

Mild disintegration of green microalgae and macroalgae



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Mild disintegration of green microalgae and macroalgae

Richard Postma

Thesis

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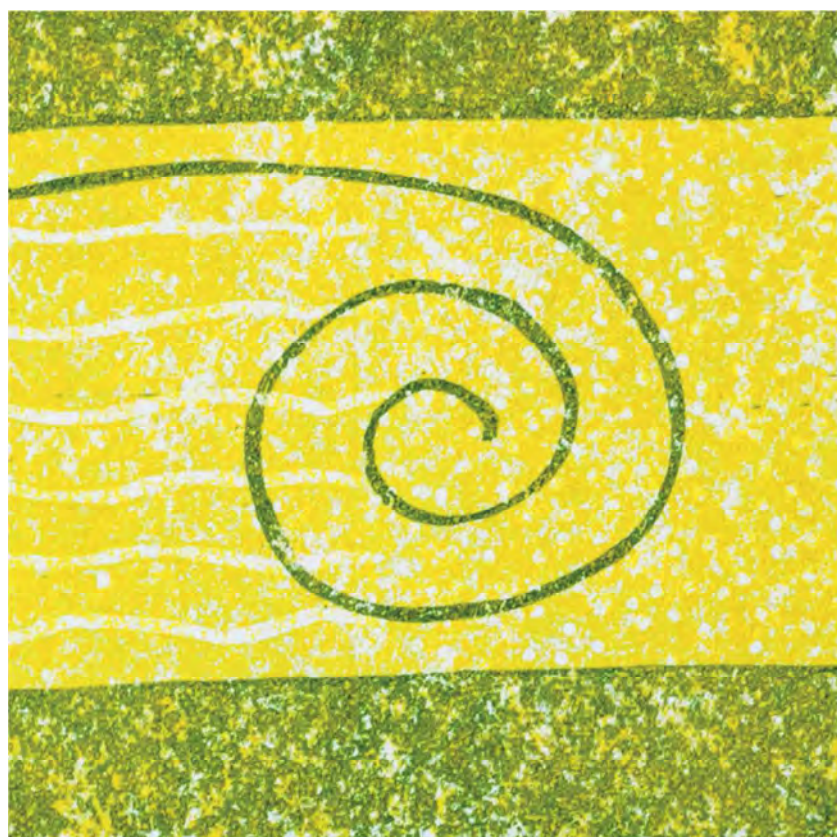
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Chapter 1

Introduction and thesis outline

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P.R. Postma*, G.P. 't Lam*, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, G. Olivieri

* both authors contributed equally

Microalgal biorefinery for bulk and high-value products: Product extraction within cell disintegration

in: D. Miklavcic (Ed.) *Handbook of Electroporation* (2016) Springer

1.1 Algae as protein source

The world population is expected to increase (Godfray et al., 2010) to 9.6 billion people by 2050 (United Nations, Department of Economic and Social Affairs, Population Division, 2015). In combination with a greater income per capita worldwide there is a large pressure on agricultural land demand (Tilman et al., 2011) to meet the protein requirements. In order to keep up with this growing demand for proteins for food and feed, novel proteins are needed. Residual agricultural waste like sugar beet leaves or novel green sources like microalgae or macroalgae could aid in this issue.

Microalgae and macroalgae are of interest because of their larger areal productivities compared to agricultural crops (Chisti, 2007, 2008; Wijffels and Barbosa, 2010) and do not require arable land. Macroalgae, better known as seaweed, can even be cultivated in open sea which covers more than 70% of the planet's surface (van den Burg et al., 2013). Both microalgae and macroalgae are good candidates for protein production (Becker, 2007; Draaisma et al., 2013; Harnedy and FitzGerald, 2011; Holdt and Kraan, 2011; Spolaore et al., 2006) for food and feed applications.

1.2 Biorefinery

According to the International Energy Agency (IEA) biorefinery is defined as '*the sustainable processing of biomass into a spectrum of marketable products and energy*' (IEA, 2009). Following this concept, for the exploitation of either microalgae or macroalgae biomass, complete biomass valorization is important to have positive economics (Ruiz et al., 2016; Vanthoor-Koopmans et al., 2013; Wijffels et al., 2010). This valorization should be performed in a cascade biorefinery strategy which comprises all downstream processing steps after the biomass cultivation and harvesting to come to the final products, being cell disintegration, extraction and fractionation. Downstream processing costs are an important part of the total production costs, and can attribute ~60% of the total manufacturing costs (Coons et al., 2014; Delrue et al., 2012; Molina Grima et al., 2003). Therefore, major effort is required to find energy efficient, yet effective, unit operations for the biorefinery. Some species have a tough cell wall, which makes them robust for outdoor cultivation, but also require hard conditions to extract the intracellular components. Literature addressing the market potential of microalgal components is for a large part focused on production of one specific product from the biomass (e.g., lipids or a single enzyme). Therefore, ad-hoc extraction methods were only developed for one specific product and the other available and valuable components in the microalgae were not valorized.

The algal components are generally stored intracellularly in organelles or bound to membranes (Günerken et al., 2015) and enclosed by cell walls made from complex polysaccharides (Gerken et al., 2013; Joubert and Fleurence, 2008; Safi et al., 2014c). Therefore, the first step after the harvesting of the biomass is the disintegration of its cell wall and macrostructure (in the case of macroalgae). It is important to perform this step under mild conditions (i.e., no high temperatures, shear, pressures or caustic agents), to make sure that the products are not negatively affected in terms of quality or functionality (Vanthoor-Koopmans et al., 2013). In the following sections, first the conventional techniques used for cell disintegration are discussed, followed by an overview of promising novel disintegration technologies.

1.2.1 Conventional cell disintegration

Traditional techniques for cell disintegration, e.g. high pressure homogenization (HPH), high speed homogenization (HSH), bead milling, heat treatment and chemical treatment are generally considered to be non-mild and so far used to obtain only single products while detrimental for the rest of the biomass (Grimi et al., 2014; Günerken et al., 2015; Vanthoor-Koopmans et al., 2013). Amongst the before mentioned techniques, HPH, bead milling and HSH are the most prevailing methods for algae disintegration. These technologies are well established, robust and scalable and have been adopted from other fields for cell disintegration (e.g., yeast and bacteria). Since in this thesis the work is focused on mild disintegration, temperature control is critical. Therefore, bead milling and HSH were considered as benchmark technologies for microalgae and macroalgae, respectively.

Bead milling

Bead mills are homogenizers originally designed for the size reduction of paint and lacquer particles but can also be used to disintegrate biomass suspensions (Kula and Schütte, 1987). The basic principle of a bead mill is the rapid stirring of small beads in the presence of a microorganism suspension. High shear forces are created due to differences in the speed of the beads, besides direct impact of the beads with a microorganism also causes cell disintegration. A bead mill consists of a horizontal or vertical jacketed grinding chamber with a shaft through the center. Disks, rotors or agitators of different designs can be mounted on this shaft and will impact the kinetic energy towards the beads. The suspension flows through the grinding chamber while the beads are retained in the chamber by a sieve or axial slot. A bead mill can be operated either in batch recirculation mode or in continuous mode. In general, an external pump is used to create the suspension flow. Figure 1.1 shows a

typical configuration of a bead mill on lab scale (batch) and pilot/industrial scale (batch/continuous).

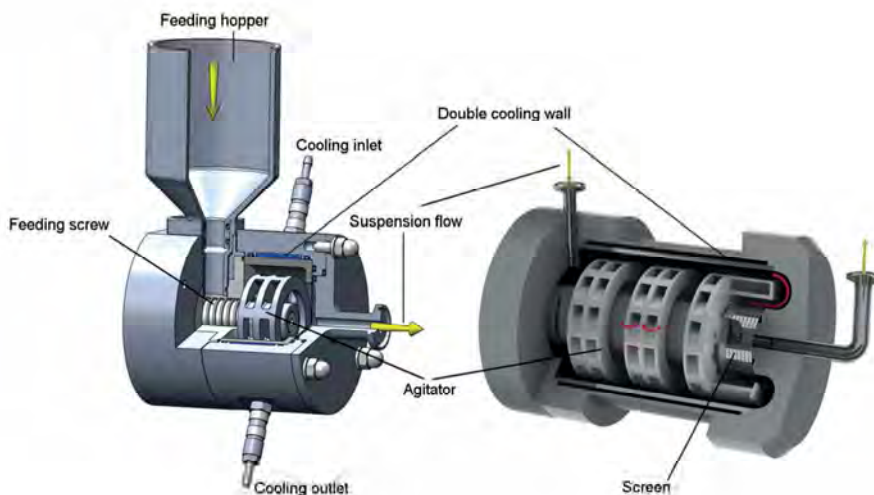


Figure 1.1 Schematic representation of lab scale (left) and large scale bead mill (right) configuration (courtesy of WAB, Muttenz, Switzerland).

For batch mode, the suspension flow rate is of minor importance (i.e., only required to assure sufficient recirculation), while the residence time distribution of the suspension inside the grinding chamber is the only important parameter directly influencing the disintegration kinetics. For continuous processing, the flow rate dictates the residence time and therefore the process kinetics.

A total of 44 process variables were described to influence the bead milling process (Möller and Hörnle, 1972). Besides the above mentioned suspension flow rate, several additional variables are most influential on the process amongst which; grinding chamber and agitator design, bead size, bead density, bead filling ratio, agitator speed and biomass concentration (Doucha and Lívanský, 2008; Kula and Schütte, 1987).

High Shear Homogenization

High shear homogenization (HSH) generally makes use of the rotor-stator principle and are commonly applied for generation of emulsions or suspensions (Hall et al., 2011; Schultz et al., 2004). In the rotor-stator principle commonly a toothed outer ring aids as the stator, while a toothed or smooth rotor is used on the inside (Figure 1.2). The rotor geometry can be adjusted depending on the desired fineness of the emulsion/particles. The mechanism of HSH relies on high shear, turbulence, cavitation and direct impact. Typically the energy

consumption of HSH is rather high and the temperature of the medium also sharply increases due to the high energy input. The geometry of the rotor and of the stator, the rotor speed and the temperature are important process variables.

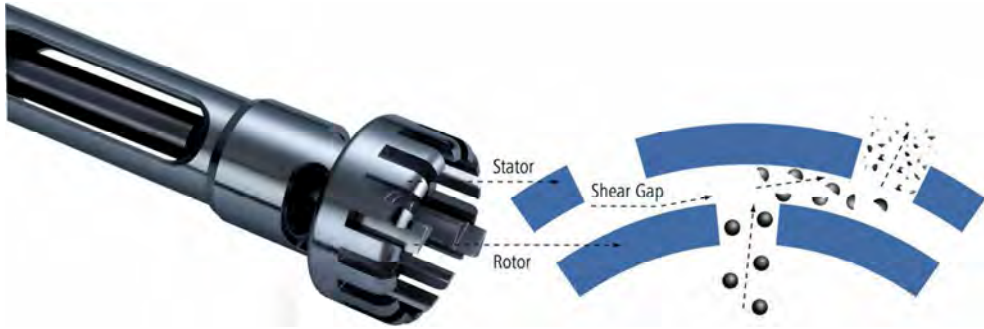


Figure 1.2 Illustration of rotor-stator principle (courtesy of IKA Works, Germany).

1.2.2 Novel cell disintegration

In contrast to the conventional disintegration technologies, specific enzyme cocktails or continuous flow technologies like Pulsed Electric Field (PEF), Ultrasound (US) and Supersonic Flow Fluid Processing (SSFF) in which an external field causes controlled pulses/shocks started to receive more attention the past decade (Günerken et al., 2015; Vanthoor-Koopmans et al., 2013). First of all, by the controlled action, only specific degradation or perforation of the cell membrane and cell wall is foreseen. This feature, in combination with very low predicted specific energy consumptions for PEF and US (Boer et al., 2012), makes these technologies very promising for a mild biorefinery approach. However, US is generally associated to local extreme temperatures. Therefore, PEF was chosen as novel cell disintegration technology in this thesis.

Pulsed Electric Field

PEF was initially not designed for cell disintegration but as an alternative for thermal food conservation techniques, a so called ‘cold-pasteurization’ (Jeyamkondan et al., 1999). Therefore this method is considered mild i.e. no use of detrimental chemicals, high temperatures or high pressures. Not only PEF shows to be an effective pasteurization method (Donsì et al., 2010; El Belghiti and Vorobiev, 2004; Fox et al., 2008; Rieder et al., 2008; Selma et al., 2003), it is also able to improve the extraction efficiency of components from plants (Bluhm and Sack, 2009; Loginova et al., 2011).

PEF works by the formation of pores in the cell membrane. By applying an electric field that induces an increase in the transmembrane potential, aqueous pores can be formed. When

the electric pulse is short, the pore can be reversible, but at a certain pulse duration the pore becomes irreversible (Kotnik et al., 2012).

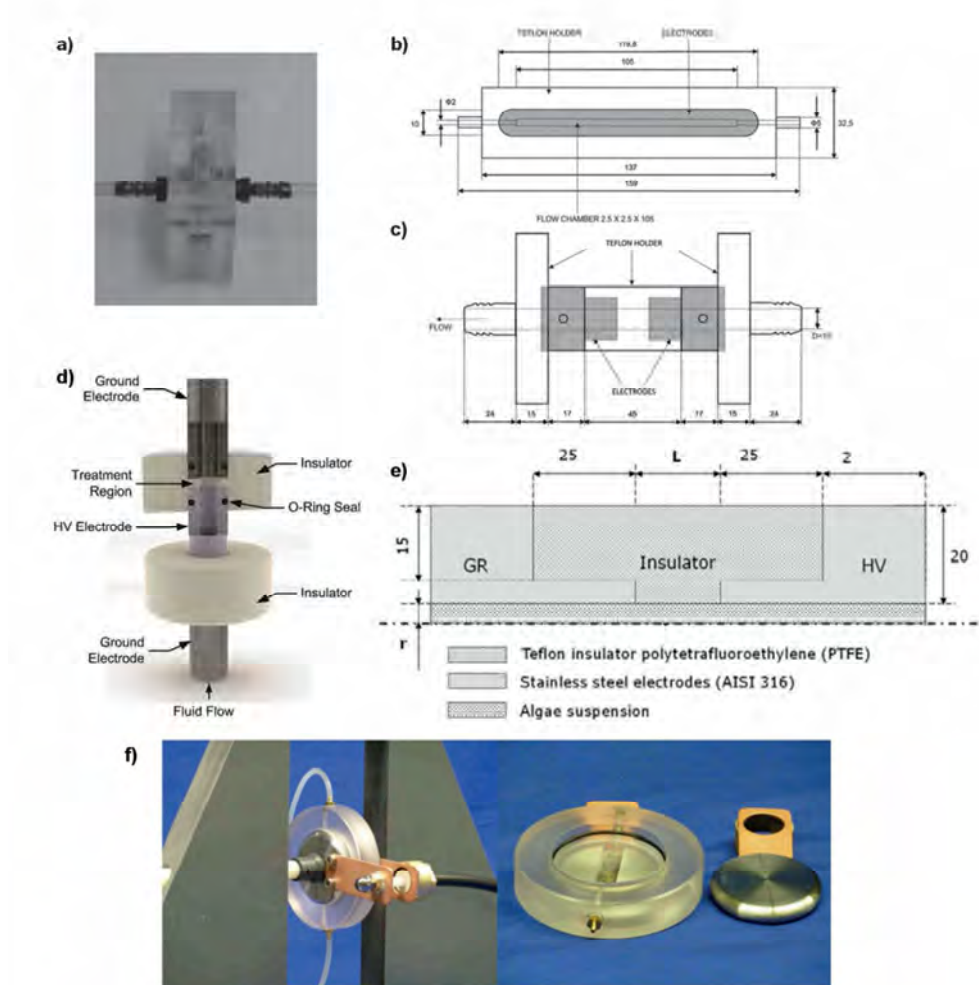


Figure 1.3 a) Flow pulsing chamber (Coustets et al., 2013), b) Cross-field chamber (Flisar et al., 2014), c) co-field chamber (Flisar et al., 2014), d) co-linear treatment chamber (Diversified Technologies, 2010), e) geometry of co-linear treatment chamber (Postma et al., 2016b), f) Cross field flow cell (Goettel et al., 2013). With permission from Springer and Elsevier.

Because microalgal cells are relatively small (3-20 μm), the required field strength is relatively high compared to animal or plant cells. To achieve those electric fields, different treatment chambers have been proposed by several research groups who are working on the application of PEF on microalgae. The most common applied chamber geometries include cross-field, co-field and co-linear treatment chambers for continuous flow processing

(Figure 1.3) (Diversified Technologies, 2010; Coustets et al., 2013; Goettel et al., 2013; Flisar et al., 2014). In addition, electroporation cuvettes or similar batch systems have been used as well (Frey et al., 2013). Next to the before mentioned electric field strength and chamber geometry, several other process variables influence the PEF process amongst which: pulse duration, pulse number/frequency, suspension conductivity, suspension flow rate (in case of continuous operation) (Kotnik et al., 2015).

1.3 Aim and thesis outline

An increased worldwide protein demand for food and feed and the necessity to release the water soluble proteins in the first stage of the cascade biorefinery require the development of mild protein extraction technologies. Cell disintegration is the first hurdle and is considered as one of the most energy consuming steps. Therefore, the aim of this thesis is to develop a mild, scalable and energy efficient cell disintegration technology for the extraction of water soluble proteins from microalgae and macroalgae.

In **Chapter 2** a benchmark procedure by means of bead milling for the disintegration of the green microalgae *Chlorella vulgaris* is presented. This benchmark was set by optimization of two major influencing process variables (agitator speed and biomass concentration) in a Design of Experiments set-up using a laboratory scale batch bead mill. The disintegration rate and water soluble protein release rate are discussed. In addition the effect of both rates on specific energy consumption of bead milling is presented.

Since only a limited number of process variables were optimized in **Chapter 2**, two other crucial parameters during bead milling were investigated in **Chapter 3**, namely the bead size and the algae species. Next to *C. vulgaris*, also *Neochloris oleoabundans* and *Tetraselmis suecica* were subjected to bead milling starting from the previous set benchmark. The effect of a decreasing bead size during bead milling for each algae species is discussed in terms of process kinetics, protein and carbohydrate yields and specific energy consumption. In addition, using the so-called stress theory, more insight was obtained in the breakage mechanism of the three algal species.

The previous two chapters involved the application of a conventional technique for cell disintegration, while in **Chapter 4** a screening study was performed on the applicability of PEF for microalgae cell disintegration. Given the little consensus in literature about the actual performance of PEF for the extraction of proteins from microalgae, a systematic screening over a wide range of operating conditions (electric field strength, pulse number, pulse duration, specific energy consumption and batch versus continuous flow mode) was

performed on both *C. vulgaris* and *N. oleoabundans* in a direct comparison with benchmark technologies.

Inactivation of microorganisms for pasteurization purposes using PEF was shown to be improved by elevated processing temperatures. Therefore, in **Chapter 5** the effect of the processing temperature during PEF treatment on the disintegration of the microalgae *C. vulgaris* was investigated. The PEF-Temperature treatment was conducted using a pilot scale continuous flow electroporation unit at specific energy consumptions competitive with the benchmark while the temperature was varied between 25 and 65°C. The effect of the PEF-Temperature treatment on the release of ions, carbohydrates and protein is discussed.

In **Chapter 6**, the focus was shifted from microalgae towards macroalgae. The first major difference between the disintegration of microalgae and macroalgae is the presence of the macro structure in seaweeds. This bottleneck limits the applied technologies (e.g., bead milling not applicable due to required bead separation) during biorefinery to batch processes, especially during the disintegration. In this chapter four batch processing technologies for macroalgae disintegration are screened and discussed, being; osmotic shock, enzyme incubation, PEF and HSH. Because of the presence of interesting carbohydrates next to the protein, the release of both products is discussed and evaluated.

Chapter 7 reflects on the major outcomes, restrictions and remaining knowledge gaps regarding the microalgae work presented in this thesis. Future research proposals are presented for microalgae disintegration and it is also discussed how selective product extraction could aid further downstream processing and what the implications are for product applications. A strategy including bottlenecks for scaling up mechanical disintegration is proposed based on the current findings. Furthermore, an economic perspective for a protein biorefinery is discussed. Finally, the thesis results are reviewed with respect to literature and a future outlook on algae biorefinery is presented.



Chapter 2

Mild disintegration of the microalgae *Chlorella vulgaris* using bead milling

The contents of this chapter have been published as:

P.R. Postma, T.L. Miron, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink
Mild disintegration of the microalgae Chlorella vulgaris using bead milling

Bioresource Technology (2015) 184, pp. 297-304

Abstract

In this work, the mild disintegration of the microalgae *Chlorella vulgaris* for the release of intracellular products has been studied. By means of bead milling the microalgae suspensions were successfully disintegrated at different biomass concentrations (25-145 g_{DW} kg⁻¹) over a range of agitator speeds (6-12 m s⁻¹). In all cases over 97% of cell disintegration was achieved resulting in a release of water soluble proteins. A clear optimum rate of disintegration and protein release was observed at an agitator speed of 9-10 m s⁻¹ regardless of the biomass concentration. Selective extraction of water soluble proteins was observed as proteins released sooner than cell disintegration took place. Proteins could be released at 85% lower energy input than for cell disintegration resulting in specific energy consumptions well below 2.5 kWh kg_{DW}⁻¹.

2.1 Introduction

There is an ongoing search for new protein sources to meet the growing global food and animal feed demand (Godfray et al., 2010; Tilman et al., 2011). Microalgae can be identified and developed as novel green sources for food and/or feed (Batista et al., 2013; Becker, 2007). The microalgae *Chlorella vulgaris* contain 48-58 % (w/w DW biomass) protein, 8-17 % carbohydrates, 14-22 % lipids and 6-9 % ash (Spolaore et al., 2006). Besides, the amino acid composition of *Chlorella vulgaris* compares favorably to other food like soybean as well as the World Health Organization reference composition (Becker, 2007).

With the current estimated production costs of microalgae being € 4-10 kg⁻¹ dry weight (Norsker et al., 2011; Wijffels et al., 2010), solely production of a single product, like lipids or proteins, from microalgae is not economically feasible. All valuable components such as proteins, lipids and carbohydrates must be used after extraction and separation from the microalgal biomass in order to become competitive as a source for commodities (Wijffels et al., 2010).

Biorefinery comprises the downstream processing of all products produced by microalgae after cultivation. It should be taken into account, that for most biotechnological processes the costs for downstream processing can be regarded as very influential on the total costs (Delrue et al., 2012) and account up to 60% of the total product production costs (Molina Grima et al., 2003). Therefore, effort should be put in minimizing the energy requirements and capital costs (Vanthoor-Koopmans et al., 2013), not only by more energy efficient processes, but also by improving the product yield of the individual process steps with respect to downstream processing.

To obtain the valuable intracellular components from the microalgae, cell disintegration is the first step to take after cell harvesting (Grimi et al., 2014; Molina Grima et al., 2003; Safi et al., 2014a). Various methods for cell disintegration have been applied, e.g. enzymatic treatment, alkali treatment, high-pressure homogenization, bead milling, microwaves, pulsed electric field and ultrasonication (Doucha and Lívanský, 2008; Gerken et al., 2013; Grimi et al., 2014; Lee et al., 2010; Safi et al., 2014a; Zheng et al., 2011). Many of those disintegration treatments expose the cells and its content to harsh conditions like high pressures, high shear levels or high temperatures. To secure the solubility and functionality of water soluble proteins, temperatures above 35°C should be avoided. Therefore, this process step needs to be performed under mild conditions i.e. avoid loss of product functionality. However, mild methods could result in low extraction yields of the products of interest (Grimi et al., 2014). On the other hand, Grimi et al. (2014) also showed that with the

use of generally regarded harsh methods, selective extraction of several microalgal components, among which water soluble proteins, is possible.

Recent design improvements and the possibility to control the temperature of the algae suspension, make the bead mill a suitable disintegration technique. A bead mill is a homogenizer originally designed for size reduction of paint or lacquer particles (Kula and Schütte, 1987). However, it has shown to be a useful technique for the mild disintegration of microbial cells and the release of proteins from these cells as well (Balasundaram et al., 2012; Balasundaram and Pandit, 2001; Chisti and Moo-Young, 1986; Kula and Schütte, 1987). Doucha and Lívanský (2008) showed that bead mills also can be used for disintegration of microalgae. Schwenzfeier (2011) used a bead mill for disintegration of microalgae, which were kept frozen before use, for the release of soluble proteins. Nevertheless, to our knowledge bead mills have so far not been used for mild release of water soluble proteins from fresh microalgae.

A bead mill can be operated under batch (recirculation) or continuous (single passage through milling chamber) conditions with respect to the biomass suspension. Many parameters affect the cell disruption efficiency, including: suspension feed rate for continuous operation and process time for batch operation (i.e., the residence time distribution), agitator speed, agitator design, milling chamber design, biomass concentration, bead diameter, bead density and the bead filling (Doucha and Lívanský, 2008). From these parameters the biomass concentration (C_x) and agitator speed (u_s) have been identified as the most influential parameters on process time, disintegration efficiency and on the energy consumption.

The aim of this work is to set the bead mill as a benchmark for mild disintegration technologies. This is achieved by evaluating the effect of biomass concentration and agitator speed during bead milling on water soluble protein extractability from *C. vulgaris*. In addition the process is optimized considering soluble protein yield, energy consumption and process time.

2.2 Methods

2.2.1 Microalgae and cultivation

The green microalgae *Chlorella vulgaris* (SAG 211-11b, EPSAG Göttingen) were grown in repeated batches in M8a-medium (Mandalam and Palsson, 1998) at 25°C. The 12 L stirred tank photobioreactor (PRB) was stirred at 300 RPM and air was supplied at 3 L min⁻¹ with on demand CO₂ supply to control the pH at 7.0. The PBR was illuminated with 50 Halogen lamps

(Philips Master Line ES, 45W, 60°), equally distributed over the reactor surface. The incident light intensity was increased from 400 to 1100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ along the course of biomass growth. For consistent biomass production, the algae of different batches were harvested during late linear growth at an optical density at 750 nm (OD_{750}) of ~ 15 (corresponding to $\sim 5 \text{ g kg}^{-1}$). The algae suspensions were stored at 4 °C for maximal seven days until further use. Prior to disintegration the algal suspensions were concentrated by centrifugation (3500 x g, 20 min and 4 °C) and the supernatant was discarded. The biomass pellet was resuspended in phosphate buffered saline (PBS) (1.54 mM KH_2PO_4 , 2.71 mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 155.2 mM NaCl at pH 7.0) to obtain suspensions with a C_x of 25, 87.5 or 145 g kg^{-1} in a total volume of 185 ml. C_x is expressed as g dried biomass per kg algae suspension.

2.2.2 Bead mill experimental setup

A Dyno-Mill Research Lab (RL) from Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland was used for disintegration experiments. The DYNO-mill consists of a horizontal milling chamber ($V_{\text{chamber}} = 79.6 \text{ ml}$) with the engine connected over a central shaft. The algal suspension is fed to a worm screw mounted on the same shaft by a feed funnel. The beads are accelerated in a radial direction opposite to the rotational direction of the shaft by a single DYNO-accelerator ($\varnothing 56.2 \text{ mm}$). Different layers of beads will be formed moving at different speeds causing the grinding effect. The major part of the energy introduced will be converted to heat due to friction. A cooling jacket integrated in the milling chamber and a cooling coil in the feed funnel were used to remove the heat and to keep the temperature below 35 °C. An external water bath was connected to the cooling jacket and coil. The algal suspension and the grinding beads are separated by a sieve plate at the outlet. After which the algal suspension is fed back to the feed funnel.

Based on the advice and experience of the manufacturer $\varnothing 1 \text{ mm}$ ZrO_2 beads (specific density 6.0 g cm^{-3} , bulk density 3.8 g cm^{-3}) (Tosoh YTZ®) were chosen at a milling chamber filling percentage of 65 % v/v. This results in a free liquid volume (V_{liquid}) inside the chamber of 46.8 ml. During initial trials these parameters showed promising results for algal cell disintegration (data not shown) and were therefore fixed for further experiments.

2.2.3 Design of experiments

Table 2.1 Parameters and values used in CCF design.

Parameters	Factor	Low value (-1)	Centre value (0)	High value (+1)
Biomass concentration (g kg^{-1})	X1	25	87.5	145
Agitator speed (m s^{-1})	X2	6	9	12

Modde v.9.1 Design of Experiments (DOE) software (MKS Umetrics, Sweden) was used as described by Desai et al. (2014a) to study the effect of C_x and u_s on the cell disintegration rate k_{dis} , soluble protein release rate k_{prot} , pigment release rate k_r , specific energy consumption E_M and soluble protein yield Y_p during bead milling. For the DOE a response surface modelling (RSM) design, or more explicit a central composite face-centered design (CCF), was used. Two independent parameters (factors), u_s and C_x at three levels were studied, which leads to 8 experiments covering the design space. To give a good estimation of the replicate error, three replicates at the center point values were conducted. The coded values for each parameter are shown in Table 2.1.

2.2.4 Analytical methods

Biomass quantification

Dry weight concentration was determined as described by Lamers et al. (2010). The cells were washed using $0.2 \mu\text{m}$ filtered deionized water. For each harvest two known dilutions were prepared of which the cell dry weight (DW) was determined as well as the OD_{750} . DW/ OD_{750} ratios were determined being $0.312 \pm 0.022 \text{ SD}$.

Sample acquisition during bead milling

During the bead milling experiments samples of a known amount of biomass (wet weight) were obtained from the feed funnel after 0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0, 45.0 and 60.0 minutes. Samples from 25, 87.5 and 145 g kg^{-1} experiments were diluted 1, 2 and 5 times respectively prior to further analysis. For water soluble proteins, the pellet and supernatant were separated by centrifugation (20 min, $20,000 \times g$) and the supernatant was stored at -80°C until further use.

Disintegration percentage analysis

To determine the efficiency of the disintegration process fresh samples of algae suspension from the bead milling experiments were analyzed using a flow cytometer (BD Accuri C6). The algae suspensions were diluted to a total volume of 1 mL using PBS buffer (pH 7.0) to acquire samples at less than $2500 \text{ events sec}^{-1}$. A fixed volume of $15 \mu\text{L}$ was measured at a fluidics

rate of $35 \mu\text{L min}^{-1}$ and a core size of $16 \mu\text{m}$. Forward scattering FSC , and Chlorophyll Auto-Fluorescence (670 nm filter) FL_{chl} were measured. " $FL_{chl} \cdot \text{count}/\mu\text{L}$ " before ($FL_{chl,0}$) and during (FL_{chl}) disintegration was used to describe the disintegration degree Dis , as:

$$Dis = \frac{FL_{chl,0} - FL_{chl}(t)}{FL_{chl,0}} \quad (2.1)$$

Protein analysis

For total protein content on dry weight basis, 6 mg of freeze dried biomass from start samples was re-suspended in 1.0 mL lysis buffer I (60 mM Tris, 2% SDS, pH 9.0) in lysing matrix E tubes (6914-500, MP Biomedicals Europe). The tubes were bead beaten for 3 cycles of 60 seconds at 6500 RPM with breaks of 120 seconds between cycles using a bead beater (Precellys 24, Bertin Technologies).

For determination of the soluble protein content in the supernatant after disintegration, the supernatant samples were unfrozen and diluted 2 times using lysis buffer II (120 mM Tris, 4% SDS, pH 9.0).

Subsequently, the samples for total and soluble protein content were incubated at 100°C for 30 minutes. The protein quantitation was performed with a commercial assay (DCTM Protein assay, Bio-Rad, U.S.) using bovine serum albumin (Sigma-Aldrich A7030) as protein standard. The microplate assay protocol was followed, where $10 \mu\text{L}$ of each standard and unknown was used. Absorbance was measured at 750 nm using a microplate reader (Infinite M200, Tecan, Switzerland) in 96-well plates (Greiner bio-one 655101). The fraction of released water soluble proteins P can be expressed as:

$$P = \frac{C_p(t)}{C_{p,biomass}} \quad (2.2)$$

in which $C_p(t)$ is the protein concentration in the supernatant at time t , and $C_{p,biomass}$ the total amount of protein inside the biomass per kg suspension.

The water soluble protein yield can be described as:

$$Y_p = \frac{C_{p,max}}{C_{p,biomass}} \quad (2.3)$$

in which $C_{p,max}$ is the maximal water soluble protein concentration at the end of the experiment.

Pigment release

The release rate of pigments was determined by measuring the absorbance between 400 and 750 nm of the supernatant using a microplate reader (Infinite M200, Tecan, Switzerland). Supernatant samples were diluted two times using PBS buffer to a total volume of 80 μL . The absorbance at 435 nm was used as a measure for the pigment (Chlorophyll) release. The fraction of released pigment R can be expressed by:

$$R = \frac{A_{435\text{nm}}(t)}{A_{435\text{nm},\text{max}}} \quad (2.4)$$

in which $A_{435\text{nm}}(t)$ is the absorption at time t during bead milling, and $A_{435\text{nm},\text{max}}$ the absorption observed at the end of the bead milling experiment.

Viscosity measurement

The viscosity of the algae suspensions was measured using an MCR 502 rheometer (Anton Paar). For algae suspensions up to 87.5 g kg^{-1} a double gap (DG26.7) measuring system was used, while a cone-plate (CP50-4) was used for suspension of 145 g kg^{-1} . The temperature was controlled at 12 $^{\circ}\text{C}$.

2.2.5 Bead milling kinetics

First order kinetics for bead milling processes (i.e., cell disintegration and cell content release) have been proposed by many authors (Chisti and Moo-Young, 1986; Doucha and Lívanský, 2008; Kula and Schütte, 1987; Limon-Lason et al., 1979). The degree of disintegration can be described by:

$$Dis = 1 - e^{-k_{dis} \cdot t} \quad (2.5)$$

where k_{dis} is the kinetic rate constant for the fraction of disintegration. Similar expressions can be obtained for the fraction of released water soluble proteins P and fraction of released pigments R :

$$P = 1 - e^{-k_{prot} \cdot t} \quad (2.6)$$

where k_{prot} is the rate constant for water soluble protein release.

$$R = 1 - e^{-k_r \cdot t} \quad (2.7)$$

in which k_r is the rate constant for the release of pigments.

Location factor

The value for the location factor is usually obtained by taking the ratio of one released product to the other (Balasundaram and Pandit, 2001). The same concept can be applied to express the relative release ratio of release rate of product i to the disintegration rate as:

$$LF_i = \frac{k_i}{k_{dis}} \quad (2.8)$$

Where k_i is the release rate of product i , where i can be either water soluble proteins (LF_{prot}) or pigments (LF_r). When the ratio of LF_i is smaller than unity, the extraction of a product is slower than the disintegration rate, this could happen when a product is hardly water soluble or is located in intracellular organelles. Hence, when the ratio becomes larger than unity, the product is released faster than the disintegration rate. For instance cytoplasmic components can already easily be released by a single rupture of the cell wall.

Residence time

The Dyno-Mill RL was operated in recirculation mode (i.e., batch operation). This means that only a part of the total volume V_{total} (~185 mL) is inside the milling chamber, with volume $V_{chamber,free}$ (46.8 mL), at all times during recirculation. The volume ratio $V_{chamber,free}/V_{total}$ can be used to describe the residence time of the algae suspension in the milling chamber. Therefore, the time t of a single experiment was corrected by multiplying with $V_{chamber,free}/V_{total}$.

2.3 Results & Discussion**2.3.1 Bead milling overall effect**

Using the chosen settings for C_x and u_s all algae suspensions could successfully be disintegrated at disintegration degrees over 97% and water soluble proteins were released. For one of the centre point runs (C_x : 87.5 g kg⁻¹, u_s : 9 m s⁻¹), the effect of bead milling on k_{dis} , k_{prot} and k_r are shown in Figure 2.1. Besides, the first-order model predictions can be observed (R^2 values see appendix A). Figure 2.1 shows that more than 99% of cell disintegration can be obtained in a milling time of about 500 seconds. Besides, after 200-250 seconds, roughly 90-95% of the cells are already disintegrated. The first order model slightly underestimates this. By fitting the data to the first order model from equation (2.5) using least square error regression (LSER) a k_{dis} of 0.0090 s⁻¹ was obtained. The maximum released protein concentration in Figure 2.1 is 17.5 mg mL⁻¹. However, the model estimates a maximum concentration of 17.8 mg mL⁻¹. A corresponding k_{prot} of 0.0095 s⁻¹ was obtained by

LSER of equation (2.6). Over 90% of the proteins can be extracted within 200 seconds, corresponding to an LF_{prot} of 1.27, which indicates that the protein can be released faster than disintegration takes place. Figure 2.1 also shows that there is a large difference between the release rate of the pigments and the disintegration rate of the cells. The estimated k_r of 0.0048 s^{-1} results in an LF_r of 0.52. These location factors show that different intracellular products can be selectively extracted with respect to the disintegration degree as also described by other authors (Balasundaram and Pandit, 2001; Grimi et al., 2014).

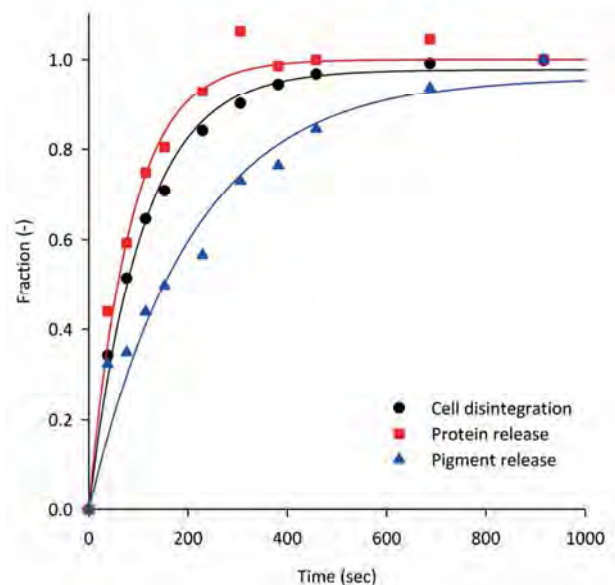


Figure 2.1 Fraction of cell disintegration (●), fraction of released protein (■) and fraction of released pigment (▲) versus time for a C_x of 87.5 g kg^{-1} and u_s of 9 m s^{-1} (experiment 6). The solid lines represent the first order model.

2.3.2 Effect of bead milling on process kinetics

Using eq. (2.5), (2.6) and (2.7) the release rates of the intracellular components, the disintegration rate and the maximum water soluble protein concentration were estimated. Different values for u_s and C_x were used to study the effect on the kinetic rate constants for disintegration, protein release and pigment release, the results are summarized in Table 2.2. R^2 values of 0.97 or higher were obtained for all disintegration and protein release fits, R^2 values for pigment release were above 0.85. First the single effects will be discussed and subsequently the interaction effects.

Table 2.2 Overview of performed experimental runs and first-order model values.

Experimental conditions			Model values				Location factors	
Exp. no.	C_x (g kg ⁻¹)	u_s (m s ⁻¹)	$C_{p,max}$ (g kg ⁻¹)	k_{dis} (s ⁻¹)	k_{prot} (s ⁻¹)	k_r (s ⁻¹)	LF_{prot} (-)	LF_r (-)
1	25	6	4.6	0.0051	0.0061	0.0025	1.22	0.50
2	25	9	4.3	0.0056	0.0087	0.0016	1.55	0.28
3	25	12	4.2	0.0056	0.0069	0.0014	1.23	0.26
4	87.5	6	14.1	0.0061	0.0079	0.0034	1.30	0.56
5	87.5	9	17.2	0.0125	0.0119	0.0029	0.95	0.23
6	87.5	9	17.8	0.0093	0.0119	0.0048	1.27	0.52
7	87.5	9	13.9	0.0090	0.0095	0.0035	1.05	0.39
8	87.5	12	17.5	0.0091	0.0113	0.0031	1.24	0.34
9	145	6	22.2	0.0075	0.0130	0.0083	1.73	1.11
10	145	9	26.6	0.0124	0.0202	0.0128	1.63	1.03
11	145	12	25.8	0.0114	0.1580	0.0062	1.39	0.54

For the first-order kinetics, the rate constant for disintegration should be independent of the initial C_x . However, it can be observed that C_x has a large impact on k_{dis} , k_{prot} and k_r . Higher values for C_x lead to a higher release rate of intercellular components and to a higher disintegration rate up to a factor 3 and 2 respectively. The rheology of the algal suspensions most likely directly influences the rate constants. Algal slurries show Newtonian behavior at a C_x below 40 g kg⁻¹, and above this concentration the behavior becomes shear thinning non-Newtonian (pseudoplastic) (Wileman et al., 2012). This pseudoplastic behavior was also found for the algal suspensions used in this work at C_x values of 87.5 and 145 g kg⁻¹ (data not shown), while the suspension of 25 g kg⁻¹ showed Newtonian behavior as expected. The effective viscosity μ_{eff} at a shear rate $\dot{\gamma}$ of 500 s⁻¹, for a C_x of 25, 87.5 or 145 g kg⁻¹ was found to be $1.77 \cdot 10^{-3}$, $8.75 \cdot 10^{-3}$ and $9.35 \cdot 10^{-2}$ Pa s, respectively. This shows that there is a large difference between the effective viscosities of the different algal suspensions. Especially the high viscous slurries at a C_x of 145 g kg⁻¹ were more difficult to handle with respect to biomass losses and mixing properties and is therefore practically limiting. Besides, the power requirement for pumping of those slurries increases significantly (Wileman et al., 2012). The viscosity of algal slurries is species depended, and therefore it is safer to say that a slurry viscosity around $9 \cdot 10^{-2}$ Pa s is practically limiting for the DYNO-mill.

The shear rates to be expected in a bead mill are in the order of 10^4 - 10^5 s⁻¹ (Limon-Lason et al., 1979), the differences in the applied u_s are expected to be negligible with respect to the high shear rates. Therefore shear rates in the same order of magnitude are assumed at u_s between 6 and 12 m s⁻¹. The energy dissipation S towards the suspension causing collision of the beads is related to the shear rate by $S = \mu_{eff} \dot{\gamma}^2$ (Gers et al., 2010). At constant shear rates, this means that S , and therefore k , is directly influenced by the suspension viscosity. Gers et al. (2010) observed that the region of high energy dissipation decreased with

increased viscosity (Reynolds number), while the maximum energy dissipation S_{max} increased.

Doucha et al. (2008) found an opposing effect of C_x on the degree of disintegration of *Chlorella* cells. For a Dyno-Mill KDL and a Labstar LS1 the degree decreased, while it increased for the MS 18 homogenizer. Mogren et al. (1974) found similar disintegration degrees for yeast cells at a varying C_x in a Dyno-Mill KD5. Nevertheless, they also observed a large dependence on the used strain. Limon-Lason (1979) found that the disintegration rate constant decreased with an increasing C_x . Marffy (1974) found increasing rate constants for an increasing C_x using a Dyno-Mill KDL.

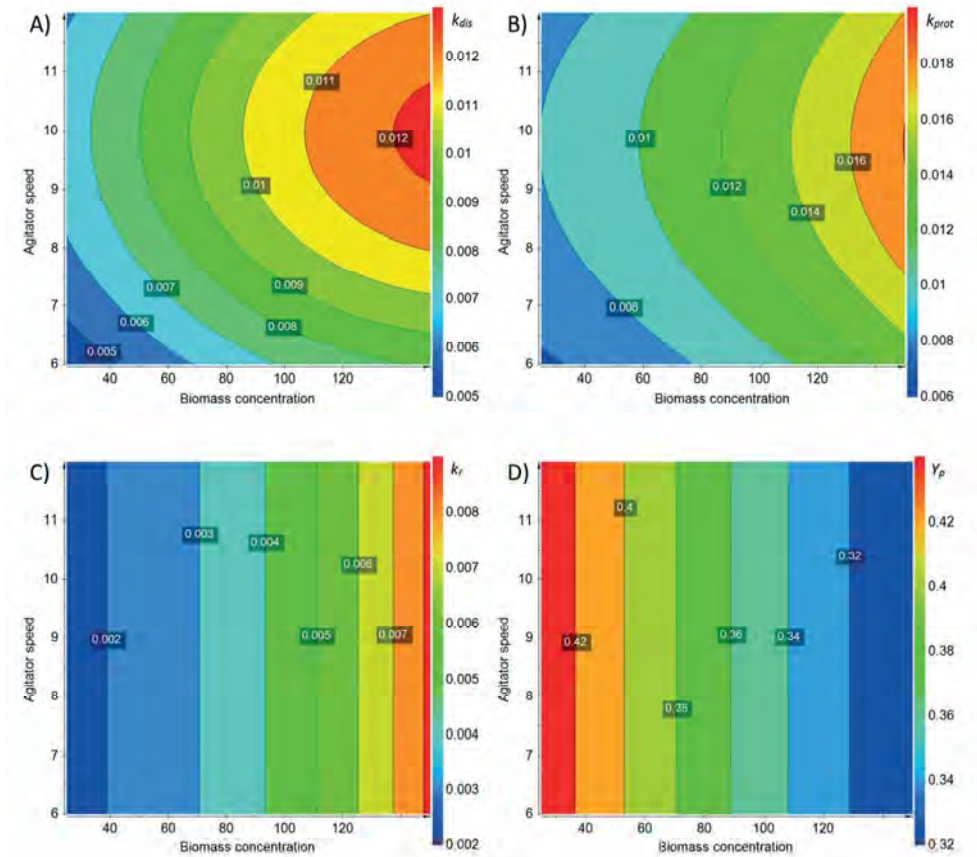


Figure 2.2 Contour plots of agitator speed u_s [$m\ s^{-1}$] and biomass concentration C_x [$g\ kg^{-1}$] on the (A) disruption rate constant k_{dis} [s^{-1}], (B) protein release rate constant k_{prot} [s^{-1}], (C) pigment release rate constant k_r [s^{-1}] and (D) protein yield Y_p .

For u_s , it can be seen that a higher speed resulted in higher disintegration rates. This effect can be explained by the increase of the impact forces and frequency with increasing u_s . However an optimum can be observed in Figure 2.2A and 2b at 9-10 m s^{-1} for k_{dis} and for k_{prot} . No significant difference ($p > 0.05$) could be found for k_r (Figure 2.2C). An optimum kinetic rate constant was also observed by other authors (Currie et al., 1972; Kula and Schütte, 1987; Limon-Lason et al., 1979; Marffy and Kula, 1974). In order to break the cells, a certain stress intensity needs to be applied, however once this amount is supplied an additional increase of stress intensity cannot be utilized for faster disintegration (Bunge et al., 1992). Nevertheless, a clear decrease in the kinetic rate constant can be observed as well at u_s above 10 m s^{-1} . During disintegration, the cell contents are released among which DNA, causing an increase of the viscosity. On the other hand, due to the temperature increase of the suspension the viscosity is decreasing. Based on the viscosity measurements it was observed that these two effects might cancel each other out and therefore the effective viscosity is approximately constant at high shear rates in the bead mill (data not shown). Yet, Gers et al. (2010) found that for increasing Reynolds numbers (i.e., dimensionless number for flow regime characteristics), which may either be caused by an increased viscosity or increased agitation speeds, a significant shift in the flow patterns occurs. With the secondary effect that S_{max} decreased for Newtonian and shear thinning non-Newtonian suspensions.

When looking to the interactions effects, Figure 2.2A and 2B show that at a high C_x and at a medium to high u_s (9-12 m s^{-1}) higher rate constants can be achieved compared to a low C_x or a low u_s .

2.3.3 Protein yield

The influence of C_x and u_s on the water soluble protein yield Y_p is shown in Figure 2.2D. No significant effect ($p > 0.05$) was observed for u_s , but it can be observed that the Y_p decreases about 25% when the C_x increased from 25 to 145 g kg^{-1} . A possible cause might be the formation of insoluble protein aggregates at a high C_x due to local high protein concentrations, shear forces and interactions with other components as suggested by Wang (2005). Temperature increase was not assumed to influencing protein aggregation since the temperature was kept below 35 °C in all experiments. Besides, protein stability was aimed to be enhanced by using a PBS buffer, the NaCl can increase protein solubility (Bondos and Bicknell, 2003). Nevertheless, the absolute amount of extracellular PBS buffer in the different algae suspensions is decreased with increasing algae concentration because the algae occupy more space in suspensions at a higher C_x . When the algae break during disintegration the medium composition shifts from PBS like towards the algae intracellular matrix. Especially at a high C_x the negative interactions with other components might be

increased causing the formation of insoluble proteins. It was observed that these proteins are not resolvable. A similar decreasing trend in protein yield was observed by Limon-Lason et al. (1979) for disintegration of packed yeast and Schwenzfeier et al. (2011) obtained a water soluble protein concentration yield of 21% by disintegration of the green microalgae *Tetraselmis* sp. at a C_x of 120 g kg^{-1} . These soluble proteins showed good techno-functional properties in comparison to other food proteins. Indicating that solubility of proteins is an important characteristic for mild disintegration.

2.4 Energy consumption

The specific energy consumption E_M of a bead mill is critical for the final process economics and sustainability. For all performed experiments, a linear increase in the specific energy consumption was observed in time. Besides, the idle energy consumption (i.e., no cells) of the Dyno-Mill accounted for 70-85% of the total energy consumption. Due to the high energy dissipation at a C_x of 25 g kg^{-1} and u_s of 12 m s^{-1} , excessive temperature increases were observed which required breaks during the process to cool down the algae suspension. No temperature issues were observed at the other conditions for a total process time of 60 minutes.

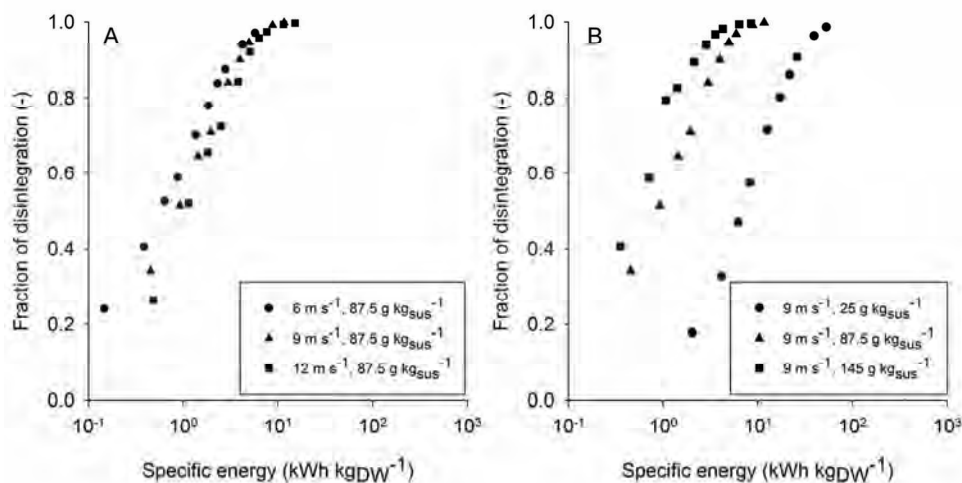


Figure 2.3 Influence of u_s [m s^{-1}] on (A) specific energy consumption and (B) influence of C_x [g kg^{-1}] on specific energy consumption [$\text{kWh kg}_{\text{DW}}^{-1}$].

Figure 2.3A shows the influence of u_s at a C_x of 87.5 g kg^{-1} on the fraction of disintegration as function of the specific energy consumption, it can be seen that the specific energy consumption almost triples when increasing u_s from 6 to 12 m s^{-1} . Looking at a constant u_s of

9 m s⁻¹ for different values of C_x in Figure 2.3B, it can be clearly observed that a higher C_x can be disintegrated quicker at lower energy expenses.

Table 2.3 gives an overview of the specific energy consumption E_M . It can be seen that the specific energy consumption decreases with increasing C_x and with decreasing u_s . The contour plot in Figure 2.4A shows that the specific energy consumption is most energy efficient at low to moderate u_s and medium to high C_x . In this case, E_M is kept below 10 kWh kg_{DW}⁻¹ which is similar to what Doucha and Lívanský (2008) reported for a disintegration degree of 90.6%. Nevertheless, when taking the kinetic rate constants into account as described above, selective extraction of water soluble proteins is possible before reaching full disintegration of the algae suspension.

Table 2.3 Overview of specific energy consumption.

Experimental conditions			Energy consumption							
Exp. no.	C_x (g kg _{sus} ⁻¹)	u_s (m s ⁻¹)	M (g _{DW})	DIS (%)	P (kWh)	E_M (kWh kg _{DW} ⁻¹)	τ (sec)	$P_{3\tau}$ (kWh)	$E_{M,3\tau}$ (kWh kg _{DW} ⁻¹)	η (%)
1	25	6	4.7	98.4	0.094	20.45	113.9	0.035	7.64	125.6
2	25	9	4.7	98.6	0.247	53.42	80.0	0.065	14.10	231.8
3	25	12	4.6	99.5	0.424	91.98	100.5	0.140	30.38	499.4
4	87.5	6	16.2	97.1	0.093	5.92	87.3	0.027	1.71	28.0
5	87.5	9	16.2	99.7	0.180	11.12	58.1	0.034	2.12	34.9
6	87.5	9	16.2	99.8	0.188	11.67	58.3	0.036	2.23	36.6
7	87.5	9	16.1	99.7	0.181	11.24	73.0	0.044	2.71	44.5
8	87.5	12	16.3	99.7	0.248	15.23	61.6	0.050	3.10	50.9
9	145	6	27.0	97.6	0.120	4.55	53.4	0.021	0.81	13.3
10	145	9	26.9	99.6	0.229	8.51	34.3	0.026	0.96	15.8
11	145	12	26.8	98.6	0.288	10.87	43.9	0.042	1.58	25.9

The residual protein half-lie time τ (i.e., characteristic time of the protein release kinetic) can be described as:

$$\tau = k_{prot}^{-1} \cdot \ln(2) \quad (2.9)$$

An overview of τ for each experiment is listed in Table 2.3. From Figure 2.3 it can be observed that disintegration degrees beyond 85-90% requires excessive amounts of additional energy which is in close relation to what was found by Mogren et al. (1974). Therefore 3 characteristic times 3τ , corresponding to 87.5% protein release, were considered to recalculate the specific energy consumption $E_{M,3\tau}$ with:

$$E_{M,3\tau} = E_M \cdot \left(\frac{3\tau}{t}\right) \quad (2.10)$$

where t is the total time of the experiment (s). This results in an energy reduction between 60 and 88%. The contour plot with the aim on protein release is shown in Figure 2.4B. The

contour line corresponding to $10 \text{ kWh kg}_{\text{DW}}^{-1}$ shifted drastically, opening the possibility to operate at a low C_x . For a C_x above 60 g kg^{-1} specific energy consumptions below $3 \text{ kWh kg}_{\text{DW}}^{-1}$ can be achieved. Doucha and Lívanský (2008) found $3.11 \text{ kWh kg}_{\text{DW}}^{-1}$ at 58% disintegration and $2.82 \text{ kWh kg}_{\text{DW}}^{-1}$ at 77% disintegration for suspensions of *Chlorella* although no values for protein release were reported. Balasundaram et al. (2012) designed a ball mill capable of disintegrating the cyanobacteria *Chlorogloeopsis fritschii* at an E_M of $1.87 \text{ kWh kg}_{\text{DW}}^{-1}$. In this study the lowest $E_{M,3\tau}$ of $0.81 \text{ kWh kg}_{\text{DW}}^{-1}$ was found at a C_x of 145 g kg^{-1} and an u_s of 6 m s^{-1} . Taking the energy content of algae as 21.9 kJ g^{-1} biomass (Weyer et al., 2010), the energy consumption per energy content η of the algae is then 13.3%. For the other experiments the ratio η is shown in Table 2.3.

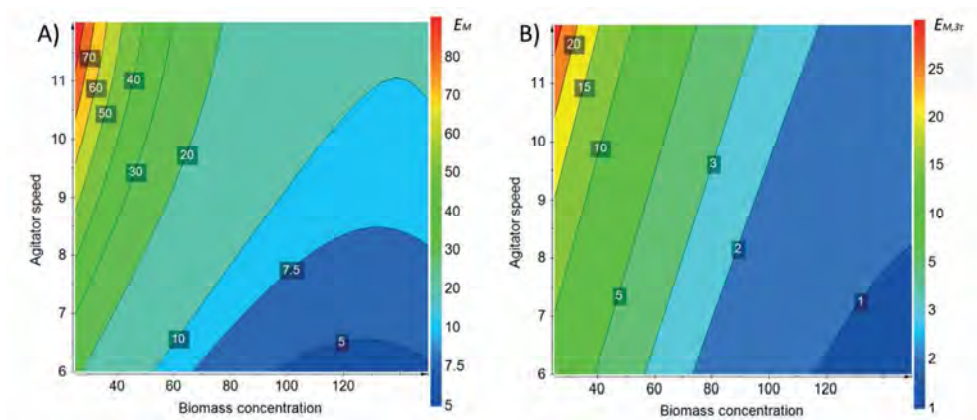


Figure 2.4 Contour plots of (A) specific energy consumption E_M [$\text{kWh kg}_{\text{DW}}^{-1}$] aimed on cell disintegration and (B) aimed on protein release for agitator speed u_s [m s^{-1}] and biomass concentration C_x [g kg^{-1}].

Many other techniques have been proposed for cell disintegration, but only few are considered mild enough to keep the intracellular components intact. Grimi et al. (2014) used pulsed electric field, high voltage electrical discharges, ultra-sonication and high pressure homogenization in sequential mode on a 1% w/w suspension of *Nannochloropsis sp.* and found protein extraction values of 5.2%, 1.15%, 1.8% and 91% respectively from a total protein yield of 70% water soluble protein (corresponding to $1.4 \text{ g protein per kg suspension}$). Pulsed electric field, high voltage electrical discharge, ultra-sonication and high pressure homogenization required up to 1.5, 1.5, 0.5 and $25.0 \text{ kWh kg}_{\text{DW}}^{-1}$ respectively. *Chlorella vulgaris* and *Nannochloropsis oculata* were treated by high pressure homogenization (2% w/w suspension) by Safi et al. (2014a) to release 51.7 and 49.5% water soluble proteins at a specific energy consumption of $7.5 \text{ kWh kg}_{\text{DW}}^{-1}$. The microalgae *Auxenochlorella protothecoides* was treated with pulsed electric field by Goettel et al. (2013)

at a specific energy consumption of 0.40 kWh kg_{DW}⁻¹. Nevertheless, hardly any proteins were released.

2.4.1 Model fit and analysis

In order to create the contour plots, a second order polynomial regression model was used for each output. These plots can visualize the effects of the two input parameters, u_s and C_x on each of the outputs (Desai et al., 2014a).

Using the Umetrics software, regression models were obtained for the k_{prot} , k_{dis} , Y_p and for the E_M . Model coefficients which were insignificant at a confidence interval of 95% were removed.

$$\log_{10}(k_{prot}) = -1.9253 + 0.1757 X_1 + 0.0489 X_2 - 0.0935 X_2^2 \quad (2.11)$$

$$R^2 = 0.90$$

$$\log_{10}(k_{dis}) = -2.0062 + 0.1369 X_1 + 0.0660 X_2 - 0.0539 X_1^2 \quad (2.12)$$

$$- 0.1025 X_2^2$$

$$R^2 = 0.802$$

$$\log_{10}(Y_{prot}) = -0.4421 - 0.0798 X_1 \quad (2.13)$$

$$R^2 = 0.84$$

$$\log_{10}(E_M) = 1.0567 - 0.3963 X_1 + 0.2403 X_2 + 0.2692 X_1^2 - 0.0822 X_2^2 \quad (2.14)$$

$$- 0.0687 X_1 X_2$$

$$R^2 = 0.99$$

$$\log_{10}(E_{M,3\tau}) = 0.3664 - 0.5709 X_1 + 0.1914 X_2 + 0.2343 X_1^2 \quad (2.15)$$

$$- 0.0773 X_1 X_2$$

$$R^2 = 0.98$$

Each of the models shows a good correlation between the experimental data and the predicted data. The models were statistically evaluated using analysis of variance (ANOVA) from which was found that each of the models is significant. Two F-tests were performed in the ANOVA both assessed at a probability of 95%. The first is for the model regression, comparing the variance in the regression to the variance of the residuals and is significant when $p < 0.05$. The second for the lack of fit, this compares the replicate error to the model error and is significant when $p > 0.05$ (appendix B).

2.5 Conclusions

A bead mill can successfully be applied for mild disintegration of microalgae slurries. Increasing biomass concentrations and agitator speeds lead to increased kinetic rate constants. Yet, an optimal agitator speed was found regardless of the biomass concentration. Water soluble protein release was found to be quicker than the biomass disintegration, while pigment release was found to be slower than the biomass disintegration. The water soluble protein yield ranged between 32-42%. By means of selective protein extraction, up to 85% energy reduction is realized. Specific energy consumptions as low as $0.81 \text{ kWh kg}_{\text{DW}}^{-1}$ can be achieved.

Acknowledgements

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Abbreviations

Symbol	Definition	units
A_{435nm}	Chlorophyll absorption at 435nm	[AU]
C_p	Protein concentration in supernatant	[g kg ⁻¹]
$C_p, biomass$	Total amount of protein in biomass per kg suspension	[g kg ⁻¹]
C_x	Biomass concentration	[g _{dw} kg ⁻¹]
C_v	Volume cell concentration	[-]
d_b	Bead diameter	[m]
Dis	Fraction disintegration cells	[-]
P	Power	[kWh]
$P_{3\tau}$	Power at 3 τ	[kWh]
E_M	Specific energy consumption	[kWh kg _{dw} ⁻¹]
$E_{M,3\tau}$	Specific energy consumption at 3 τ	[kWh kg _{dw} ⁻¹]
FL_{chl}	Chlorophyll auto-fluorescence	[AU]
k_{dis}	Disintegration first order kinetic constant	[s ⁻¹]
k_{prot}	Protein release first order kinetic constant	[s ⁻¹]
k_r	Pigment release first order kinetic constant	[s ⁻¹]
LF_i	Location factor of protein of pigment	[-]
M	Mass of biomass on dry weight	[g]
n	Agitator speed (revolutions)	[s ⁻¹]
P	Fraction released water soluble proteins	[-]
R	Fraction released pigment	[-]
S	Energy dissipation	[Pa]
t	Disruption/milling time	[s]
u_s	Agitator tip speed	[m s ⁻¹]
$V_{Chamber, free}$	Volume milling chamber free for liquid	[mL]
V_{total}	Total milled suspension volume	[mL]
μ_{eff}	Effective viscosity	[Pa s]
$\dot{\gamma}$	Shear rate	[s ⁻¹]
Y_{carb}	Carbohydrate yield	[%]
Y_{prot}	Protein yield	[%]
ε	Bead bulk porosity	[-]
φ_b	Bead filling ratio	[-]
ρ_b	Specific density beads	[kg m ⁻³]
τ	Characteristic time of process kinetic	[s]

Appendix A: R^2 values

Table 2.A.1 R^2 values for first order model fits.

Exp. no.	C_x (g kg _{sus} ⁻¹)	u_s (m s ⁻¹)	R^2 k_{dis}	R^2 k_{prot}	R^2 k_r
1	25	6	0.998	0.995	0.979
2	25	9	0.999	0.999	0.968
3	25	12	0.993	0.997	0.954
4	87.5	6	0.989	0.992	0.988
5	87.5	9	0.989	0.997	0.977
6	87.5	9	0.995	0.987	0.955
7	87.5	9	0.992	0.988	0.988
8	87.5	12	0.998	0.996	0.997
9	145	6	0.982	0.971	0.985
10	145	9	0.997	0.978	0.855
11	145	12	0.999	0.998	0.945

Appendix B: ANOVA tables

Table 2.B.1 ANOVA table of Disintegration rate (k_{dis}).

	DF	SS	MS (variance)	F	p	SD
Total	11	48.3268	4.39334			
Constant	1	48.1188	48.1188			
Total Corrected	10	0.208027	0.0208027			0.144231
Regression	4	0.183253	0.0458133	11.0956	0.006	0.21404
Residual	6	0.0247737	0.00412896			0.064257
Lack of Fit (Model Error)	4	0.0124236	0.00310591	0.502977	0.749	0.0557307
Pure Error (Replicate Error)	2	0.0123501	0.00617505			0.0785815
	N = 11	Q2 =	0.626	Cond. no. =	3.082	
	DF = 6	R2 =	0.881	RSD =	0.06426	
		R2 Adj. =	0.802			

Table 2.B.2 ANOVA table of Protein release rate (k_{prot}).

	DF	SS	MS (variance)	F	p	SD
Total	11	43.2019	3.92745			
Constant	1	42.9615	42.9615			
Total Corrected	10	0.240395	0.0240395			0.155047
Regression	3	0.223444	0.0744812	30.7573	0.000	0.272912
Residual	7	0.016951	0.00242158			0.0492095
Lack of Fit (Model Error)	5	0.0105714	0.00211429	0.662828	0.693	0.0459814
Pure Error (Replicate Error)	2	0.0063796	0.0031898			0.0564783
	N = 11	Q2 =	0.855	Cond. no. =	2.739	
	DF = 7	R2 =	0.929	RSD =	0.04921	
		R2 Adj. =	0.899			

Table 2.B.3 ANOVA table of Protein Yield (Y_p).

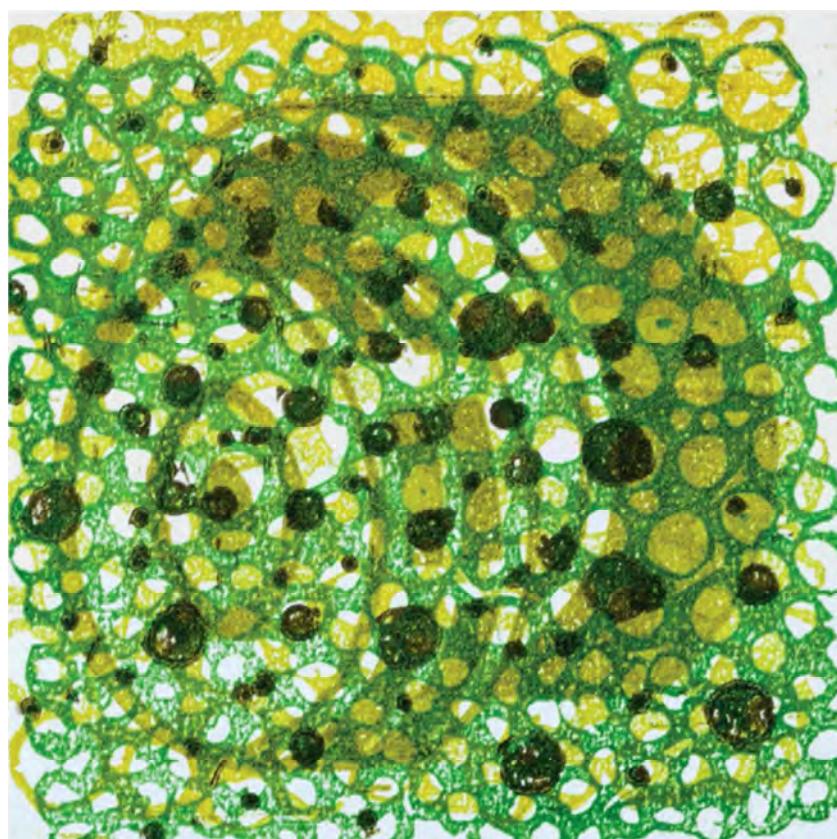
	DF	SS	MS (variance)	F	p	SD
Total	11	2.19455	0.199505			
Constant	1	2.14974	2.14974			
Total Corrected	10	0.044812	0.00448115			0.066941
Regression	1	0.038242	0.0382415	52.386	0	0.195554
Residual	9	0.00657	0.00073			0.027018
Lack of Fit (Model Error)	7	0.005027	0.0007182	0.931171	0.608	0.026799
Pure Error (Replicate Error)	2	0.001543	0.00077129			0.027772
	N = 11	Q2 =	0.783	Cond. no. =	1.354	
	DF = 9	R2 =	0.853	RSD =	0.02702	
		R2 Adj. =	0.837			

Table 2.B.4 ANOVA table of Specific Energy Consumption (E_M).

	DF	SS	MS (variance)	F	p	SD
Total	11	16.2628	1.47844			
Constant	1	14.7673	14.7673			
Total Corrected	10	1.49549	0.149549			0.386716
Regression	5	1.49148	0.298296	371.885	0.000	0.546165
Residual	5	0.00401059	0.000802118			0.0283217
Lack of Fit (Model Error)	3	0.00376821	0.00125607	10.3644	0.089	0.0354411
Pure Error (Replicate Error)	2	0.000242381	0.000121191			0.0110087
	N = 11	Q2 =	0.973	Cond. no. =	3.082	
	DF = 5	R2 =	0.997	RSD =	0.02832	
		R2 Adj. =	0.995			

Table 2.B.5 ANOVA table of Specific Energy Consumption ($E_{M,3\sigma}$).

	DF	SS	MS (variance)	F	p	SD
Total	11	5.05778	0.459798			
Constant	1	2.68665	2.68665			
Total Corrected	10	2.37113	0.237113			0.486942
Regression	4	2.34899	0.587248	159.2	0.000	0.766321
Residual	6	0.0221325	0.00368874			0.060735
Lack of Fit (Model Error)	4	0.015792	0.003948	1.24533	0.491	0.0628331
Pure Error (Replicate Error)	2	0.00634047	0.00317023			0.0563048
	N = 11	Q2 =	0.950	Cond. no. =	2.739	
	DF = 6	R2 =	0.991	RSD =	0.06074	
		R2 Adj. =	0.984			



Chapter 3

Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates

The contents of this chapter have been accepted as:

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*Energy efficient bead milling of microalgae: Effect of bead size on
disintegration and release of proteins and carbohydrates*

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Abstract

The disintegration of three industry relevant algae (*Chlorella vulgaris*, *Neochloris oleoabundans* and *Tetraselmis suecica*) was studied in a lab scale bead mill at different bead sizes (0.3 mm-1 mm). Cell disintegration, proteins and carbohydrates released into the water phase followed a first order kinetics. The process is selective towards proteins over carbohydrates during early stages of milling. In general, smaller beads led to higher kinetic rates, with a minimum specific energy consumption of $\leq 0.47 \text{ kWh kg}_{\text{DW}}^{-1}$ for 0.3 mm beads. After analysis of the stress parameters (stress number and stress intensity), it appears that optimal disintegration and energy usage for all strains occurs in the 0.3-0.4 mm range. During the course of bead milling, the native structure of the marker protein Rubisco was retained, confirming the mildness of the disruption process.

3.1 Introduction

There is a growing demand for sustainable protein sources and bio-based products as an alternative for traditional agricultural crops. Microalgae are a potential source of renewable high value proteins, carbohydrates, lipids and pigments for food, feed and chemical industries (Vanthoor-Koopmans et al., 2013). Such products are typically located intracellularly, either in the cytoplasm, in internal organelles or bound to cell membranes, and in most cases, the cells need to be disintegrated before extraction. This step can be done by chemical hydrolysis, high pressure homogenization, ultrasonication, pulsed electric fields or bead milling (Doucha and Lívanský, 2008; Goettel et al., 2013; Grimi et al., 2014; Günerken et al., 2015; Montalescot et al., 2015; Postma et al., 2016b, 2015; Safi et al., 2014a).

Bead mills are commonly applied in the chemical industry for the manufacture of paints/lacquers and grinding of minerals (Kula and Schütte, 1987) and have been successfully applied for the disintegration of yeast, cyanobacteria and microalgae for release of intracellular products, under low energy inputs and mild conditions (Balasundaram et al., 2012; Bunge et al., 1992; Günerken et al., 2016; Postma et al., 2015). The efficiency of cell disintegration in bead mills depends on several parameters, such as chamber and agitator geometry, biomass concentration, agitator speed (i.e., tip speed of agitator), suspension flow rate, bead filling ratio, bead type and bead diameter. An overview of relevant investigations on the disruption of algae using bead mills, with particular emphasis on the effect of bead size on the process performance, is presented in Table 3.1.

To describe the comminution of cells in bead mills as a function of different process parameters, Kwade and Schwedes (Kwade and Schwedes, 2002) and Bunge (Bunge et al., 1992) presented a very clear description of the so-called Stress Model (SM). The SM assumes that the disruption process in stirred media mills (e.g., bead mill) is governed by the number of stress events (i.e., bead to bead collisions) and by the intensity of such events. Quantitatively, this is expressed by the Stress Number (SN) (Eq. (3.1)), (3.2)) and the Stress Intensity (SI) (Eq. (3.4)) (Bunge et al., 1992; Kwade and Schwedes, 2002); two types of behaviors are also recognized: 1) disintegration/deagglomeration of cells, characterized by the fact that a cell is either intact or disrupted; and 2) grinding of crystalline materials, applicable for materials in which the particle size decreases during the milling process.

Table 3.1 Overview of bead milling studies; the effect of the bead size on experimental outcome

Bead Sizes (mm)	Microorganism	Biomass concentration ($\text{g}_{\text{DW}} \text{L}^{-1}$)	Agitator speed (m s^{-1})	Flow rate (kg h^{-1})	Beads filling (% v/v)	Specific energy input ($\text{kWh kg}_{\text{DW}}^{-1}$)	Results	Reference
0.2–0.3 (glass)		107	14	20	82	n.a.	92.5% disintegrated cells	
0.42–0.58 (glass)			14	15, 40		n.a.	97, 90% disintegrated cells	
0.3–0.4 (ZrO_2)	<i>Chlorella</i> sp.	102.5		35	85	10.03	90.6% disintegrated cells	(Doucha and Livansky, 2008)
		124.4	13	35	85	8.37	82.8% disintegrated cells	
0.6–0.8 (ZrO_2)		124.4		35	85	7.51	85.2% disintegrated cells	
0.35–0.5 (glass)	<i>Scenedesmus obliquus</i> /						90% disintegrated cells	
0.5–0.7 (glass)	<i>Scenedesmus quadricauda</i>	50	1450 rpm	40	50	0.5 ^a	80% disintegrated cells	(Hedenskog et al., 1969)
1.0–1.2 (glass)							50% disintegrated cells	
0.375, 0.625, 1.3, 2.15 (glass)	<i>Porphyridium cruentum</i>	2.5–3.7				4.2·10 ³ –10.6·10 ³	5–48% disintegrated cells	
	<i>Nannochloropsis oculata</i>	0.7–1	8	9–10.2	35–75	55·10 ³ –222·10 ³	23–35% disintegrated	(Montalescot et al., 2015)
1.0 (ZrO_2)	<i>Chlorella vulgaris</i>	25–145	6–12	n.a.			99% disintegrated cells	(Postma et al., 2015)
0.205–1.280 (glass)		10 ^b	8	n.a.	65	0.81–30.38	32–48% protein yield	
0.110–1.500 (glass)	<i>Arthro bacter</i> sp.	20 ^b	4	n.a.	80	13.89	50–100% disintegrated cells	(Bunge et al., 1992)
0.5–2.3 (ZrO_2), 1mm (glass)			900 rpm			0.69	50–85% disintegrated cells	
2.0–2.5 (glass & ZrO_2)	<i>Chlorogloeopsis fritschii</i>	30.7		n.a.	48	1.87	50–100% polysaccharide yield	(Balasundaram et al., 2012)
			500 rpm				98–100% polysaccharide yield	
0.4–0.6 (ZrO_2)	<i>Tetraselmis</i> sp.	120	n.a.	n.a.	65	n.a.	100% disintegrated cells	(Schwenzfeier et al., 2011)
0.4–0.6 (ZrO_2)	<i>Neochloris oleoabundans</i>	30	n.a.	n.a.	65	7.5	21% protein yield	
							100% disintegrated cells	(Günkeren et al., 2016)
							35 ^c –59 ^d % protein yield	

^a: preliminary calculations (Hedenskog et al., 1969)^b: 10% w/v wet biomass, assumption: 90% moisture^c: Nitrogen replete cultivated^d: Nitrogen deplete cultivated

n.a.: not available

Accordingly, the SN (-) can be calculated for Disintegration (SN_D) and for Grinding (SN_G) as:

$$SN \propto \frac{\varphi_b(1 - \varepsilon)}{\{1 - \varphi_b(1 - \varepsilon)\}c_V} \frac{nt}{d_b} \propto C \cdot SN_D \quad (3.1)$$

$$SN \propto \frac{\varphi_b(1 - \varepsilon)}{\{1 - \varphi_b(1 - \varepsilon)\}c_V} \frac{nt}{d_b^2} \propto C \cdot SN_G \quad (3.2)$$

with

$$C = \frac{\varphi_b(1 - \varepsilon)}{\{1 - \varphi_b(1 - \varepsilon)\}c_V} \quad (3.3)$$

where φ_b is the bead filling ratio (-), ε is the bead bulk porosity (-), c_V the volume cell concentration (-), n the agitator revolutions (s^{-1}), t the milling time (s) and d_b the bead diameter (m).

Furthermore, the SI (Nm) can be regarded as the magnitude of the kinetic energy of a single bead and can be calculated as:

$$SI \propto d_b^3 \rho_b u_s^2 \quad (3.4)$$

in which ρ_b is the specific density of the beads ($kg\ m^{-3}$) and u_s is the agitator tip speed ($m\ s^{-1}$). A cell can only be intact or disintegrated upon the release of the intracellular products. Therefore, an optimal stress intensity SI_{opt} can be considered. At or above SI_{opt} cells break with a single stress event, below SI_{opt} , multiple stress events are required to break the cell.

Consequently, the theoretical specific energy input is proportional to the product of the number of stress events times the energy of such events:

$$\tilde{E}_M \propto \frac{SN \cdot SI}{M} \quad (3.5)$$

where M is the mass of biomass (kg_{DW}) in the system and \tilde{E}_M is the theoretical specific energy input ($kWh\ kg_{DW}^{-1}$).

The SM was first applied to microalgae by Montalescot et al. (2015). However, to our knowledge, it has not been applied in combination with the release of water soluble microalgae components. In large scale disruption trials for yeasts and bacteria, Schütte et al. (1983) observed that cytoplasmic enzymes were better solubilized by smaller beads, and that periplasmic enzymes were more easily released by larger beads. We therefore hypothesize that smaller beads could interact more effectively with internal organelles over larger beads and thus are better able to release proteins (e.g., Rubisco) from the pyrenoids and carbohydrates from the cell wall or starch granules. If the process is operated above

Sl_{opt} , smaller beads would also lead to higher kinetics, higher yields and lower energy consumption.

The aim of this work is to investigate the effect of the bead size on the disintegration, release of water soluble components and energy consumption during the bead milling of *C. vulgaris*, *N. oleoabundans* and *T. suecica*.

3.2 Methods

3.2.1 Microalgae, cultivation and harvesting

Chlorella vulgaris (SAG 211-11b, EPSAG Göttingen, Germany) was cultivated according to Postma et al. (2016b).

Neochloris oleoabundans (UTEX 1185, University of Texas Culture Collection of Algae, USA) was cultivated using a fully automated 1400L vertical stacked tubular photo- bioreactor (PBR) located in a greenhouse (AlgaePARC, The Netherlands). The algae were cultivated in Bold's Basal medium (CCAP, 2015) at a pH value of 8.0 and the temperature was controlled at 30°C. The light intensity was set at an average of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Tetraselmis suecica (UTEX LB2286, University of Texas Culture Collection of Algae, USA) was cultivated in repeated batches in a 25L flat panel PBR (AlgaePARC, The Netherlands) at 20°C. Ten fluorescent lamps (Philips 58W/840) provided a continuous incident light intensity of 373 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The PBR was located in a greenhouse and thus, it was also exposed to natural light during the period October 2015 -January 2016 (Wageningen, The Netherlands). Mixing and pH control (pH 7.5) were provided by sparging gas (0.254 vvm) composed of a mix of air and 5 % v/v CO_2 . Walne medium was supplied at a ratio of 8.8 mL L^{-1} medium (Michels et al., 2014).

To obtain a biomass paste, *C. vulgaris* was centrifuged (4000x g, 15 min) using a swing bucket centrifuge (Allegra X-30R, Beckman Coulter, USA) while *N. oleoabundans* and *T. suecica* were centrifuged (80Hz, ~3000x g, 0.75 $\text{m}^3 \text{h}^{-1}$) using a spiral plate centrifuge (Evodos 10, Evodos, The Netherlands). After centrifugation, the biomass paste of all three algae was stored at 4°C in the dark and used within two days. Prior to disintegration experiments, the biomass paste was resuspended in phosphate buffered saline (PBS) (1.54 mM KH_2PO_4 , 2.71 mM $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 155.2 mM NaCl at pH 7.0) to obtain a biomass concentration (C_x) of about 90 g kg^{-1} . C_x is expressed as g dried biomass per kg algae suspension.

3.2.2 Bead mill experimental procedure

The bead mill experiments were performed in a horizontal stirred bead mill (Dyno-Mill Research Lab, Willy A. Bachofen AF Maschinenfabrik, Switzerland) operated in batch recirculation mode. The operation procedure was previously described by Postma et al. (2015). In brief, the mill consists of a milling chamber ($V_{chamber}$ 79.6 mL) in which the beads are accelerated by a single DYNO®-accelerator (\varnothing 56.2 mm). To maintain the feed temperature at 25°C, a cooling water bath connected to a cooling jacket integrated in the milling chamber and a cooling coil in the feed funnel were used. Yttrium stabilized ZrO₂ beads (Tosoh YTZ®) with four diameters (0.3, 0.4, 0.65 and 1 mm, specific density ρ_b of 6 g cm⁻³, bulk density ϵ of 3.8 g cm⁻³) were applied. The beads were loaded at a constant filling volume of 65% v/v.

First order release kinetics was used to calculate the kinetic constant k_i for the disintegration percentage (k_{dis}), protein release (k_{prot}) or carbohydrate release (k_{carb}) as:

$$\frac{X_i(t)}{X_{i,max}} = 1 - e^{-k_i t} \quad (3.6)$$

where $X_i(t)$ represents the degree of disintegration (Dis), the protein concentration or the carbohydrate concentration at time t , and $X_{i,max}$ represents the maximal degree of disintegration, protein concentration or carbohydrate concentration in the liquid phase.

3.2.3 Analytical methods

Biomass quantification. The dry weight concentration was determined as described by Lamers et al. (2010). The DW/OD₇₅₀ ratio for *C. vulgaris*, *N. oleoabundans* and *T. suecica* were determined experimentally to be 0.312, 0.350 and 0.537, respectively. These ratios were used to calculate the initial biomass concentration. The cell size and cell number were measured with a cell counter (Beckman Coulter Multisizer 3, USA). The samples were diluted using Coulter® Isoton® II dilution buffer. The cell size and cell number were used to calculate the total cell volume.

Disintegration, protein and carbohydrate analysis. The disintegration percentage, protein analysis on dry weight (DW) and the water soluble protein after bead milling were analyzed as described by Postma et al. (2015). The carbohydrate content on DW and the water soluble carbohydrates after bead milling were also determined as described by Postma et al. (2016b).

The protein and carbohydrate yield after bead milling are expressed as:

$$Y_i = \frac{C_i(t) - C_i(0)}{C_{i,Biomass}} \quad (3.7)$$

where $C_i(t)$ and $C_i(0)$ are the concentrations of component i in the supernatant at time t and 0, respectively. $C_{i,Biomass}$ is the total content of component i within the total biomass (DW), where i can be protein or carbohydrates.

Starch analysis. To determine the total starch content on biomass DW, lyophilized algae were dissolved in 1 mL 80% ethanol and bead beaten at 6000 RPM for 3 cycles with 120 s breaks in between cycles, after which the total starch content was determined using a commercial kit (Total Starch, Megazyme International, Ireland). The absorbance was measured at a wavelength of 510 nm with a spectrophotometer (DR6000, Hach Lang, USA).

Scanning Electron Microscopy. 150 μ L microalgae suspension was applied on poly-L-lysine coated cover slips (\varnothing 8 mm) and incubated for 1h. Subsequently the samples were rinsed in fresh PBS and fixed for 1h in 3% glutaraldehyde in PBS. After washing twice in PBS, the samples were post-fixed in 1% OsO₄ for one hour, rinsed with demineralized water and dehydrated in a graded (30-50-70-90-100-100%) ethanol series. Subsequently, the samples were critical point dried with CO₂ (EM CPD 300, Leica, Wetzlar, Germany). The cover slips were attached to sample holders using carbon adhesive tabs (EMS, Washington, USA) and sputter coated with 10 nm Wolfram (EM SCD 500, Leica, Wetzlar, Germany). The samples were analyzed in a high resolution scanning electron microscope at 2 KV at room temperature (Magellan 400, FEI, Eindhoven, The Netherlands). Images were contrast enhanced with Photoshop CS5.

Native PAGE analysis. Native PAGE analysis was performed as described by Postma et al. (2016b). In addition, scanned gels were analyzed by ImageJ (IJ 1.46r) to convert the Rubisco band intensity in a density chromatogram. Subsequently, the chromatogram was integrated. From the peak areas the relative density was determined over the course of bead milling:

$$Relative\ density\ (-) = \frac{A_{Peak,final}}{A_{Peak,t}} \quad (3.8)$$

where $A_{Peak,final}$ is the peak area of Rubisco in the final sample and $A_{Peak,t}$ the peak area of Rubisco at time t .

3.2.4 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA). When groups were significantly different at an α level of 0.05, Tukey's honest significance test was performed to find which groups differed.

3.3 Results and discussion

The overall effect of bead size is studied first in terms of kinetic rates and product yields. The mechanism of disintegration is then analyzed using the stress model and subsequently the specific energy consumption and selective protein release are presented.

3.3.1 Disintegration and product release kinetics

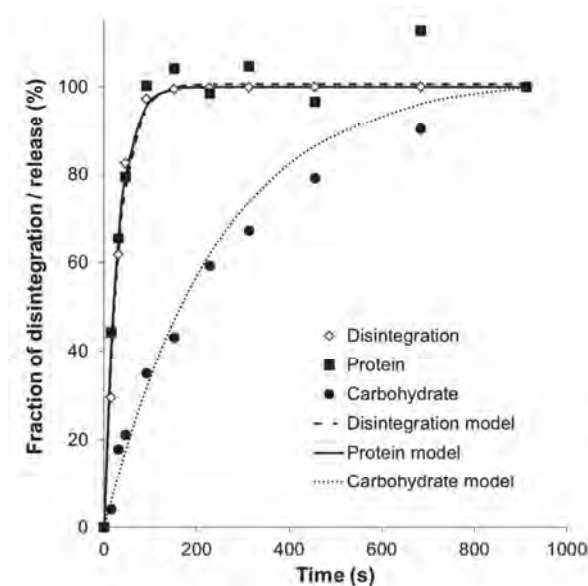


Figure 3.1 Relative disintegration and release of protein and carbohydrate for *T. suecica* using 0.3 mm beads.

As a follow up to Postma et al. (2015) in which a benchmark for the disintegration of *C. vulgaris* was set using 1 mm ZrO_2 beads, one goal of this study was to understand the effect of decreasing the bead size during bead milling of microalgae.

Figure 3.1 shows the fraction of cell disintegration, protein and carbohydrate release for *T. suecica* for 0.3 mm beads. It can be observed that a disintegration percentage and protein release of over 99% can be reached in a total processing time of 400 s. Nevertheless, the maximal amount of carbohydrate was only found at the end of the experiment without reaching a plateau. For the other bead sizes and algae species, disintegration percentages >99% were obtained. By means of Least Square Error Regression (LSER) the first order model (Eq. (3.6)) was fitted to the experimental disintegration percentage, protein and carbohydrate release data. In all cases, the coefficient of correlation ranged between 0.8856 and 0.9997.

Figure 3.2A-C presents an overview of kinetics constants for disintegration, proteins and carbohydrates release, respectively, for all algae strains and bead sizes. *Chlorella vulgaris* showed a clear optimum bead size of 0.4 mm for disintegration and release of carbohydrates. The k_{dis} of $0.041 \pm 0.003 \text{ s}^{-1}$ from this study represents a significant four-fold increase ($p < 0.05$) with respect to the benchmark of 1 mm beads ($k_{dis} = 0.009 \pm 0.001 \text{ s}^{-1}$). The protein release constant (Figure 3.2B) using the 1 mm beads was similar to the previously determined benchmark (Postma et al., 2015). It can be observed that *C. vulgaris* shows an increasing trend in the protein release rate for a decreasing bead size.

For *N. oleoabundans* a clear upward trend in the k_{dis} and k_{prot} was observed when decreasing the bead size, but no evident optimum bead size appeared. The best disintegration results obtained with a k_{dis} of $0.025 \pm 0.001 \text{ s}^{-1}$ was 3 fold faster using 0.3 mm beads than the 1 mm beads. The carbohydrate release for *N. oleoabundans* did not show significant differences ($p = 0.44$). *Neochloris oleoabundans* and *C. vulgaris* exhibit several structural similarities, including cell size (average 3.3 μm and 3.2 μm , respectively) and morphology. Yet, we observed different kinetic constants, in particular, for carbohydrates. This is most likely caused by differences in the cell composition and cell wall structure of both algae, which contain cellulose-like polymer structures. The genus *Chlorella* is known to have amino sugars as constituents in the rigid cell wall, and it is suspected that chitin-like glycans are present (Kapaun and Reisser, 1995). To our knowledge, no literature exists on the polymeric links present in *N. oleoabundans*.

Among the strains tested, the disintegration rates of *T. suecica* were higher, and statistically independent of bead size. This clearly suggests a weaker cell structure. A maximum k_{dis} of $0.050 \pm 0.009 \text{ s}^{-1}$ was determined, which is almost five-fold higher than the rates for *C. vulgaris* and *N. oleoabundans* (1 mm beads), but only 60% of the rate obtained by Halim et al. (2013) when disrupting *T. suecica* using ultrasound for similar batch volume and processing time. No significant trend was observed in the protein or carbohydrate release rate with respect to the bead size. The similar trends and magnitudes of k_{dis} and k_{prot} for all strains suggest that most of the proteins that were measured in the soluble phase are released to the bulk directly upon cell bursting. Differences in k_{dis} and k_{prot} are probably due to diffusion limiting transport. The absolute carbohydrate release rate was lower for *T. suecica* compared to *C. vulgaris* and *N. oleoabundans*. From the literature it is known that *T. suecica* can accumulate significant amounts of carbohydrates in the form of starch granules (Kermanshahi-pour et al., 2014), which are hardly soluble. For *T. suecica* the measured starch-carbohydrate ratio was always between 0.5 and 0.9, while for *C. vulgaris* and *N. oleoabundans* it was below 0.3 and 0.1, respectively.

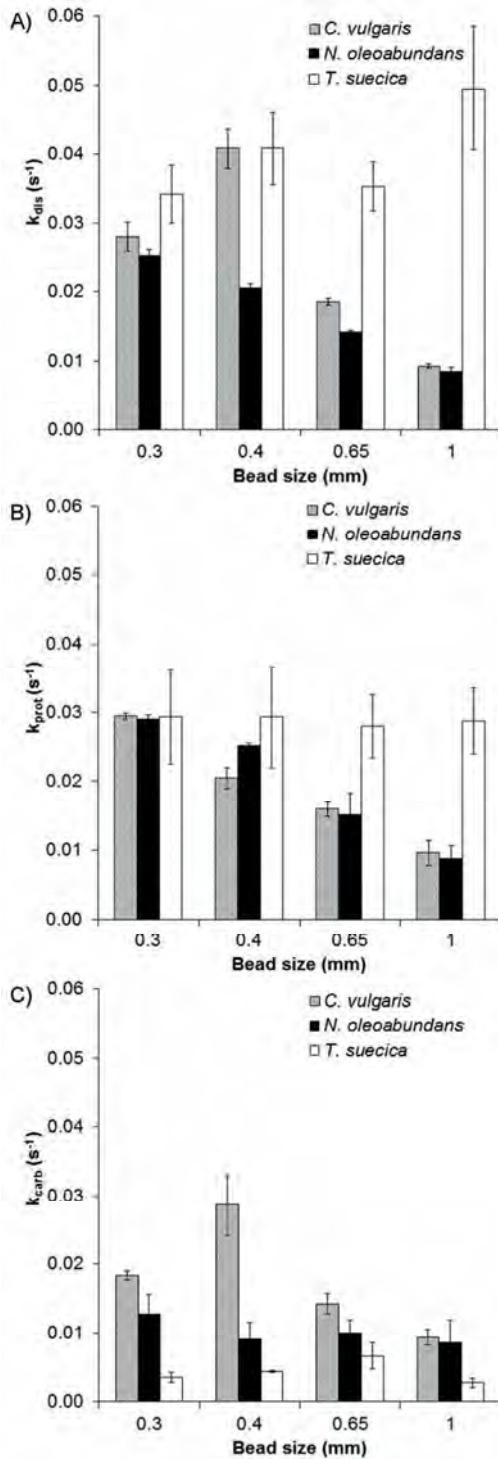


Figure 3.2 Kinetic constants for disintegration k_{dis} (A), protein k_{prot} (B) and carbohydrate k_{carb} (C) release as a function of the bead size (mm) for *C. vulgaris*, *N. oleoabundans* and *T. suecica*.

Montalescot et al. (2015) reported no difference in the kinetic constant for bead diameters of 0.325 and 0.625 mm for the disintegration of the microalgae *N. oculata* (algae diameter 3 μm). However, the obtained k_{dis} of $\sim 0.006 \text{ s}^{-1}$ was on average a factor 5-6 lower than the k_{dis} values obtained in the current study for similar bead sizes (0.3 – 0.65 mm).

3.3.2 Protein and carbohydrate yield

Different algae batches were used per bead size, and the composition (total protein, total carbohydrate and starch on biomass DW) of each batch was measured to calculate the product yield using Eq. (3.7), an overview is shown in Table 3.2.

Table 3.2 Overview of biomass composition, protein and carbohydrate yield for each bead size. The protein and carbohydrate yield correspond to 87.5% of the maximal release (i.e., 3τ).

Experimental conditions		Biomass composition			Product yield	
Algae	d_b (mm)	Protein % _{DW} \pm SD	Carbohydrate % _{DW} \pm SD	Starch % _{DW} \pm SD	$Y_{Prot} \pm \text{SD}$ (%)	$Y_{Carb} \pm \text{SD}$ (%)
<i>T. suecica</i>	0.3	43.3 \pm 2.7	21.2 \pm 2.1	18.7 \pm 0.1	17.4 \pm 1.3	51.7 \pm 15.2
	0.4	29.0 \pm 0.0	33.7 \pm 2.3	28.1 \pm 0.9	23.1 \pm 3.1	69.9 \pm 0.7
	0.65	36.9 \pm 0.3	16.0 \pm 1.7	8.6 \pm 0.1	15.7 \pm 4.9	24.4 \pm 7.3
	1	40.7 \pm 3.5	23.6 \pm 2.2	12.4 \pm 0.2	20.8 \pm 0.2	40.6 \pm 3.4
<i>C. vulgaris</i>	0.3	53.1 \pm 1.7	17.9 \pm 0.6	4.6 \pm 0.0	27.9 \pm 0.8	62.1 \pm 1.9
	0.4	57.0 \pm 1.4	14.0 \pm 0.7	2.8 \pm 0.3	36.3 \pm 0.6	65.5 \pm 1.5
	0.65	53.4 \pm 1.1	15.7 \pm 0.3	5.3 \pm 0.4	23.2 \pm 1.1	67.7 \pm 0.1
	1	51.6 \pm 3.2	15.7 \pm 0.4	3.4 \pm 0.4	31.5 \pm 0.4	55.4 \pm 3.9
<i>N. oleoabundans</i>	0.3	47.3 \pm 5.8	11.5 \pm 2.1	1.3 \pm 0.2	31.9 \pm 1.1	40.3 \pm 1.3
	0.4	50.8 \pm 6.3	12.2 \pm 1.3	1.0 \pm 0.0	30.2 \pm 0.1	31.7 \pm 1.7
	0.65	51.2 \pm 1.9	17.1 \pm 2.5	1.5 \pm 0.2	25.5 \pm 3.0	47.1 \pm 2.3
	1	55.6 \pm 0.6	11.4 \pm 1.7	0.9 \pm 0.1	23.2 \pm 5.2	22.4 \pm 0.2

According to Postma et al. (2015), 2.5 – 8 times more energy is required for continuing the bead milling process beyond 85-90% protein release in order to reach the maximum release. Therefore the yields in Table 3.2 are presented at 87.5% of the maximal release, which corresponds to 3τ (i.e., characteristic time of the protein/carbohydrate release kinetic). τ can be described as:

$$\tau = k_i^{-1} \cdot \ln(2) \quad (3.9)$$

Considering the difference in protein and carbohydrate release kinetics, it is important to note that the time at which 87.5% release of each component is achieved is different for each species and bead size. For *C. vulgaris*, the highest water soluble protein yield of 36.3% was obtained using 0.4 mm beads. For both *T. suecica* and *N. oleoabundans*, no significant

differences ($p > 0.05$) between the protein yields were found at different bead sizes. The protein yields obtained for *C. vulgaris* using the 1 mm beads were similar to the yields found in previous work (Postma et al., 2015) under the same operating conditions.

Schwenzfeier et al. (2011) found a water-soluble protein yield of 21% using *Tetraselmis* sp., which is similar to the average yields found for *T. suecica* in this work. For *N. oleoabundans* under nitrogen replete cultivation conditions, up to 35% of water-soluble protein was released after bead milling (Günerken et al., 2016). In addition, 't Lam et al. (2016) found protein yields up to 50% after bead milling of *N. oleoabundans*. These studies, however, aimed at completed disintegration rather than optimizing energy consumption.

On average, a carbohydrate yield of $62.7 \pm 4.5\%$ and $46.6 \pm 17.2\%$ was observed for *C. vulgaris* and *T. suecica*, respectively, independent of the bead size (based on Tukey's test). For *N. oleoabundans*, the carbohydrate yield improved ($p < 0.05$) from 22.4% to 40.3% from 1 mm to 0.3 mm, respectively. However, a clear trend could not be observed with decreasing bead sizes. Large variations in the carbohydrate yields were observed for *T. suecica*, which might be explained by natural variation or stress factors that altered the biomass composition. Analysis of the total starch content (Table 3.2) on biomass DW revealed that *T. suecica* contained considerably more starch with the same fluctuation as the yield, compared to *C. vulgaris* and *N. oleoabundans*. As was observed in the previous section (3.3.1), the carbohydrate release kinetics behaved independent of the bead size and were not influenced by the biomass composition.

3.3.3 Disintegration mechanism

The disintegration of microalgae cells and the breakage of organelles and internal structures to release water-soluble biomolecules are the result of the shear generated by collisions of beads in the mill chamber. Using Eq. (3.4), the *SI* was calculated to be $5.8 \cdot 10^{-6}$, $1.4 \cdot 10^{-5}$, $5.9 \cdot 10^{-5}$ and $2.2 \cdot 10^{-4}$ Nm per bead for 0.3, 0.4, 0.65 and 1 mm beads, respectively. Since the same agitator tip speed and volumetric bead filling was used for each bead size, the total kinetic energy for each bead size should be equal, under the assumption that all beads acquire the agitator's tip speed. This explains the statistically similar rates observed for *T. suecica*, but cannot clarify why higher kinetic constants were measured at lower bead sizes for *C. vulgaris* and *N. oleoabundans*.

In this study, the *SN* (Eq. (3.1) and (3.2)), which quantifies the amount of stress events during bead milling, is a function solely of bead size. The corresponding *SN* for the case of disintegrations (Eq. (3.1)) for all strains is presented in Figure 3.3A-C. For *C. vulgaris* and *N. oleoabundans*, the disintegration percentage can be described by a single curve, independent of the bead size. This clearly indicates that the theoretical amount of shear (*SI*)

and corresponding energy delivered by all beads sizes go beyond an optimal disruption barrier (SI_{opt}). A similar behavior was found for the disintegration of yeast cells by Bunge et al. (1992), where the stress frequency (similar to stress number) was found to describe the disintegration percentage by a single curve. On the contrary, for *T. suecica*, a dependence on the bead size with respect to the stress number can be observed (Figure 3.3A). For a constant stress number (e.g., $1 \cdot 10^7$), an increase in the bead size (i.e., increase in stress intensity) caused a larger disintegration percentage. This confirms the apparent trend (i.e., increased rate with increased bead size) in the disintegration kinetics (Figure 3.2A). Furthermore, it shows that SI was below SI_{opt} because under the investigated bead diameters, bead sizes below 1 mm only gave the same level of disintegration when the stress number was increased.

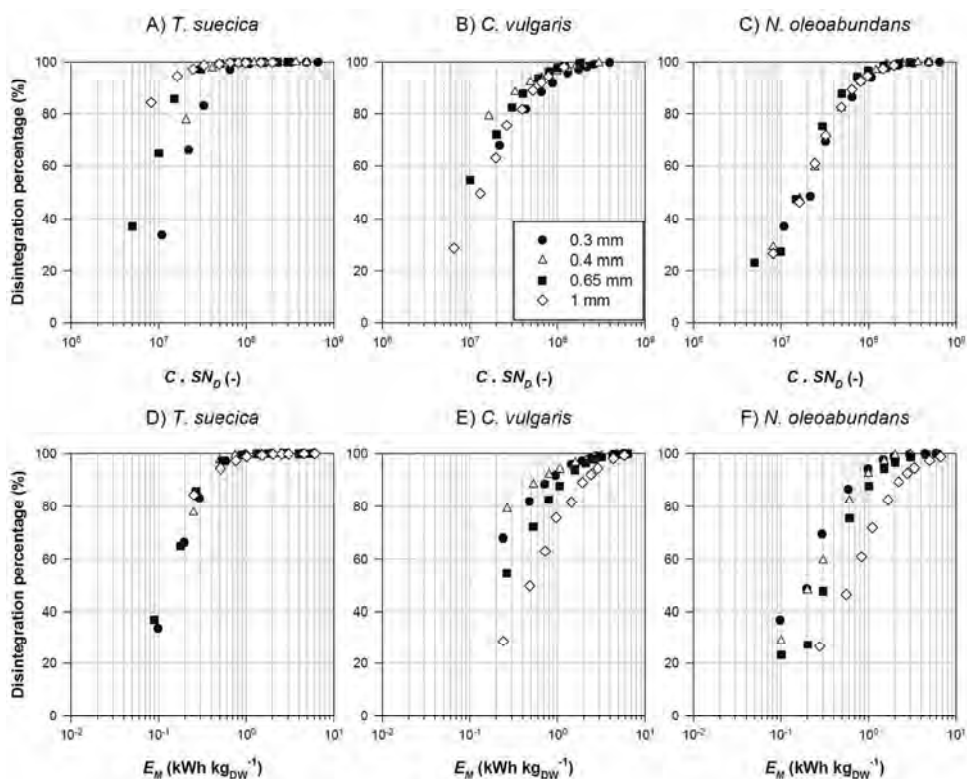


Figure 3.3 Semi-log plot of disintegration percentage (%) as a function of stress number $C \cdot SN_D (-)$ for *T. suecica* (A), *C. vulgaris* (B) and *N. oleoabundans* (C). Semi-log plot of disintegration percentage as a function of the specific energy consumption $E_M (kWh kg_{DW}^{-1})$ for *T. suecica* (D), *C. vulgaris* (E) and *N. oleoabundans* (F).

When plotting the disintegration percentage as a function of the measured specific energy consumption E_M (Figure 3.3D-F), the data for *T. suecica* are described by a single curve. Regardless of the bead size, the same energy is used to reach equal levels of disintegration. This is explained by the fact the specific energy consumption is proportional to the SI and SN

(Eq. (3.5)). For *C. vulgaris* and *N. oleoabundans*, on the contrary, in order to achieve similar disintegration percentages with different beads, a higher energy consumption is required; for both algae, the small range of beads (0.3-0.4 mm) leads to the lowest energy consumptions (i.e., optimal energy utilization was achieved). Figure 3.3 supports the idea that *N. oleoabundans* is the strongest alga, followed by *C. vulgaris*, and *T. suecica* shows to be the weakest algae.

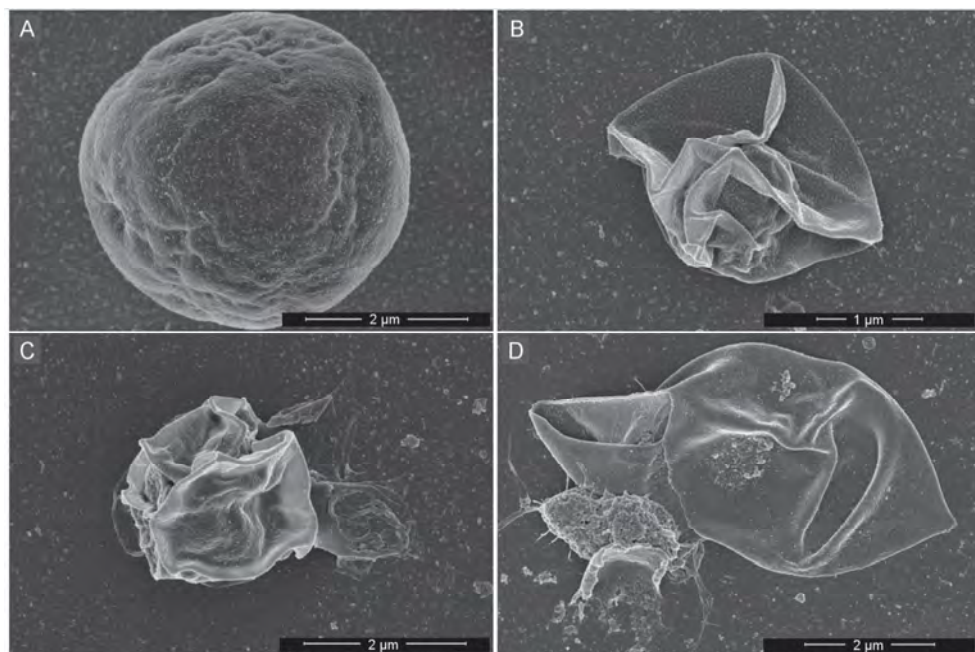


Figure 3.4 SEM micrographs of *C. vulgaris* before disintegration (A), after 50% disintegration using 1 mm beads (B), 87.5% disintegration using 1 mm beads (C) and 87.5% disintegration using 0.3 mm beads (D).

Scanning electron microscopy (SEM) micrographs of *C. vulgaris* were made prior to bead milling and after 50% and 87.5% disintegration using both 1 mm and 0.3 mm beads (Figure 3.4). The cells appear to be cracked upon bead impact after which the cell content was released, leaving an empty cell wall envelope (Figure 3.4B-D). During disintegration, no visual differences could be observed in the breakage mechanism between bead sizes (Figure 3.4C, D) at the same disintegration rate of 87.5%.

For the release of proteins, a similar behavior was obtained with respect to the disintegration when plotting the fraction of release (normalized with respect to $Y_{Prot,max}$ of individual experiment) versus the SN or E_M (appendix Figure 3.A.1). This suggests that most of the soluble proteins are present in the cytoplasm or inside weak organelles. Upon

disintegration, all proteins quickly migrate to the bulk medium. Conceptually, the process of protein release is similar to disintegration.

On the other hand, the release of carbohydrates revealed a different tendency (appendix Figure 3.A.2). For all three algae species, the release of carbohydrates was found to depend on both SN and SI when plotting the release fraction versus $C \cdot SN_G$ (appendix Figure 3.A.2 A-C). Furthermore, it was observed that the release of carbohydrates can be described using the specific energy consumption at a first glance by a single curve (appendix Figure 3.A.2 D-F). Similar behavior was also observed for weak/medium-hard crystalline materials like limestone (Kwade and Schwedes, 2002), from which we hypothesize that carbohydrates from the cell wall and starch granules behave like crystalline material. During the course of the disintegration process the cell wall debris and starch granules are stressed multiple times, breaking off polymers, oligomers and monomers, thereby solubilizing simple sugars.

3.3.4 Specific energy consumption

The specific energy consumption for the release of 87.5% (3τ) of the maximal protein release ($E_{M,3\tau}$) for the benchmark set with *C. vulgaris* was $1.71 \text{ kWh kg}_{\text{DW}}^{-1}$ (Postma et al., 2015). This was confirmed in the current work for *C. vulgaris* and *N. oleoabundans* (which behave similarly) with a specific energy consumption of $1.42 \text{ kWh kg}_{\text{DW}}^{-1}$ and $1.78 \text{ kWh kg}_{\text{DW}}^{-1}$, respectively. An overview of τ and $E_{M,3\tau}$ is given in Table 3.3, in which it can be observed that *T. suecica* can give the same protein release regardless of the bead size using the same specific energy consumption on average $0.47 \text{ kWh kg}_{\text{DW}}^{-1}$ ($p = 0.65$). In this regard, Lee et al. (2013) measured that the minimum specific energy $E_{M,\min}$ required to break up one kg *T. suecica* is $1.87 \cdot 10^{-4} \text{ kWh kg}_{\text{DW}}^{-1}$, and compared it with an energy efficient disruption process (hydrodynamic cavitation) with an E_M of $9.2 \text{ kWh kg}_{\text{DW}}^{-1}$ for a 1% w/w yeast suspension. In contrast, our findings (E_M : $0.47 \text{ kWh kg}_{\text{DW}}^{-1}$) show a twenty-fold improvement of E_M compared to that process. Nonetheless, it is clear that mechanical disintegration methods are highly energy inefficient since a large fraction of the total energy is used to displace beads and fluid and another fraction is lost due to mechanical dissipation. According to Eq. (3.5), the specific energy consumption of the system is proportional to the SI and SN . The theoretical specific energy input of the beads $\tilde{E}_{M,3\tau}$ at 87.5% release of the protein content was calculated for each experiment. The ratio of $\tilde{E}_{M,3\tau} / E_{M,3\tau}$ gives an indication of how much energy was utilized to give the beads momentum and which part of the energy was dissipated. For 0.3, 0.4, 0.65 and 1 mm beads (for all algae), this ratio was below 1%, 2%, 5% and 11%, respectively, showing that the total required bead energy decreases with bead size. This might be caused by the increased probability of impact at lower bead sizes due to a high SN (i.e., more beads colliding in the mill) while maintaining an SI above SI_{opt} . Moreover,

this shows that running a bead milling process close to Sl_{opt} provides extra potential energy savings.

Table 3.3 Overview of characteristic process time τ , specific energy consumption $E_{M,3\tau}$ protein concentration C_{Prot} carbohydrate concentration C_{Carb} and selectivity S at 87.5% protein release.

Experimental conditions		Energy consumption		Product release		
Algae	d_b (mm)	$\tau \pm SD$ (s)	$E_{M,3\tau} \pm SD$ (kWh kg _{DW} ⁻¹)	$C_{Prot} \pm SD$ (g L ⁻¹)	$C_{Carb} \pm SD$ (g L ⁻¹)	S (C_{Prot}/C_{Carb})
<i>T. suecica</i>	0.3	24.3 ± 5.7	0.47 ± 0.11	6.7 ± 0.5	2.4 ± 0.7	2.8 ± 0.4
	0.4	24.4 ± 6.1	0.48 ± 0.12	5.7 ± 0.8	6.7 ± 1.5	0.9 ± 0.8
	0.65	25.1 ± 4.1	0.45 ± 0.07	5.3 ± 1.7	1.5 ± 0.3	3.4 ± 0.8
	1	24.4 ± 4.1	0.49 ± 0.06	7.7 ± 0.0	1.8 ± 0.0	4.4 ± 0.0
<i>C. vulgaris</i>	0.3	23.6 ± 0.3	0.45 ± 0.01	13.3 ± 0.4	8.3 ± 0.1	1.6 ± 0.2
	0.4	34.0 ± 2.6	0.72 ± 0.05	19.3 ± 0.3	9.2 ± 0.1	2.1 ± 0.2
	0.65	43.5 ± 2.9	0.92 ± 0.06	11.0 ± 0.6	9.1 ± 0.1	1.2 ± 0.3
	1	73.7 ± 13.7	1.42 ± 0.26	14.7 ± 0.2	7.8 ± 0.7	1.9 ± 0.4
<i>N. oleoabundans</i>	0.3	23.9 ± 0.5	0.47 ± 0.01	13.5 ± 0.5	2.8 ± 0.3	4.8 ± 0.3
	0.4	27.4 ± 0.4	0.55 ± 0.01	13.8 ± 0.0	2.1 ± 0.2	6.7 ± 0.1
	0.65	46.7 ± 9.4	0.94 ± 0.19	11.9 ± 1.4	6.3 ± 0.4	1.9 ± 0.7
	1	80.8 ± 17.3	1.78 ± 0.38	11.3 ± 2.5	2.2 ± 0.4	5.2 ± 1.3

Figure 3.3E and 3.3F show that the energy utilization of the bead mill can be improved when smaller beads are applied during bead milling for *C. vulgaris* and *N. oleoabundans*. Decreasing the bead size from 1 to 0.3 mm can improve the energy utilization by a factor 3.3 and 3.9 ($p < 0.05$) for *C. vulgaris* and *N. oleoabundans*, respectively (Table 3.3). The lowest specific energy input found in this study was 0.45 kWh kg_{DW}⁻¹ for *C. vulgaris* using a bead diameter of 0.3 mm resulting in a Y_{Prot} of 28% and a Y_{Carb} of 52%. Doucha and Lívanský (2008) reported energy consumptions between 2.8 and 10.0 kWh kg_{DW}⁻¹ at 77.7% or 90.6% disintegration of *Chlorella sp.*, though no product release was reported. Furthermore, $>55 \cdot 10^3$ kWh kg_{DW}⁻¹ was required for 90% disintegration of *N. oculata* by Montalescot et al. (2015). Safi et al. (2014a) reported a Y_{Prot} 49.6% for an E_M of 7.5 kWh kg_{DW}⁻¹ for *C. vulgaris* using high pressure homogenisation. In addition, Postma et al. (2016b) reported an E_M of only 0.55 kWh kg_{DW}⁻¹ for disintegration of *C. vulgaris* using pulsed electric field, though Y_p was below 5%.

With respect to the estimated energy content of a microalgae being 6.82 kWh kg_{DW}⁻¹, and the assumption that no more than 10% of the energy content of the algae should be used for extraction/disintegration (National Algal Biofuels Technology Roadmap target, (U.S. DOE, 2010)), the total energy for extraction should not exceed 0.682 kWh kg_{DW}⁻¹ (Coons et al., 2014). The $E_{M,3\tau}$ values presented in this work show that the specific energy consumption for

bead milling can drop below this target, especially with the smaller 0.3 mm beads. To our knowledge, this is the first study to present such figures using fresh biomass.

As described above, it is known that mechanical disintegration techniques are energy inefficient processes in which a large part of the energy is not utilized for the effective breakage of cells. In a first assumption, the total energy is used to move parts (agitator), to displace beads and fluid, and dissipated into heat, which needs to be removed from the system by means of cooling. For the energy-efficient hydrodynamic cavitation process proposed by Lee et al. (2013), this means that only 0.002% of the required energy is utilized. Therefore, almost all energy needs to be removed as heat and thereby inevitably doubles the effective utilized energy. This would be true for all processes in which only an algae suspension is “moved” (e.g., high pressure homogenization, hydrodynamic cavitation, and ultrasound). However, in a bead milling process, not only is an algae suspension moved, but also the beads require energy to be displaced. The actual energy required for cooling of an algae suspension was also measured for *T. suecica*, using 0.4 mm beads and the conditions described in section 2.2. During the course of one experiment (1h) a ΔT of 18.2 °C was measured, which in terms of power, only corresponds to 4.2% of the E_M . Although the energy needed to cool down the engine is not yet included, it is evident that the cooling requirements could be ignored by running the bead milling shorter times (i.e., at 3τ) and by considering that after bead milling for 3τ (5 min) the suspension has heated up from 21.6 °C up to 24.3 °C, at which mild processing is still assured.

3.3.5 Selective protein release

An overview of the protein and carbohydrate concentration at 3τ protein release in the water-soluble phase is given in Table 3.3. *C. vulgaris* gives on average the highest absolute protein and carbohydrate concentration in the supernatant. High product concentrations are desirable if further fraction/purification is required, which reduces the amount of water that needs to be removed. Table 3.3 also provides an overview of the selectivity S (i.e., concentration ratio of released protein and released carbohydrate). In general, the process is selective towards proteins, in particular, at early stages of disruption ($S > 1$ for all times). The protein selectivity was highest for *N. oleoabundans*, followed by *T. suecica* and *C. vulgaris*. S can be regarded as a quality parameter for the bead milling process, i.e., a higher selectivity for the desired product makes further processing easier (e.g., less impurities). Therefore, S could be used to tune the desired properties of the end product. Schwenzfeier et al. (2011) found that “algae juice” (i.e., supernatant after bead milling), “crude protein isolate” and “purified protein isolate” from *Tetraselmis sp.* have good solubility at pH values (5.5 - 6.5), a range where seed protein isolates show low solubility. These extracts exhibit a

selectivity factor of around 2. In addition, it was shown that the carbohydrate fraction contributes considerably to the high emulsion and foam stability over a large pH range (Schwenzfeier et al., 2014). This suggests that the protein-carbohydrate concentrates found in the current work might possess similar functionality.

The proteins released by bead milling were analyzed by means of Native PAGE to provide insight about the size of the released proteins and whether they were negatively affected (i.e., degradation or aggregation) (Figure 3.5A-C). Overall, it can be observed that the microalgal proteins have a large size distribution. To investigate the hypothesis that smaller beads are favorable over larger beads to specifically release products from intracellular organelles, Rubisco (Ribulose-1,5-biphosphate carboxylase oxygenase) was chosen as a biomarker. Moreover, it is mainly located in an intracellular organelle called the pyrenoid (Meyer et al., 2012), which is present in the investigated strains. Rubisco consists of 8 large subunits (~56 kDa) and 8 small subunits (~14 kDa) making a native size of ~540 kDa. As can be observed from Figure 3.5A-C, Rubisco is released over time during the bead milling process. The release of native and active Rubisco was also observed in a previous study (Postma et al., 2016b).

The band intensities of Rubisco were graphically processed to a density chromatogram allowing peak identification and integration. With respect to the maximal amount of Rubisco obtained, a relative density plot was created (Figure 3.5D-F). The most distinct difference was observed between the 1 mm and the 0.3 mm beads. With respect to the different microalgae used in this study, *T. suecica* (Figure 3.5D) did not reveal any difference in the specific release of Rubisco. This could be due to the starch sheaths which *T. suecica* synthesizes around the pyrenoid structure, which make the pyrenoid not easily accessible (van den Hoek et al., 1995). Both *C. vulgaris* (Figure 3.5E) and *N. oleoabundans* (Figure 3.5F) showed that with a smaller bead size of 0.3 mm the release of Rubisco can be enhanced which further supports our hypothesis.

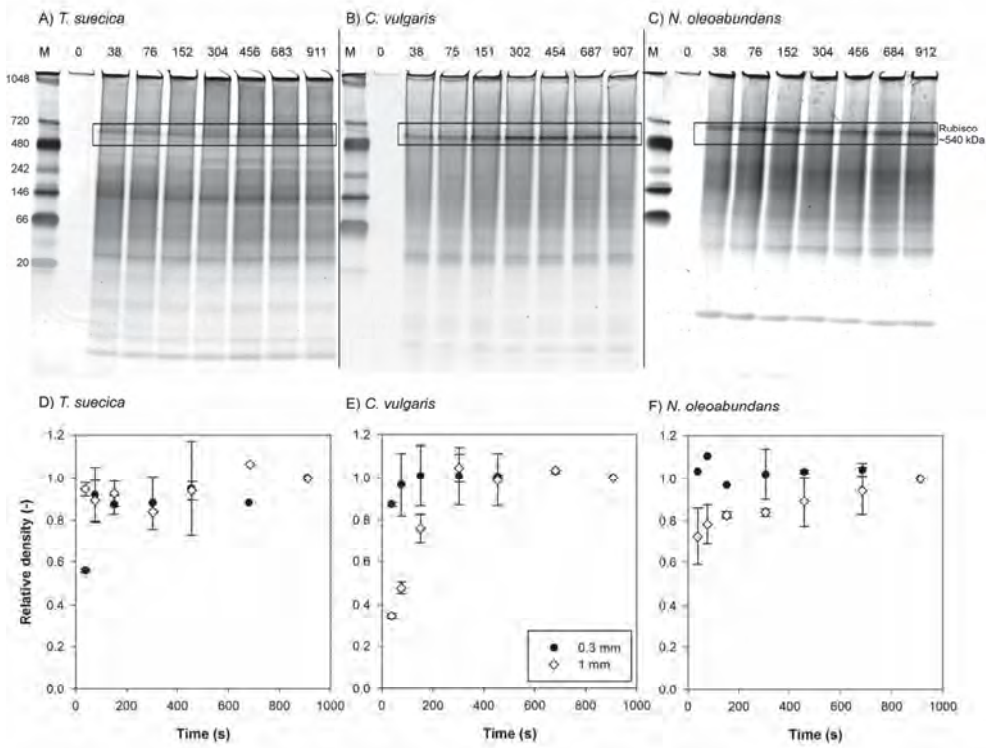


Figure 3.5 Native PAGE gel after bead milling using 1 mm beads for *T. suecica* (A), *C. vulgaris* (B) and *N. oleoabundans* (C). Values on left in kDa, M: marker. Lanes are indicated by the time in s after start of bead milling. The black box marks the Rubisco band (~540 kDa). Relative density (-) versus time of bead milling (s) for *T. suecica* (D), *C. vulgaris* (E) and *N. oleoabundans* (F).

3.3.6 Conclusions

Three main effects were observed when decreasing the bead size during bead milling: first, the kinetics of disintegration and component release (carbohydrates and proteins) were improved for *C. vulgaris* and *N. oleoabundans*, but remained unaffected for *T. suecica*; second, the specific energy consumption was reduced to $\leq 0.47 \text{ kWh kg}_{\text{DW}}^{-1}$ for all strains; and third, the protein yields remained constant. In addition, it was demonstrated that by running the milling process until significant component release is achieved (i.e., 87.5% of total), cooling requirements are minimized. Analysis of the stress parameters revealed that the bead mill was operated close to an optimum in terms of bead size for *C. vulgaris* and *N. oleoabundans*, in the range 0.3 mm-0.4 mm beads. In addition, it appeared clear that *T. suecica* is a significantly weaker alga. Finally, selective protein release was achieved in early stages of disintegration, and the relative Rubisco content could be enhanced for

C. vulgaris and *N. oleoabundans* using smaller beads. Under all conditions and processing times the native protein structure was retained.

Acknowledgements

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Abbreviations

Symbol	Definition	units
A	Peak area	[AU]
C	Constant	[-]
C_i	Concentration of component i in supernatant	[g L ⁻¹]
$C_{i,Biomass}$	Total concentration of component i in biomass	[g L ⁻¹]
C_V	Volume cell concentration	[-]
C_x	Biomass concentration	[g _{DW} kg ⁻¹]
d_b	Bead diameter	[m]
E_M	Specific energy consumption	[kWh kg _{DW} ⁻¹]
\bar{E}_M	Theoretical specific energy consumption	[kWh kg _{DW} ⁻¹]
$E_{M,3\tau}$	Specific energy consumption at 3τ	[kWh kg _{DW} ⁻¹]
$E_{M,min}$	Minimal specific energy consumption	[kWh kg _{DW} ⁻¹]
k_{carb}	Carbohydrate release first order kinetic constant	[s ⁻¹]
k_{dis}	Disintegration first order kinetic constant	[s ⁻¹]
k_{prot}	Protein release first order kinetic constant	[s ⁻¹]
M	Mass of biomass on dry weight	[kg]
n	Agitator speed (revolutions)	[s ⁻¹]
SN	Stress Number	[-]
SN_D	Reduced Stress Number for disintegration	[-]
SN_G	Reduced Stress Number for grinding	[-]
SI	Stress Intensity	[J] / [Nm]
SI_{opt}	Optimal Stress Intensity	[J] / [Nm]
t	Disruption/milling time	[s]
u_s	Agitator tip speed	[m s ⁻¹]
V	Volume	[mL]
X_i	Degree of disintegration (Dis), protein concentration or carbohydrate concentration	[-]
$X_{i,max}$	Maximal degree of disintegration (Dis), protein concentration or carbohydrate concentration	[-]
Y_{carb}	Carbohydrate yield	[%]
Y_{prot}	Protein yield	[%]
ε	Bead bulk density	[kg m ⁻³]
φ_b	Bead filling ratio	[-]
ρ_b	Specific density beads	[kg m ⁻³]
τ	Characteristic time of process kinetic	[s]

Appendix A

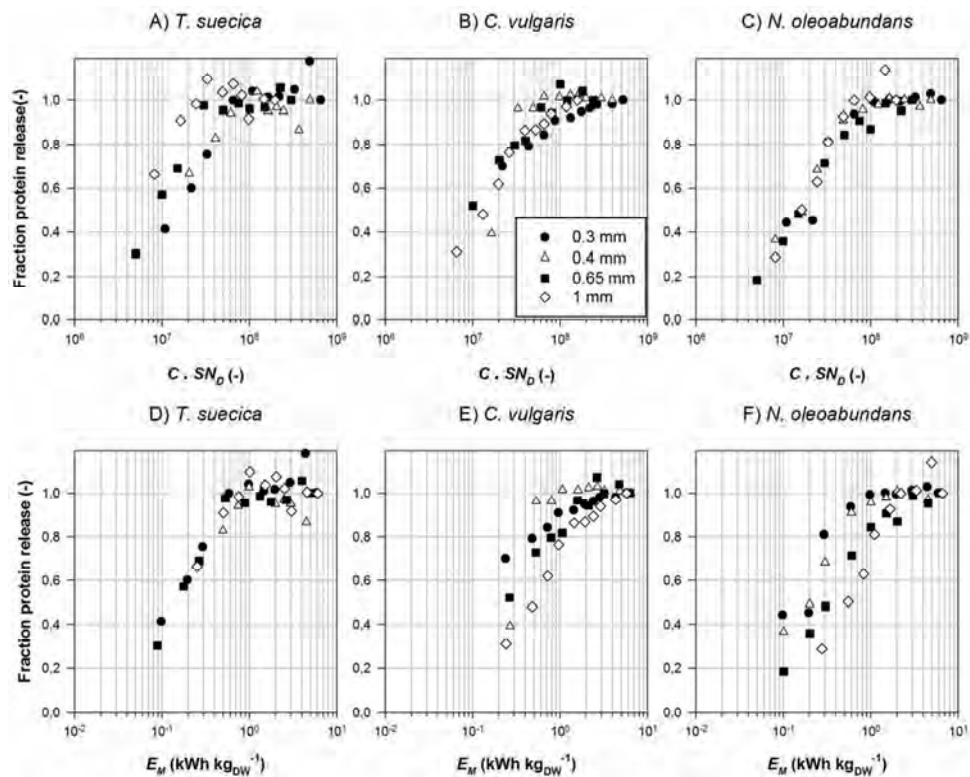


Figure 3.A.1 Semi-log plot of fraction protein release as a function of stress number $C \cdot SN_D$ (-) for *T. suecica* (A), *C. vulgaris* (B) and *N. oleoabundans* (C). Semi-log plot of fraction protein release as a function of the specific energy consumption E_M (kWh kg_{DW}⁻¹) for *T. suecica* (D), *C. vulgaris* (E) and *N. oleoabundans* (F).

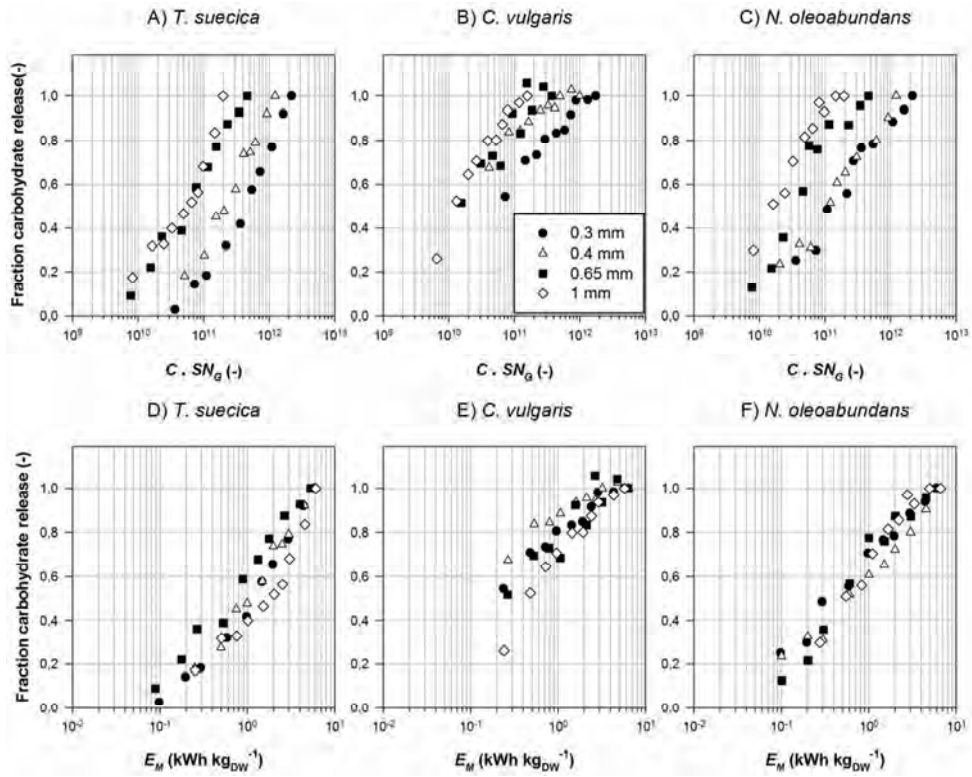
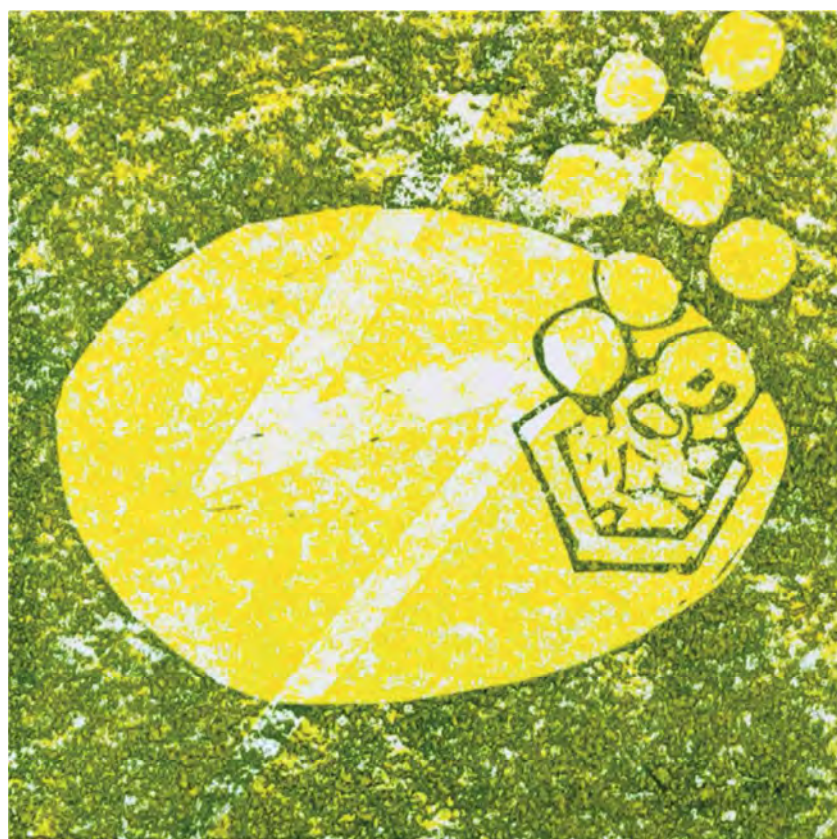


Figure 3.A.2 Semi-log plot of fraction carbohydrate release as a function of stress number $C \cdot SN_G$ (-) for *T. suecica* (A), *C. vulgaris* (B) and *N. oleoabundans* (C). Semi-log plot of fraction carbohydrate release as a function of the specific energy consumption E_M ($\text{kWh kg}_{\text{DW}}^{-1}$) for *T. suecica* (D), *C. vulgaris* (E) and *N. oleoabundans* (F).



Chapter 4

Pulsed Electric Field for protein release from the microalgae *Chlorella vulgaris* and *Neochloris oleoabundans*

The contents of this chapter have been submitted as:

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Pulsed Electric Field for protein release of the microalgae Chlorella vulgaris and Neochloris oleoabundans

Abstract

Pulsed Electric Field (PEF) is currently discussed as promising technology for mild and scalable cell disintegration of microalgae. In this study *Chlorella vulgaris* and *Neochloris oleoabundans* have been subjected to batch and continuous PEF treatments under a wide range of operating conditions (1 – 40 pulses, 0.05 – 5 ms pulses, 7.5 – 30 kV cm⁻¹, 0.03 – 150 kWh kg_{DW}⁻¹). In many cases after treatment, both algal species show release of ions, which indicates that PEF treatment resulted in permeabilization of the algal cell. However, the poration effect was not sufficient to substantially release intracellular proteins. Even at severe energy input (10 to 100 times higher than the benchmark bead milling) only up to 13% of proteins released from the cells in comparison to 45-50% after bead milling.

4.1 Introduction

Microalgae are a promising feedstock for the production of bulk commodities because of their interesting composition (Becker, 2007; Popovich et al., 2012; Wijffels et al., 2010). It has been proposed in literature to increase the potential value of the biomass by adopting a biorefinery approach instead of a single-product isolation (Eppink et al., 2012; Singh and Gu, 2010; Vanthoor-Koopmans et al., 2013; Wijffels et al., 2010; Yen et al., 2013). By applying biorefinery, all the components, such as proteins, pigments and carbohydrates, can be valorized (Vanthoor-Koopmans et al., 2013). Though, the biorefinery should be mild to maintain the integrity of the components (Schwenzfeier et al., 2014, 2013a, 2013b, 2011; Vanthoor-Koopmans et al., 2013).

The majority of these components are present in the cytoplasm or in internal organelles (e.g., chloroplast) and they are difficult to access due to the rigid algae cell walls (Günerken et al., 2015). On the other hand, harsh cell disintegration technologies are not preferred if especially proteins are foreseen to be extracted in their native form (Vanthoor-Koopmans et al., 2013).

PEF has already been mentioned as a promising technology for mild cell disintegration in literature (Eing et al., 2009; Kotnik et al., 2015; Luengo et al., 2014). By applying short electrical pulses, the cell membrane can be charged sufficiently to cause a rearrangement of the lipid membrane, resulting in pore formation (Kotnik et al., 2015). Due to the short electrical pulses applied, this technology requires a low energy input. In addition, the method is mild for the molecules that should be released because there is a limited temperature increase and limited shear forces during the treatment.

An overview of studies on the application of PEF for disintegration of microalgal and cyanobacterial biomass for the release of proteins and lipids is presented in Table 4.1. From this overview, it can be observed that not only various experimental approaches, but also various results have been obtained. When looking to the protein yields, it can be seen that over a wide range of specific energy inputs ($0.02 - 239 \text{ kWh kg}_{\text{DW}}^{-1}$) very low to low protein yields have been obtained.

On the other hand, PEF was shown to be an effective technology for the disintegration of cyanobacteria to enhance the extraction of lipids (Table 4.1). Therefore, it is difficult to create a consensus about the performance of PEF for the disintegration of microalgal biomass. The low protein yields contradict the current view in literature on the general feasibility of PEF (Kotnik et al., 2015). In addition, even though PEF is regarded as a promising technology for releasing hydrophilic proteins, an elaborate study that evaluates PEF over a similar range of processing conditions in direct comparison to benchmark disintegration

technologies is not presented yet. Further, some studies applied marine cultivated microalgae, although the effect of desalination prior to the PEF treatment has not been addressed yet (Coustets et al., 2013, 2015; Grimi et al., 2014; Parniakov et al., 2015a).

Table 4.1 Literature overview of previous performed PEF studies.

Microorganism	Product	Conditions	Outcome	Reference
<i>Nannochloropsis salina</i>	Proteins	15.4 - 30.9 kWh/kg, 37 °C outflow temperature, 0.0545 - 0.109 % DCW	4 fold more extraction with water than methanol extraction of untreated cells	(Coustets et al., 2013)
<i>Chlorella vulgaris</i>	Proteins	2.3 kWh/kg, 37 °C outflow temperature, 0.73 % DCW	2 fold more extraction with water than methanol extraction of untreated cells	(Coustets et al., 2013)
<i>Auxenochlorella protothecoides</i>	Lipids	0.15 - 0.6 kWh/kg, 10 % DCW	Over 3 fold more extraction with ethanol	(Eing et al., 2013)
<i>Auxenochlorella protothecoides</i>	Proteins	0.15-0.6 kWh/kg, 14-22 °C temperature increase, 3.6-16.7%DCW	2 µg/L of protein release in the supernatant	(Goettel et al., 2013)
<i>Nannochloropsis salina</i>	Proteins	0.4-1.5 kWh/kg, 1.0 % DCW	3.6% protein release after PEF treatment	(Grimi et al., 2014)
<i>Nannochloropsis salina</i>	Proteins	0.02-14 kWh/kg, 5.74-34.45 °C temperature increase, 1%DCW	Protein release in the supernatant of 10%	(Parniakov et al., 2015a)
<i>Chlorella vulgaris</i>	Proteins/ Carbohydrates	0.6 – 1.1 kWh/kg, 2.5% DCW, continuous flow (33 mL min ⁻¹)	4.9% ^{protein} release after PEF treatment	(Postma et al., 2016b)
<i>Synechocystis PCC 6803</i>	Lipids	59.7 – 239 kWh/kg, 0.03% DCW	25-75% increased lipid recovery	(Sheng et al., 2012)
<i>Scenedesmus spp.</i>	Lipids	6.9 kWh/kg, 0.44% DCW	3.1 fold increase in lipid recovery	(Lai et al., 2014)

This work therefore presents a systematic screening of the operating conditions required to spontaneously release ions and proteins from the fresh water species *Chlorella vulgaris* and the marine water cultivated species *Neochloris oleoabundans* using two different PEF devices in a wide range of operating conditions. The results obtained with PEF are compared with those found for bead milling as a mechanical benchmark (Postma et al., 2015). By doing so, a quantitative insight on the current state-of-development of PEF compared to a benchmark technology for both freshwater and marine cultivated microalgae is obtained.

4.2 Material and Methods

4.2.1 Study Design

This study is divided in three different parts: biomass pre-treatment, batch PEF operation and continuous PEF operation. The biomass pre-treatment describes the effect of washing and concentrating on the integrity of both microalgal strains. After the pre-treatment, using a batch mode PEF, various experiments were performed to determine the effect of operating conditions, and the energy input on the release of ions and proteins. Finally, to eliminate an effect of the equipment design, additional experiments using a continuous mode PEF were performed.

4.2.2 Biomass supply and preparation

Chlorella vulgaris (SAG 211-11b, EPSAG Göttingen, Germany) was cultivated for 7 days according to Postma et al. (2015) using repeated batch cultivation in a fully controlled 12L stirred tank reactor. The light intensity was increased during the cultivation from 400 up to 1100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The temperature was kept constant at 25 °C and *C. vulgaris* was cultivated in M8a medium at pH 7.0 according to Kliphuis et al. (2010). The microalgae were harvested each time at late linear growth phase at an $\text{OD}_{750\text{nm}}$ of ~ 15 .

Neochloris oleoabundans (UTEX 1185, Austin, USA) was cultivated for 4 days in a continuous mode operated 3L stirred tank reactor. During cultivation the incident light intensity was kept constant at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Temperature and pH were kept constant at 25 °C and 7.5 respectively. *N. oleoabundans* was cultivated in artificial sea-water according to Breuer et al. (2012). After harvesting, the biomass of both species was stored in a cooled (4°C) and dark environment for maximum 72 hours.

Samples were centrifuged at 4000 x g for 15 minutes and the pellet was washed with Milli-Q water (*N. oleoabundans*) or with a 0.04% NaCl solution (*C. vulgaris*) to adjust the conductivity of the samples to a conductivity of maximum 1.5 mS cm^{-1} prior to PEF treatment. After washing the biomass, the concentration was adjusted to the desired concentration. The effect of a possible osmotic shock after washing the algal biomass was determined by analysis of protein release before and after washing.

4.2.3 Batch mode PEF treatment

Batch mode screening of PEF conditions was performed in a lab-scale electroporator (Gene-Pulser XcellTM Bio-Rad, USA), also commonly used for electrotransformation of algae cells (Chow and Tung, 1999; Kilian et al., 2011; Run et al., 2016), using cuvettes with gap distances of 1, 2 and 4 mm (Bio-Rad, Hercules, CA, USA). By altering the voltage between 1.6 and 3.0

kV the electric field strength could be varied between 7.5 and 30 kV cm⁻¹. Further, 1 - 40 square wave pulses with various lengths (0.05 – 5 ms) were applied each 5 s. For *N. oleoabundans*, the cuvettes they were cooled to a temperature of 4 °C after filling, before PEF treatment. Electroporation of *C. vulgaris* was always conducted at room temperature. After treatment, the temperature was measured and it never exceeded 40 °C for all experiments of both algae.

The treated samples were gently mixed for 1 hour to allow intracellular components to diffuse out of the biomass. After mixing, the suspension was centrifuged (20,000 x g, 10 min) and the release of intracellular components was measured in the supernatant.

4.2.4 Continuous flow PEF treatment

Continuous flow PEF experiments were performed on a previously described lab-scale PEF system (2014) as a downscaled copy of a pilot-scale PEF equipment (Timmermans et al., 2011). Special attention was paid to downscale criteria to guarantee electric field homogeneity. In short, the algae suspension (20 °C) was pumped at a flowrate of 13 ml min⁻¹ through two co-linear treatment zones placed in series with a diameter of 1 mm and a gap distance of 2 mm, resulting in a total residence time of 13.5 ms in the treatment chambers. Directly after leaving the treatment chambers, the suspension was cooled down within 3 seconds to a temperature below 20 °C. PEF processing was applied using square wave monopolar pulses at an electric field strength of 20 kV cm⁻¹ with a pulse duration of 2 µs. The pulse waveform, voltage and intensity were monitored with a digital oscilloscope (Rigol DS1102, Beaverton, USA). By varying the pulse frequency, the total number of applied pulsed was changed leading to different maximum temperatures (Table 4.2).

Table 4.2 Process conditions used for PEF treatment of algae suspensions on continuous flow system.

Suspension	Frequency (Hz)	Number of pulses	Electrical field strength (kV cm ⁻¹)	T _{in} (°C)	T _{out} (°C)	dT (°C)
<i>C. vulgaris</i>	964	14.0	20.6	21.7	30.4	8.7
	390	5.7	20.4	21.8	25.7	3.9
	120	1.7	20.1	21.8	23.2	1.4
	0	0	0.0	21.9	21.9	0.0
<i>N. oleoabundans</i>	964	14.0	19.7	20.8	31.4	10.6
	390	5.7	20.3	21.0	25.3	4.3
	120	1.7	20.7	21.2	22.7	1.5
	0	0	0.0	21.3	21.3	0.0

Temperature increase for each condition was measured using thermocouples placed just before and after the treatment chambers. Furthermore, it could be calculated, based on Eq. (4.1):

$$dT = \frac{E^2 \cdot \sigma \cdot \tau}{\rho \cdot c_p} \quad (4.1)$$

where E is electric field strength (V m^{-1}), σ is conductivity (S m^{-1}), τ is pulse duration (s), ρ is density of the algae suspension, c_p is the specific heat ($\text{kJ (kg} \cdot \text{K)}^{-1}$), being $4.12 \text{ kJ (kg} \cdot \text{K)}^{-1}$. The used biomass concentration in this experiment was 25 g kg^{-1} for both algae, resulting in a specific energy input of 0, 0.05, 0.165 and $0.41 \text{ kWh kg}_{\text{DW}}^{-1}$.

4.2.5 Bead mill experiments

The protein release after bead milling reported for *C. vulgaris* by Postma et al. (2015) was used to evaluate the performance of using PEF for this species. For *N. oleoabundans*, additional bead mill experiments were performed similar to Postma et al. (2015). A Dyno® - Mill ECM-AP 05 bead mill was operated using zirconia beads with bead sizes of 0.3 and 0.5 mm. The treatment chamber was filled for 70% and the applied tip speed was 8 m s^{-1} . Biomass concentrations ranging between 50 and $100 \text{ g}_{\text{DW}} \text{ kg}^{-1}$ were treated in different modes of operation: single pass, double pass and with a batch recirculation. In all experiments the liquid throughput was 10 kg h^{-1} . After treatment, the protein release in the supernatant was measured. To determine the increase in conductivity, lab scale experiments using beat beating were performed.

4.2.6 Conductivity measurement

Before and after every treatment, the conductivity of the supernatant was measured at room temperature using a Mettler Toledo® SevenCompact™ probe without temperature compensation. All samples were analyzed at the same temperature (room temperature). As a positive control, bead beaten biomass was measured and results were used for further calculations.

4.2.7 Protein analysis

The total protein content on biomass dry weight (DW) was determined according to de Winter et al. (2013). In short, the biomass was freeze dried and then beat beaten in a cell lysis buffer to solubilize all proteins. After bead beating the samples were incubated for 30 min at 100°C .

Modified Lowry protein assay kits (Thermo Scientific and Bio-rad) were used to measure the total protein content and the soluble protein release before and after PEF treatment. The absorbance was measured at 750nm. Bovine serum albumin was used as a proteins standard.

4.2.8 Determination of the specific energy input

The volumetric specific energy input (W_V), previously described as the treatment intensity (TI) by Salerno et al. (2009) and Sheng et al. (2012), was calculated based on the operating conditions (electrical field strength, pulse number) and the conductivity before PEF as:

$$W_V \text{ (kWh } m^{-3}) = \frac{E^2 \cdot t_p \cdot N \cdot \sigma}{3600000} \quad (4.2)$$

in which E is the electrical field strength in $V \text{ m}^{-1}$, t_p is the pulse length (s), N are the number of pulses and σ is the initial conductivity at room temperature ($S \text{ m}^{-1}$).

The mass specific energy input (W_M) was subsequently calculated as:

$$W_M \text{ (kWh } kg_{DW}^{-1}) = \frac{W_V}{C_x} \quad (4.3)$$

in which C_x is the biomass concentration ($kg_{DW} \text{ m}^{-3}$).

4.2.9 Determination of the relative ion yield and protein yields

The permeabilization of the cell membrane was monitored by measurement of the electrical conductivity (Donsì et al., 2010). Similar to other studies, the relative ion yield (σ_R) was expressed as the specific increase in conductivity with PEF over the specific conductivity increase after bead beating. The conductivity increase after bead beating was measured to be 0.98 mS cm^{-1} for *C. vulgaris* and 1.06 mS cm^{-1} for *N. oleoabundans*.

$$\sigma_R (\%) = \frac{(\sigma_{\text{after PEF}} - \sigma_{\text{before PEF}})}{(\sigma_{\text{after bead beating}} - \sigma_{\text{before beat beating}})} \quad (4.4)$$

Finally, the amount of released proteins was expressed as the increase in released proteins divided over the total protein content of the biomass:

$$\text{Protein yield } (\%) = \frac{PR_{\text{sup}} (\%_{\text{dw}})}{\text{total protein content } (\%_{\text{dw}})} \quad (4.5)$$

In which the 'proteins released in supernatant (PR_{sup})' are expressed as:

$$PR_{\text{sup}} (\%_{\text{dw}}) = PR_{\text{sup after PEF}} (\%_{\text{dw}}) - PR_{\text{sup before PEF}} (\%_{\text{dw}}) \quad (4.6)$$

By using ' $PR_{\text{sup before PEF}}$ ', and not the initial amount of proteins present in the supernatant, the effect of the osmotic shock can be distinguished from the effect of the PEF treatment.

4.2.10 Experimental design and statistical analysis

To exclude possible effects of the equipment design and to confirm the obtained results in the batch mode PEF, additional experiments were performed under continuous mode PEF. During continuous mode experiments, next to performing all analysis in technical replicates,

drifts in the pulse delivery were eliminated by ensuring steady state operation prior to sampling.

To ensure reliability of the experimental data, all analytical procedures have been performed in at least technical duplicates. During the batch-electroporator campaign of experiments, additional tests at extreme conditions ($W_M > 90 \text{ kWh kg}_{\text{DW}}^{-1}$) were performed. An independent samples t-test with a significance level of $p = 0.05$ (assuming equal variances) was used for statistical analysis.

4.3 Results and Discussion

In this section, first the results obtained using the batch mode PEF are presented followed by the results of the continuous flow PEF. Finally, the current state-of-development is discussed.

4.3.1 Batch mode PEF

Effect of pulse parameters on PEF

Pre-treatment of *C. vulgaris* by resuspending in 0.04% NaCl did not release any proteins, even if an osmotic shock occurred. Figure 1 presents the specific ion release and the protein yield for *C. vulgaris* after applying a PEF treatment at three different energy consumptions for a fixed biomass concentration of 25 g kg^{-1} . At each energy input, the pulse length, and number of pulses were changed to determine the effect of these individual parameters. The used energy inputs were; 0.4, 1.4 and $14 \text{ kWh kg}_{\text{DW}}^{-1}$. With an increasing pulse length, the number of pulses was decreased proportionally at a given specific energy input. (Figure 4.1).

The results in Figure 4.1 show that with a specific energy input similar to the ones reported for bead milling (Postma et al., 2015), a substantial increase in conductivity was obtained. These results imply that small components such as ions can be successfully released using PEF-treatments. Even though high amounts of ions were released, the protein yields were at best 6-8 fold lower in comparison to the mechanical benchmark bead milling (Postma et al., 2015). Noteworthy are the results by Sheng et al. (Sheng et al., 2012, 2012) and Ganeva et al. (Ganeva et al., 2003a), who treated the cyanobacteria *Synechocystis* PCC68003 and the yeast *Saccharomyces cerevisiae*, respectively. A volumetric specific energy input (i.e., treatment intensity) W_V of $\sim 30 \text{ kWh m}^{-3}$ appeared in their study sufficient to successfully disintegrate the cyanobacteria and yeast cells. Yet, this work showed that in the case of eukaryotic microalgae, a W_V of 35 kWh m^{-3} ($1.4 \text{ kWh kg}_{\text{DW}}^{-1}$) or even 350 kWh m^{-3} ($14 \text{ kWh kg}_{\text{DW}}^{-1}$) was merely enough to release small ionic substances.

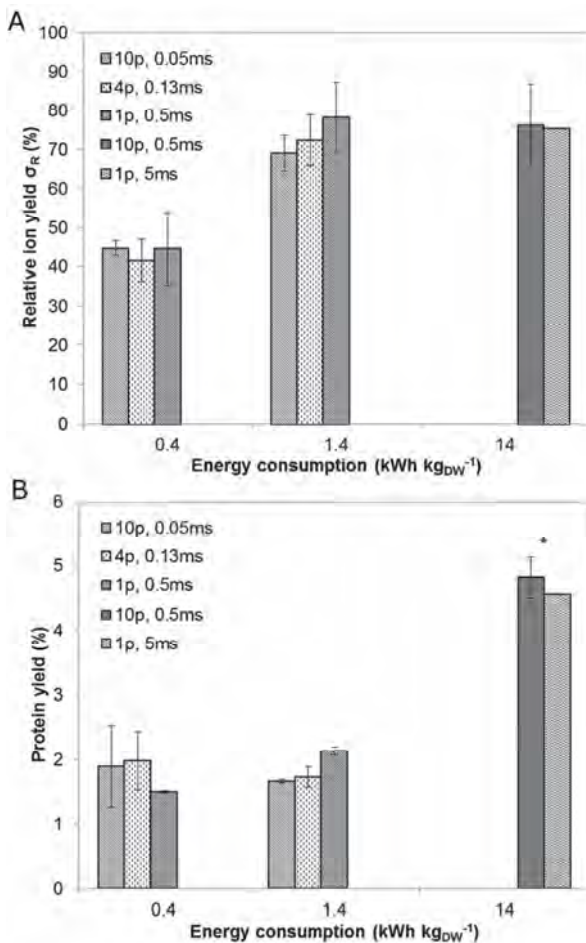


Figure 4.1 Relative ion yield (σ_R) after PEF treatment as a function of pulse parameters for *C. vulgaris* (A). Protein yield measured 1h after PEF as a function of pulse parameters for *C. vulgaris* (B). The electric field strengths was 8, 15 and 15 kV cm^{-1} for 0.4, 1.4 and 14 $\text{kWh kg}_{\text{DW}}^{-1}$ respectively. * 14 $\text{kWh kg}_{\text{DW}}^{-1}$ significant different from 0.4 and 1.4 $\text{kWh kg}_{\text{DW}}^{-1}$. Errors bars show standard deviation ($n=2$).

Next to the release of proteins, Figure 4.1B also illustrates that individually varying the pulse length or number of pulses did not affect the protein yield. Instead, it appears that only the energy input affects the performance of PEF, as being illustrated the increase in release from about 1.8% at 0.4 $\text{kWh kg}_{\text{DW}}^{-1}$ up to 4.8% at 14 $\text{kWh kg}_{\text{DW}}^{-1}$ ($p < 0.05$). No difference could be observed between 0.4 and 1.4 $\text{kWh kg}_{\text{DW}}^{-1}$ ($p = 0.82$). This suggests that the specific energy input is the most important parameter affecting the operation. Similar results have been reported by Coustets et al. (2015). In their study 30 pulses of 1 ms and 15 pulses of 2 ms resulted in the same protein release at a fixed field strength of 4.5 kV cm^{-1} .

4.3.2 Release of intracellular components

The results of Figure 4.1 showed that only the specific energy input affects the overall performance of PEF (under the same biomass concentration). Therefore, additional experiments were performed in which the ion release and the protein yield were investigated as a function of the energy input. The goal of these experiments was to identify operating conditions at which both a high release of ions and a high release of proteins could be obtained. This was done by extending the energy input range from 0.03 up to 150 kWh kg_{DW}⁻¹. In these experiments, both *C. vulgaris* and the seawater cultivated *N. oleoabundans* were subjected to a PEF treatment.

Prior to PEF-treatment, also *N. oleoabundans* was washed similar to the washing applied on *C. vulgaris*. The washing resulted in a decrease of medium conductivity from 45 mS cm⁻¹ to less than 0.5 mS cm⁻¹. The protein release caused by this pre-treatment was at maximum 4.8%_{DW} after washing (3.4%_{DW}) and concentrating (1.4%_{DW}).

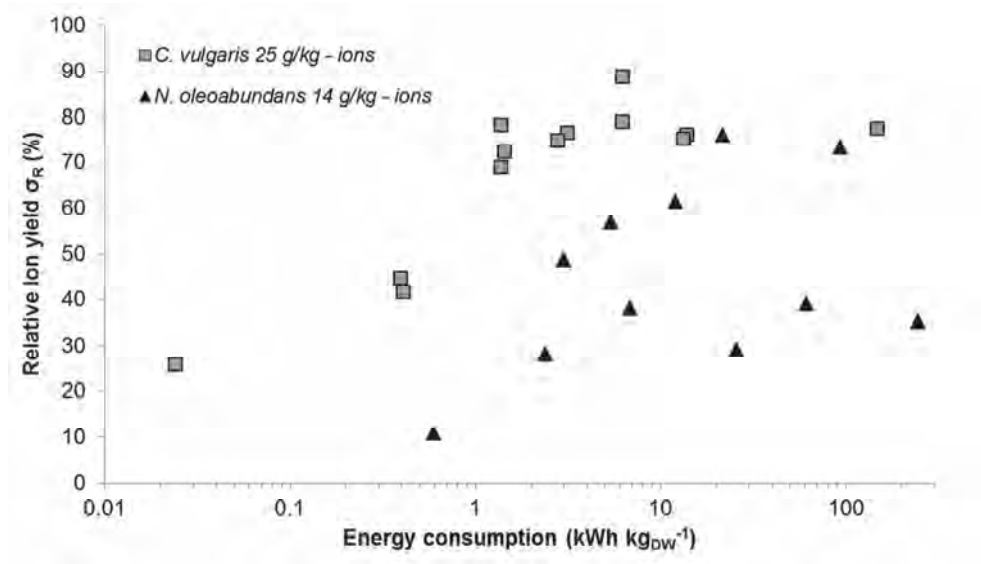


Figure 4.2 Relative ion yield (σ_R) for *C. vulgaris*, and *N. oleoabundans* after PEF treatment. Part of *C. vulgaris* originates from Figure 4.1.

In Figure 4.2, the ion-yield for both microalgae is presented as a function of the mass specific energy input W_M . The results show that due to the PEF treatment, a relative increase up to 87% and 81% with *C. vulgaris* and *N. oleoabundans* compared to beat beating was obtained, respectively. These results suggest that only small pores were formed in the cell membrane and cell wall allowing ions to be released.

Similar results were reported by Goettel et al. (2013), after PEF treatment and 6 hours of resting time, an increase in conductivity of 1 mS cm^{-1} was observed using biomass concentrations ranging between 36 and $167 \text{ g kg}_{\text{DW}}^{-1}$. Also in the study of Eing et al. (Eing et al., 2013), a conductivity increase of 1 mS cm^{-1} at a biomass concentration of $100 \text{ g kg}_{\text{DW}}^{-1}$ was obtained. Although a relative increase (σ_R) was not calculated in those studies, the absolute increase in conductivity after PEF treatment was in the same order of magnitude as the increase obtained in this study.

Next to achieving a reasonable high ion-yield, part of the aim was to further enhance the protein release. Figure 4.3 shows the protein yield as a function of the mass specific energy input W_M .

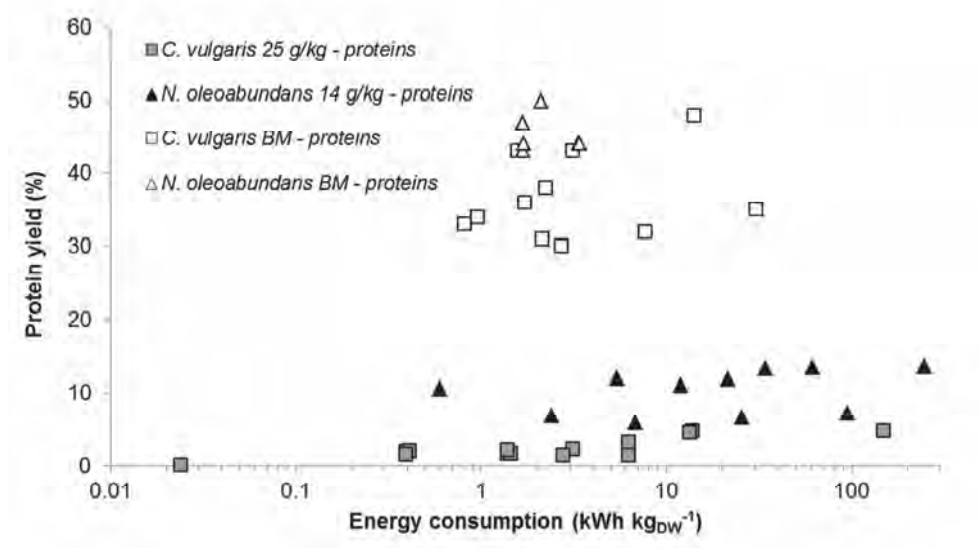


Figure 4.3 Protein yield as function of the specific energy input. Protein release measured 1h after application of PEF. Specific energy consumption calculated based on initial conductivity at 25 °C. Benchmark by bead milling BM for *C. vulgaris* (Postma et al., 2015) and *N. oleoabundans* (this study).

From Figure 4.3 it can be observed that for both strains treated with PEF, the protein release did not exceed 13%. With bead milling however, the protein release ranged between 30-50% for both *C. vulgaris* and *N. oleoabundans*. Moreover, in the study of Safi et al. (2014a), a protein release of 51.7% was observed after high pressure homogenization of *C. vulgaris*. These results are in agreement with the protein release presented in Figure 4.2. Even at energy inputs higher than applied during bead milling, no protein release close to the one by mechanical disintegration was observed ($p < 0.05$).

The results obtained with PEF as shown in Figure 4.3 are in agreement with results reported in other studies as well. Parniakov et al. (2015a) reported with *Nannochloropsis salina* a protein yield of maximal 10%. In addition, Goettel et al. (2013) reported a protein yield of < 1% with *Auxenochlorella protothecoides* (assuming a total protein content of 50% on DW). Also in the study of Postma et al. (2016b), which investigated the effect of processing temperature during PEF-treatment, for *C. vulgaris*, similar protein yields to the ones reported in this study were obtained. Furthermore, Grimi et al. (2014) obtained a protein yield of 3.6% with *N. salina*. Coustets et al. (2013) measured proteins after PEF-treatment as well. Although it was not possible to calculate a yield, the protein concentrations in the supernatant were equal, or lower than the protein concentrations measured in this study. In addition as already illustrated by Table 1, the degree of protein release or disintegration was not provided in all studies. Instead only absolute concentrations of components such as carbohydrates, pigments or 'total organic components' were provided (Eing et al., 2013; Luengo et al., 2014). It is therefore difficult to compare our results elaborately with other work.

Overall, the results presented in Figure 4.1, Figure 4.2 and Figure 4.3 suggest that small pores were formed allowing ions to be liberated through the cell wall and membrane. The performance of PEF with respect to protein release was not as efficient as with bead milling limited by the pore formation and/or disintegration (Postma et al., 2016a).

4.3.3 Continuous flow PEF

To quantify the impact of the PEF apparatus design on the observed yields, a continuous flow PEF unit was used and compared to the batch PEF unit. Based on the results presented in Figure 4.1, only the specific energy input was varied in this experiment. By varying the pulse frequency the specific energy input was varied, while keeping the field strength and biomass concentration constant at 20 kV cm⁻¹ and 25 g kg_{DW}⁻¹, respectively.

Figure 4.4 shows that a protein yield between 2.5 and 3.2% was obtained for *C. vulgaris* and between 1.9 and 2.5% for *N. oleoabundans*. These yields are in the same order of magnitude as the ones presented in Figure 4.3, and remained substantially lower than the yields obtained after bead milling. With a similar specific energy consumption of 0.4 and 0.6 kWh kg_{DW}⁻¹ for *C. vulgaris* and *N. oleoabundans* during batch mode PEF yields up to 2.3% and 10.5% were obtained, respectively. So, for *N. oleoabundans* even lower protein yields were obtained as with the batch mode PEF. The results of Figure 4.3 and Figure 4.4 imply that regardless of the energy input and the pulse length (2 μs for continuous PEF and 0.05-5 ms for batch PEF) similar results were obtained.

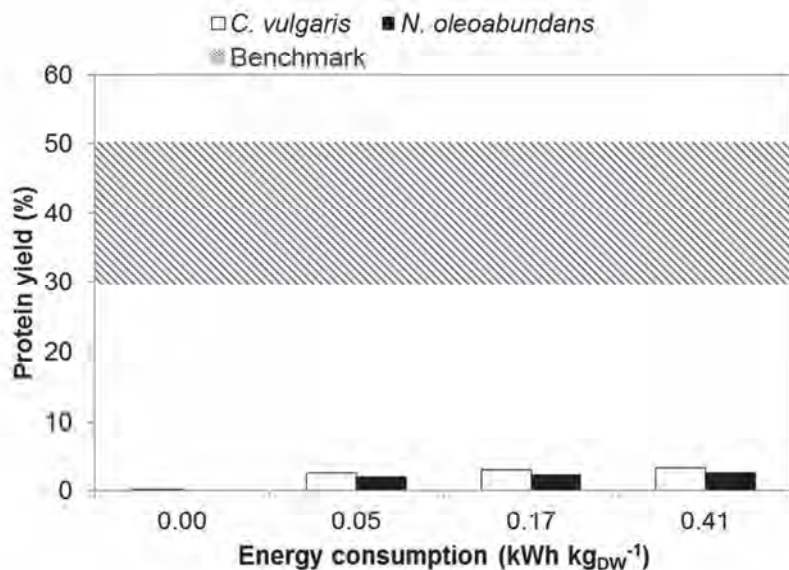


Figure 4.4 Protein release yield measured after 1h versus specific energy consumption for continuous flow PEF. Marked area represents benchmark yields range.

Both strains were cultivated in fresh water for the experiments shown in Figure 4 instead of using artificial seawater medium for *N. oleoabundans*. No proteins were released before treatment as can be observed in Figure 4.4 at 0 kWh kg_{DW}⁻¹, whereas washing of marine cultivated *N. oleoabundans* did release proteins and thus caused an osmotic shock (see paragraph 4.3.2). In any case, the results of Figure 4.4 confirm the general trend that proteins remained entrapped intracellular.

4.4 General discussion

In this study, the highest yield of proteins of 13% was obtained with *N. oleoabundans* cultivated in seawater medium in a batch mode PEF. Despite the effect of an osmotic shock that *N. oleoabundans* suffered during the washing treatment, no yields similar to bead milling were obtained. Also in other studies, similar protein yields after PEF were observed (Goettel et al., 2013; Grimi et al., 2014; Parniakov et al., 2015a).

This study showed, that regardless of the high amount of released ions, PEF was not feasible yet for either a complete disintegration, or for selectively releasing proteins. Although only low protein yields were observed after PEF, several other studies already reported that increased lipid yields could be obtained using extraction after PEF-treatment for both microalgae and also cyanobacteria (Eing et al., 2013; Sheng et al., 2012; Zbinden et al., 2013). It may be that the electroporation performed in this study is sufficient to allow enhanced lipid extraction, making PEF an interesting technology for lipid-scenarios. However, the native state of the soluble proteins is most likely negatively affected diminishing the total biomass value. Therefore, we believe that for a successful biorefinery strategy, first native proteins should be released.

Besides the reported enhanced lipid extraction from microalgae and cyanobacteria, other work showed that PEF was successful in opening cell membranes to inactivate/disintegrate microorganisms lacking a cell wall (Frey et al., 2013; Timmermans et al., 2014). However, microalgae often have an additional rigid cell wall. Recently, Scholz et al. (Scholz et al., 2014) proposed for example that the *Eustigmatophyceae Nannochloropsis gaditana* has a bilayered cell wall composed of a thick layer of cellulose and algaenans. It may be that other microalgae such as the species used in this study have similar properties, limiting the performance of PEF. This observation was also done by Azencott et al. (Azencott et al., 2007) who found that the cell wall of *Chlamydomonas reinhardtii* was limiting the uptake of relatively large (66 kDa) protein molecules.

Next to the protein yield, the energy consumption is influencing the feasibility of PEF. By assuming a total energy content of $6.82 \text{ kWh kg}_{\text{DW}}^{-1}$ in combination with an energy input less than 10%, the resulting energy consumption should be equal or lower than $0.682 \text{ kWh kg}_{\text{DW}}^{-1}$ (Coons et al., 2014). According to this criterion, next to low protein yields, the belonging energy input with PEF was substantially higher than $0.682 \text{ kWh kg}_{\text{DW}}^{-1}$.

4.5 Conclusion

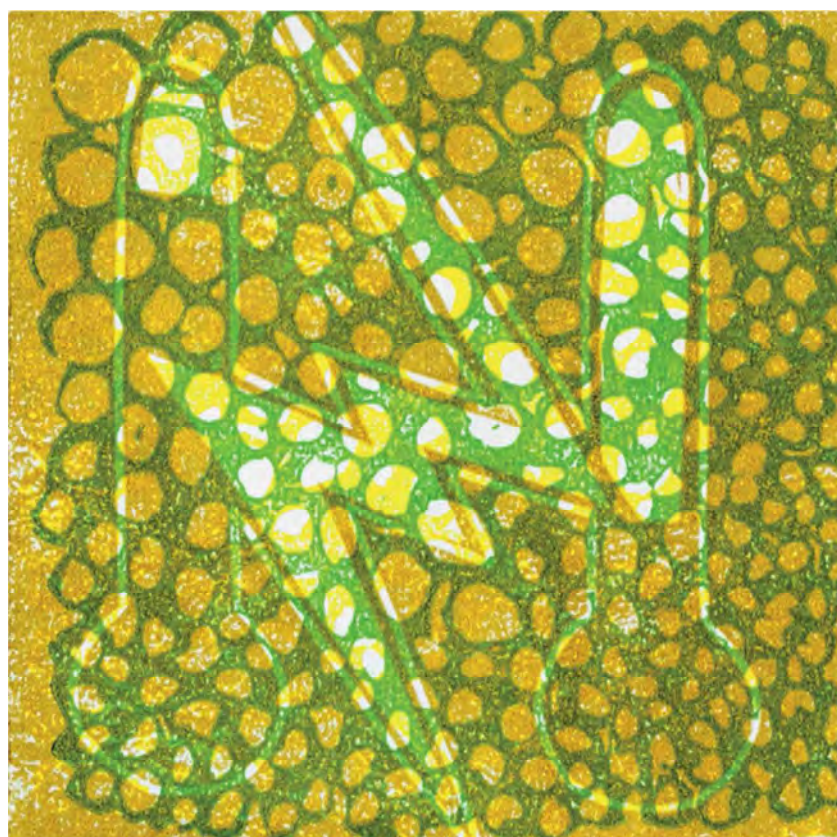
The high release of ions illustrated that the application of PEF for the disintegration of fresh and marine cultivated microalgae, resulted in a weakening of the cell membrane suggesting the formation of pores. Nevertheless, with respect to the mechanical benchmark, no sufficient amounts of protein were liberated by the application of PEF. Moreover, the required energy input for PEF was higher than the mechanical benchmark.

Acknowledgements

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Abbreviations

Symbol	Definition	units
c_p	Specific heat capacity	[kJ kg ⁻¹ K ⁻¹]
C_x	Biomass concentration	[kg m ⁻³]
E	Electrical field strength	[V m ⁻¹]
N	Number of pulses	[-]
PR	Proteins in supernatant	[% _{dw}]
t_p	Pulse duration	[s]
T	Temperature	[°C]
W_V	Volumetric specific energy input	[kWh m ⁻³]
W_M	Mass specific energy input	[kWh kg _{dw} ⁻¹]
ρ	Density of suspension	[kg m ⁻³]
σ	Conductivity	[S m ⁻¹]
σ_R	Relative ion yield	[%]



Chapter 5

Selective extraction of intracellular components from the microalga *Chlorella vulgaris* by combined Pulsed Electric Field-Temperature treatment

The contents of this chapter have been published as:

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*Selective extraction of intracellular components from the microalga Chlorella vulgaris
by combined Pulsed Electric Field-Temperature treatment*

Bioresource Technology (2016) 203, pp. 80-88

Abstract

The synergistic effect of temperature (25-65 °C) and total specific energy input (0.55-1.11 kWh kg_{DW}⁻¹) by pulsed electric field (PEF) on the release of intracellular components from the microalgae *Chlorella vulgaris* was studied. The combination of PEF with temperatures from 25-55 °C resulted in a conductivity increase of 75% as a result of cell membrane permeabilization. In this range of temperatures, 25-39% carbohydrates and 3-5% proteins release occurred and only for carbohydrate release a synergistic effect was observed at 55 °C. Above 55 °C spontaneous cell lysis occurred without PEF. Combined PEF-temperature treatment does not sufficiently disintegrate the algal cells to release both carbohydrates and proteins at yields comparable to the benchmark bead milling (40-45% protein, 48-58% carbohydrates).

5.1 Introduction

Microalgae are promising for the production of multiple components like proteins, carbohydrates, lipids and pigments to be used as functional additives for cosmetic, nutraceutical, chemical, food and feed products as well as for the production of biofuels (Batista et al., 2013; Vanthoor-Koopmans et al., 2013). In order to have an economically feasible production process all intracellular components have to be used in a biorefinery approach (Günerken et al., 2015; Wijffels et al., 2010).

Biorefinery comprises the downstream processing (i.e., recovery, fractionation and purification) of added value ingredients from biomass. Most interesting products from microalgae are commonly stored either in the cytoplasm or in internal organelles. The complex cell structure of microalgae, comprises several organelles such as the chloroplast, mitochondria, Golgi apparatus, nucleus etc., and all these organelles have a different composition and structure (Eppink et al., 2012). In a cascade biorefinery approach, the first step after harvesting is the use of a cell disintegration technique able to break the cell wall and cell membranes facilitating the release of these high value added components from the cytoplasm and the internal organelles (Günerken et al., 2015; Vanthoor-Koopmans et al., 2013). However, cell disintegration should be done avoiding the use of severe processing conditions that could negatively affect the quality and purity of the extracts, diminishing the product value. Conventional cell disintegration techniques may cause complete cell disruption, thus fostering the non-selective release of all cell components. This will reduce the quality and purity of the extracts complicating the subsequent fractionation phase. Moreover, these techniques could be very energy intensive. For instance bead milling is known to be a high-energy-consuming-technique requiring specific energy levels of 0.81, 1.71 or 7.64 kWh kg_{DW}⁻¹ for a biomass concentration of 145, 87.5 and 25 kg m⁻³, respectively (Postma et al., 2015). Therefore, there is a growing interest in finding mild cell disintegration methods, but effective enough to facilitate the release of the target compounds from the inner parts of the cells with low specific energy consumption.

In the past decade, pulsed electric field (PEF) has been claimed to be a promising mild technique able to induce the permeabilization of the microalgal cell membranes by electroporation and to enhance the spontaneous release of intracellular components (Toepfl et al., 2006). Recently, Goettel et al. (2013) investigated for the first time the use of PEF for the release of multiple intracellular components (protein, carbohydrates and lipids) from microalgae. Later on, other researchers (Coustets et al., 2015; Grimi et al., 2014; Lai et al., 2014; Luengo et al., 2015; Parniakov et al., 2015b) have studied the effect of PEF on the recovery of single cell products, where a total specific energy consumption ranging between

0.4–30.9 kWh kg_{DW}⁻¹ was applied. Originally PEF was widely investigated and applied in the medical field for electrochemotherapy (Jaroszeski et al., 2000) and gene transfer (i.e., electrotransformation) of microorganisms and plant cells (Kandušer and Miklavčič, 2009). Additionally, it has been applied in the food industry for either microbial inactivation (cold-pasteurization) or on mass transfer of liquids and valuable compounds from the inner parts of plant cells (extraction, drying) (Álvarez et al., 2006; Donsì et al., 2010; Kotnik et al., 2015; Pataro et al., 2011; Vorobiev and Lebovka, 2010). Moreover, Ganeva et al. (2003b) showed that PEF can be a mild technique to disintegrate yeast, where 70-90% of the total enzyme activity was maintained and large proteins up to 250 kDa could be released with a yield up to 50%. To our knowledge, these yields of large proteins have so far not been obtained for microalgae.

Several parameters influence the PEF efficacy, which mainly include electric field strength and total specific energy input (Pataro et al., 2014). In general, depending on the settings of these parameters reversible or irreversible pores are formed (Kotnik et al., 2015). However, in some cases, it is necessary to apply intense process conditions (high field strengths and energy inputs) to obtain a sufficient high permeabilization degree of the cell membrane (Pataro et al., 2011). Therefore, in order to obtain the required permeabilization effect with less severe processing conditions, or to achieve higher efficacy at the same treatment conditions, PEF has also been applied in a hurdle approach. For example, additive or synergistic effects have been observed by combining PEF with moderate heating above 35 °C for microbial inactivation (Timmermans et al., 2014). As per the literature survey, only Luengo et al. (2015) described the effect of temperature (10-40 °C) on the release of the pigment lutein during PEF treatment of microalga *Chlorella vulgaris*. They found that, at 25 kV cm⁻¹, increasing the temperature of the biomass from 10 °C to 20 °C increased the extraction yield by 35%, but an increase lower than 10% was observed by raising the treatment temperature from 30 to 40 °C. Therefore, further studies are necessary to better elucidate the interactions between electric field and temperature and the dependence of the extraction yield of the target components on the process parameters of the combined treatment. To our knowledge this is the first attempt to investigate the effect of a combined PEF-Temperature treatment on the selective release of multiple water soluble components from microalgae within a microalgae biorefinery concept.

The objective of this work was to investigate the effect of the processing temperature during PEF treatment of the microalga *Chlorella vulgaris* on the selective release of intracellular components. Release of ions (conductivity), carbohydrates and proteins was followed. Additionally, the effect of this treatment on the product quality was measured with gel electrophoresis and a Rubisco activity assay.

5.2 Methods

Chlorella vulgaris (SAG 211-11b) was obtained from the Culture Collection of Algae at Göttingen University and was cultivated in M-8a medium as described by Kliphuis et al. (2010) using a 12L stirred tank photobioreactor (Postma et al., 2015). After harvesting, the biomass was concentrated by means of centrifugation ($4000 \times g$, 15min and 4°C) up to a final concentration (C_x) of $25 \text{ kg}_{\text{DW}} \text{ m}^{-3}$ with an initial conductivity (σ) of about 0.6 mS cm^{-1} at 25°C (Conductivity meter HI 9033, Hanna Instrument, Milan, Italy).

The concentrated biomass was pre-packed in high-density polyethylene bottles (Nalgene) and cooled to 4°C . The transport of the biomass to ProDAI Scarl (University of Salerno, Fisciano (SA), Italy) was conducted by courier within 24 hours in an EPS box in which the refrigerated temperature was maintained using gel-packs. PEF treatments were performed on the delivery day. A sample of the concentrated biomass was taken and analyzed at Wageningen University on the shipment day as well as the delivery day. Results showed no influence of the microalgae transport to Salerno on the cell components of interest (proteins and carbohydrates) for this work (data not shown).

5.2.1 PEF experimental set-up

PEF experiments were conducted in a bench-scale continuous flow PEF system of which a schematic overview is shown in Figure 5.1. The PEF unit design was based on the unit described by Pataro et al. (2014), but adjusted in order to include two PEF treatment zones. In short, a peristaltic pump (Pump Drive PD5201, Heidolph Instruments GmbH, Germany) provided a continuous flow Q of 33 mL min^{-1} of the algae biomass suspension. Prior to entering each of the two PEF treatment zones, the algae suspension flowed through a stainless steel coil immersed in a water heating bath to control the temperature between $25 - 65^\circ\text{C}$. Each PEF treatment zone consisted of a module made of two co-linear cylindrical treatment chambers, hydraulically connected in series, with an inner radius (r) of 1.5 mm and a gap distance (L) of 4 mm . The geometry of each treatment chamber was previously described by (Pataro et al., 2012) and is schematically shown in Figure 5.1B.

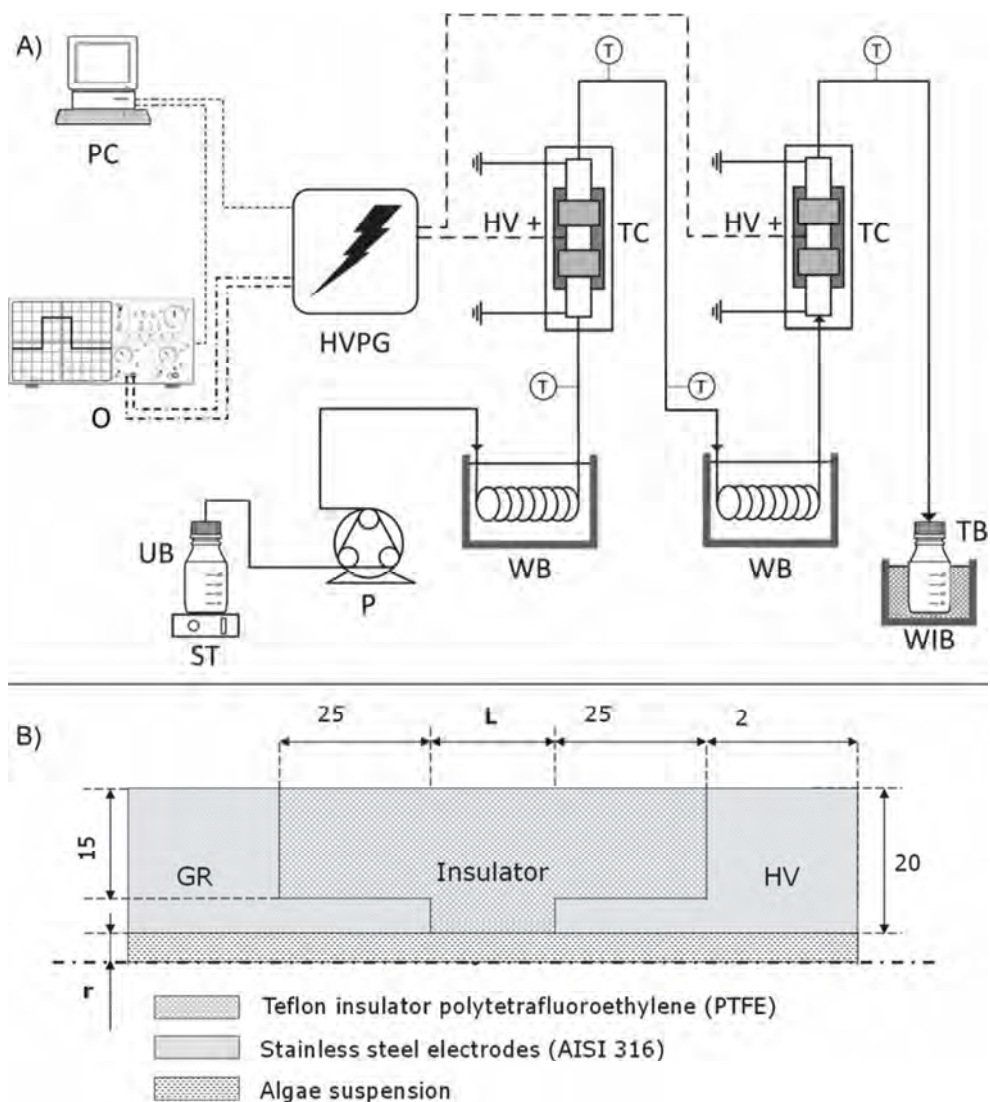


Figure 5.1 A) Schematic overview of continuous flow PEF system. O: oscilloscope, UB: untreated biomass, ST: magnetic stirrer, HVPG: high voltage pulse generator, P: peristaltic pump, WB: water bath, HV+: high voltage, T: thermocouple, TC: treatment chamber, TB: treated biomass, WIB: water ice bath; and B) Dimensions and geometry of a single co-linear PEF treatment chamber in axis-symmetrical configuration. GR: ground electrode; HV: High Voltage electrode; L: gap distance (4 mm); r: inner radius (1.5 mm) (adapted from Pataro et al., (2012)).

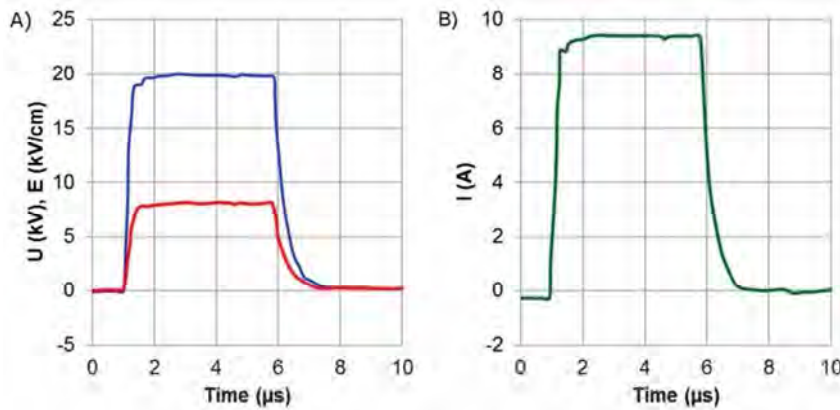


Figure 5.2 Typical pulse waveforms captured at the treatment chamber. A) Voltage U (blue) and Electric field strength E (red) and B) current I (green). $E=U(t)/L$.

Monopolar square wave electric pulses were supplied by a high voltage pulse generator (Diversified Technology Inc., Bedford, WA, USA). The voltage and current signals at the treatment chamber were measured by a high voltage probe (P6015A, Tektronix, Wilsonville, OR, USA) and a Rogowsky coil (2-0.1W, Stangenes, Inc., USA), respectively. The measurements were recorded and displayed using a 300 MHz digital oscilloscope (TDS 3034B, Tektronix, Wilsonville, OR, USA) connected to a PC. An example of typical pulse waveforms at the treatment chamber is shown in Figure 5.2. The theoretical electric field intensity (E) was evaluated as the applied voltage (U) divided by the gap between electrodes (L). However, it is known that the actual electric field strength in the PEF treatment zone of a co-linear chamber is often lower than the theoretical value (E) and suffers from a non-uniform distribution and intensity peaks near the insulator edges (Buckow et al., (2011). Therefore, in this work, the actual average electric field strength E_{av} (kVcm⁻¹) applied in the treatment zone was evaluated according to the following equation previously proposed by Buckow et al. (2011):

$$E_p \cdot E_{av}^{-1} = 1 + \alpha \cdot r \cdot L^{-1} \quad (5.1)$$

where E_p (kV cm⁻¹) is the theoretical peak electric field strength evaluated as the peak voltage divided by the inter-electrode gap, and α is a coefficient which assumes a value of 0.4475 for an inner radius of 1.5 mm (Pataro et al., 2012). The total specific energy input W_{PEF} (kWh kg_{DW}⁻¹) was calculated as a function of the total number of pulses n applied in the four treatment chambers, the volume v_i (mL) of each treatment zone and the biomass concentration C_x (kg_{DW} m⁻³). $U(t)$ and $I(t)$ represent the voltage across the electrodes and the current intensity through the product at time t (s), respectively:

$$W_{PEF} = \frac{n}{3.6 \cdot v_i \cdot C_x} \int_0^{\infty} U(t) \cdot I(t) dt \quad (5.2)$$

$$\text{in which: } n = \frac{v_i \cdot N_{TC}}{\Phi} \cdot f \quad (5.3)$$

where, N_{TC} is the total number of treatment chambers, Φ is the flow rate (mL s^{-1}) and f is the pulse repetition frequency (Hz). During the treatment, a single voltage value of 8 kV was set resulting in a theoretical peak electric field strength (E_p) of 20 kV cm^{-1} which corresponds, according to Eq. (5.1), to an average electric field (E_{av}) of 17.1 kV cm^{-1} .

The pulse length was fixed to $5 \mu\text{s}$ and the pulse frequency was adjusted between 50 – 200 Hz to provide a specific energy input (W_{PEF}) of 0.55 and 1.11 kWh kg^{-1} dry weight ($\text{kWh kg}_{\text{DW}}^{-1}$) at each processing temperature investigated ($25\text{--}65^\circ\text{C}$). Four thermocouples were used to measure the product temperature at the inlet and outlet of the PEF chamber module.

Table 5.1 Overview of conducted PEF experiments and process conditions.

Sample	E (kV cm^{-1})	W_{PEF} ($\text{kWh kg}_{\text{DW}}^{-1}$)	Temperature ($^\circ\text{C}$)
C-25	0	0	25
P1-25	20	0.55	25
P2-25	20	1.11	25
C-35	0	0	35
P1-35	20	0.55	35
P2-35	20	1.11	35
C-45	0	0	45
P1-45	20	0.55	45
P2-45	20	1.11	45
C-55	0	0	55
P1-55	20	0.55	55
P2-55	20	1.11	55
C-65	0	0	65
P1-65	20	0.55	65
P2-65	20	1.11	65

An overview of the conducted experiments and process conditions is shown in Table 5.1. At the exit of the unit the treated algae suspension was collected in plastic tubes and placed in an ice water bath. After cooling, the samples were allowed to stand for 1 hour at 25°C under shaking at 140 rpm to allow intracellular components to diffuse out of the cells. After this resting time, the cell suspensions were centrifuged (10 min, $5300 \times g$) and the supernatant was transferred to fresh tubes and stored at -20°C until further analysis.

5.2.2 Synergy of PEF and temperature

Energy was provided via two ways in the current study: (1) heating (increased processing temperature using water heating baths) and (2) via electrical pulses, abbreviated as W_T and W_{PEF} , respectively. For the algae suspension (97.5% w/w water, 2.5% w/w algae) a specific heat capacity of $1.16 \cdot 10^{-3} \text{ kWh (kg K)}^{-1}$ was used assuming a suspension of only water. When increasing the temperature from 25 up to 65 °C, up to an additional $1.83 \text{ kWh kg}_{DW}^{-1}$ energy is consumed. Where W_{PEF} was 0.55 or $1.11 \text{ kWh kg}_{DW}^{-1}$, on the laboratory scale plant used in this work, this had a substantial effect (i.e., $W_T \approx W_{PEF}$) on the total amount of consumed energy for a single operating condition. Nevertheless, on an industrial scale, heat exchangers could be used to recover the energy from the outlet stream. Besides, the energy input of the PEF (W_{PEF}) also increased the temperature of the suspension (5-10 °C). This increase can be sufficient to exchange enough energy between the outlet and inlet, reducing the amount additional W_T to a minimum. Therefore, only W_{PEF} was considered in comparison to other studies.

5.2.3 Bead mill experimental procedure

The bead mill (Dyno Mill Research Lab, WAB AG Maschinenfabrik, Muttenz, Switzerland) operation procedure was previously described (Postma et al., 2015). In short, 1 mm ZrO_2 beads (Tosoh YTZ®) were used at a filling percentage of 65% v/v. The bead mill was operated with an agitator speed of 6, 9 or 12 m s^{-1} and a biomass concentration of $25 \text{ kg}_{DW} \text{ m}^{-3}$ was used. The specific energy consumption for bead milling is expressed as $W_{BM} \text{ (kWh kg}_{DW}^{-1})$.

5.2.4 Analytical methods

Protein analysis

The total protein content on biomass dry weight (DW) and the water soluble protein content of supernatants before and after PEF treatment were determined according to Postma et al. (2015). In short, for total protein content on DW, 6 mg of freeze dried algae were bead beaten in 1.0 mL lysis buffer I (60 mM Tris, 2% SDS, pH 9.0) in a lysing matrix E tube (6914-500, MP Biomedicals Europe, France). The tubes were beaten using a bead beater (Precellys 24, Bertin Technologies, France) for 3 cycles of 60 s at 6500 RPM with 120 s breaks between cycles.

For analysis of the water soluble protein content, supernatant obtained from PEF-treated samples was diluted 2 times using lysis buffer II (120 mM Tris, 4% SDS, pH 9.0).

Subsequently, samples for both total protein content on DW and water soluble protein content from supernatant were incubated at 100 °C for 30 min before quantification using a

commercial kit (DCTM Protein assay, Bio-Rad, USA) similar to the Lowry assay (Lowry et al., 1951). Bovine serum albumin (A7030, Sigma-Aldrich, USA) was used as protein standard. The absorbance was measured at 750 nm. The protein yield (Y_p) was expressed as:

$$Y_p = \frac{C_{p,sup}}{C_{p,biomass}} \quad (5.4)$$

Where $C_{p,sup}$ is the protein content in the supernatant (%_{DW}) and $C_{p,biomass}$ is the total protein content on DW (%_{DW}).

Carbohydrate analysis

In order to analyze the carbohydrate content on biomass DW, ~1 mg of algae DW was hydrolyzed in 1 mL 2.5 M HCl in a heat block at 100 °C for 3 hours. Samples were neutralized using 1 mL of 2.5 M NaOH.

Both, hydrolyzed samples and supernatant of PEF treated samples were analyzed according to DuBois et al. (1956). 0.2 mL of 5% w/w phenol and 1 mL of concentrated sulfuric acid were added to 0.2 mL of (diluted) sample (hydrolyzed sample or supernatant obtained from PEF). The samples were incubated at 35 °C for 30 minutes before reading of the absorbance at 485 nm against a blank of 0.2 mL 5% w/w phenol, 1 mL concentrated sulfuric acid and 0.2 mL of deionized water. Glucose was used as a standard. The carbohydrate yield (Y_c) was expressed as:

$$Y_c = \frac{C_{c,sup}}{C_{c,biomass}} \quad (5.5)$$

In which $C_{c,sup}$ is the carbohydrate content in the supernatant (%_{DW}) and $C_{c,biomass}$ is the total carbohydrate content on DW (%_{DW}).

Polyacrylamide gel electrophoresis

Supernatant samples from PEF experiments were thawed and 6x concentrated using Amicon Ultra-0.5 3K (Merck Millipore, USA) centrifugal tubes. Concentrated samples were kept on ice until further use.

Native PAGE was conducted using a 4-20% Criterion TGX gel (#567-1094, Biorad). 50 µl of Native sample buffer (#161-0738, Biorad) and 125 µg of protein was mixed and made up to 100 µL using Milli-Q®. 25 µg of protein was loaded per lane. NativeMarkTM (LC0725, life technologies) was used as marker for size estimation. Tris/Glycine (#161-0734, Biorad) was used as running buffer at 200 V constant for 35 min.

Bio-Safe Coomassie stain (#161-0787, Biorad) was used to stain the Native PAGE gel for 120 min followed by overnight rinsing with de-ionized water to increase background contrast before scanning.

Rubisco activity assay

The Rubisco activity was analyzed spectrophotometrically according to Desai et al. (2014b). NADH oxidation was measured at 340 nm using quartz cuvettes over a period of 6 minutes. The Rubisco activity was calculated using a molar extinction coefficient of 6.22 mM^{-1} . The final reaction mixture (3 mL) contains 259 mM Tris, 5 mM magnesium chloride, 67 mM potassium bicarbonate, 0.2 mM β -nicotinamide adenine dinucleotide (reduced form), 5 mM adenosine 5' -triphosphate, 5 mM glutathione (reduced form), 0.5 mM D-ribulose 1,5-di-phosphate, 5 units alpha-glycerophosphate dehydrogenase trios phosphate isomerase, and 5 units glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase. Either, PEF treated supernatant or bead mill supernatant was added just before the measurement, a reaction mixture without Rubisco was used as blank.

5.3 Results and Discussion

The biomass composition of *C. vulgaris* used in this study was quantified as follows: 61.1 %_{DW} protein and 16.2 %_{DW} carbohydrates. The results of this study will be presented in terms of extraction yields expressed with respect to this composition according to Eq. (5.4) and (5.5). Cell membrane permeabilization was monitored by measurement of electrical conductivity (Donsi et al., 2010). The release of relatively small and large molecules was monitored by the release of carbohydrates and proteins, respectively. Furthermore, a detailed analysis on the size distribution and activity of the released protein is presented in comparison to our benchmark bead milling. Subsequently, the role of PEF in a biorefinery approach is discussed.

5.3.1 Effect of combined PEF-temperature treatment on the release of macromolecules

Permeabilization of the algal cells

In order to quantify the maximal increase in conductivity after combined PEF-temperature treatment, a sample of the microalgal suspension was subjected to a beat beater step followed by measurement of the conductivity. The microalgal suspension had a maximal absolute increase of 1.0 mS cm^{-1} with respect to the fresh sample (0.6 mS cm^{-1}). This maximal conductivity increase was further used as a benchmark value to calculate the

relative increase of conductivity for PEF treated samples 1 h after PEF treatment. From the results shown in Figure 5.3A it can be observed that there was a strong increase in conductivity after PEF treatment under all conditions applied. This is a clear indication that PEF caused permeabilization of the algal cell resulting in leakage of small ions as also observed by Goettel et al. (2013) and Grimi et al. (2014). Besides, it can be observed that processing temperatures between 25 and 55 °C did not result in additional spontaneous release of ions without PEF treatment. However, when the algae were heated up to 65 °C without PEF treatment, a strong increase of the conductivity up to 62.5% of the maximal possible was observed. As a result of the PEF treatment at 25 – 55 °C, the conductivity increased up to 60-75% of the maximal conductivity value. Applying PEF at an energy input of 0.55 or 1.11 kWh kg_{DW}⁻¹ at 65 °C only resulted in a further increase of the conductivity up to 65.5% or 67.5% respectively. The maximal relative conductivity increase was 75% achieved after a PEF treatment at 1.11 kWh kg_{DW}⁻¹ at 45 °C. From these results, which are in agreement with the findings of Grimi et al. (2014), it can be concluded that, under the processing conditions investigated, no complete extraction of ionic components was achieved.

Release of carbohydrates

The analysis of the carbohydrates in the supernatant (Figure 5.3B) shows that only a small fraction (Y_C : < 5% of biomass carbohydrate content) of the total carbohydrates content in the microalgae was released at processing temperatures between 25 and 55 °C without any PEF treatment. However, when the temperature was further increased up to 65 °C, a substantial increase in the release of carbohydrates was observed. This was likely due to the thermal disintegration of the cell membranes, resulting in an extraction yield of more than 35% of the total carbohydrate content. Figure 5.3B also shows the carbohydrate release yields achieved after the combined PEF-temperature treatments. When PEF was applied at processing temperatures between 25 and 45 °C, carbohydrate yields between 22 and 25% were obtained. Nevertheless, no positive interaction between PEF and temperature can be noted in this range of temperatures. Further increase of the processing temperature up to 55°C, instead, showed a clear synergistic effect of the combined treatment leading to an increase of the carbohydrate yield up to 39%. However, no difference could be detected at any temperature when the total specific energy input was increased from 0.55 to 1.11 kWh kg_{DW}⁻¹. This synergistic effect was most likely caused by a less stable cell membrane due to the increased temperature, making the lipid bilayer of the cell membrane more sensitive for electric pulses (Timmermans et al., 2014) allowing a larger amount of carbohydrates to be released. This effect could even be further enhanced because at increased temperature

values, diffusivity and solubility of carbohydrates tend to be higher. In contrast, no further release of carbohydrates was observed at a processing temperature of 65 °C when PEF was applied. This is likely due to the fact that, at this high temperature value, the thermal effect was enough to break the cell membranes masking the PEF effect. Goettel et al. (2013) reported a carbohydrate release of about 8 g L⁻¹ using a specific energy input of 0.40 kWh kg_{DW}⁻¹ from a suspension of 109 g_{DW} kg⁻¹ with the microalgae *Auxenochlorella protothecoides*. Assuming a carbohydrate content of 33% on DW (Bohutskyi et al., 2015), a carbohydrate yield of 22% was achieved which is in the same range as the current study.

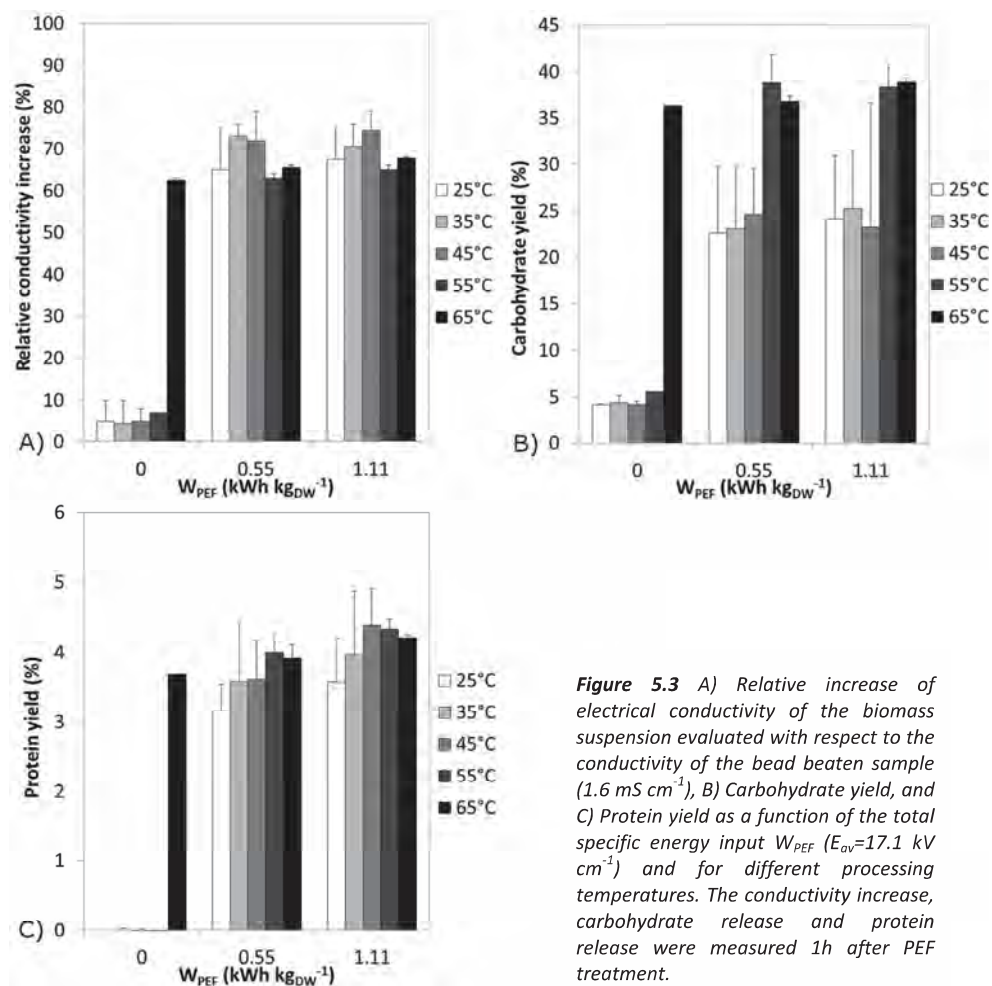


Figure 5.3 A) Relative increase of electrical conductivity of the biomass suspension evaluated with respect to the conductivity of the bead beaten sample (1.6 mS cm⁻¹), B) Carbohydrate yield, and C) Protein yield as a function of the total specific energy input W_{PEF} ($E_{av}=17.1$ kV cm⁻¹) and for different processing temperatures. The conductivity increase, carbohydrate release and protein release were measured 1h after PEF treatment.

5.3.2 Release of water soluble protein

Figure 5.3C shows the protein yield achieved after either heating or combined PEF-temperature treatment. The results show that no or hardly any protein could be detected in the supernatant of the suspension when no PEF treatment was applied at a processing temperature between 25 and 55 °C. This is either because no protein was released or the protein content was below the limit of quantitation (0.05 mg mL^{-1}). However, similarly to the results on conductivity (Figure 5.3A) and release of carbohydrates (Figure 5.3B), a substantial increase in the released protein up to an extraction yield of 3.7% was found at a processing temperature of 65 °C without any PEF treatment. The application of a PEF treatment at room temperature resulted in an extraction yield of 3.2% at $0.55 \text{ kWh kg}_{\text{DW}}^{-1}$ and 3.6% at $1.11 \text{ kWh kg}_{\text{DW}}^{-1}$. A slight synergistic effect was observed when the electrical treatment was combined with heating of the biomass. However, the absolute maximum yields are still a tenfold lower than the yields obtained with the bead mill (Y_p : 40-45% of biomass protein content at $25 \text{ kg}_{\text{DW}} \text{ m}^{-3}$) (Postma et al., 2015). Nevertheless, it appeared that above a certain critical temperature, no further improvement could be observed. For the PEF treatment at 0.55 and $1.11 \text{ kWh kg}_{\text{DW}}^{-1}$, the maximum protein extraction was obtained at 55 °C (Y_p : 4%) and 45 °C (Y_p : 4.4%), respectively.

Although for mild treatments generally temperatures below 35 °C are used to prevent any damage to the protein structure, this is only a prerequisite if long treatment times (i.e., order of minutes to hours) are applied. In the current work, the residence time of the biomass inside the treatment chambers was only 0.22 s, while the total residence time at each processing temperature was lower than 25 s. Moreover, the treated algae suspension collected at the exit of the PEF unit was immediately cooled in a water-ice bath. Studies on the denaturation kinetics of whey proteins showed that it takes over 200 seconds at 65 °C to denature 1% of β -lacto-globulins or α -lactalbumin (Dannenberg and Kessler, 1988).

In recent PEF studies, similar protein yields and corresponding specific energy inputs (W_{PEF}) to the results presented in this study were reported. Grimi et al. (2014) found a relative yield of 3.6% with an energy consumption of $1.50 \text{ kWh kg}_{\text{DW}}^{-1}$ using *Nannochloropsis sp.* Parniakov et al. (2015b) even obtained a protein yield between 5-10% although this required $4.00 \text{ kWh kg}_{\text{DW}}^{-1}$. In addition, these authors used frozen/thawed *Nannochloropsis sp.* algae prior to the application of PEF, which most likely weakened/damaged the cell structure. On the other hand, Goettel et al. (2013) were able to enhance the spontaneous release of protein slightly (from $8 \text{ } \mu\text{g L}^{-1}$ before PEF to $10.5 \text{ } \mu\text{g L}^{-1}$ after PEF) from fresh cells of *A. protothecoides* at a specific energy input of $0.40 \text{ kWh kg}_{\text{DW}}^{-1}$.

5.3.3 Protein size distribution and activity

Since low protein yields (Y_p : < 5 % of biomass protein content) were obtained in comparison to the yields obtained with the bead mill (Y_p : 40 - 45% of biomass protein content at a C_x of 25 kg_{DW} m⁻³) (Postma et al., 2015), and a substantial amount of algal protein is water soluble (Safi et al., 2014b), it is expected that only small proteins were released. Therefore, the size distribution of the released proteins was determined via gel electrophoresis. This gives better understanding on the release behavior and location of the proteins.

Native PAGE

Native PAGE provides understanding whether the released protein is negatively affected in size (i.e., degradation or aggregation). Figure 5.4 shows the Native PAGE gel in which bead mill samples (lanes 14-16) are compared to the PEF treated samples at an energy input of 1.11 kWh kg_{DW}⁻¹ at 25- 65 °C (lane 2-10, 12, 13). From lanes 2-13 it can be observed that a wide range of proteins of different molecular sizes was released by combined PEF-temperature treatment. From 45 °C onwards, a more intense group of proteins is visible between 20-66 kDa, and at 65°C even additional bands occur. This indicates that elevated processing temperatures do negatively affect the native state of the released protein, irreversibly damaging the protein structures, despite the short processing time. Conclusively, only processing temperatures up to 35 °C should be applied if native proteins are desired. Comparing the overall profile of the proteins released by bead milling and PEF, it can be observed that the bead mill samples reveal a strong band at ~540 kDa (i.e., the size of native Rubisco) next to a large range of proteins in different sizes, both larger and smaller. The samples subjected to PEF also reveal a band at ~540 kDa although less distinct. Based on the soluble protein (Lowry) assay, equal amounts of protein were loaded on the gel per lane. However, it can be observed that the higher molecular weight proteins are more distinct in the bead mill samples than in the PEF samples. Instead, below 20 kDa an intense band of low molecular weight protein material can be observed which is more pronounced for the PEF samples. To summarize, according to the results, it appears that PEF releases more small proteins rather than large proteins.

Similar to the observations in this work, Azencott et al. (2007) found that Bovine Serum Albumin (BSA) was able to move across the cell wall and cell membrane of a wild-type *Chlamydomonas reinhardtii* when subjected to electrical pulses. Although, virtually all cells were able to take up the fluorescent dye calcein (~0.6 kDa), only a fraction of the much larger BSA (~66 kDa) was taken up.

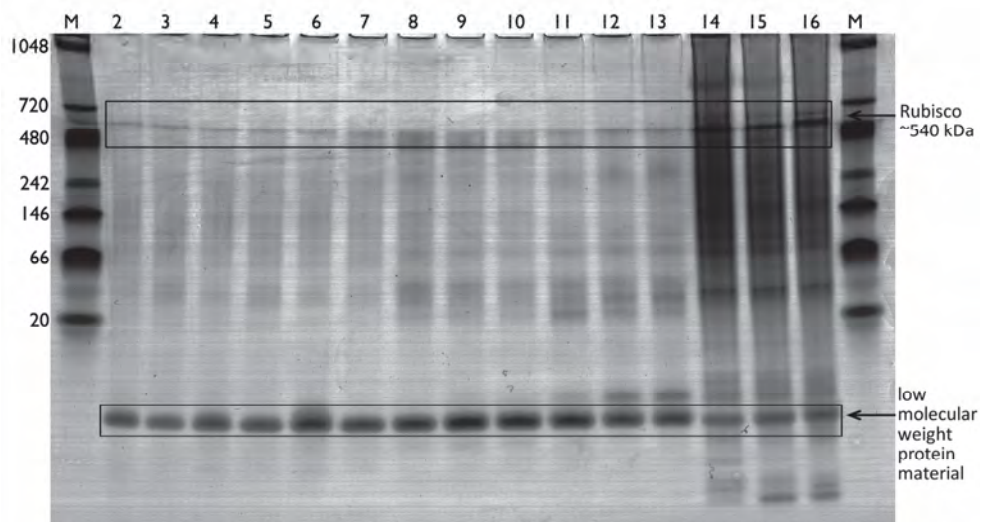


Figure 5.4 Native PAGE gel for PEF samples treated at W_{PEF} 1.11 kWh kgDW⁻¹. Values in kDa. M: marker; 2: 25 °C; 3: 25 °C; 4: 35 °C; 5: 35 °C; 6: 45 °C; 7: 45 °C; 8: 45 °C; 9: 55 °C; 10: 55 °C; 11: 65 °C (no PEF); 12: 65 °C; 13: 65 °C; 14: bead mill 6 m s⁻¹; 15: bead mill 9 m s⁻¹; bead mill 12 m s⁻¹.

Rubisco activity

To confirm whether the released multimeric Rubisco was still biologically active a Rubisco activity assay was performed. Figure 5.5 shows the specific Rubisco activity of the protein released in the supernatant after PEF treatment (processing temperature: 35 °C, W_{PEF} : 1.11 kWh kgDW⁻¹) in comparison to a supernatant sample obtained from bead milling (agitator speed: 9 m s⁻¹, W_{BM} : 14.1 kWh kgDW⁻¹). It can be observed that the specific activity of the bead mill sample was about 10 times higher than the PEF sample, indicating that the purity (i.e., amount of Rubisco per amount of total protein) of the PEF sample was lower. This confirms the observations from the Native PAGE gel leading to the suggestion that during PEF treatment less intracellular organelles were disintegrated than during bead milling. Bead milling completely disintegrates the algal cells including larger and smaller internal organelles. Therefore, also Rubisco stored in the chloroplast (free in stroma and/or inside pyrenoids) would be released. Nevertheless, since active Rubisco was observed after combined PEF-temperature treatment, it is likely that part of the chloroplast was disintegrated causing free Rubisco from the stroma to diffuse out of the cell. Though, this hypothesis cannot be confirmed based on the current results.

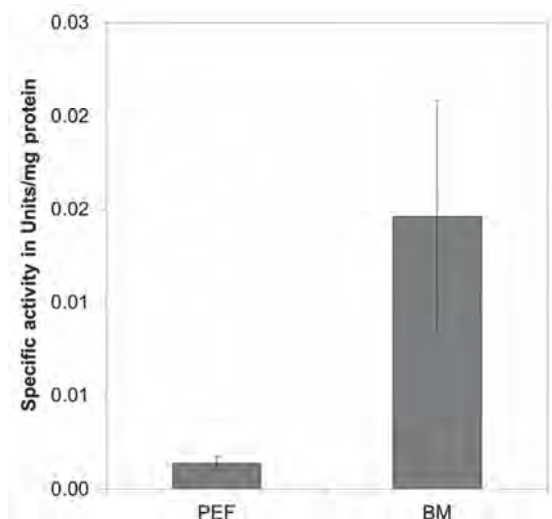


Figure 5.5 Specific Rubisco activity (Units/mg protein) of supernatant obtained from PEF (17.1 kV cm⁻¹, 1.11 kWh kg_{DW}⁻¹, and 35 °C), and from bead milling (BM) (9 m s⁻¹).

5.4 The role of PEF in a cascade biorefinery

A multi-stage biorefinery should exploit the full potential of the cell in terms of compartmentalization and products and, at the same time, require a low energy input (Eppink et al., 2012). Mahnič-Kalamiza et al. (2014) envisioned the application of PEF as a first disintegration step in such a multi-stage biorefinery followed by two extraction stages. In the first stage, water soluble components should be extracted and in a second stage an environmentally friendly solvent could be applied to extract pigments or other hydrophobic components. In addition, Coons et al. (2014) described that no more than 10% of the total available energy from the produced algae \bar{W}_{Algae} (6.82 kWh kg_{DW}⁻¹) should be utilized for the disintegration, extraction and fractionation, abbreviated as $\bar{W}_{Biorefinery}$.

Our findings indicate that maximum 39% of the carbohydrates and less than 5% of the proteins could be released, but only at a combined PEF-temperature treatment at 55 °C and 0.55 or 1.11 kWh kg_{DW}⁻¹. Even though, at a specific energy input of 0.55 kWh kg_{DW}⁻¹ the energy target $\bar{W}_{Biorefinery}$ of 0.682 kWh kg_{DW}⁻¹ is exceeded, the results of the native PAGE showed that from 45 °C onwards the proteins seem to be negatively affected. Albeit a lower processing temperature of 35 °C yields active Rubisco, a carbohydrate and protein yield of only 25% and 3.8% were obtained, respectively.

In the ideal case, PEF would allow selective release of small soluble molecules (e.g., carbohydrates) resulting in relative pure fractions without negatively harming the other components. This requires the optimization of the PEF processing conditions in order to further increase the extraction yield of these small molecules. For this purpose, it should be

taken into account that our results were obtained by combining moderate temperatures with a PEF treatment applied at a relatively low theoretical field strength value (20 kV cm^{-1}). In addition, this value was further reduced by 15% due to the intrinsic non-uniform distribution of the electric field inside the treatment zone of the co-linear chamber used in this work, so that the actual average electric field strength applied was 17.1 kV cm^{-1} . Which is consistent with the results found for co-linear chamber by Buckow et al. (2011). Because of this lower E_{av} , further experimental work should be carried in order to test whether, for example, the combination of moderate temperature with higher field strength as well as improvements of the treatment uniformity by modifications of the chamber geometry (e.g., insulator and electrode ring shape) may results in a higher extraction yield of small molecules like carbohydrates, without negatively affecting the other components.

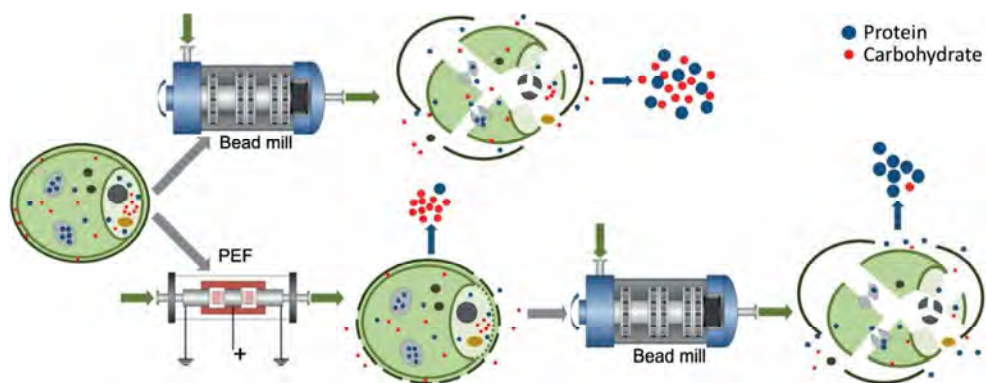


Figure 5.6 A schematic representation of a single disintegration approach (top) compared to a multi-stage or cascade biorefinery approach (bottom).

In this way extensive fractionation of complex protein and carbohydrate mixtures (e.g., by membrane filtration) could be omitted while reducing $\bar{W}_{Biorefinery}$. After the PEF stage, effort should be made in order to recover the high-value water soluble proteins and likewise to allow the recovery of the remaining carbohydrates in a subsequent stage before any solvent is applied (Figure 5.6). Bead milling has shown to be a promising candidate to release up to 45% of the water soluble protein (Postma et al., 2015) and 48-58% of carbohydrates (data not shown).

To conclude, further studies are necessary in order to optimize the processing conditions of the combined PEF-temperature treatment as well as its integration in a cascade biorefinery in order to maximize the selective recovery of the high value components with minimal energy consumption.

5.5 Conclusion

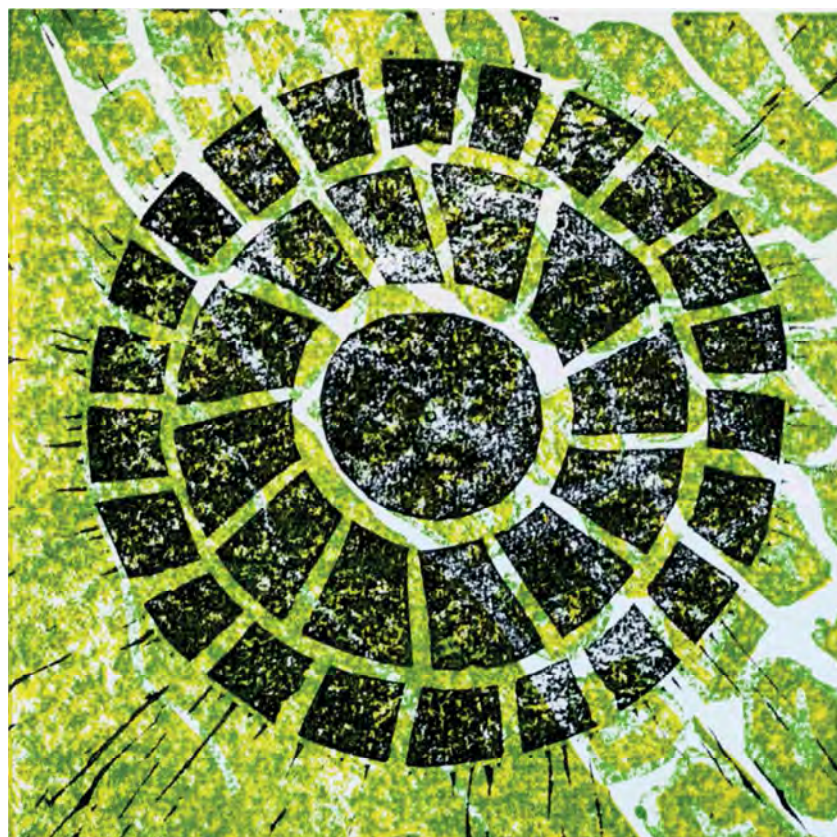
PEF showed to be an effective technique to release small ionic solutes and carbohydrates up to 75% and 39%, respectively. Nevertheless, permeabilization was not effective enough to release high quantities of large molecules such as protein (< 5%). Though, the released protein fraction contained the biologically active multimeric Rubisco showing that PEF is a mild technique at 35 °C. In conclusion, under the processing conditions investigated in this work, the combined PEF-temperature treatment seems not able to sufficiently disintegrate the algal cells to release both carbohydrates and proteins at yields comparably to the benchmark bead milling (40-45% protein, 48-58% carbohydrates).

Acknowledgements

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Abbreviations

Symbol	Definition	units
$C_{c,sup}$	Carbohydrate content supernatant	[% _{dw}]
$C_{c,biomass}$	Carbohydrate content biomass	[% _{dw}]
$C_{p,sup}$	Protein content supernatant	[% _{dw}]
$C_{p,biomass}$	Protein content biomass	[% _{dw}]
C_x	Biomass concentration	[kg _{DW} m ⁻³]
E	Electrical field strength	[kV cm ⁻¹]
E_{av}	Average applied electric field strength	[kV cm ⁻¹]
E_p	Theoretical peak electric field strength	[kV cm ⁻¹]
f	Pulse frequency	[Hz]
I	Current	[A]
L	Treatment chamber gap distance	[mm]
n	Number of pulses	[-]
N_{TC}	Number of treatment chamber	[-]
r	Treatment chamber radius	[mm]
t_p	Pulse duration	[s]
T	Temperature	[°C]
U	Voltage	[V]
\bar{W}_{Algae}	Total energy available from algae	[kWh kg _{DW} ⁻¹]
$\bar{W}_{Biorefinery}$	Maximal energy use for biorefinery	[kWh kg _{DW} ⁻¹]
W_{BM}	Specific energy consumption bead mill	[kWh kg _{DW} ⁻¹]
W_{PEF}	Specific energy consumption PEF	[kWh kg _{DW} ⁻¹]
W_T	Specific energy consumption by temperature	[kWh kg _{DW} ⁻¹]
Y_C	Carbohydrate yield	[%]
Y_P	Protein yield	[%]
α	Coefficient	[-]
Φ	Flow rate	[mL s ⁻¹]
ρ	Density of suspension	[kg m ⁻³]
σ	Conductivity	[S m ⁻¹]
σ_R	Relative ion yield	[%]
v_i	Treatment zone volume	[mL]



Chapter 6

Biorefinery of the macroalgae *Ulva lactuca*: extraction of proteins and carbohydrates by mild disintegration

The contents of this chapter have been submitted as:

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*Biorefinery of the macroalgae Ulva lactuca: extraction
of proteins and carbohydrates by mild disintegration*

Abstract

The effect of osmotic shock, enzymatic incubation, pulsed electric field and high shear homogenization on the release of water soluble proteins and carbohydrates from *Ulva lactuca* was investigated. For osmotic shock both temperature and incubation time had a significant influence on the release with an optimum at 30°C for 24 h incubation. For enzymatic incubation, pectinase showed to be the most promising enzyme for both protein and carbohydrate release. Pulsed electric field treatment was most optimal at an electric field strength of 7.5 kV cm⁻¹ with 0.05 ms pulses and a specific energy input relative to the released protein as low of 6.6 kWh kg_{prot}⁻¹. With respect to literature, this study reported the highest protein (~40%) and carbohydrate (~51%) yields of the four technologies using high shear homogenization. Besides, an energy reduction up to 86% was achieved by applying a novel two-phase (macrostructure size reduction and cell disintegration) technique.

6.1 Introduction

A growing world population, with an estimated 9.6 billion residents by 2050 (United Nations, Department of Economic and Social Affairs, Population Division, 2015), demands a growth in protein sources to feed these people. The available arable land is limiting and will even decrease per capita worldwide (FAO, 2011). The sustainable production of food and feed at sea could be an option since the oceans cover more than 70% of the planet's surface (van den Burg et al., 2013) and could thereby address one of the main issues of securing the food production on earth (Godfray et al., 2010).

Macroalgae are a potential renewable source of proteins, carbohydrates, chemical building blocks, nutraceuticals and bioenergy (Hal, van et al., 2014; Holdt and Kraan, 2011; van den Burg et al., 2013; van der Wal et al., 2013). They can be divided in three major types, being red (*Rhodophyta*), brown (*Phaeophyta*) and green (*Chlorophyta*) algae. Each type has a typical composition which can considerably vary depending on the moment of harvest due to seasonal variation (Holdt and Kraan, 2011). From the three types, the red and green algae show the highest protein contents (Harnedy and FitzGerald, 2011), in combination with the highest areal yields (20 tons DM/ha) for green macroalgae (van den Burg et al., 2013), the green macroalga *Ulva lactuca* was selected as model species in this work.

Microalgal products are stored inside the cell cytoplasm or are bound to cell membranes and require disintegration before extraction. In addition to the rigid cell wall, macroalgae possess an additional barrier to obtain the intracellular products, namely: the macrostructure. On the one hand, this macrostructure aids in the ease of harvesting after cultivation decreasing the costs of dewatering. On the other hand, it could be imagined that this macrostructure hinders the use of continuous liquid flow processes to disintegrate the cells. Therefore, focus should be put more towards batch or semi-batch systems in which both the macrostructure and the cell wall are disintegrated. In addition, similar to microalgae, all products should be valorized in a biorefinery approach to make the biomass production economically feasible (Wijffels et al., 2010).

Upon disintegration of the biomass, the mildness of the applied conditions should be taken into account (Vanthoor-Koopmans et al., 2013) to prevent negative influences on the product quality. Especially proteins are sensitive to detrimental conditions like extreme shear forces, elevated temperatures (i.e., long heating above 35°C) or chemicals which cause loss of functional properties. Yet, the complex structure composed of often charged polysaccharides complicates the extraction of intracellular products (Joubert and Fleurence, 2008).

The past decades, many techniques have been applied towards macroalgae biomass for the extraction of carbohydrates (e.g., ulvans), amino acids, peptides and proteins, pigments and DNA (Alves et al., 2013; Barbarino and Lourenço, 2005; Coste et al., 2015; Fleurence et al., 1995; Harnedy and FitzGerald, 2013, 2011; Joubert and Fleurence, 2008; Polikovskiy et al., 2016; Sun et al., 2009). To extract those components, mechanical grinding, acid and alkaline treatment, polysaccharidase treatment, high shear forces, osmotic shock, ultrasound and pulsed electric field have been applied. In many of these investigations mild dried, frozen or freeze dried biomass was used which does help to have a consistent supply of biomass. Nonetheless, the biomass might already partially be disclosed or permeabilized and could therefore mask part of effect of the applied disintegration and extraction method. Therefore no quantitative data is available from literature on extraction yields of protein of carbohydrates from fresh macroalgae. Moreover, pre-drying or freeze drying of the biomass is an energy intensive process considering a biomass which consists ~80% of water. With the water heat of evaporation being 0.63 kWh kg^{-1} , about $2.5 \text{ kWh kg}_{\text{DW}}^{-1}$ ($4 \text{ kg}_{\text{water}}/\text{kg}_{\text{biomass}} \times 0.63 \text{ kWh kg}^{-1}$) is required to remove the water which is equal to about 60% of the biomass energy density (Bruhn et al., 2011). Therefore, the disruption and extraction of proteins and carbohydrates from freshly harvested *U. lactuca* was studied.

The objective of this work was to provide a strategy to disintegrate *U. lactuca* for the release of water soluble proteins and carbohydrates using several (non)mechanical methods, including osmotic shock, high shear homogenization, enzymatic treatment and pulsed electric field.

6.2 Methods

6.2.1 Macroalgae cultivation and harvest

U. lactuca (origin: Wierderij, Oosterschelde, The Netherlands N 51°41'34.8", E 3°48'27.5") was cultivated in 1 m^3 tanks filled with filtered seawater (Oosterschelde, The Netherlands) at the greenhouse facilities of Wageningen University and Research (Nergena, Wageningen, The Netherlands). The seawater was replaced on a monthly basis. Algae were cultivated and harvested over the period of September 2014 – May 2016. After harvest the biomass was washed using tap water to remove extracellular salts and was drained from extracellular water using a salad spinner. Subsequently the fresh biomass was directly used or stored no longer than three days in sealed bags at 4°C in the dark.

For each extraction procedure 5, 10 or 15 g_{DW} biomass per L was prepared in the corresponding medium (see following sections for details), based on the fresh algae moisture content.

6.2.2 Osmotic shock

Osmotic shock experiments to disintegrate *U. lactuca* were conducted as described by Fleurence et al. (1995) and Harnedy and Fitzgerald (2013) with some modifications. Washed *U. lactuca* thallus was suspended in de-ionized water at a biomass concentration of 10 g_{DW} kg⁻¹ in a total mass of 5 g (liquid and algae), and stirred gently up to 24 h (during overnight period no stirring) at 4°C, 22°C (RT) or 30°C. Samples were taken after 1, 4 and 24 h after the start of the osmotic shock. Experiments were performed in duplicate.

6.2.3 Enzyme treatment

Three commercial available enzymes, Cellulase Onozuka RS (C0615), pectinase Macerozyme R10 (P2401) and β-Glucuronidase (SRE0022) were bought from Sigma-Aldrich (USA) and a freeze dried powder from *Abalone* gut was kindly provided by Aroma NZ Ltd. (New Zealand). Washed *U. lactuca* thallus was suspended in acetate buffer (pH 4: 82 mM HAC, 18 mM NaAc; pH 5: 29.5 mM HAC, 70.5 mM NaAc) at a biomass concentration of 10 g_{DW} kg⁻¹ in a total mass of 5 g (liquid, algae and enzyme). Enzyme incubation experiments were conducted for 4 h according to Table 6.1 in a shaking water bath. After incubation the biomass was removed by centrifugation (20000x g, 10 min) and the supernatant was stored at -20°C until further analysis.

Table 6.1 Overview of enzyme incubation experiments.

Exp.	Enzyme	Abbreviation	pH	Temperature (°C)	Concentration (% _{DW})		
a	Cellulase Onozuka RS	CO-RS	5	30	0	0.5	2
b	Pectinase Macerozyme R10	PMC-R10	4	25	0	0.5	2
c	Cellulase Onozuka RS + Pectinase Macerozyme R10	CO-RS + PMC-R10	5	30	0	0.25 (each enzyme)	1 (each enzyme)
d	β-Glucuronidase	β-G	4	30	0	0.5	2
e	<i>Abalone</i> powder	<i>Ab</i>	5	30	0	0.5	2

6.2.4 Pulsed Electric Field

A batch electroporator (Gene Pulser X-Cell™, Bio-Rad, USA) with cuvettes (gap distance 4 mm, PulseStar, Westburg, The Netherlands) was used for the electroporation of the *U. lactuca* thallus. The effect of the electric field strength (*E*) was assessed by altering the voltage between (1.2 and 3.0 kV). Further, the treatment time was varied by changing the pulse duration (0.05, 0.5 or 5 ms) of the square wave pulses for a fixed pulse number (2 pulses). The *U. lactuca* thallus was suspended at a fixed biomass concentration of 10 g_{DW} L⁻¹

in a 0.04% NaCl solution to obtain a solution with a conductivity of $1250 \mu\text{S cm}^{-1}$. After electroporation, product release was measured after 1 h of resting time allowing the intracellular products to diffuse in the aqueous bulk.

The specific energy consumption per unit of volume W_V (Frey et al., 2013) and the specific energy consumption per unit of mass W_M are calculated according to:

$$W_V (\text{kWh m}^{-3}) = \frac{E^2 \cdot t_p \cdot N \cdot \sigma}{3600000} \quad (6.1)$$

$$W_M (\text{kWh kg}^{-1}) = \frac{W_V}{C_x} \quad (6.2)$$

in which E is the electric field strength (V m^{-1}), t_p is the pulse length (s), N is the number of pulses, σ the electrical conductivity (S m^{-1}) and C_x is the biomass concentration (kg m^{-3}).

6.2.5 High shear homogenization

High shear homogenization (HSH) was performed using an Ultra Turrax (T-50, IKA Works, Germany) equipped with a G65F rotor-static dispersing element. Two independent quantitative parameters (biomass concentration C_x and rotor tip speed u_s) were studied at three levels in a Design of Experiments similar to Postma et al. (2015). Modde v.9.1 (Umetrics, Sweden) DOE software was used to study the effect on C_x and u_s using a central composite face-centered design (CCF). The experimental range and parameters are shown in Table 6.2.

Table 6.2 Parameters of experiments for CCF design of HSH experiment.

Parameters	Factor	Low Value (-1)	Centre Value (0)	High Value (+1)
Biomass concentration (g kg^{-1})	X1	~5.4	~10.7	~16.1
Rotor tip speed (m s^{-1})	X2	11	16	21

A third (multilevel) parameter (X3), pre-treatment by manual cutting, was also evaluated to assess the ability of the HSH to deal with the macro structure of the *U. lactuca* thallus. The thalli were either left intact (F; X3: 240 cm^2), cut to pieces of $\sim 3 \times 4 \text{ cm}$ (FC; X3: 12 cm^2) or cut to pieces of 1 cm^2 (FC2; X3: 1 cm^2). For each of the pre-cut conditions the above mentioned CCF was conducted. For all experiments the macroalgal biomass was suspended in phosphate buffered saline (PBS) (1.54 mM KH_2PO_4 , 2.71 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 155.2 mM NaCl at pH 7.0).

The temperature during the treatment was controlled by placing the beaker glass in a water-ice suspension, consequently the temperature never exceeded 35°C for an experiment of maximal 40 min.

Energy consumption was measured by means of an energy logger (Energy Logger 4000, Voltcraft®, Germany). The specific energy consumption E_M is defined as the consumed energy per kg of dry biomass ($\text{kWh kg}_{\text{DW}}^{-1}$).

6.2.6 Analytical methods

Biomass dry weight content. Biomass dry weight determination was conducted by taking a known amount of washed fresh weight *U. lactuca* (~2 grams) and put in pre-weighted aluminum cups. The cups were incubated overnight at 105 °C and re-weighted. Dry weight determination was performed in triplicate.

Protein analysis. Water soluble protein release and total protein content on biomass DW was determined according to Postma et al. (2015), with the exception that lysing matrix D tubes (6913-500, MP Biomedicals Europe) were used. For soluble proteins, protein values found in control experiments with enzyme but without algae were subtracted from the water soluble protein release samples.

Carbohydrate analysis. Water soluble carbohydrate and total carbohydrate on DW analysis were conducted as described before (Postma et al., 2016b).

6.2.7 Scanning Electron Microscopy

Macroalgae thallus treated by HSH was fixed on poly-L-lysine coated cover slips (\varnothing 8 mm) by applying a drop of 150 μL on the cover slip and incubated for 1h. Hereafter the glasses with attached cells and thalli treated by enzymes were rinsed by dipping in fresh PBS and subsequently fixed for 1h in 3% glutaraldehyde in PBS. After a 2 times wash in PBS the samples were postfixed in 1% OsO_4 for one hour, rinsed with demineralized water and dehydrated in a graded (30-50-70-90-100-100%) ethanol series. Subsequently, cover slips and thalli were critical point dried with carbon dioxide (EM CPD 300, Leica, Wetzlar, Germany). The cover slips with cells and the thalli were attached on sample holders using carbon adhesive tabs (EMS, Washington, USA) and sputter coated with 10 nm Wolfram (EM SCD 500, Leica, Wetzlar, Germany). The cells and thalli were analyzed in a high resolution scanning electron microscope at 2 KV at room temperature (Magellan 400, FEI, Eindhoven, The Netherlands). Images were contrast enhanced with Photoshop CS5.

6.2.8 Statistical analysis

Statistical analysis was performed in the Modde DOE software (Umetrics, Sweden) or by analysis of variance (ANOVA) in Excel (Microsoft, USA). Significant differences within groups were determined by independent samples t-tests at a significance level of 95%.

6.3 Results and discussion

The protein and carbohydrate yields presented in this work are based on the measured total protein and carbohydrate composition of the *U. lactuca* biomass. First the results of extraction by means of osmotic shock are presented, followed by the effects of enzymatic disintegration, PEF treatment and high shear homogenization.

6.3.1 De-watering

Preliminary experiments using mild (35°C, 48 h) pre-dried biomass prior to disintegration by HSH showed a lower protein yield ($5.6\% \pm 1.7$, $n=6$) compared to the use of fresh biomass ($15.9\% \pm 6.7$, $n=6$). ANOVA revealed that this was a significant ($p= 0.004$) difference. Therefore, in subsequent experiments only fresh biomass was used.

6.3.2 Osmotic shock

The results of the osmotic shock on the release of proteins and carbohydrates are shown in Figure 6.1. Both the temperature and the duration had a substantial effect on the release of soluble proteins and carbohydrates. Using the independent samples t-test it was found that increasing the time of the osmotic shock from 4 h to 24 h significantly increased the product release at 4°C ($p = 0.017$), 22°C ($p = 0.037$) and 30°C ($p = 0.024$). An increase of the temperature from 4°C to 22°C did not result in significant improvement of the product release. The highest protein (19.5%) and carbohydrate (44.7%) yield were observed when increasing the temperature up to 30°C in combination with an incubation time of 24h. Harnedy and Fitzgerald (2013) found only little effect of both the temperature (4°C or 22°C) and the duration (3, 7 or 16 h) on the aqueous extraction of protein from *Palmaria palmata* following osmotic shock. A maximal yield of 5.9% was found at a temperature of 4°C after 7 h incubation, while in this work a similar yield was only achieved after 24 h. Yet, at a temperature of 22°C and a duration of 24 h the yield of 4.3% in this work is similar to the yield of 4.7% found by Harnedy and Fitzgerald (2013) at 22°C after 16 h.

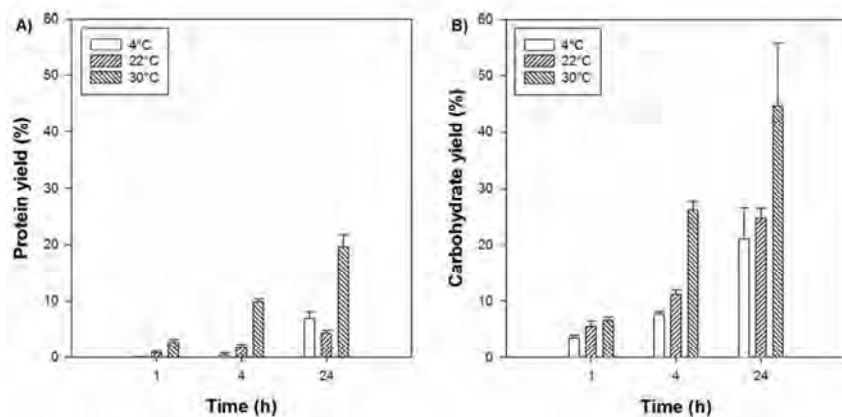


Figure 6.1 Overview of results of effect of temperature and duration following osmotic shock on protein (A) and carbohydrate (B) yield.

6.3.3 Enzymatic degradation of cell wall

Enzymatic incubation was assessed as a mild approach with respect to HSH because of its high presumed energy consumption. Four different enzymes and one mixture were screened and the results of the protein and carbohydrate yield are shown in Figure 6.2. The control experiments in which no enzyme was added to the fresh *U. lactuca* thallus show a relative high yield with respect to the enzyme incubated experiments. The major contributor to this was the low osmolarity of the applied acetate buffer (20 mOsm) while seawater has a typical osmolarity of 1000-1200 mOsm. This low osmolarity most likely caused an osmotic shock due to which a part of the cells break.

The applied CO-RS and PMC-R10 show the highest yields (~25-30%) for both protein and carbohydrates at a crude enzyme concentration of 2% (Figure 6.2A, B). Increasing the crude enzyme concentration from 0.5% to 2% did not result in a proportional increase of the protein or carbohydrate yield (i.e., a four-fold increase in enzyme did not result in a four-fold yield increase). Worth mentioning are the results of Reddy and Fujita (1991) who found that the addition of 2% PMC-R10 did not result in protoplast release, moreover it was mentioned that no effect on the cell wall was observed at al. Remarkably, the mixture of both enzymes (CO-RS and PMC-R10) as a cocktail (Figure 6.2C) did not result in an improvement of the yield. This is in line with the work of Reddy et al. (2006), who applied 2% CO-RS and a mixture of 2% CO-RS + 2% PMC-R10 to several *Ulva* sp. and found that the generation of protoplasts was more effective without pectinases. β -Glucuronidase (β -G) is an enzyme which is isolated from the entrails of *Abalone* (*Ab*), a natural grazer of *U. lactuca*.

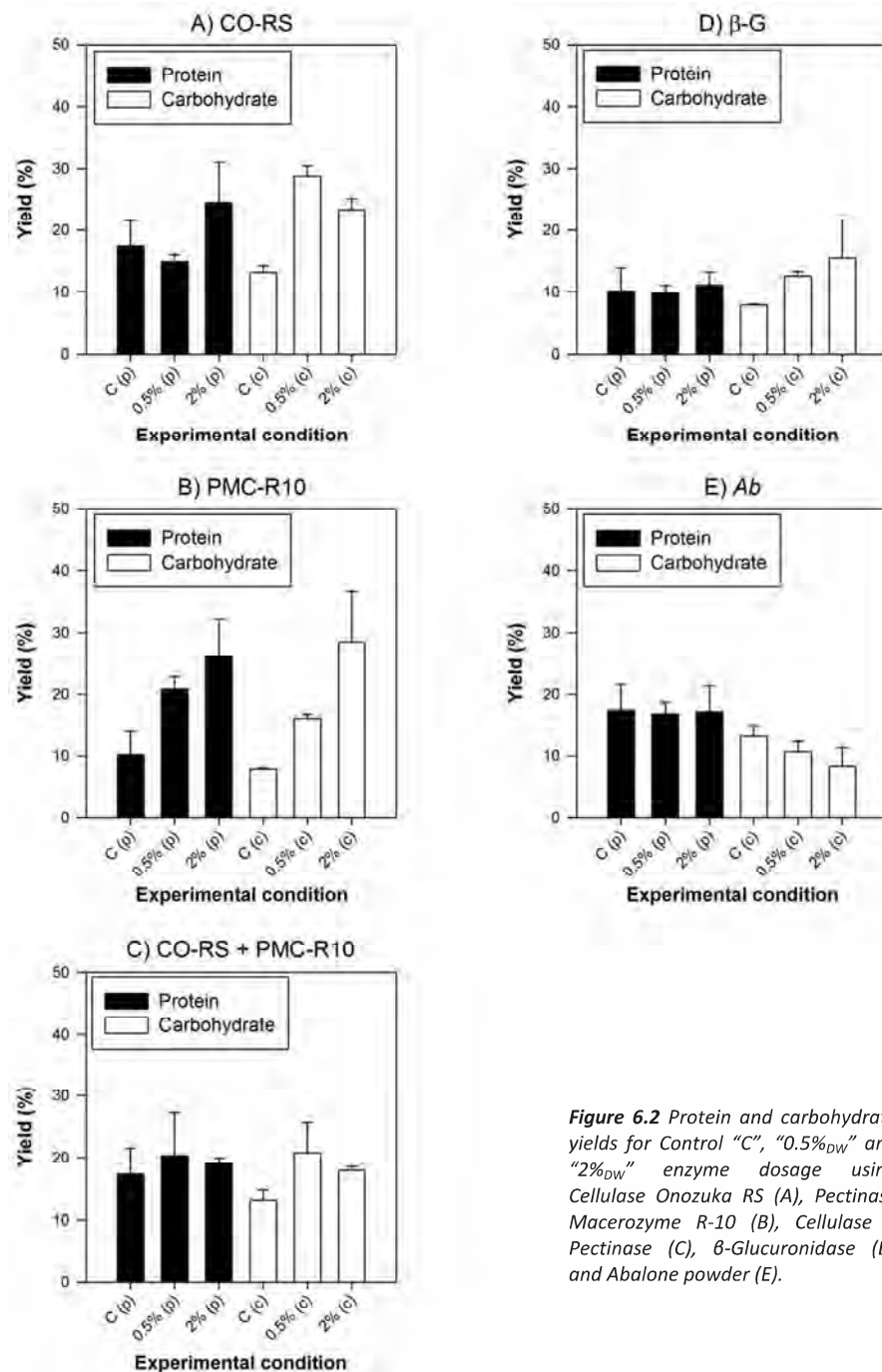


Figure 6.2 Protein and carbohydrate yields for Control “C”, “0.5%_{DW}” and “2%_{DW}” enzyme dosage using Cellulase Onozuka RS (A), Pectinase Macerozyme R-10 (B), Cellulase + Pectinase (C), β -Glucuronidase (D) and Abalone powder (E).

However, the protein and carbohydrate yield was relatively low (~10%), moreover no improvement with respect to the control was observed. *Ab* powder also showed poor yields (<16%) at the same level as the control experiment. This *Ab* powder was received dried, possibly enzymes were denatured during extraction of the entrails prior to delivery or the enzyme specificity is not able to disintegrate the *U. lactuca* macrostructure. In contrast, Reddy and Fujita (1991) found that *Abalone* powder was able to degrade the cell wall of three *Enteromorpha* spp. (taxonomic synonym for certain *Ulva* spp.; but not *U. lactuca*). Fleurence et al. (1995) found a mixture of cellulase, hemicellulase and β -glucanase to be effective on *Ulva rigida* and *Ulva rotundata* with protein yields of 18.5 and 22.0%, respectively. On the contrary, they observed protein yields below 1% for a commercial cellulase.

6.3.4 Pulsed Electric Field

The effect of a PEF treatment on the release of water soluble protein and carbohydrate is shown in Figure 6.3. Under all pulse conditions an increase in the water soluble protein (Figure 6.3A) content was observed in the supernatant with respect to the control (0 kV cm^{-1}), ANOVA revealed that for each condition the effect of the PEF treatment was significant ($p < 0.05$). At both 3 and 5 kV cm^{-1} , the pulse duration had no significant effect on the protein yield. The highest protein yield of 15.1% was achieved at 7.5 kV cm^{-1} using 0.05 ms pulses. Moreover, at 7.5 kV cm^{-1} and 0.05 ms pulses (pulse number: 2), the t-test showed a significant higher protein yield compared to 0.5 ($p = 0.019$) or 5 ($p = 4.95 \cdot 10^{-4}$) ms pulses. When looking only to the effect of the electric field strength at a fixed pulse duration of 0.05 ms, at 7.5 kV cm^{-1} a higher protein yield was obtained compared to 3 ($p = 0.015$) or 5 ($p = 0.004$).

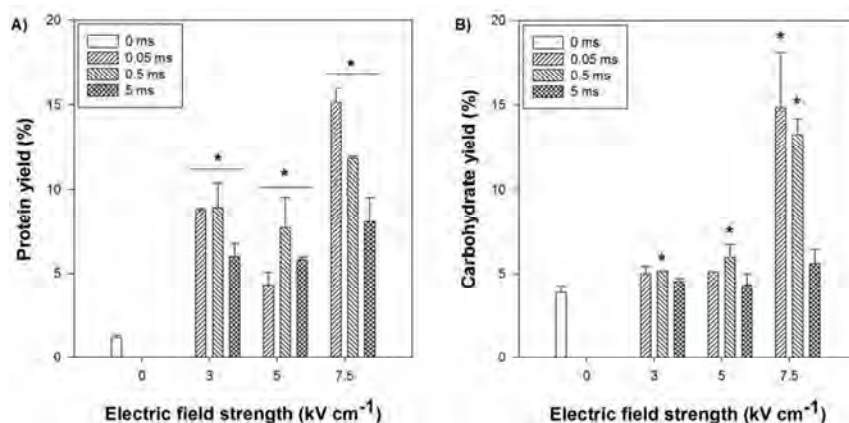


Figure 6.3 Protein yield (A) and carbohydrate yield (B) as function of electric field strength during PEF treatment. * Significant different from control (0 kV cm^{-1}).

Polikovskiy et al. (2016) applied an electric field strength of 2.964 kV cm^{-1} and found a specific energy input relative to the extracted protein of $251 \pm 3 \text{ kWh kg}_{\text{PROT}}^{-1}$. Taking into account the highest protein yield in this study (15.1%) with the corresponding specific energy input of $0.2 \text{ kWh kg}_{\text{DW}}^{-1}$, a specific energy input relative to the extracted protein of only $6.6 \pm 0.28 \text{ kg}_{\text{PROT}}^{-1}$ was obtained.

The carbohydrate release (Figure 6.3B) on the other hand, showed a less significant effect. With respect to the control, a pulse duration of 0.5 ms in combination with an electric field strength of 3, 5 or 7.5 kV cm^{-1} resulted in a significant increase of the carbohydrate yield ($p < 0.05$). The highest carbohydrate yield was obtained at the same condition as the highest protein yield, namely 0.05 ms and 7.5 kV cm^{-1} .

The spontaneous release of protein and carbohydrates in the control could be caused by the lack of osmotic pressure since the osmolality of the used PEF medium was only 13 mOsm (0.04 % w/w NaCl solution). Such a low osmolality was required to ensure a low conductivity of the PEF medium in order to avoid sparking. When looking to the osmotic shock data from section 6.3.2, the time scale during PEF was much shorter, yet the incubation afterwards (at RT) to allow diffusion was 1 h which resulted in similar yields.

6.3.5 High shear homogenization

To find out the ability of the HSH to disintegrate both, the macro structure and the cellular structure (i.e., cell wall), the fresh biomass was either left intact ("F") or manually pre-cut to pieces of 3×4 ("FC") or $1 \times 1 \text{ cm}$ ("FC2") preceding the HSH treatment. The results of the protein and carbohydrate yields are shown in Figure 6.4.

The first observation made for the uncut "F" biomass (Figure 6.4A, B) was that the thallus could not be effectively reduced in size under all conditions. Especially at low or moderate rotor speeds the thallus remained intact and thus did also not result in disintegration of the cell wall to release proteins and carbohydrates (Y_p : 0%). Increased rotor speeds up to 21 m s^{-1} in combination with low biomass concentrations resulted in a maximal protein and carbohydrate yield of 34% and 65%, respectively.

When the *U. lactuca* thallus was pre-cut to pieces of $3 \times 4 \text{ cm}$ "FC" (Figure 6.4C, D), it was observed that the thallus was more easily reduced in size, except for the combination of high biomass concentration and a low rotor speed. A low to moderate biomass concentration combined with a low to moderate rotor speed resulted in the highest protein yields up to 37%, while a high rotor speed was still favorable for the release of carbohydrates up to 57% yield. This shows that the maximal yield did not improve, however the thallus and cell wall were more easily disintegrated over a broader range of operating conditions.

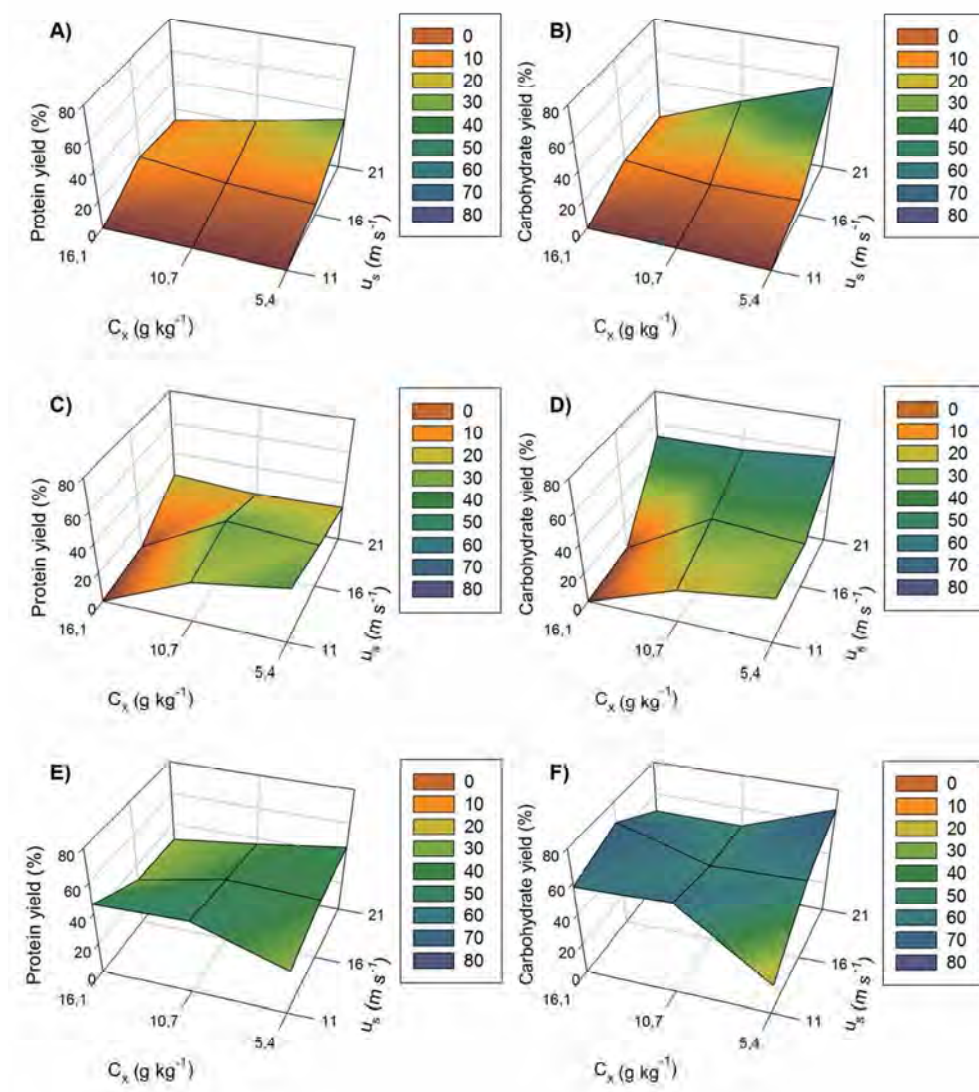


Figure 6.4 3D mesh plots of protein yield using uncut “F” (A), 3x4 cm pieces “FC” (C), 1x1 cm pieces “FC2” (E) biomass and carbohydrate yields using uncut “F” (B), 3x4 cm pieces “FC” (D), 1x1 cm pieces “FC2” (F) biomass. Color coding in the legend represents the protein and carbohydrate yield (%). Protein and carbohydrate content measured in the supernatant after 40 min of disintegration.

For the smallest pieces of 1x1 cm “FC2” (Figure 6.4E, F) under all conditions the thallus was disintegrated. The highest protein yields were obtained for either a moderate to high biomass concentrations combined with a relative low rotor speed or a low to moderate biomass concentration and relative high rotor speed. The maximal protein yield improved up

to 48% for a biomass concentration of 10.7 g kg^{-1} and a rotor speed of 11 m s^{-1} . The carbohydrate yields improved under all conditions except for a high rotor speed and low biomass concentration with a maximal yield of 68% for a low biomass concentration of 5.4 g kg^{-1} and a rotor speed of 21 m s^{-1} . Harnedy and Fitzgerald (2013) studied the extraction of protein from pre-frozen and dried (50°C) *Palmaria palmate*. Using a similar HSH principle a water soluble protein yield of 3-4% was achieved which could be improved up to 40% in combination with a sequential alkaline extraction which disintegrates the proteins as well due to hydrolyses.

The results from Figure 6.4 show that the macrostructure of *U. lactuca* was initially limiting the release of the water soluble proteins and carbohydrates. When the thallus was manually pre-cut the release of both proteins and carbohydrates was improved. Moreover, the usage of high rotor speeds increases the energy consumption as 40 min disintegration at 11, 16 or 21 m s^{-1} with a biomass concentration of 10.7 g kg^{-1} required 30.4, 57.3 or $77.8 \text{ kWh kg}_{\text{DW}}^{-1}$. To reduce the energy consumption and to overcome the manual pre-cutting, a two-phase HSH strategy was developed. This two-phase strategy included a cutting (i.e., thallus size reduction) phase of 3.5 min at high rotor speed (21 m s^{-1}) and a disintegration phase to release the proteins and carbohydrates at either 11 or 16 m s^{-1} .

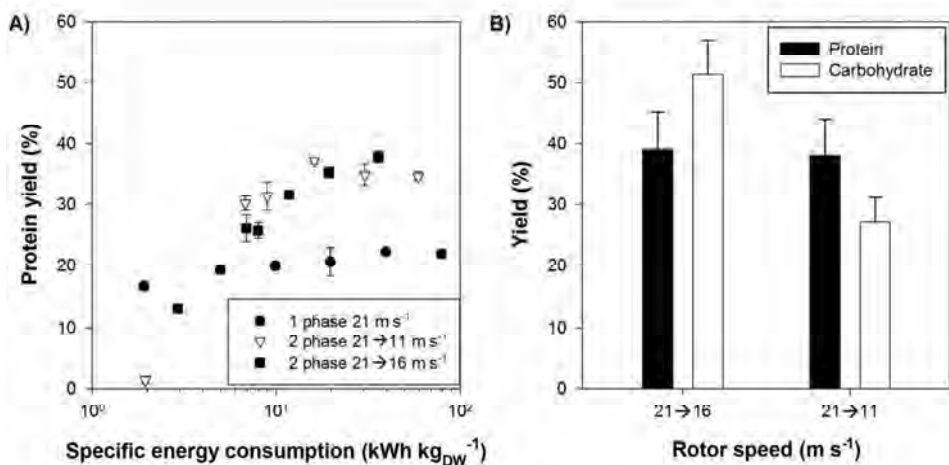


Figure 6.5 Protein yield (%) as function of the specific energy consumption ($\text{kWh kg}_{\text{DW}}^{-1}$). Single rotor speed (1 phase) of 21 m s^{-1} compared to two-phase (A) and maximal protein and carbohydrate yield for the two-phase experiments at $21\text{-}16 \text{ m s}^{-1}$ and $21\text{-}11 \text{ m s}^{-1}$ (B) for a fixed biomass concentration of 10.7 g kg^{-1} .

Figure 6.5A shows the protein yield as function of the specific energy consumption. Similar to what was observed in the single phase experiments where the thallus was manually pre-cut (Figure 6.4C-F), the maximum protein yields were higher in the two-phase experiment than in the single phase experiment at high speed (Figure 6.4A). Showing that the initial pre-

cutting phase of 3.5 min was sufficient to reduce the size of thallus and allowing milder disintegration of the cell wall to release the water soluble proteins and carbohydrates. Furthermore, it can be observed that both two-phase set-ups resulted in similar protein yields ($p > 0.87$) at equal energy consumption. This is because the set-up at which a moderate speed disintegration phase was used was faster compared to the low speed. On the other hand, when looking also to the carbohydrate yield (Figure 6.5B), the maximal carbohydrate yield did differ significantly ($p < 0.05$) with yields of 51% and 27% for the “21→16” m s^{-1} and “21→11” m s^{-1} set-up, respectively. A possible explanation is that the high shear forces not only release natively soluble carbohydrates from the cytosol, but also chop the polysaccharide structure of the thallus. This effect was also observed in Figure 6.4D and 3F, the carbohydrate yield was lower at 11 m s^{-1} compared to 16 m s^{-1} .

Beyond ± 10 min of disintegration during the two-phase experiments (11 – 16 kWh kgDW^{-1}), no further increase in the protein yield was taking place. Compared to a full experiment of 40 min at 21 m s^{-1} (77.8 kWh kgDW^{-1}) with intact thallus this corresponds to an energy reduction of 79% to 86%. Taking into account the protein yield after 10 min of disintegration, a specific energy input relative to the extracted protein of 318 ± 48 and 313 ± 71 $\text{kWh kg}_{\text{PROT}}^{-1}$ for the “21→16” m s^{-1} and “21→11” m s^{-1} set-up was obtained, respectively.

Based on the design of experiments made in the MODDE software, an indicative model was created to predict the protein and carbohydrate yield, the details about this model can be found in the appendix A.

6.3.6 Scanning Electron Microscopy

To get a better understanding behind the mechanism of disintegration by enzymatic disintegration and HSH, SEM micrographs from *U. lactuca* thallus before and after treatment were taken; untreated thallus after harvest, PMC-R10 and *Abalone* powder enzymatic digestion and after HSH. Figure 6.6A-D shows the untreated *U. lactuca* thallus. A nice and structured arrangement of the individual cells is visible (Figure 6.6A, B) with a clear turgor (Figure 6.6C). A detailed picture of the cell wall (Figure 6.6D) shows a rough surface.

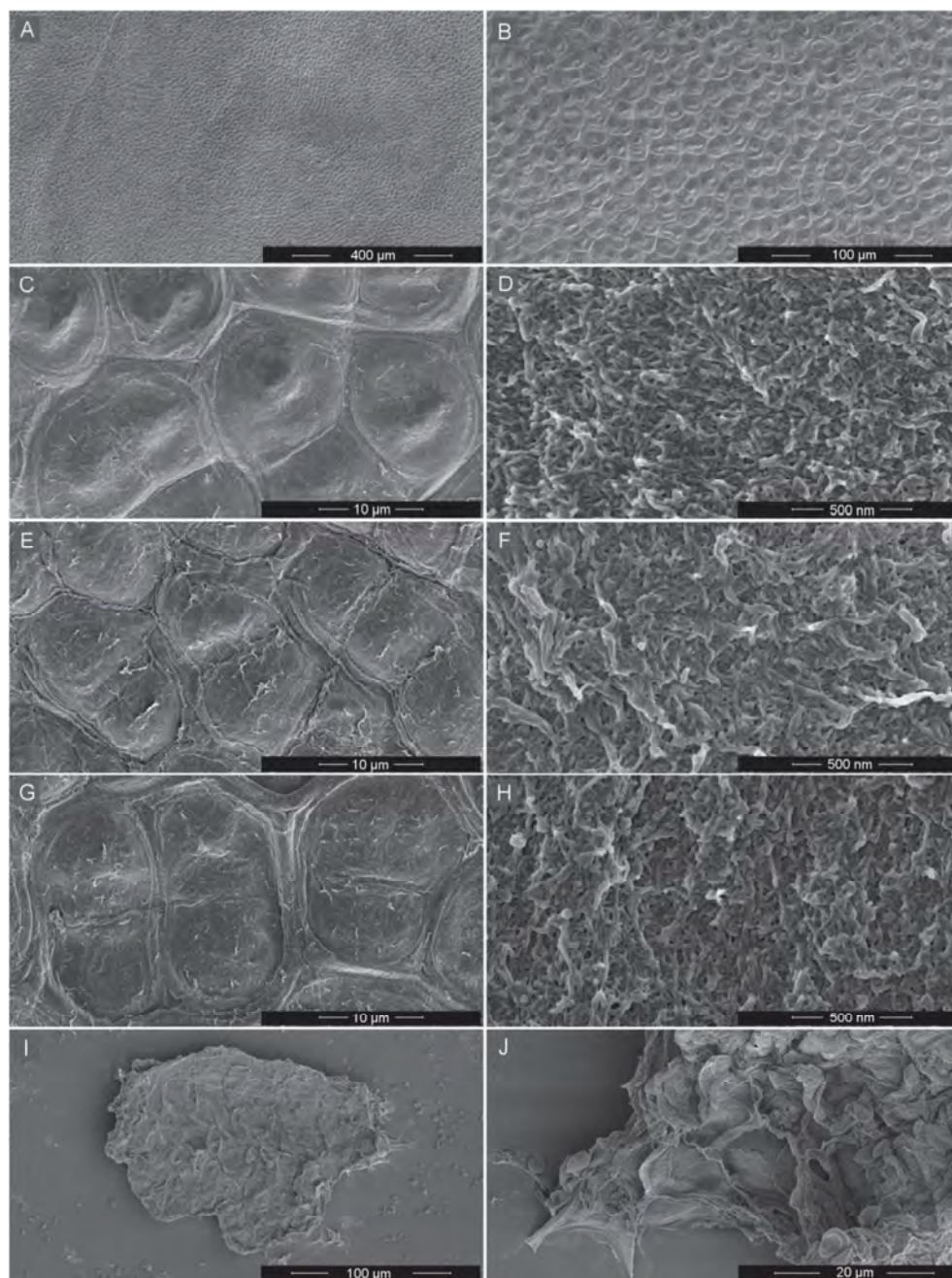


Figure 6.6 SEM micrographs of *U. lactuca* thallus before treatment (A, B, C, D), after PMC-R10 treatment (E, F), after Abalone treatment (G, H) and after HSH (I, J).

Treatment of the thallus with the pectinase PMC-R10 revealed a loss of the cell turgor (Figure 6.6E), yet no clear destruction of the cells could be observed. Besides, the cell wall surface (Figure 6.6F) does not show substantial differences with respect to the control. Which is in line with the above mentioned findings of Reddy and Fujita (1991), who also did not observe any effect of PMC-R10 on the cell wall surface. Contradictory, the cell wall must be permeable in order to release the proteins and carbohydrates up to a yield of almost 30%. Similar observations were made for the Abalone powder treatment, with a loss of turgor and no modifications on the cell wall surface (Figure 6.6G, H).

HSH of the thallus lead to the highest yields in this work, and is clearly demonstrated by the small thallus pieces observed (Figure 6.6I). Moreover, broken and emptied individual cells could be observed (Figure 6.6J).

6.3.7 Perspective of macroalgae disintegration for biorefinery

Macroalgae show a large potential in terms of securing world protein supply, and possess interesting carbohydrates. The main bottleneck is to retrieve these interesting components because of the low digestibility. Only few studies exist on the evaluation of macroalgal disintegration and protein/carbohydrate extraction (Fleurence et al., 1995; Harnedy and FitzGerald, 2013; Joubert and Fleurence, 2008). This study evaluated four different methodologies in which the release of both proteins and carbohydrates were investigated. A summary of the best results obtained in this work and literature is given in Table 6.3.

Osmotic shock would merely require that the biomass is washed with demineralized water and incubation for 24h at 30°C. However, such long holding times might become an issue when conducted for large volumes at industrial scale.

Enzymatic disintegration of *U. lactuca* is an effective method to release protein and carbohydrates. Yet, it was observed that the applied buffer might cause an osmotic shock depending on the chosen conditions. A slight dosage effect was observed (e.g., for PMC-R10). However, no proportional increase was observed when increasing the enzyme concentration from 0.5 to 2% of biomass DW. Therefore, further optimization of the applied buffer system (e.g., osmolarity) and enzyme concentration should be subject of further study.

PEF showed initial promising results, though the screening revealed that an electric field strength of 7.5 kV cm⁻¹ gives the highest results, which is contradicting the results of Polikovskiy et al. (2016) who only required a field strength of ~3 kV cm⁻¹. This might be due to the difference in the used PEF equipment and thus requires further investigation.

Table 6.3 Overview of best product yields achieved by osmotic shock, enzyme incubation, PEF and HSH from current work and literature.

Technique	Algal species	Procedure	Protein yield (%)	Carbohydrate yield (%)	Reference
Osmotic shock	<i>U. rigida</i>	Overnight, 4 °C	9.7 ± 0.6	n/a	(Fleurence et al., 1995)
	<i>U. rotundata</i>	Overnight, 4 °C	14.0 ± 1.8	n/a	
	<i>P. palmata</i>	7 h, 4°C	5.9 ± 0.4	n/a	(Harnedy and FitzGerald, 2013)
	<i>U. lactuca</i>	24 h at 30°C	19.5 ± 2.1	44.7 ± 11.2	(This work)
Enzyme incubation	<i>U. rigida</i>	2h, 3% Cellulase A, 30°C	18.5 ± 2.1	n/a	(Fleurence et al., 1995)
	<i>U. rotundata</i>	2h, 3% Cellulase A, 30°C	22.0 ± 1.5	n/a	
	<i>P. palmata</i>	48·10 ³ U Shearzyme 500L + Celluclast 1.5L	18.4 ± 1.7	n/a	(Harnedy and FitzGerald, 2013)
	<i>U. lactuca</i>	4 h, 2% PMC-R10, 30°C	26.1 ± 6.0	28.1 ± 8.1	(This work)
PEF	<i>U. lactuca</i>	E: 3 kV cm ⁻¹ , 75 pulses of 5.7 µs	< 1% ^a	n/a	(Polikovskiy et al., 2016)
	<i>U. lactuca</i>	E: 7.5 kV cm ⁻¹ , 2 pulses of 0.05 ms	15.1 ± 0.7	14.8 ± 3.3	(This work)
HSH	<i>P. palmata</i>	24000 RPM, 1h post incubation	4.3 ± 0.1	n/a	(Harnedy and FitzGerald, 2013)
	<i>U. lactuca</i>	2 phase set-up "21→16" m s ⁻¹	39.0 ± 6.2	51.3 ± 5.6	(This work)

This research revealed that HSH is the most effective technique among the tested methods, to reduce the size of the thallus, disintegrate the cell wall and result in the highest yields. The applicability of this method on larger scale should be validated (according to manufacturer website scaled-up equipment is available). Furthermore, the process revealed to be energy intensive (≥ 11 kWh kgDW⁻¹) with respect to the biomass energy density ~ 4.8 kWh kgDW⁻¹ (on ash free dry matter) (Bruhn et al., 2011). The applied biomass concentrations were rather low compared to microalgae disintegration (Doucha and Lívanský, 2008; Goettel et al., 2013), an increase might be the solution to overcome the high specific energy consumption.

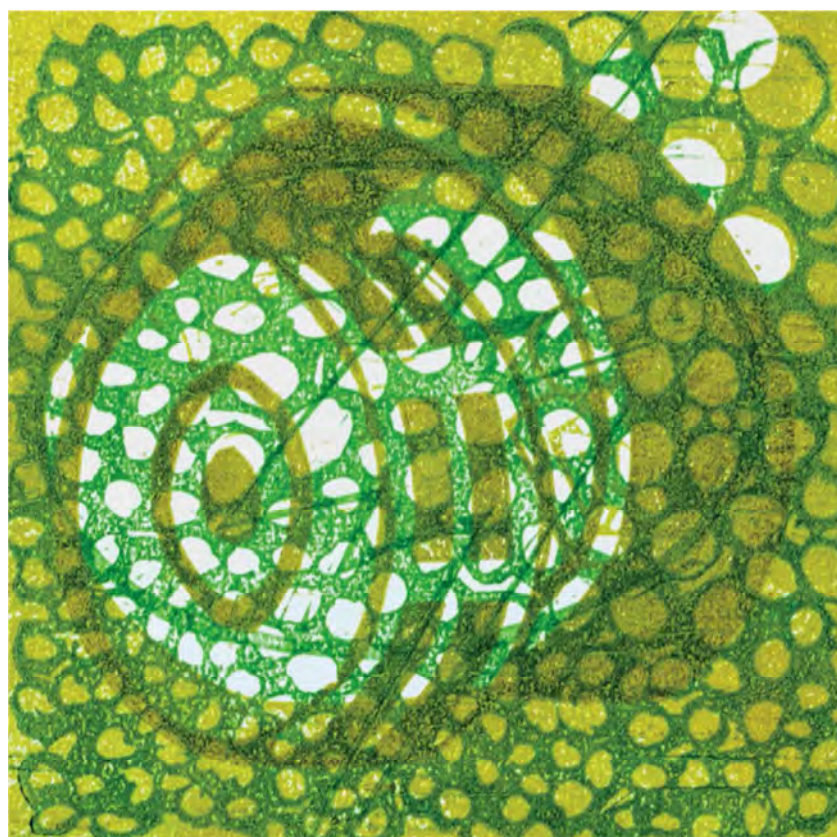
To conclude, in descending order the highest carbohydrate yields per treatment; HSH ($\sim 51\%$) > Osmotic shock ($\sim 45\%$) > enzyme degradation ($\sim 28\%$) > PEF ($\sim 15\%$) and in descending order the highest protein yields per treatment; HSH ($\sim 40\%$) > enzyme degradation ($\sim 25\%$) > osmotic shock ($\sim 20\%$) > PEF ($\sim 15\%$). Nevertheless, PEF (6.6 kWh kg_{prot}⁻¹) did show a more promising specific energy consumption with respect to the extracted protein compared to HSH (313-318 kWh kg_{prot}⁻¹).

Acknowledgements

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Abbreviations

Symbol	Definition	units
C_x	Biomass concentration	$[\text{kg m}^{-3}]$
E	Electrical field strength	$[\text{V m}^{-1}]$
F	Fresh uncut biomass	[-]
FC	Fresh cut biomass (3x4 cm)	[-]
$FC2$	Fresh cut biomass 2 (1x1 cm)	[-]
N	Number of pulses	[-]
t_p	Pulse duration	[s]
W_V	Volumetric specific energy input	$[\text{kWh m}^{-3}]$
W_M	Mass specific energy input	$[\text{kWh kg}_{\text{DW}}^{-1}]$
σ	Conductivity	$[\text{S m}^{-1}]$



Chapter 7

General discussion

7.1 Introduction

Worldwide there is a growing demand for proteins for food and animal feed (Godfray et al., 2010). Moreover, 68% of the total protein demand in the EU is imported (FAO, 2013). To keep up with the growing demand, novel protein sources like microalgae and macroalgae have been identified (van den Burg et al., 2013; Wijffels et al., 2010). To utilize microalgae and macroalgae, the products which are commonly stored intracellularly need to be extracted. This should be done using a biorefinery approach to be able to valorize the whole biomass (Ruiz et al., 2016; Vanthoor-Koopmans et al., 2013). The proteins in this algal biomass can be characterized by a water soluble and insoluble fraction. The water soluble proteins can be used as food additives because solubility is an important techno-functional property (Boland et al., 2013; Kinsella and Melachouris, 1976). The remaining (insoluble) proteins have a good amino acid profile and consequently nutritional value (Becker, 2007; Harnedy and FitzGerald, 2011) and can be used for bulk food and animal feed. To achieve maximal biomass valorization (i.e., economic value) it is important to maintain the valuable techno-functional properties of the water soluble protein fraction (Ruiz et al., 2016). Therefore, the water soluble proteins should be isolated in the first phase of the biorefinery consisting of mild (i.e., low temperature, no destructive solvents) disintegration and extraction technologies. In addition, there is a continuous strive to make processes more energy efficient and to improve process yields (Vanthoor-Koopmans et al., 2013).

Before products can be extracted the cell wall needs to be disintegrated. After cell disintegration, water soluble components can readily be extracted in the aqueous phase. For that reason, the research in this thesis focused on finding a mild, scalable and energy efficient disintegration technology for water soluble protein extraction without negatively affecting product quality. In this chapter, first a summary of the major results achieved in this thesis on microalgae disintegration is given, bottlenecks are discussed and opportunities for future research are presented. Subsequently, the effect of cell disintegration on the selective product release is discussed, after which a scale up strategy for microalgae cell disintegration is proposed. Finally, an economic evaluation is made and a future outlook on microalgae disintegration is provided.

7.2 Cell disintegration

In this section the major outcomes on microalgae disintegration by bead milling and pulsed electric field (PEF) are discussed and future research opportunities are proposed.

7.2.1 Bead milling

As described in **Chapter 1**, conventional disintegration techniques (e.g., bead milling) were not considered to be mild and require much energy (Vanthoor-Koopmans et al., 2013). However, no data were available about the “mildness” of these conventional technologies. Bead milling was already applied for disintegration of several microorganisms such as bacteria, yeast and also microalgae at specific energy consumption over 10 kWh kg_{DW}⁻¹ (Doucha and Lívanský, 2008; Kula and Schütte, 1987; Middelberg, 1995). In **Chapter 2** we developed a benchmark for microalgal cell disintegration using bead milling. It was shown that bead milling could actually be applied as a mild and energy efficient technology for the disintegration of the green microalgae *Chlorella vulgaris*. It was also found that proteins were released before the cell disintegration appeared, most likely caused by single cracks in the cells wall allowing the cytoplasmic content to be released. This led to the conclusion that it is more important to follow the actual product release than solely looking at the disintegration. The specific energy consumption was reduced over a factor 12 compared to literature data, to values as low as 0.81 kWh kg_{DW}⁻¹. Furthermore, between 32% and 48% of the total protein was released as water soluble protein after cell disintegration. Although in other studies higher water soluble protein yields were reported (51-63%) after high pressure homogenization, the specific energy consumption (7.5-25 kWh kg_{DW}⁻¹) was much larger (Grimi et al., 2014; Safi et al., 2014b).

In **Chapter 2** the influence of the biomass concentration and the agitator speed on the disintegration during bead milling of *C. vulgaris* was investigated. In literature little consensus was available on the effect of the beads size during disintegration (Table 3.1). Therefore, in **Chapter 3** the influence of the bead size on disintegration efficiency of several microalgae species was studied. The microalgae *C. vulgaris*, *Neochloris oleoabundans* and *Tetraselmis suecica* were bead milled with four different bead sizes each. The major observation in this study was that the use of smaller bead sizes, as low as 0.3 mm, resulted in a further decrease of the specific energy consumption to 0.45 kWh kg_{DW}⁻¹. In addition, we observed that both proteins and carbohydrates can be effectively released by means of bead milling. In general, the protein release was faster than the carbohydrate release. We hypothesized that the carbohydrates are still associated to starch granules and the cell wall debris, and therefore require a longer and more intensive milling process. Depending on the used algae species and the time of the milling process, the selectivity (i.e., ratio of proteins over carbohydrates concentration) could be altered up to a factor 6.7.

The release of both carbohydrates and proteins after bead milling does not negatively affect the product quality (i.e., techno-functional properties) and therefore further purification is

not required as was demonstrated by Schwenzfeier et al. (2011, 2014). Currently, food production processes make use of highly purified ingredients. These isolates are obtained from raw materials like grain. Subsequently, the different isolates are mixed again to form a product or ingredient with the right properties (e.g., foam capacity, texture). The use of functional fractions (less purified concentrates) in which several different components are present, potentially decreases water and energy use for extensive separation and fractionation (van der Goot et al., 2016). Therefore, the released proteins and carbohydrates after bead milling might be directly applied as a concentrate without further refinement.

Next to the differences in product release, also differences in disintegration behavior were observed between the three algae species. This might be explained by differences in the cell wall structure and morphology of the algae. *C. vulgaris* (taxonomical order *Chlorellales*) and *N. oleoabundans* (taxonomical order *Sphaeropleales*) have a very similar morphology but did show different breakage behavior. Algae belonging to the order of *Sphaeropleales* are known to have a trilaminar outer cell wall which contains sporopollenin (Atkinson et al., 1972). The possible presence of sporopollenin in *N. oleoabundans* might hint for its greater toughness. Moreover, *C. vulgaris* does not contain sporopollenin (Reisser, 1984), but its cell wall contains chitin-like polysaccharides (Kapaun and Reisser, 1995). Cell morphology and cell wall composition seem to be important for the efficiency of bead milling. Morphology and composition vary per strain and are depending on cultivation conditions. Further research is required on the effect of cell morphology and cell wall structures to understand the disruption behavior during bead milling. Not only the polysaccharide linkages but also the cell wall thickness and the cell shape play an important role in the overall cell strength. By means of nanoindentation or atomic force microscopy the minimal specific energy requirement could be determined. The minimal shear stress at which the cells disintegrate could be determined using a shear cylinder (Michels et al., 2015).

Mechanical disintegration processes generally have a large energy inefficiency (Lee et al., 2013; Günther et al., 2016). A large part of the energy input is dissipated into heat or friction. As was discussed in **Chapter 3**, one kg of *T. suecica* can be disintegrated ($E_{M,min}$) using only $1.87 \cdot 10^{-4}$ kWh kg_{DW}⁻¹ (Lee et al., 2013) while a kg of *C. vulgaris* requires $6.88 \cdot 10^{-4}$ – $2.52 \cdot 10^{-3}$ kWh kg_{DW}⁻¹ (Günther et al., 2016). This represents a difference in the range of 10^2 – 10^3 compared to the lowest energy consumptions obtained in this study for *T. suecica* and *C. vulgaris*, being 0.47 kWh kg_{DW}⁻¹ and 0.45 kWh kg_{DW}⁻¹, respectively.

The energy is not only used for disintegration of the cells. Energy is also used to move the mass of beads and water. In our experiments only 4.4% of the total mass in the bead mill are algae (196.6 g beads, ~170 g water, ~17g_{DW} algae; at biomass concentration: 87.5 g_{DW} kg⁻¹).

Therefore, only 4.4% of the energy supplied is used to disintegrate the algae, the rest is used for moving the beads and the water. The corrected specific energy consumption ($E^*_{M,3\tau}$) with respect to the fraction of the total mass can be determined as:

$$E^*_{M,3\tau} = \frac{P_{BM} \cdot \frac{M_x}{M_b + M_w + M_x}}{M_x} = \frac{P_{BM}}{M_b + M_w + M_x} \quad (7.1)$$

in which P_{BM} is the power consumed by the mill (kWh), M_b is the mass of beads (kg), M_w is the mass of water (kg) and M_x is the mass of algae (kg).

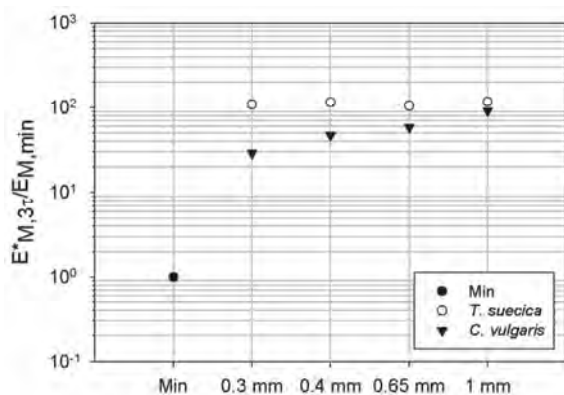


Figure 7.1 Overview of $E^*_{M,3\tau}/E_{M,min}$ ratio with minimal specific energy input “Min” and different bead sizes for *T. suecica* and *C. vulgaris*.

Figure 7.1 shows the ratio of $E^*_{M,3\tau}$ over the minimal required energy consumption $E_{M,min}$. In the best case scenario where $E^*_{M,3\tau}$ is equal to $E_{M,min}$ this ratio is unity which is depicted in Figure 7.1 by “Min”. Using Eq. ((7.1), $E^*_{M,3\tau}$ was determined to be between $1.97 \cdot 10^{-2}$ - $2.16 \cdot 10^{-2}$ and $1.96 \cdot 10^{-2}$ - $6.42 \cdot 10^{-2}$ kWh kg_{DW}⁻¹ for *T. suecica* and *C. vulgaris*, respectively. With respect to $E_{M,min}$ (as cited above), the ratio is two orders of magnitude larger (105 - 116x) for *T. suecica* and only one order of magnitude larger (29 - 91x) for *C. vulgaris*. This shows that the specific energy consumption after correction is close to the minimal value of cell wall disintegration and that the energy efficiency only can be increased if less energy is used for moving water and beads.

Future research should focus on closing the gap between the applied energy and the minimal required energy. As described in **Chapter 1**, a limited number (nine) out of 44 (Möller and Hörnle, 1972) identified process variables is deemed to be most influential (Kula and Schütte, 1987), from these nine only three have been optimized in this thesis. First of all, the energy consumption could be improved by using a higher biomass concentration (**Chapter 2**). To further reduce the specific energy consumption, the stress intensity (SI) should be closer to the optimal value (SI_{opt}) (**Chapter 3**). An important factor influencing the SI is the agitator speed, which could be further reduced, hereby both the applied SI and the specific

energy consumption will be decreased. Development of beads with a lower specific density than the used ZrO_2 beads (3.8 g cm^{-3}), but with higher wearing resistance than glass, could also lead to a closer approximation of the Sl_{opt} . Furthermore, efficiency improvement of hardware (e.g., electrical motor and bearing friction) could also lead to a reduction of energy consumption.

As described above, further energy reduction could be achieved when less water and beads are moved in the bead mill. Ideally, technologies are developed which involve hardly any moving parts (i.e., like beads, agitator, valves) or on external fields which do not dissipate energy in the medium to close the gap towards $E_{M,min}$. Two technologies which could aid in this and involve no moving parts are microfluidic principles and hydrodynamic cavitation. Both rely on the Venturi effect. When an incompressible liquid flows through a narrowed section in a channel, the fluid velocity *must increase* while the pressure *must decrease* (i.e., Bernoulli's law). If such a principle is achieved in a microfluidic device or at this scale, very high velocities and thus shear rates could be achieved. To generate hydrodynamic cavitation, the narrowed section should be designed such that the sudden pressure drop causes the pressure to decrease below the vapor pressure subsequent to increase the pressure. Several patents have been filed for designs on hydrodynamic cavitation cylinders, e.g. Gordon et al. (2010). Operation pressures of maximum 35 MPa (e.g., $>>200 \text{ MPa}$ in HPH) with single passage and an assumed biomass concentration equal to the bead mill benchmark ($87.5 \text{ g}_{\text{DW}} \text{ kg}^{-1}$) would potentially result in an energy consumption of only $0.1 \text{ kWh kg}_{\text{DW}}^{-1}$. A third option applying a different type of external field could be laser induced lysis which cause local cavities (Günkerken et al., 2015; Nan et al., 2014) and therefore could initiate selective disintegration of the cells. Nonetheless, for disintegration by microfluidic and technologies inducing cavitation, further research is required on the sudden extreme local temperatures and its effects on the internal products.

7.2.2 Pulsed Electric Field

Pulsed Electric Field (PEF) has been used in the past decade to disintegrate cells by permeabilization of the microalgal cell membrane and cell wall. Up to 27, 80 and 53% more protein, chlorophyll and carotenoids were released from *C. vulgaris* after PEF treatment, respectively (Toepfl et al., 2006). These relative numbers suggested very positive perspectives for PEF to be applied for microalgae disintegration. However, the absolute data from **Chapter 4 and 5** show the opposite. In **Chapter 4** of this thesis we investigated the effect of pulse parameters in a screening study on *C. vulgaris* and *N. oleoabundans* using two different PEF setups. Although for both algae effective permeabilization of the cell

membrane was observed, the protein yield never exceeded 13% with specific energy consumptions ranging from 0.03 to 150 kWh kg_{DW}⁻¹.

To increase the performance of PEF, in **Chapter 5** the effect of the treatment temperature (25–65°C) during PEF was studied using a continuous flow pilot scale setup. Studies on the inactivation of microorganisms in fruit juices and extraction of pigments from microalgae showed a positive effect of the temperature during electroporation (Luengo et al., 2015; Timmermans et al., 2014). In this study, the specific energy consumption of the PEF treatment (0.55 – 1.11 kWh kg_{DW}⁻¹) was set equivalent to the energy consumption found for bead milling (**Chapter 2 and 3**). Also in this study a high ion release close to 80% was observed, showing that effective permeabilization was achieved. Nevertheless, the protein yields did not exceed 5%, independent of processing temperature and specific energy consumption. The results of **Chapter 4 and 5** show that it is with PEF not possible to sufficiently disintegrate the algal cells to achieve water soluble protein yields similar to bead milling at similar or higher specific energy consumption.

In **Chapter 4 and 5** we observed that about 87–95% of the protein was retained inside the cells after PEF. Yet, in **Chapter 5** we did find that up to 39% of the carbohydrates could be released at a processing temperature of 55°C making PEF a carbohydrate selective method (temperature treatment at 55°C without PEF resulted in a carbohydrate yield of 5%). Figure 7.2 shows a graphical representation of the product selectivity of both the bead mill and the PEF.

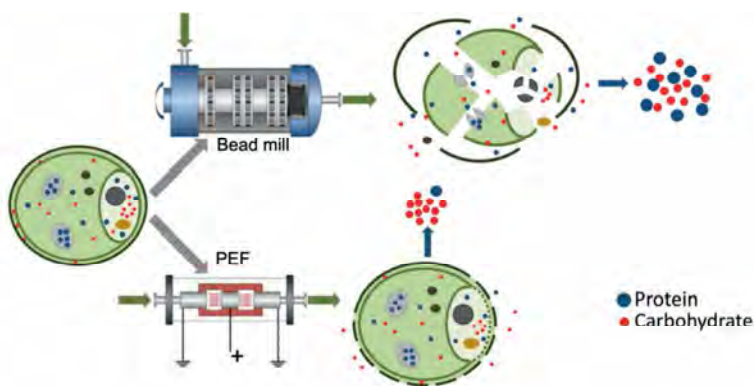


Figure 7.2 Graphical presentation of selectivity from bead mill and PEF.

In **Chapter 5** we discussed the possibility of applying a bead milling step after the application of PEF. In that way, a more pure carbohydrate fraction could be obtained directly after PEF, while the bead mill would release the proteins and also result in a more pure fraction. A complete different approach would be to exchange the biorefinery steps, i.e. first bead mill

followed by PEF. After bead milling, 60-70% of the total protein content was not released as soluble protein, while other studies even showed soluble protein yields of >50% (Grimi et al., 2014; Safi et al., 2014b). Since PEF showed to have merely an influence on the cell membrane and not on the cell wall, we hypothesize that the subsequent application of PEF after bead milling could allow the release of proteins associated to the membranes.

Where bead milling showed to be a suitable technique to release protein and PEF was not, the question arises what could be the bottleneck. Typically, the required electric field strength for small cells is relatively high to overcome the transmembrane potential for effective electroporation (Kotnik et al., 2015). The induced transmembrane potential U_m applied in the PEF treatment chamber can be calculated using Schwan's equation: $U_m = 1.5 \cdot r \cdot E$; where r is the radius of the cell (m) and E is the applied electric field strength (V m^{-1}) (Azencott et al., 2007; Vorobiev and Lebovka, 2008). With a typical diameter of 3.2 and 3.3 μm for *C. vulgaris* and *N. oleoabundans*, and a transmembrane potential of 1 V (Weaver et al., 2012), the critical electric field strength (E_{crit}) can be calculated: 4 – 4.2 kV cm^{-1} . It is known that the effective electric field strength often deviates from the theoretical field strength (Buckow et al., 2010, 2011). For co-field or co-linear treatment chambers this means that only 75-81% of the theoretical field strength can be achieved (Buckow et al., 2011). In both **Chapter 4 and 5**, the E_{crit} was exceeded taking into account the non-homogeneous electric field strength. Nevertheless, the permeabilization was only effective to release small molecules like ions and carbohydrates, while the vast majority of the larger biomolecules –proteins– remained inside the cell. This is most likely caused by the strong algal cell wall which is not affected by the electrical field as was also demonstrated by Azencott et al. (2007).

In contrast to the limiting cell wall which suggests that only small holes were made, in **Chapter 5** native page revealed that little amounts of Rubisco could be released by PEF. Taking into account the size of Rubisco being ~540 kDa, relatively large pores have been formed in a fraction of the cells to allow the release of this protein. Further studies are necessary to understand why some of the Rubisco is released.

7.3 Scale up strategy and bottlenecks for bead milling

Results obtained in experiments at a laboratory scale need to be translated to larger scale (pilot or industrial). The bead mill applied in **Chapter 2 and 3** is a specifically designed lab scale apparatus to test whether a product can be efficiently milled in a bead mill (WAB, 2013, personal communication). When comparing the specific energy consumptions E_M (Table 2.3) to larger scale systems in literature (Doucha and Lívanský, 2008), the results

(**Chapter 2**) initially indicated similar outcomes in terms of disintegration levels and energy consumption. This showed that the lab scale bead mill we used is a representative downscaled model system for laboratory scale bead milling. In **Chapter 2 and 3** the bead milling process was further optimized at lab scale to specific energy consumptions as low as $0.45 \text{ kWh kg}_{\text{DW}}^{-1}$, which is also promising for larger scale systems.

Bead mills do not scale linearly because the distance between the tip of the agitator and the grinding chamber wall gets larger with increasing scale (i.e., speed of beads decreases closer to the grinding chamber wall). Therefore, the agitator speed applied at small scale cannot be directly applied at larger scale. As stated in **Chapter 3**, Eq. (3.4), the *SI* can be described as $SI \propto d_b^3 \cdot \rho_b \cdot u_s^2$. To scale the bead mill, the assumption that the bead velocity (v_b) is equal to the agitator tip speed (u_s) only holds for equal geometry. Therefore, to keep the velocity profile –i.e., the *SI*– of the beads equal, a higher tip speed is most likely required (WAB, 2013, personal communication) at the cost of higher energy consumption (**Chapter 2**). Appendix Figure 7.A.1 shows a power law correlation ($R^2 = 0.995$) between the chamber volume and the agitator speed (WAB, 2013, personal communication). This manufacturer data allows scale up of bead mills in the range from 0.5 – 60 L grinding chambers for grinding of hard crystalline materials (e.g., paints, lacquers) and has not yet been validated for microalgae. Extrapolation of this power law to the small chamber of the lab scale bead mill used in this thesis ($V_{\text{chamber}} = 0.08 \text{ L}$) showed that a maximal agitator speed of 6.4 m s^{-1} is still scalable. The results found in this thesis at 6 m s^{-1} should therefore be scalable applying this power law.

However, in **Chapter 3** we discussed the effect of the stress number *SN* and the *SI*. As long as the *SI* is above SI_{opt} at which the cells just break only the *SN* determines the process. The *SN* can be influenced by both the agitator speed and the total processing time which have a contradicting effect on the energy consumption (increased agitator speed means decreased processing time or vice versa). This SI_{opt} might even be reached at slightly lower agitator speeds, which might result in a further decrease of the specific energy consumption. Therefore, additional research is required to fine-tune the agitator speed, calculated from the power law for large scale, at which SI_{opt} is just reached.

Additionally, to get a fundamental understanding on the bead velocity profiles and stress events during bead milling, computational fluid dynamics (CFD) coupled to discrete element method (DEM) might be used. As stated above, for successful scale up, the average *SI* should be equal for each bead mill size. Therefore, the velocity profile of the beads should be equal. Previous work by Jayasundara et al. (2012) and Beinert et al. (2015) showed the successful application of CFD coupled to DEM for different types of stirred media mills.

Finally, for large scale algae processing, batch mode bead milling is envisioned over continuous mode. The energy consumption in a bead mill process is dictated for a large part by the idle energy consumption of the mill (70-85% of total, **Chapter 2**). Therefore, shorter processing times are desired to reduce operational costs.

7.4 Concluding remarks and future outlook on algae disintegration

In this thesis the disintegration of fresh microalgae for the mild extraction of water soluble proteins was investigated. At the start of this project, bead milling consumed on average 10 kWh kg_{DW}⁻¹ (Boer et al., 2012), which is currently reduced to 0.45 kWh kg_{DW}⁻¹ (**Chapter 3**). To date, bead milling is the only technique with which fresh biomass can be disintegrated and proteins released at a specific energy consumption of less than 10% of the biomass energy content (U.S. DOE, 2010) (Figure 7.3). Therefore, one of the core issues addressed for microalgae biorefinery –cell disintegration– (Vanthoor-Koopmans et al., 2013) has been overcome.

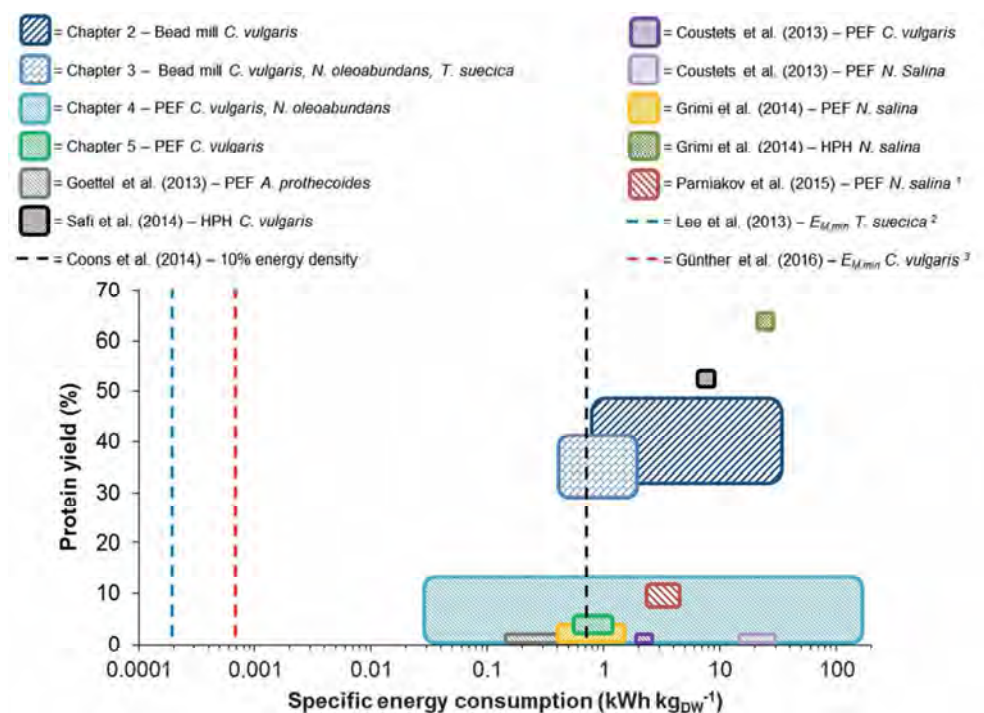
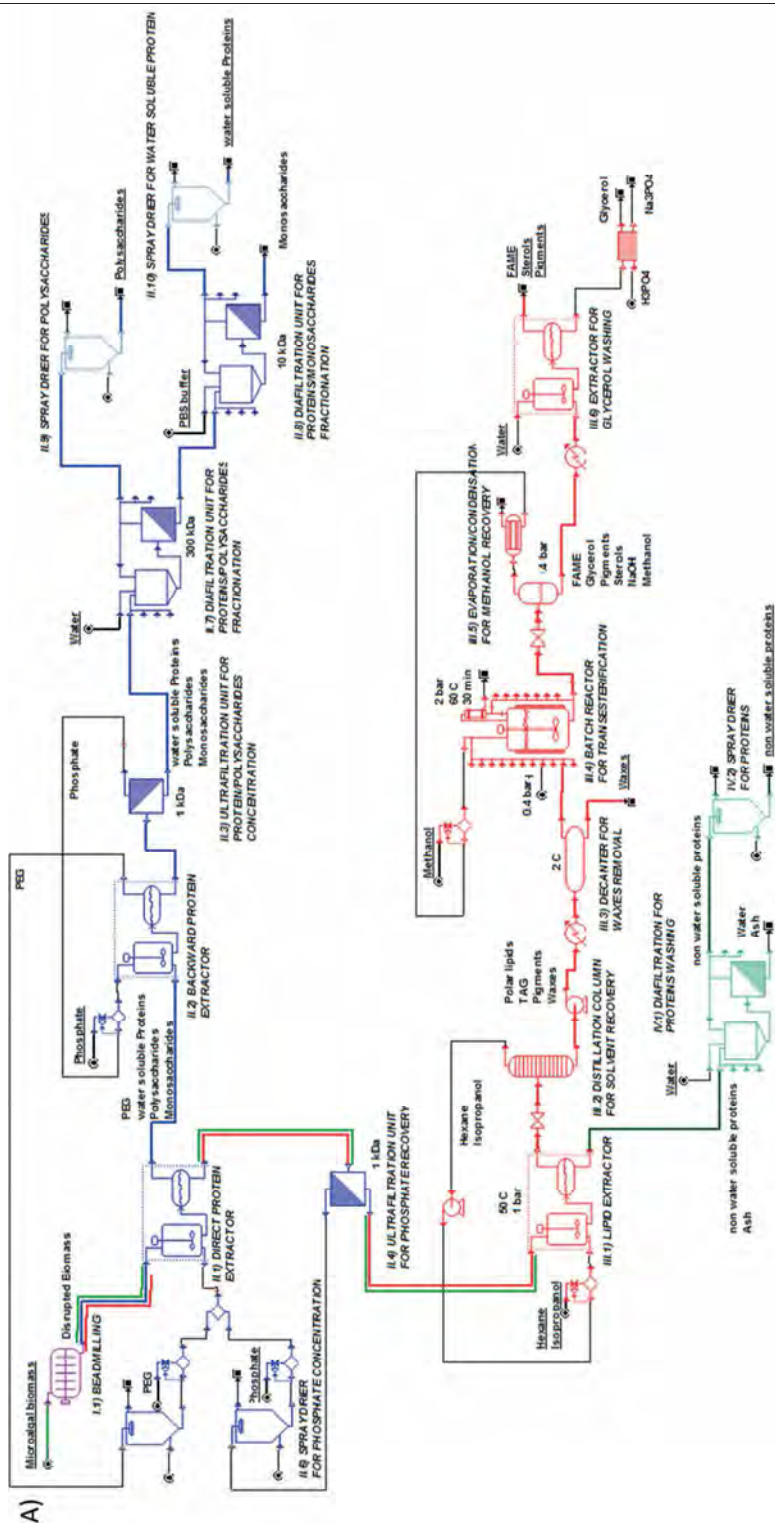


Figure 7.3 Concluding figure summarizing major reference works on pulsed electric field (PEF) and high pressure homogenization (HPH) and the results in this thesis on bead milling and PEF (Chapter 2-5). ¹ frozen alga used, ² minimal specific energy consumption by AFM, ³ minimal specific energy consumption by nanoindentation.

For the overall feasibility of microalgae valorization, to date only complete biorefinery or high value markets create positive economics with a realistic return on investment. The market price for high values products is $\sim 30 \text{ € kg}^{-1}$ with total production costs of $\sim 7.4 \text{ € kg}^{-1}$. For commodity products like biofuels, chemicals or bulk food/feed protein the production costs ($\sim 4.4 \text{ € kg}^{-1}$) are lower, but also the market value is substantially lower ($\sim 1 \text{ € kg}^{-1}$) (Ruiz et al., 2016). Five market scenarios ranging from bulk commodities to complete biorefinery for high value markets were described by Ruiz et al. (2016). In addition to these market scenarios, we propose a new market scenario in which bead milling is applied after the cultivation step followed by a simple solid-liquid separation step (centrifugation) to separate the released techno-functional fraction (soluble proteins and carbohydrates) from the insoluble fraction (cell debris and insoluble proteins), followed by spray-drying of the soluble and insoluble fraction. No further fractionation is used in this scenario and is hereafter described as the “techno-functional” (“TF”) scenario. For comparison, the complete biorefinery (hereafter indicated as “C”) and “TF” scenario flowsheets are illustrated in Figure 7.4 and show a five-fold decrease in the amount of unit operations.

This new “TF” market scenario was evaluated against the “C” scenario for high value products. A complete production cost calculation for $10,000 \text{ MT}_{\text{biomass}} \text{ y}^{-1}$ was performed based on flow-sheeting software (SuperPro Designer® v9.0, Intelligen, Inc., USA) in which capital investment (CAPEX) and operational costs (OPEX) were calculated similar to the approach of Ruiz et al. (2016). A detailed breakdown of the costs can be found in appendix B (Table 7.B.1–7.B.4). The costs for cultivation (3.40 € kg^{-1}) and the complete “C” biorefinery costs (3.0 € kg^{-1}) were obtained from Ruiz et al. (2016). Based on literature and this thesis, the total bead milling costs were recalculated for the “C” scenario. The bead milling costs were based on a specific energy consumption of $10 \text{ kWh kg}_{\text{DW}}^{-1}$ for the *old* case (before this thesis) and $0.45 \text{ kWh kg}_{\text{DW}}^{-1}$ for the *new* case (after this thesis). The bead mill optimization achieved in this thesis (**Chapter 3**) not only resulted in a reduction of the energy consumption (i.e., OPEX reduction) but also in process time which allows a higher throughput/smaller equipment (i.e., OPEX and CAPEX reduction) (see Appendix Table 7.B.2). Figure 7.5 shows the total production costs per kg algae for each of the scenarios, specified for the cultivation, bead milling costs and biorefinery costs (excluding bead milling) for the “C” and “TF” scenario. For the “C” scenario, the old total production costs were 7.79 € kg^{-1} which are reduced to 6.32 € kg^{-1} by the bead mill optimization. However, this reduction does not change anything about the market potential for complete biorefinery. Given the market price (30.4 € kg^{-1} , black dashed line in Figure 7.5) for high valuables, even with the old bead milling energy consumption ($10 \text{ kWh kg}_{\text{DW}}^{-1}$), the complete biorefinery scenario would have given positive economics.



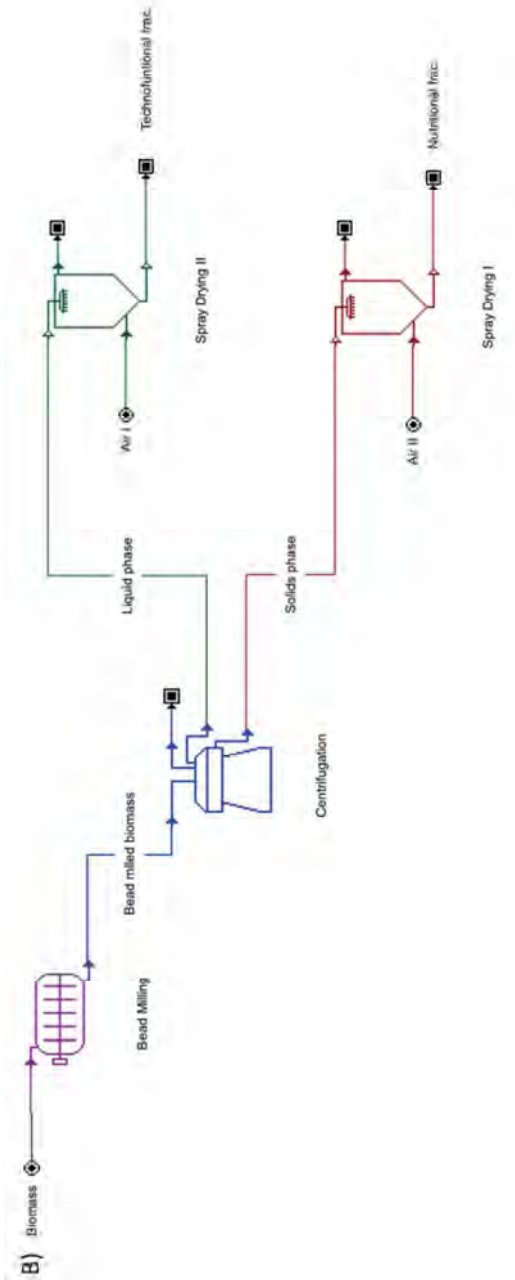


Figure 7.4 Flowsheet for (A) complete biorefinery “C”, 20 unit operations (Ruiz et al., 2016) and (B) techno-functional biorefinery “TF”, 4 unit operations.

For the “TF” scenario no complete biorefinery was applied which significantly decreases the overall production costs to 3.72 € kg⁻¹ (Appendix Table 7.B.2 and 7.B.3). However, the “TF” scenario for bulk food and feed gives a turnover of 1.40 € kg⁻¹ (red dashed line in Figure 7.5, Appendix Table 7.B.4) which in each of the scenarios is not enough to make a profitable business case because of the relatively high microalgae cultivation costs which remains the main bottleneck for overall process economics (Wijffels and Barbosa, 2010; Norsker et al., 2011; Ruiz et al., 2016).

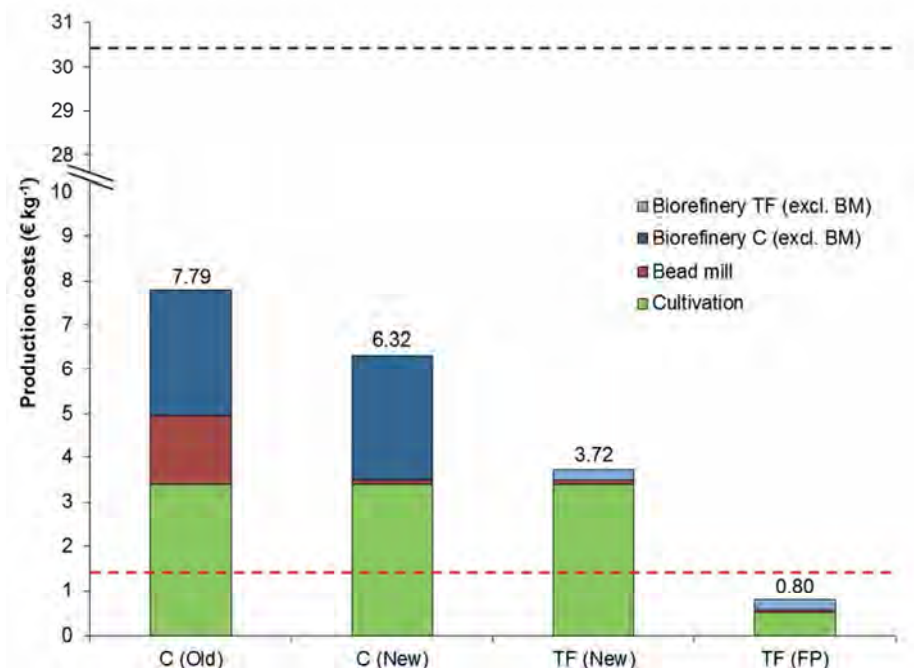


Figure 7.5 Production (€ kg⁻¹) costs for four scenarios specified for the cultivation, bead milling and biorefinery costs (excluding bead milling); complete “C” biorefinery with specific energy consumptions for bead milling (BM) of 10 (Old) or 0.45 (New) kWh kg_{DW}⁻¹; techno-functional “TF” biorefinery for 0.45 (New) or 0.32 (FP) kWh kg_{DW}⁻¹; Black dashed line: “C” market price (30.4 € kg⁻¹); red dashed line: “TF” market price (1.40 € kg⁻¹).

Ruiz et al. (2016) projected a future algae cultivation cost of 0.50 € kg⁻¹. In addition, the bead mill energy consumptions from **Chapter 3** are for small scale and do not yet include translation to large scale which are expected to be benefit from scale-economy. First of all since electrical engines are more energy efficient at larger scale, and also the grinding chamber wall area to volume ratio improves (which decreases attrition of beads to the wall and therefore reduces the energy consumption). In **Chapter 2**, on average a 30% energy efficiency improvement was achieved with increasing biomass concentration. In case this is extrapolated to the data of **Chapter 3**, the future projection for bead milling would be 0.32 kWh kg_{DW}⁻¹. The total production costs for the “TF” scenario would reduce to 0.80 € kg⁻¹

showing the future potential of a simple protein-first biorefinery aimed at techno-functional food additives and nutritional bulk food and feed. From this we can conclude that focus is required on reducing the algal cultivation costs, and that a simple biorefinery procedure in which the algal biomass is disclosed followed by a solid-liquid separation step and drying of the products, is not the bottleneck for bulk proteins.

Appendix A

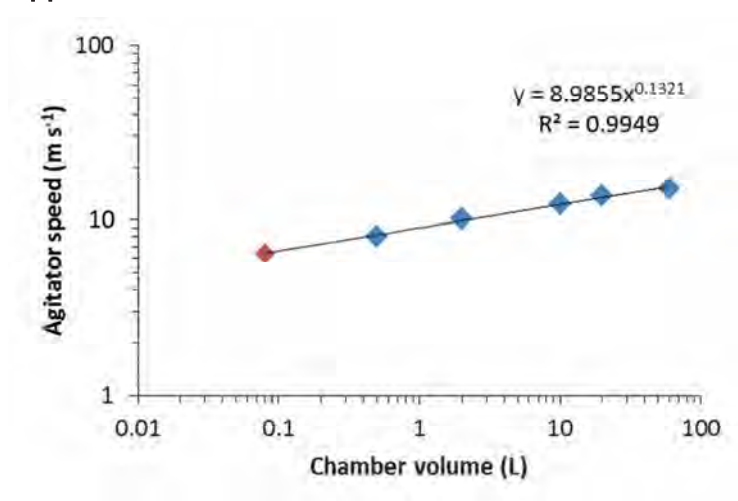


Figure 7.A.1 Power law correlation between chamber volume (L) and agitator tip speed (m s⁻¹) (blue diamonds) (WAB, 2013, personal communication) extrapolated to lab scale bead mill (red diamond).

Appendix B

Table 7.B.1 Process conditions for “TF” scenario and specification of CAPEX and OPEX.

Process conditions			
General	Biomass throughput (Q_M)		10,000 MT y^{-1}
	Biomass concentration (C_x)		150 g L^{-1}
	Liquid throughput (Q_V)		66667 $m^3 y^{-1}$
	Operational time		300 d y^{-1}
Bead mill	Free volume degree		60%
	Max chamber volume		600 L
	Residence time		See table 7.B.3
	Energy consumption		See table 7.B.3
	Product release percentage		87.5% (Chapter 3)
	Protein yield		40%
	Carbohydrate yield		50%
Centrifuge	Dissipation to heat (bead mill)		100%
	Power		55 kW
	Max capacity		10 $m^3 h^{-1}$
	Concentration solids in pellet		250 g L^{-1}
Spray dryers	Specific power uptake		0.02 kWh kg_{feed}^{-1}
	Specific utility amount		2.0 $kg_{evaporated}^{-1}$
	Evaporation rate		100 $(kg h^{-1}) m^{-3}$
CAPEX			
	Overall Lang factor		4.97
	Process lifetime		15 y
	Working capital		2 months of OPEX
OPEX (Energy + Utilities + Labor)			
Energy	Electricity cost		0.122 € kWh $^{-1}$
	Utility type		Cooling water (20-25 °C)
	Temperature increase		5 °C
	Specific enthalpy		4.18 kJ $kg^{-1} °C^{-1}$
Utilities	Utility cost		0.05 € MT $^{-1}$
	Utility type		Steam (152 °C)
	Specific enthalpy (steam)		2107 kJ kg^{-1}
	Heat of vaporization (water)		2230 kJ kg^{-1}
Labor	Utility cost		11.30 € MT $^{-1}$
	Operator		0.2 labor h h^{-1}
	Supervisor		28.3 € labor h $^{-1}$
	Manager		0.03 labor h h^{-1}
			40.6 € labor h $^{-1}$
			0.01 labor h h^{-1}
			63.3 € labor h $^{-1}$
Direct labor time			70%

Table 7.B.2 Detailed breakdown of biorefinery costs for scenarios.

Scenario	Cost breakdown	
C (Old)	Energy consumption BM (Boer et al., 2012)	10 kWh kg _{DW} ⁻¹
	Residence time BM	24.8 min
	# BM units ($V_{chamber}$)	11 (580 L)
	BM Equipment costs ^a	344.1 k€ unit ⁻¹
	Original biorefinery CAPEX + OPEX (excl. BM) (Ruiz et al., 2016)	2.85 € kg ⁻¹
	BM CAPEX + OPEX	1.550 € kg ⁻¹
	BM Energy costs	1.22 € kg ⁻¹
	BM Utilities cost	0.086 € kg ⁻¹
	BM Labor cost	0.103 € kg ⁻¹
	Recalculated biorefinery costs (incl. BM) CAPEX + OPEX	4.39 € kg ⁻¹
	Cultivation costs (Ruiz et al., 2016)	3.40 € kg ⁻¹
	Production costs	7.79 € kg ⁻¹
C (New)	Energy consumption BM (Chapter 3)	0.45 kWh kg _{DW} ⁻¹
	Residence time BM	1.2 min
	# BM units ($V_{chamber}$)	1 (309 L)
	BM Equipment costs ^a	250.9 k€ unit ⁻¹
	Original biorefinery CAPEX + OPEX (excl. BM) (Ruiz et al., 2016)	2.85 € kg ⁻¹
	BM CAPEX + OPEX	0.077 € kg ⁻¹
	BM Energy costs	0.055 € kg ⁻¹
	BM Utilities cost	0.004€ kg ⁻¹
	BM Labor cost	0.009 € kg ⁻¹
	Recalculated biorefinery costs (incl. BM) CAPEX + OPEX	2.92 € kg ⁻¹
	Cultivation costs (Ruiz et al., 2016)	3.40 € kg ⁻¹
	Production costs	6.32 € kg ⁻¹
TF (New)	Energy consumption BM (Chapter 3)	0.45 kWh kg _{DW} ⁻¹
	Residence time BM	1.2 min
	# BM units ($V_{chamber}$)	1 (309 L)
	BM Equipment costs ^a	250.9 k€ unit ⁻¹
	Biorefinery costs (centrifuge after BM + spray drying) (Table 7.B.3)	0.24 € kg ⁻¹
	BM CAPEX + OPEX	0.077 € kg ⁻¹
	BM Energy costs	0.055 € kg ⁻¹
	BM Utilities cost	0.004€ kg ⁻¹
	BM Labor cost	0.009 € kg ⁻¹
	Cultivation costs (Ruiz et al., 2016)	3.40 € kg ⁻¹
	Production costs	3.72 € kg ⁻¹
TF (FP)	Energy consumption BM (Chapter 7)	0.32 kWh kg _{DW} ⁻¹
	Residence time BM	0.8 min
	# BM units ($V_{chamber}$)	1 (217 L)
	BM Equipment costs ^a	210.1 k€ unit ⁻¹
	Biorefinery costs (centrifuge after BM + spray drying) (Table 7.B.3)	0.24 € kg ⁻¹
	BM CAPEX + OPEX	0.058 € kg ⁻¹
	BM Energy costs	0.039 € kg ⁻¹
	BM Utilities cost	0.003 € kg ⁻¹
	BM Labor cost	0.009 € kg ⁻¹
	Cultivation costs (Ruiz et al., 2016)	0.50 € kg ⁻¹
	Production costs	0.80 € kg ⁻¹

^a: "bead mill cost" [$V_{chamber} > 60L$] = $14218 \cdot V_{chamber}^{0.5011}$ (SuperPro Designer v9.0, 2016)

Table 7.B.3 "TF" scenario biorefinery cost breakdown for centrifuge after bead mill (BM) and spray dryers.

Equipment	Cost breakdown	
Centrifuge after BM	Energy consumption	0.04 kWh kg _{dw} ⁻¹
	# units (Q)	1 (9259 L h ⁻¹)
	Equipment costs ^a	1691.3 k€ unit ⁻¹
	Centrifuge CAPEX + OPEX	0.070 € kg⁻¹
	Energy costs	0.004 € kg ⁻¹
	Utilities cost	n/a
	Labor cost	0.009 € kg ⁻¹
Spray dryers	Q _{solids} (cell debris and insoluble proteins/carbohydrates)	3888 L h ⁻¹
	Q _{liquid} (soluble proteins and carbohydrates)	5370 L h ⁻¹
	Water removal	99.55%
	Capacity	29.0 m ³
	Spray dryer I	
	Equipment costs ^b	181 k€
	Spray dryer II	
	Capacity	49.3 m ³
	Equipment costs ^b	187 k€
	Spray dryers CAPEX + OPEX	0.169 € kg⁻¹
	Energy costs	0.014 € kg ⁻¹
	Utilities cost	0.135 € kg ⁻¹
	Labor cost	0.019 € kg ⁻¹
Total	Biorefinery costs CAPEX + OPEX	0.24 € kg⁻¹

^a: "centrifuge cost" [$Q_V: L h^{-1}$] = $18788 \cdot Q_V^{0.4927}$ (SuperPro Designer v9.0, 2016)

^b: "spray dryer cost" [$V_{\text{spray dryer}}: m^3$] = $143.04 \cdot V_{\text{spray dryer}}^{0.0693}$ (SuperPro Designer v9.0, 2016)

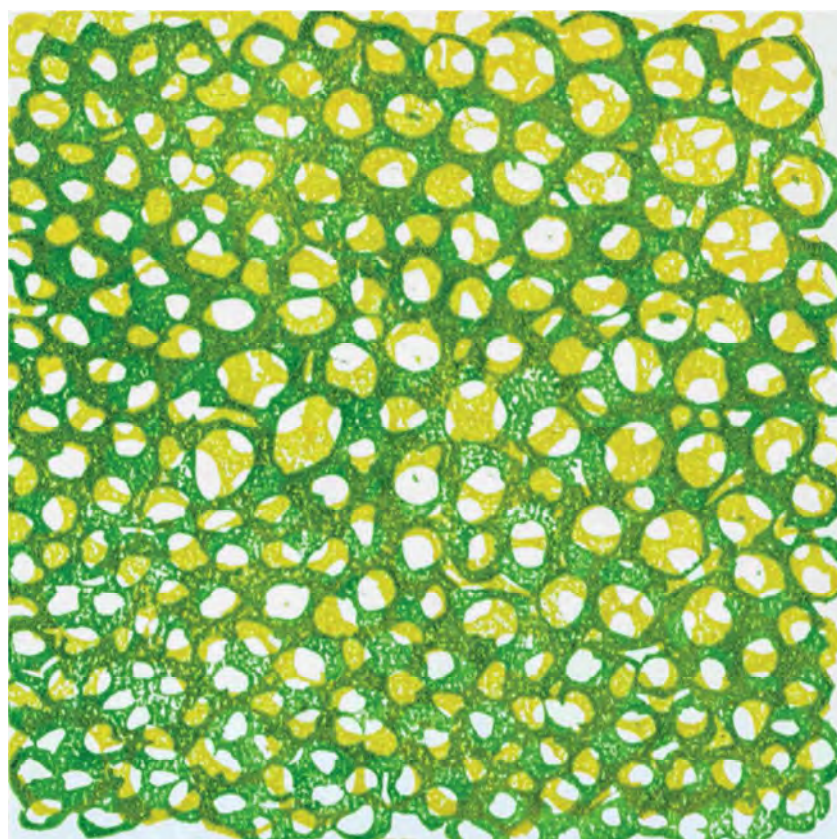
Table 7.B.4 Biomass composition and market value adapted from Ruiz et al. (2016).

Component	Percentage	Market value	
Lipids	20%	n/a	
Proteins	50%		
Soluble	20%	3.3 € kg ^{-1 a}	0.66 €
Insoluble	30%	1.1 € kg ^{-1 a}	0.33 €
Carbohydrates	20%		
Soluble ^b	10%	3.3 € kg ^{-1 a}	0.33 €
Insoluble ^b	10%	1.1 € kg ^{-1 a}	0.075 €
Pigments	3%	n/a	
Ashes	7%	n/a	
Total	10%	1.40 € kg⁻¹	

n/a: not applicable

^a: soluble fraction and insoluble fraction soled as a whole, therefore equal market price assumed

^b: soluble and insoluble carbohydrate fractions based on outcomes of Chapter 3



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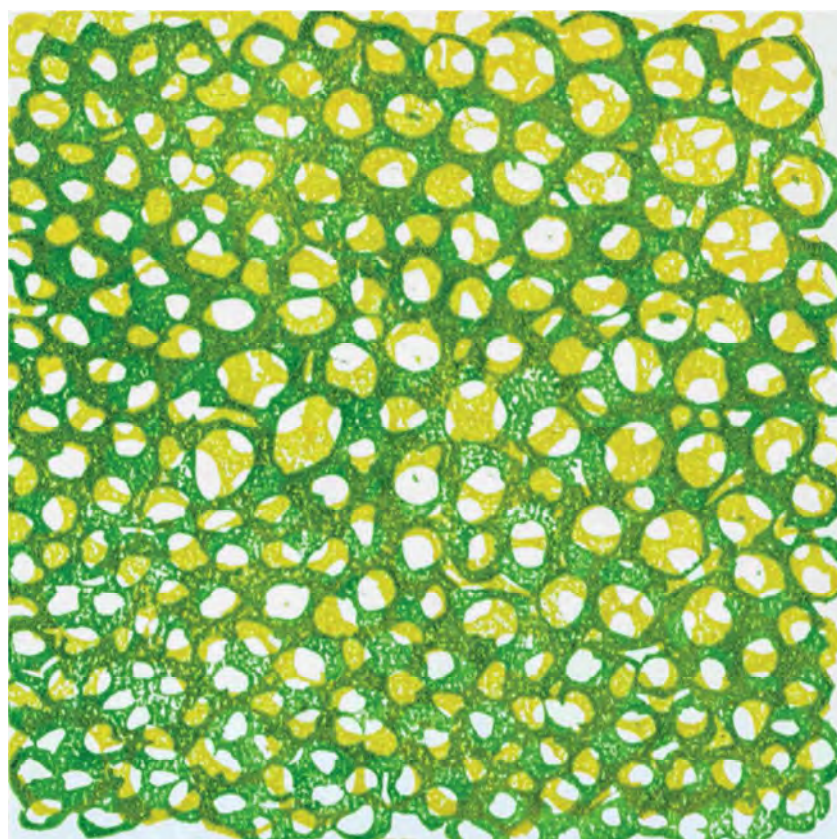
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Summary

The growing world population demands a higher quantity of proteins for food and feed. These proteins should be obtained in a sustainable manner without using more arable land. Both, microalgae and macroalgae (i.e., seaweeds) are known to have a higher productivity than common agricultural crops. Moreover, for cultivation of microalgae and seaweed no arable land is needed. Production and processing of algae and seaweed is still too expensive and for a positive business case costs of production and processing need to be reduced and complete valorization of the biomass is required following a biorefinery concept. Biorefinery comprises all steps after the cultivation, i.e., harvesting, cell disintegration, extraction and fractionation.

In general the algal components are stored intracellularly, either freely in the cytoplasm or in organelles or bound to membranes. Therefore the first step towards the algal products is cell disintegration. It is important to perform this step under mild (i.e., low temperatures, shear etc.) conditions to prevent negative effects on the product quality. The aim of this thesis was to develop a mild, scalable and energy efficient disintegration technology for the extraction of water soluble protein from microalgae and macroalgae. To fulfill this goal, conventional as well as novel cell disintegration technologies were studied and optimized in terms of product yield and energy usage.

For microalgae disintegration, a clear reference in which both energy use and product yield were presented was missing from literature. Therefore in **Chapter 2** a benchmark was set by means of bead milling for the release of water soluble protein from the green microalgae *Chlorella vulgaris*. Nine out of 44 process variables are deemed most influential during bead milling. In this study, two of those major influencing process variables were investigated, being the agitator speed ($6 - 12 \text{ m s}^{-1}$) and the biomass concentration ($25 - 145 \text{ g}_{\text{DW}} \text{ kg}^{-1}$). Under all experimental conditions over 97% cell disintegration was achieved which resulted in a release of 32-48% water soluble protein of the total protein content. Cell disintegration and protein release both followed first order disintegration kinetics. It was observed that the proteins actually released faster than the disintegration appeared to takes place, this was attributed to the occurrence of single cracks in the cells upon bead impact allowing the intracellular content to leak out of the cell. Moreover, a clear optimum protein release rate was observed around an agitator speed of $9 - 10 \text{ m s}^{-1}$, yet the lowest specific energy consumptions were achieved at a speed of 6 m s^{-1} . Also an increased biomass concentration caused a lower specific energy consumption since more units of biomass were disintegrated for the same basal energy expenditure. Overall, protein yields between 32 and 42% were achieved, while the energy consumption was reduced with 85% by selective protein

extraction to values as low as $0.81 \text{ kWh kg}_{\text{DW}}^{-1}$. Remarkably, the benchmark was much better than expected.

In **Chapter 3**, two additional bead milling process variables were investigated, being the bead size (0.3 – 1 mm) and the algal species (*C. vulgaris*, *Neochloris oleoabundans* and *Tetraselmis suecica*). It was hypothesized that smaller beads are more effectively interacting with internal organelles over larger beads, and therefore are better at releasing proteins or carbohydrates from pyrenoids or starch granules, respectively. In addition, to be able to better understand the disintegration mechanism, the so-called stress model was applied. This model describes the comminution process in a bead mill as function of the amount of bead contacts and the force of each impact. For all algae, cell disintegration, protein release and carbohydrate release followed first order disintegration kinetics. However, a clear differentiation could be made between the release rates of proteins and carbohydrates allowing selective protein release. This was most likely caused by the nature of the products where a lot of protein is freely available in the cytoplasm, the majority of carbohydrates are bound to the cell wall or starch granules. The release kinetics could be improved and thereby the specific energy consumption could be reduced to $0.45\text{--}0.47 \text{ kWh kg}_{\text{DW}}^{-1}$ by using 0.3 mm beads for all algae. Moreover, the protein yields remained unaffected. The stress model revealed that the bead milling process was operated close to an optimum for *C. vulgaris* and *N. oleoabundans* around 0.3 – 0.4 mm beads. It also became clear that *T. suecica* was the weaker algal species of the three. Finally, the hypothesis about the smaller bead sizes interacting more effectively with internal organelles was confirmed for *C. vulgaris* and *N. oleoabundans*, since the relative amount of Rubisco (which is generally stored in the pyrenoid) increased when applying smaller beads.

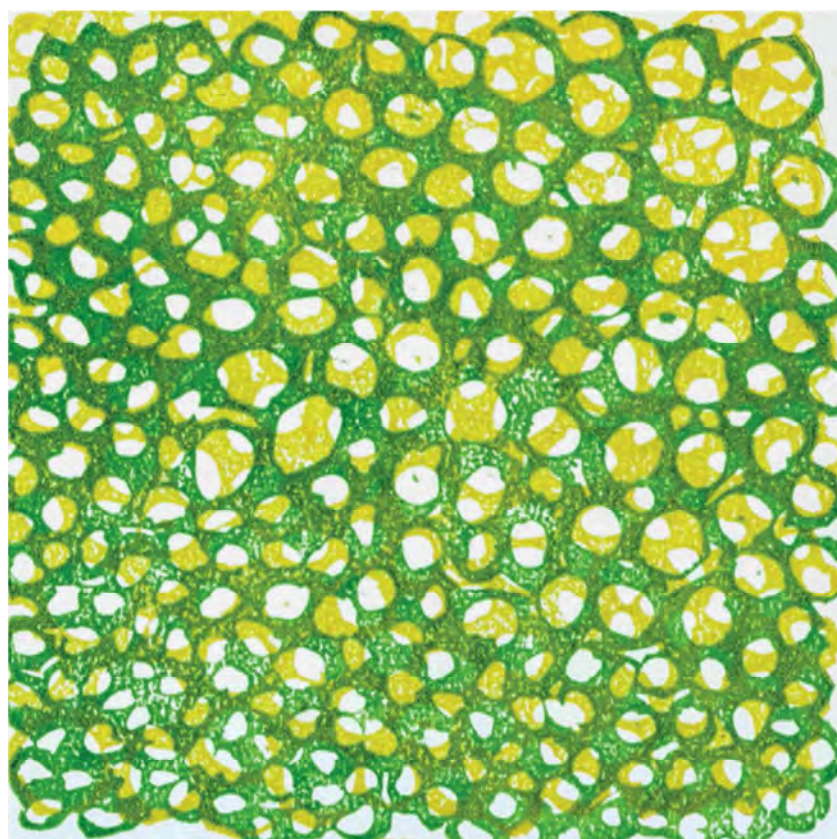
Very little consensus was available in literature about the actual performance of Pulsed Electric Field (PEF) for the extraction of protein from microalgae. Therefore, a screening study over a broad range of operating conditions on the application of PEF for the disintegration of *C. vulgaris* and *N. oleoabundans* was conducted and is presented in **Chapter 4**. The influence of the electric field strength, pulse duration, pulse number, suspension conductivity, cultivation condition and specific energy consumption were investigated. Besides, the effect of batch mode PEF versus continuous flow PEF was investigated. It was found that PEF it is possible to effectively permeabilize the microalgal cells since a sharp increase in the electrical conductivity was observed which shows that ions could be released. With respect to the release of water soluble protein, the first remarkable result was that the pulse duration and pulse number had no independent influence on the PEF outcome while the specific energy consumption did. Secondly, for both algae species the protein yield never exceeded 13% even if an osmotic shock caused cell damage prior to the application of PEF.

This led to the conclusion that PEF is not suitable to release water soluble proteins, not even at specific energy consumptions much higher than those for the benchmark, bead milling.

In **Chapter 5** an attempt was made to improve the performance of PEF by investigating the synergistic effect with the processing temperature. From literature it was known that an increased processing temperature had a positive effect on the inactivation of microorganisms during PEF treatment. The PEF experiments were performed using a pilot scale continuous flow electroporation unit in which the processing temperature was controlled between 25 – 65 °C. Directly after the PEF treatment, at a specific energy consumption of 0.55 or 1.11 kWh kg_{DW}⁻¹, the suspension was cooled to room temperature. The results showed that effective cell permeabilization was obtained during PEF treatment at processing temperatures from 25 – 55 °C. Yet, spontaneous cell lysis occurred at a processing temperature of 65°C without PEF. Carbohydrates could be released up to a yield of 25% with a PEF-Temperature treatment between 25 – 45 °C, while the yield further increased to 39% when the PEF processing temperature was increased to 55 °C. Despite the observation of an apparent trend, no substantial protein yields were obtained (< 5%) for all tested processing conditions. Remarkably some Rubisco was released which still maintained its native activity, showing that PEF is a mild technique at 35 °C. In conclusion, under the tested conditions, the combined PEF-Temperature treatment did not result in substantial disintegration of the algal cells to effectively release water soluble proteins in comparison to the benchmark.

In addition to the microalgae, macroalgae were subject of investigation in the search for new protein sources in **Chapter 6**. The macrostructure of these algae limit the potential disintegration technologies to batch systems. Therefore, four batch technologies were used to disintegrate the green macroalgae *Ulva lactuca*, being; osmotic shock, enzyme incubation, PEF and High Shear Homogenization (HSH). The results showed that mild pre-dried biomass released significantly less water soluble proteins than fresh biomass. Therefore, subsequent experiments were performed with fresh biomass only at a fixed biomass concentration (10 g_{DW} L⁻¹). For osmotic shock the maximum release was obtained at 30 °C during 24 h of incubation with a protein yield of ~20%. Enzyme incubation yielded ~25% protein by applying a pectinase. PEF gave the lowest protein yield of ~12% at an electric field strength of 7.5 kV cm⁻¹, but the specific energy consumption with respect to the released protein was lowest with 6.6 kWh kg_{protein}⁻¹. In contrast, for HSH the protein yield was the highest (~45%) by applying a novel two-phase strategy, but the protein specific energy consumption was between 313 – 318 kWh kg_{protein}⁻¹. It was concluded that HSH is the most effective technique but requires improvement in terms of energy consumption and scalability should be validated.

Finally, in **Chapter 7** the main results and remaining bottlenecks of this thesis were discussed. First, the performance of bead milling for mild disintegration and water soluble protein release was discussed. It was concluded that, to date, bead milling is the only technology able to disintegrate fresh microalgae at specific energy consumptions below 10% of the total energy available from the algae and release substantial amounts of water soluble protein. Yet, a gap of a factor 10^2 - 10^3 exists between the minimum specific energy requirement and the found specific energy requirement for bead milling which gives possibilities for future improvement. Subsequently, the low performance of PEF due to the limiting cell wall was discussed. PEF showed to be able to permeabilize the algal cells, but is not a suitable technology to release water soluble proteins from algae. Furthermore, the implications of selective product release for downstream processing and product development were evaluated and a scale up strategy for bead milling is presented. The effect of the bead milling costs on *complete biomass biorefinery* and *biorefinery for only food and feed proteins* were evaluated. The costs for bead milling before this work were over 1.5 € kg⁻¹ and contributed for 20% to the total production costs. The costs for bead milling are unimportant with the improvements in this study (0.08 € kg⁻¹) and contribute at maximum 2% to the production costs. For a bulk food and feed proteins scenario, complete biorefinery can be omitted which significantly reduces the costs. This economic evaluation also revealed that the cultivation costs are still the main bottleneck for bulk food and feed from microalgae. Therefore, future focus should be on the cultivation as there is a lot to improve.



Samenvatting

De wereldpopulatie groeit en daarom is er een steeds grotere hoeveelheid eiwitten nodig voor voeding. Van belang is ook om deze eiwitten duurzaam te produceren. Een mogelijke oplossing zou de productie van algen kunnen zijn. Zowel microalgen als macroalgen (beter bekend als zeewieren) staan bekend om hun hogere productie van eiwitten dan traditionele landbouwgewassen zoals mais of soja. Bovendien is er voor de ontwikkeling van microalgen en zeewieren geen landbouwgrond nodig. Echter productie en verwerking van microalgen en zeewieren is momenteel nog te duur om economisch interessant te zijn. Met behulp van bioraffinage zouden alle componenten uit de biomassa benut kunnen worden om zo de verwerking rendabel te maken.

Bioraffinage behelst de processtappen na de cultivatie: oogst, celdisruptie (celdesintegratie), extractie en fractionering. De algencomponenten zijn opgeslagen binnenin de cel, vrij in het cytoplasma of in organellen, of gebonden in membranen. De eerste stap tot het verkrijgen van de componenten is daarom de desintegratie van de algencel. Het is belangrijk om deze stap onder zogenaamde milde condities (lage temperaturen, weinig schuifspanning etc.) uit te voeren ter voorkoming van negatieve gevolgen op de productkwaliteit zoals denaturatie van eiwitten.

Het doel van dit proefschrift is derhalve het ontwikkelen van een efficiënte technologie voor celdesintegratie voor de extractie van wateroplosbare-eiwitten uit micro- en macroalgen, die mild, schaalbaar en energiezuinig is. Om dit doel te behalen zijn zowel conventionele (b.v. parelmolen) als nieuwe (b.v. Pulsed Electric Field) desintegratietechnologieën bestudeerd en geoptimaliseerd ten aanzien van de productopbrengst en het energieverbruik.

Een duidelijke referentie voor energieverbruik en productopbrengst ontbrak in de literatuur voor de desintegratie van microalgen. In **Hoofdstuk 2** is, gebruikmakend van een roerwerk-kogelmolen (parelmolen), een ijkpunt bepaald voor de extractie van wateroplosbare-eiwitten uit de groene microalg *Chlorella vulgaris*. Slechts negen van de in totaal 44 parelmolen-procesvariabelen werden invloedrijk bevonden. In deze studie zijn twee van deze invloedrijke variabelen onderzocht, namelijk de roersnelheid ($6 - 12 \text{ m s}^{-1}$) en de biomassaconcentratie ($25 - 145 \text{ g}_{\text{DM}} \text{ kg}^{-1}$). Onder alle experimentele condities is meer dan 97% celdesintegratie behaald, waardoor 32 – 48% van de totale hoeveelheid eiwitten vrijkwamen. Het viel op dat de eiwitten sneller vrijkwamen dan de desintegratie van de cellen visueel kon worden waargenomen met een microscoop. Dit is toe te schrijven aan het voorkomen van scheuren in de cellen na botsing met een parel, waardoor de celinhoud naar buiten kon lekken. Er werd een duidelijk optimum waargenomen voor de eiwitvrijlating bij een roersnelheid van $9 - 10 \text{ m s}^{-1}$, het laagste energieverbruik werd echter behaald bij

6 m s⁻¹. Ook het gebruik van een hogere biomassaconcentratie leidt tot een lager energieverbruik, omdat meer eenheden massa werden gedesintegreerd met hetzelfde energiegebruik. Een eiwitopbrengst van tussen de 32% en 48% is behaald, waarbij het energieverbruik met 85% is gereduceerd, door middel van selectieve eiwitextractie, tot waarden zo laag als 0.81 kWh kg_{DM}⁻¹. Opmerkelijk is het dat hiermee het ijkpunt veel beter, lager energiegebruik en hoge eiwitopbrengst, werd dan verwacht.

In **Hoofdstuk 3** zijn twee extra parelmolen procesvariabelen onderzocht, namelijk de parelgrootte (0.3 – 1 mm) en de algensoort (*C. vulgaris*, *Neochloris oleoabundans* en *Tetraselmis suecica*). De hypothese was dat kleinere parels beter in staat zijn tot effectieve interactie met de interne organellen dan grotere parels. Hierdoor zouden ze beter eiwitten en suikers vrij kunnen maken uit organellen zoals pyrenoiden (eiwitkorrels) of zetmeelkorrels. Daarnaast is het zogenoemde stress-model toegepast om een beter begrip te krijgen van de celdesintegratie mechanismen. Dit model beschrijft het maalproces in een parelmolen als functie van de totale hoeveelheid parelcontact en de kracht van iedere botsing. Voor elk van de drie algen werden de celdesintegratie, eiwit- en suiker-vrijlating beschreven. Er kon een duidelijk onderscheid worden gemaakt tussen snelheid van de eiwit- en suiker-vrijlating. Hierdoor was selectievere vrijlating van eiwitten mogelijk. Dit is zeer waarschijnlijk veroorzaakt door de aard van de componenten. Een groot deel van de eiwitten is vrij beschikbaar in het cytoplasma terwijl het overgrote deel van de suikers zich in de celwand of de zetmeelkorrels bevindt. Door het gebruik van 0.3 mm grote parels kon het energieverbruik verder worden verlaagd tot 0.45 – 0.47 kWh kg_{DM}⁻¹ voor elk van de algen. Bovendien bleef de eiwitopbrengst gelijk met het gebruik van deze kleinere parels. Het stress-model heeft laten zien dat de desintegratie van *C. vulgaris* en *N. oleoabundans* bij het gebruik van 0.3 – 0.4 mm parels rond het optimum zit. Ook werd duidelijk dat *T. suecica* de zwakkere alg is van de drie en dat er geen onderscheid te zien is tussen de verschillende groottes van de parels tijdens celdesintegratie. Tenslotte kon de hypothese dat kleinere parels efficiënter zijn tot interactie worden bevestigd voor *C. vulgaris* en *N. oleoabundans*. De fractie Rubisco (wat normaal vaak in het pyrenoïd zit) kon namelijk worden vergroot door middel van het gebruik van kleinere parels.

Zoals hierboven beschreven is er naast de conventionele parelmolen technologie ook een nieuwe technologie onderzocht, namelijk pulserend elektrische velden (*Pulsed Electric Field*, PEF). In de literatuur kon weinig overeenstemming worden gevonden over de werkelijke prestaties van PEF voor de extractie van eiwitten uit microalgen. Daarom bevat **Hoofdstuk 4** een screeningstudie over een breed scala van experimentele condities voor de toepassing van PEF om *C. vulgaris* en *N. oleoabundans* te desintegreren. De invloed van de elektrische veldsterkte, pulsduur, pulsaantal, suspensiegeleidbaarheid, cultivatiecondities en

energieverbruik zijn onderzocht. Bovendien is de invloed van *batch* of continue stroom verwerking onderzocht. De resultaten lieten zien dat het met PEF mogelijk is om de algencellen doorlaatbaar te maken. Dit is waargenomen door een sterkte toename van de geleidbaarheid van de suspensie na PEF, wat laat zien dat ionen zijn vrijgekomen. Bij de wateroplosbare eiwitten werd als eerste duidelijk dat de pulsduur en het aantal pulsen geen onafhankelijke invloed hadden, terwijl het energieverbruik dat wel had. Voor beide algen was de totale eiwitopbrengst echter nooit hoger dan 13%, zelfs niet met het extra effect van een osmotische schok voor de PEF-behandeling. Dit heeft tot de conclusie geleid dat PEF geen geschikte techniek is om wateroplosbare eiwitten vrij te maken uit microalgen, zelfs niet bij een energieverbruik vele malen hoger dan gebruikt voor de parelmolen.

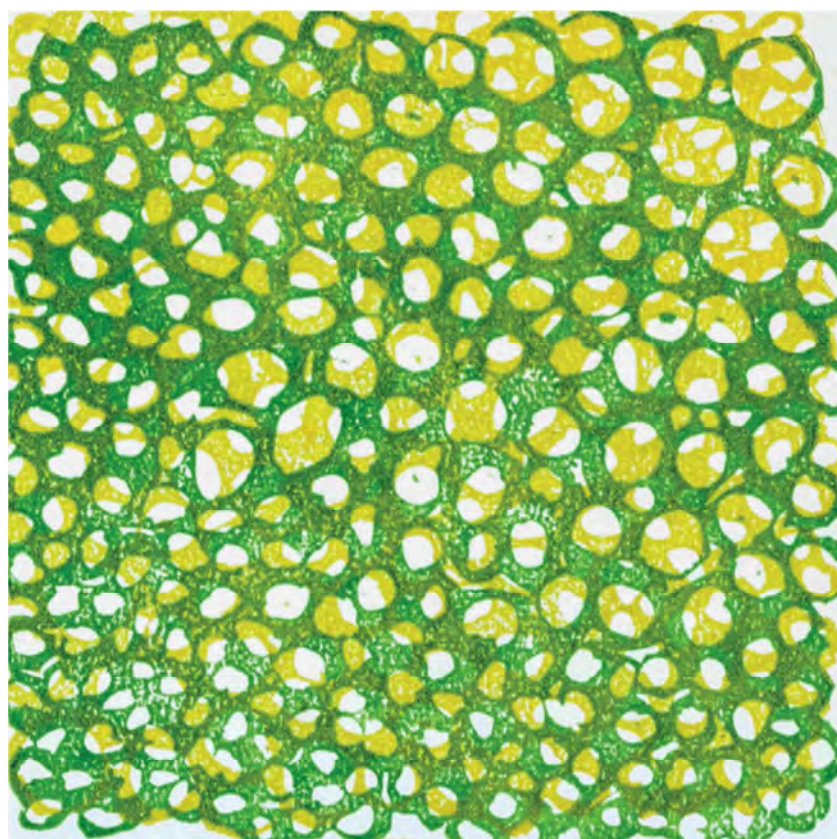
In **Hoofdstuk 5** is getracht om de resultaten van PEF te verbeteren door het synergistische effect van de procestemperatuur te onderzoeken. Uit de literatuur was het al bekend dat een hogere procestemperatuur een positief effect had op de inactivatie van micro-organismen gedurende een PEF-behandeling. De PEF-experimenten in deze studie zijn gedaan met een pilot-schaal elektroporatie-unit waarin de procestemperatuur werd geregeld tussen de 25 en 65 °C. Direct na de PEF-behandeling (met een energieverbruik van 0.55 of 1.11 kWh kg_{DM}⁻¹) werd de algensuspensie gekoeld tot kamertemperatuur. De resultaten lieten zien dat de algen effectief doorlaatbaar zijn geworden door PEF bij een procestemperatuur van 25 – 55 °C. Echter bij 65 °C gingen de cellen spontaan kapot zonder PEF-behandeling. Tussen 25 – 45 °C werd een suikers-opbrengst van 25% behaald door middel van de PEF-temperatuurbehandeling, terwijl de opbrengst naar 39% ging bij een temperatuur van 55 °C. Ondanks de ogenschijnlijke trend was de eiwit-opbrengst niet substantieel (< 5%) voor alle geteste condities. Opmerkelijk genoeg kon er toch actief Rubisco worden vrijgemaakt wat aantoonde dat PEF een milde techniek zou zijn bij 35 °C. Onder de geteste condities heeft de PEF-temperatuurbehandeling niet tot een substantiële desintegratie geleid van de algencellen om effectief wateroplosbare eiwitten vrij te maken in vergelijking met de parelmolen.

In de zoektocht naar een nieuwe eiwitbron zijn in dit proefschrift naast de microalgen ook de macroalgen onderwerp van onderzoek (**Hoofdstuk 6**). De macrostructuur van deze algen beperkt de potentiële desintegratietechnieken in eerste instantie tot *batch*-systemen. Daarom zijn in deze studie vier *batch*-technologieën bestudeerd om de groene macroalg *Ulva lactuca* ("zee-sla") te desintegreren, namelijk; osmotische schok, enzymatische afbraak, PEF en *High Shear* Homogenisatie (HSH). Eerste resultaten lieten zien dat mild gedroogde biomassa significant minder eiwit vrij liet dan het geval is met verse biomassa. Daarom is de rest van het onderzoek alleen verricht met vers geoogste biomassa. Met osmotische schok kon de maximale eiwitopbrengst van ~20% worden bereikt na 24 h incubatie bij 30 °C. Voor

enzymatische afbraak is pectinase het meest geschikt wat een eiwitopbrengst van ~25% geeft. PEF gaf de laagste eiwitopbrengst (~12%). De eiwitopbrengst van HSH was het hoogst (~45%) met een nieuwe twee-staps strategie, maar het energieverbruik ten opzichte van de eiwitopbrengst was $313 - 318 \text{ kWh kg}_{\text{eiwit}}^{-1}$. Uit deze studie kon worden geconcludeerd dat HSH de meest effectieve techniek is voor het vrijmaken van eiwitten uit macroalgen, maar dat het energieverbruik verbetering vereist en daarnaast de schaalbaarheid moet worden onderzocht.

Tot slot zijn in **Hoofdstuk 7** de voornaamste resultaten en knelpunten uit dit proefschrift nader beschouwd. Allereerst zijn de prestaties van de parelmolen voor milde desintegratie en eiwitopbrengst beschreven. De conclusie is dat een parelmolen tot op heden de enige techniek is die verse microalgen kan desintegreren bij een energieverbruik lager dan 10% van de energiedichtheid van de alg zelf en daarnaast een substantiële eiwitopbrengst heeft. Er bestaat echter nog steeds een gat met een factor $10^2 - 10^3$ tussen het minimaal benodigde energieverbruik en het werkelijke energieverbruik, wat nog ruimte geeft voor verbetering. Vervolgens zijn de prestaties van PEF beschreven met betrekking tot de limiterende celwand. Met PEF kunnen de algencellen worden geperforeerd, maar het is op dit moment geen geschikte techniek om wateroplosbare eiwitten vrij te maken uit algen.

In dit hoofdstuk worden ook de gevolgen van selectieve productextractie op de productverwerking en -ontwikkeling besproken. Tevens wordt een opschalingsstrategie voor de parelmolen gepresenteerd. Als laatste is een economische evaluatie gemaakt van het effect van de parelmolen kosten op *complete bioraffinage* en *bioraffinage voor alleen eiwit*. De kosten voor het gebruik van de parelmolen waren voor dit proefschrift ruim 1.5 € kg^{-1} en maakten voor 20% deel uit van de totale productiekosten. De kosten voor het parelmolengebruik met de verbeteringen in dit proefschrift zijn gemarginaliseerd (0.08 € kg^{-1}) en maken nog maximaal 2% deel uit van de totale kosten. Bij bulk levensmiddelen en diervoeder eiwit scenario's kan complete bioraffinage worden vermeden, wat de kosten significant verminderd. De economische evaluatie laat tevens zien dat de ontwikkelings(cultivatatie)kosten van algen het voornaamste knelpunt zijn. Vervolgonderzoek zou daarom gericht moeten zijn op de kosten van cultivatie.



Gearfetting

De wrâldbefolking groeit en dêrom is hieltyd mear aaiwynt nedich foar iten. Fan belang is ek dat aaiwynt duorsum te produsearjen. In mooglike oplossing soe de produksje fan algen wêze kinne. Sawol mikro-algen as makro-algen (better bekend as seewieren) steane bekend om har hegere produksje fan aaiwynt as tradisjonele lânbougewaaksen sa as mais en soja. Boppedat is foar de ûntwikkeling fan mikro-algen en seewieren gjin lânbougrûn nedich. Produksje en ferwurking fan mikro-algen en seewieren is lykwols op dit momint noch te djoer om ekonomysk ynteressant te wêzen. Mar mei help van bioraffinaazje soene alle komponinten fan de biomassa brûkt wurde kinne om sa de ferwurking rendabel te meitsjen.

Bioraffinaazje omfiemet de prosesstappen nei de produksje fan de algen: rispje, seldisruptsje (seldesyntegraasje), ekstraksje en fierdere suvering. De algekomponinten binne opslein binnenyn de sel, frij yn it sytoplasma of yn organellen, of bûn yn membranen. De earste stap ta it krijen fan de komponinten is dêrom de desyntegraasje fan de algesel. It is belangryk om dizze stap ûnder saneamde mylde kondysjes (lege temperatueren, lege skospanning ('*schuifspanning*') etc.) út te fieren mei as doel it tefoaren kommen fan negative gefolgen foar de produktkwaliteit, lykas denaturaasje fan aaiwiten.

It doel fan dit proefskrift is dus it ûntwikkeljen fan in effisjinte technology foar seldesyntegraasje foar de ekstraksje fan yn wetter oplosber aaiwynt út mikro- en makro-algen, dy't myld en enerzjysunich is en op gruttere skaal tapasber ('*schaalbaar*'). Om dat doel te berikken binne sawol konvinsjonele (bygelyks pearelmûne) as nije (bygelyks Pulsed Electric Field) desyntegraasjetechnologyen bestudearre en optimalisearre oangeande de produktopbringst en it enerzjyferbrûk.

In dúdlike referinsje foar enerzjyferbrûk en produktopbringst ûntbriek yn de literatuer foar de desyntegraasje fan mikro-algen. Yn **Haadstik 2** is, gebrûk meitsjend fan in rearwurkkûgelmûne (pearelmûne '*parelmolen*'), in itigingspunt ('*ijkpunt, norm*') set foar de ekstraksje fan yn wetter oplosber aaiwynt út de griene mikro-alg *Chlorella vulgaris*. Fan net mear as njoggen fan de yn totaal 44 pearelmûneprosesfariabelen koe konstataarre wurde dat se ynvloedryk wiene. Yn dizze stúdzje binne twa fan dy ynfloedrike fariabelen ûndersocht, nammentlik de rearsnelheid ($6 - 12 \text{ m s}^{-1}$) en de biomassakonsintraasje ($25 - 145 \text{ g}_{\text{DM}} \text{ kg}^{-1}$). Under alle eksperimintele kondysjes is mear as 97% seldesyntegraasje behelle, dêr't 32 - 48% fan it totale aaiwynt by frijkaam. It foel op, dat it aaiwynt hurder frijkaam asdat de desyntegraasje fan de sellen fisueel ûnderskieden wurde koe mei in mikroskoop. Dat is ta te skriuwen oan it foarkommen fan skuorren yn de sellen nei yn botsing mei in pearle, wêrtroch't de selynhâld nei bûten lekke koe. Der waard in dúdlik optimum foar de aaiwytfrijlitting waarnommen by in rearsnelheid fan $9 - 10 \text{ m s}^{-1}$; it leechste enerzjyferbrûk waard lykwols behelle by 6 m s^{-1} . Ek it brûken fan in hegere biomassakonsintraasje liedt ta in

leger enerzjyferbrûk, om't mear ienheden massa desyntegrearre waarden mei itselde enerzjyferbrûk. In aawytopbringst fan tusken de 32% en 48% is behelle (wêrby't it enerzjyferbrûk mei 85% redusearre is) troch middel fan selektive aaiwytekstraksje, oant wearden sa leech as $0.81 \text{ kg}_{\text{DM}} \text{ kWh}^{-1}$. Opmerklik is dat hjirmei de nije noarm folle better, it enerzjyferbrûk leger en de aaiwitopbringst heger wiene as ferwachte.

Yn **Haadstik 3** binne twa ekstra prosesfariabelen fan de pearelmûne ûndersocht, nammentlik de pearलगrutte (0.3 – 1 mm) en de algesoarte (*C. vulgaris*, *Neochloris oleoabundans* en *Tetraselmis suecica*). De hypoteze wie dat lytsere pearles better by steat binne om effektive ynteraksje te hawwen mei de ynterne organellen as gruttere pearles. Dêrtroch soene sy better aaiwyt en sûkers frijmeitsje kinne út organellen sa as pyrenoïden (aaiwytkerrels) of setmoalkerrels. Boppedat is it saneamde stressmodel tapast om in better begryp te krijen fan de meganismen fan seldesyntegraasje. Dat model beskriuwt it mealproses yn in pearelmûne as funksje fan it totale pearlekontakt en de krêft fan elke botsing. Foar elk fan de trije algen waarden de seldesyntegraasje en de aaiwyt- en sûkerfrijlitting beskreaun. Der koe in dúdlik ûnderskie makke wurde tusken snelheid fan de aaiwyt- en sûkerfrijlitting. Dêrtroch wie selectivere frijlitting fan aaiwyt mooglik. Dat is heechstwierskynlik feroarsake troch de aard fan de komponinten. In grut part fan it aaiwyt is frij beskikber yn it sytoplasma, wylst fierwei it grutste part fan de sûkers yn 'e selwand of de setmoalkerlen sit. Troch it brûken fan 0.3 mm grutte pearles koe it enerzjyferbrûk fierder ferlege wurde ta 0.45 – 0.47 $\text{kWh kg}_{\text{DM}}^{-1}$ foar elk fan de algen. Boppedat bleau de aaiwytopbringst mei it brûken fan dy lytsere pearles gelyk. It stressmodel hat sjen litten dat de desyntegraasje fan *C. vulgaris* en *N. oleoabundans* by it brûken fan 0.3 – 0.4 mm pearles om it optimum hinne sit. Ek waard dúdlik dat *T. suecica* de swakkere alg is fan de trije en dat der gjin ûnderskied te sjen is tusken de ûnderskate ôfmjittings fan de pearles yn de seldesyntegraasje. By einsluten koe de hypoteze dat lytsere pearles effisjinter binne foar ynteraksje befêstige wurde foar *C. vulgaris* en *N. oleoabundans*. De fraksje Rubisco (dat normaal faak yn it pyrenoïd sit) koe nammentlik fergrutte wurde troch it brûken fan lytsere pearles.

Lykas hjirboppe beskreaun, is neist de konvinsjonele pearelmûnetechнологy ek in nije technology ûndersocht, nammentlik pulsearjende elektryske fjilden (*Pulsed Electric Field*, PEF). Yn de literatuer wie net folle oerienstimming te finen oer de werklike prestaasjes fan PEF foar de ekstraksje fan aaiwyt út mikro-algen. **Haadstik 4** befettet dêrom in screeningstúdzje oer in breed skala fan eksperimintele kondysjes foar it tapassen fan PEF om *C. vulgaris* en *N. oleoabundans* te desyntegrearjen. De ynfloed fan de elektryske fjildsterkte, pulsdooer, pulsoantal, elektrysk liedfermogen ('geleidbaarheid') fan de suspinsje, algeproduksjekondysjes en enerzjyferbrûk binne ûndersocht. Boppedat is de ynfloed fan 'batch' of kontinue stroomferwurking ûndersocht troch ferskillende PEF-apparaten te

betsjinjen. De resultaten lieten sjen dat it mei PEF mooglik is om de algesellen trochlitber te meitsjen. Dat is waarnommen troch in grutte ferheging fan it liedfermogen fan de suspinsje nei PEF, wat sjen lit dat ioanen frijkaam binne. By yn wetter oplosber aaiwyt waard as earste dúdlik dat de pulsdouer en it tal pulsen gjin ûnôfhinklike ynfloed hiene, wylst it enerzjyferbrûk dat wol hie. Foar beide algen wie de totale aaiwytopbringst lykwols nooit heger as 13%, sels net mei it ekstra effekt fan in osmotyske skok foar de PEF-behandeling. Dat hat laat ta de konklúzje dat PEF gjin gaadlike technyk is om yn wetter oplosber aaiwyt frij te meitsjen út mikro-algen, sels net by in enerzjyferbrûk hiel wat kearen heger as brûkt foar de pearelmûne.

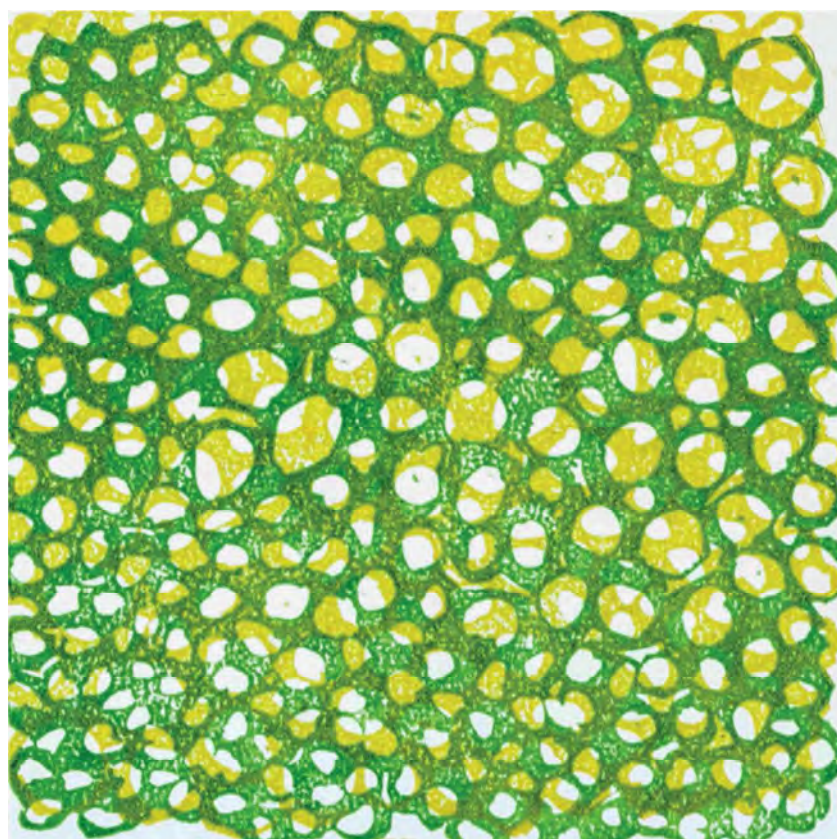
Yn **Haadstik 5** is besocht om de resultaten fan PEF te ferbetterjen troch it synergistyske effekt fan de prosestemperatuer te ûndersykjen. Ut de literatuer wie al bekend dat in hegere prosestemperatuer in posityf effekt hat op de ynaktivaasje fan mikro-organismen yn in PEF-behandeling. De PEF-eksperimenten yn dizze stúdzje binne dien mei in elektroporaasje-unit op pilotskaal dêr't de prosestemperatuer yn regele waard tusken de 25 en 65 °C. Fuort nei de PEF-behandeling (mei in enerzjyferbrûk fan 0.55 of 1.11 kWh kg_{DM}⁻¹) waard de algesuspinsje kuolle nei keamertemperatuer. De resultaten lieten sjen dat de algen effektyf trochlitber wurden binne troch PEF by in prosestemperatuer fan 25 – 55 °C. Lykwols, by 65 °C giene de sellen spontaan stikken sûnder PEF-behandeling. Tusken 25 – 45 °C waard in sûkeropbringst fan 25% helle troch middel fan de PEF-temperatuerbehandeling, wylst de opbringst nei 39% gyng by in temperatuer fan 55 °C. Hoewol't de trend sa te sjen oars wie, wie de aaiwytopbringst net substansjeel (< 5%) foar alle kondysjes dy't test binne. Opmerklik genôch koe der dochs aktyf Rubisco frijmakke wurde, wat oantoande dat PEF in mylde technyk wêze soe by 35 °C. Under de teste kondysjes hat de PEF-temperatuerbehandeling net laat ta in substansjele desyntegraasje fan de algesellen om effektyf yn wetter oplosber aaiwyt frij te meitsjen yn ferliking mei de pearelmûne.

Yn de syktocht nei in nije aaiwytboarne binne yn dit proefskrift njonken de mikro-algen ek de makro-algen ûnderwerp fan ûndersyk (**Haadstik 6**). De makrostruktuer fan dy algen beheint de potinsjele desyntegraasjetechniken yn earste ynstânsje ta batchsystemen. Dêrom binne yn dizze stúdzje fjouwer batchtechnologyen bestudearre om de griene makro-alg *Ulva lactuca* 'see-slaad' te desyntegrearjen, nammentlik osmotyske skok, enzymatyske ôfbraak, PEF en 'High Shear Homogenisaasje' (HSH). Earste resultaten lieten sjen dat myld drûge biomassa signifikant minder aaiwyt frijliet asdat it gefal is mei farske biomassa. Dêrom is de rest fan it ûndersyk allinne dien mei farsk rispe biomassa. Mei osmotyske skok koe de maksimale aaiwytopbringst fan ~20% berikt wurde nei 24 h inkubaasje by 30°C. Foar enzymatyske ôfbraak is pektinaze it meast geskikt, wat in aaiwytopbringst fan ~25% jout. PEF joech de leechste aaiwytopbringst (~12%). De aaiwytopbringst fan HSH wie it heechst (~45%)

mei in nije twastappe-stratezjy, mar it enerzjyferbrûk yn ferliking ta de aaiwytopbringst wie 313 – 318 kWh kg_{aaiwyt}⁻¹. Ut dizze stúdzje koe konkludearre wurde dat HSH de meast effektive technyk is foar it frijmeitsjen fan aaiwiten út makro-algen, mar dat ferbettering fan it enerzjyferbrûk nedich is en dat it effekt by skaalfergrutting fierder ûndersocht wurde moat.

As lêste binne yn **Haadstik 7** de wichtichste resultaten en knyp punten út dit proefskrift fierder besjoen. Yn it foarste plak binne de prestaasjes fan de pearelmûne foar mylde desyntegraasje en aaiwytopbringst beskreaun. De konklúzje is dat in pearelmûne oant hjoed de dei ta de iennichste technyk is dy't farske mikro-algen desyntegrearje kin by in enerzjyferbrûk leger as 10% fan de enerzjyynhâld fan de alg sels, en dêrneist in substansjele aaiwytopbringst hat. Der sit lykwols noch altyd in gat mei in faktor $10^2 - 10^3$ tusken it minimaal nedige enerzjyferbrûk en it werklike enerzjyferbrûk, wat noch romte jout foar ferbettering. Dêrnei binne de prestaasjes fan PEF beskreaun yn relaasje ta de limitearjende selwand. Mei PEF kinne de algesellen perforearre wurde, mar dat is op dit stuit gjin gaadlike technyk om yn wetter oplosber aaiwyt frij te meitsjen út algen.

Yn dit haadstik wurde ek de gefolgen fan selektive produktekstraksje op de produktferwurking en -ûntwikkeling besprutsen. Tagelyk wurdt in opskalingsstratezjy foar de pearelmûne presintearre. As lêste is in ekonomyske evaluaasje makke fan it effekt fan de pearelmûnekosten op folsleine bioraffinaazje en bioraffinaazje foar allinnich aaiwyt. De kosten foar it brûken fan de pearelmûne wiene foar dit proefskrift goed 1.5 € kg⁻¹ en makken foar 20% diel út fan de totale produksjekosten. De kosten foar it brûken fan de pearelmûne mei de ferbetteringen yn dit proefskrift binne marginalisearre (0.08 € kg⁻¹) en meitsje noch op syn heechst 2% diel út fan de totale kosten. By senario's foar bulk libbensmiddels en bistefoer kin folsleine bioraffinaazje mijd wurde, wat de kosten signifikant ferminderet. De ekonomyske evaluaasje lit ek sjen dat de ûntwikkelings(produksje)kosten fan algen it foarnaamste knyp punt binne. Fierder ûndersyk soe dêrom rjochte wêze moatte op de kosten fan algekultivaasje.



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- Richard -

About the author

Richard Postma was born on 12 January 1989 in Leeuwarden, The Netherlands. He followed his secondary education at Piter Jelles Montessori in Leeuwarden where he obtained his VWO diploma (major *Natuur en Techniek + Natuur en Gezondheid*) in 2007. Afterwards he started his BASc Biotechnology at Van Hall Larenstein in Leeuwarden. He did his internship and graduation (2010) at MSD in Oss about cultivation and purification of recombinant enzymes from *Escherichia coli*. In 2010 he started his MSc study biotechnology with a specialization



in process engineering. Richard did a MSc thesis about cultivation and modeling of microalgae in a flat-plate-photobioreactor at the department of bioprocess engineering. He finalized his MSc in 2012 with an internship at Synthon Biopharmaceuticals B.V. in Nijmegen. The topic was about the implementation of single-use TFF cassettes for ultrafiltration and diafiltration in a monoclonal antibody process.

In October 2012, he started his PhD research on microalgae biorefinery as part of the Wageningen University IPOP Proteins from Green Sources. He investigated the disintegration of green microalgae and macroalgae to release water soluble proteins. As part of the research, Richard made an international collaboration with the University of Salerno in the framework of a COST action (td 1104). The results of his PhD Research are described in this thesis.

In November 2016, Richard started working for CNC Grondstoffen B.V. in Milsbeek as process technologist. CNC produces and delivers substrates for the mushroom industry. In his role he is involved in developing new projects, process optimization, conducting feasibility studies and monitoring and analyzing production processes internally and externally at mushroom farmers.

List of publications

P.R. Postma, T.L. Miron, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink (2015). Mild disintegration of the microalgae *Chlorella vulgaris* using bead milling. *Bioresource Technology* (184) 297-304

P.R. Postma*, E. Suarez-Garcia*, C. Safi, K. Yonathan, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink (*submitted*). Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates

G.P. 't Lam*, **P.R. Postma***, D.A. Fernandes, R.A.H. Timmermans, M.H. Vermuë, M.J. Barbosa, M.H.M. Eppink, R.H. Wijffels, G. Olivieri (*submitted*). Pulsed Electric Field for protein release of the microalgae *Chlorella vulgaris* and *Neochloris oleoabundans*

P.R. Postma, G. Pataro, M.M. Capitoli, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, G. Olivieri, G. Ferrari (2016). Selective extraction of intracellular components from the microalga *Chlorella vulgaris* by combined pulsed electric field-temperature treatment. *Bioresource Technology* (203) 80-88

P.R. Postma, O. Cerezo-Chinarro, R.J. Akkerman, G. Olivieri, R.H. Wijffels, W.A. Brandenburg, M.H.M. Eppink (*submitted*). Biorefinery of the macroalgae *Ulva lactuca*: extraction of proteins and carbohydrates by mild disintegration

P.R. Postma*, G.P. 't Lam*, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, G. Olivieri. (2016). Microalgal biorefinery for bulk and high-value products: Product extraction within cell disintegration in: D. Miklavcic (Ed.) *Handbook of Electroporation*, Springer

W. Blanken, **P.R. Postma**, L. de Winter, R.H. Wijffels, M. Janssen (2016). Predicting microalgal growth. *Algal Research* (14) 28-38

T.L. Miron, **P.R. Postma**, G. Bosch, M.H.M. Eppink (*submitted*). Preliminary evaluation of aqueous protein extraction from black soldier fly larvae (*Hermetia illucens* L.)

* Both authors contributed equally

Overview of completed training activities

Discipline specific activities

Courses

Biorefinery Training School	2012
Advanced Course Downstream Processing	2013
Industrial Proteins ¹	2013
Microalgae Process Design	2013
Training WAB (Switzerland)	2013
COST Action td1104 Salerno (Italy)	2014

Conferences

BFF2013, Wageningen, The Netherlands ¹	2013
ESBES-IFBIOP, Lille, France ¹	2014
World Congress Electroporation, Portorož, Slovenia ²	2015
Netherlands Biotechnology Conference, Wageningen, The Netherlands ²	2016
RRB-12, Ghent, Belgium ^{1,2}	2016

General courses

VLAG PhD Week, Baarlo	2012
PhD Competence Assessment	2012
Project and Time Management	2013
Teaching and Supervising thesis students	2013
Basic statistics	2014
PhD Workshop carousel	2014
Career Perspectives	2016

Optionals

Brainstorm/PhD-student days, Bioprocess Engineering group ²	2012-2016
IPOP meetings, Wageningen ²	2012-2016
PhD Excursion, Portugal ^{1,2}	2015

Teaching

MSc course Transfer Processes	2012-2015
Invited lecture Microalgae Biorefinery, Wageningen ²	2016

¹ Poster presentation

² Oral presentation

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