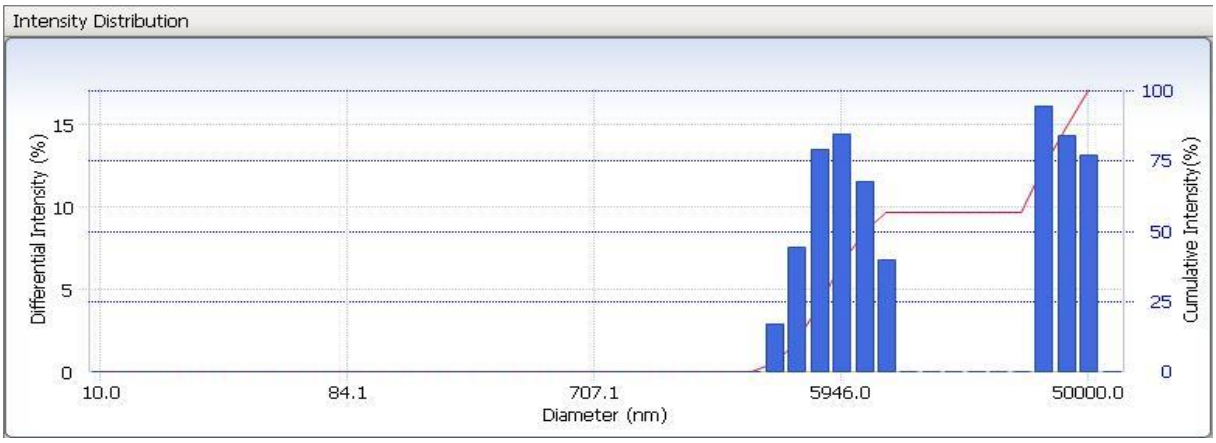



Figure 77: DLS measurement of *S. obliquus* culture

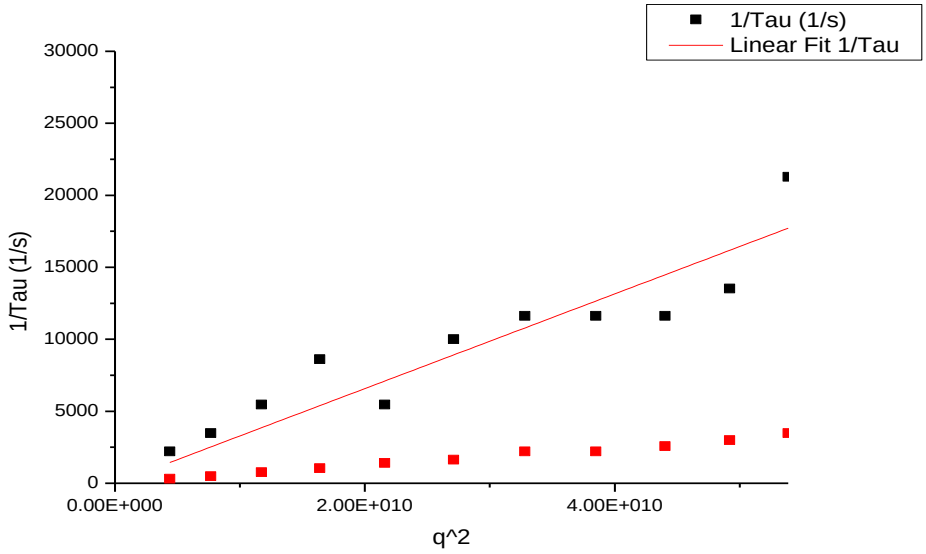

Figure 78: DLS measurement of *S. obliquus* culture containing 3%wt Triton X-114

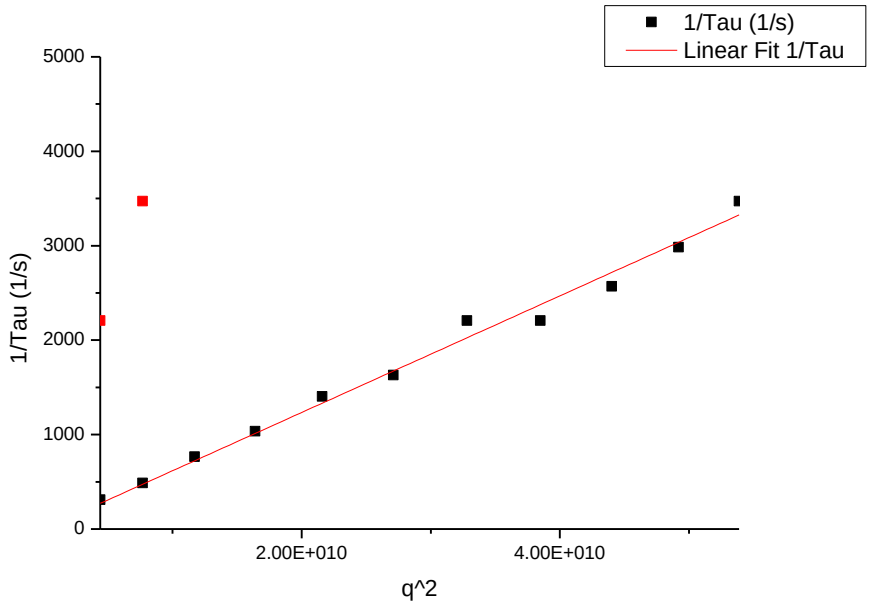
4 wt. % Triton X-114 in water at t= 20°C

Diffusion coefficient D2 = $3.3*10^{-7}$ $cm^2/sec$

Hydrodynamic radius Rh = 6.5 nm

D1=$6.2*10^{-8}cm^2/sec$



## Lebenslauf

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