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# Combined bead milling and enzymatic hydrolysis for efficient fractionation of lipids, proteins, and carbohydrates of *Chlorella vulgaris* microalgae

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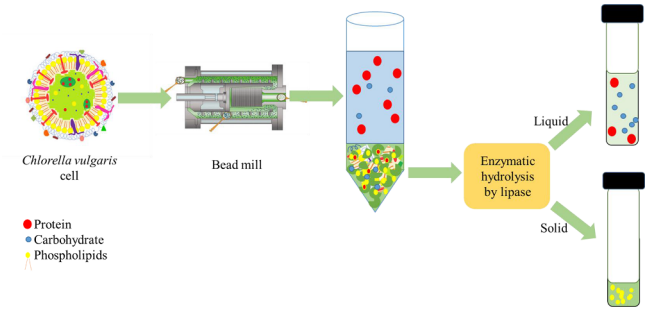
## GRAPHICAL ABSTRACT

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## ABSTRACT

A combined bead milling and enzymatic hydrolysis process was developed for fractionation of the major valuable biomass components, i.e., proteins, carbohydrates, and lipids from the microalgae *Chlorella vulgaris*. The cells were treated by bead milling followed by hydrolysis with different hydrolytic enzymes, including lipase, phospholipase, protease, and cellulase. Without enzymatic hydrolysis, the recovery yield of lipids, carbohydrates, and proteins for bead milled biomass was 75%, 31%, and 40%, respectively, while by applying enzymatic treatments these results were improved significantly. The maximum recovery yield for all components was obtained after enzymatic hydrolysis of bead milled biomass by lipase at 37 °C and pH 7.4 for 24 h, yielding 88% lipids in the solid phase while 74% carbohydrate and 68% protein were separated in the liquid phase. The recovery yield of components after enzymatic hydrolysis of biomass without bead milling was 44% lower than that of the milled biomass.

## 1. Introduction

Microalgae have received increasing attention as novel bio-based crops, because of the high productivity of proteins and lipids ('t Lam et al., 2018). Sustainability will further increase if all microalgae components are valorized in an optimal way (William and Laurens, 2010). Complete fractionation and valorization of the microalgal biomass can be performed in a multi-product biorefinery concept (Eppink

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et al., 2019). In a multi-product biorefinery, all major components (lipid, pigments, carbohydrates, and proteins) need to be fractionated instead of focusing on obtaining one specific component. To achieve valuable intracellular ingredients, it is necessary to pass the barriers such as the cell wall and cell membrane (Safi et al., 2015). There are three different cell disruption methods to release the intracellular compounds: 1) (bio) chemical cell disruption (enzymes or alkali/ thermal treatment); 2) mechanical cell disruption (bead milling and high-pressure homogenization); 3) physical cell disruption (pulse electric field or ultrasonic treatment) (Günerken et al., 2015; Postma et al., 2015).

The selection of the disruption method depends on the thickness of the cell wall and product localization in the cytoplasm (Eppink et al., 2019). Additionally, for maintaining the native structure of individual cell components, mild conditions of cell disruption are necessary ('t Lam et al., 2018). For example, there are some reports of water-soluble proteins in intact form which have high-value properties (Günerken et al., 2015). So controlled cell disruption (initially disruption of the cell wall followed by organelle disruption) was proposed in biorefineries (Eppink et al., 2019). Among different introduced cell disruption approaches, mechanical processes have recently gained renewed interest because of high efficiency in disruption of the cell barriers and release of high-value products (Günerken et al., 2016; Postma et al., 2016a). In addition, no harsh conditions like high temperature and high or low pH, and from this point of view they are mild to keep the intracellular components intact (Schwenzfeier et al., 2011). After breaking the cell wall, the separation of all fractions without losing any product is the main object in biorefinery (Vanthoor-Koopmans et al., 2013; Eppink et al., 2019). Among valuable fractions in the microalgae cells, protein and carbohydrate are hydrophilic components, while lipids and pigments are hydrophobic. In the past, extraction and separation of lipids/ pigments were done with organic solvent-based processes (Ghasemi Naghdi et al., 2014; Mahmood et al., 2017). The separation of lipids/pigments by organic solvents not only needs additional unit operations to recover solvents ('t Lam et al., 2018) but proteins, as the other high-value components of microalgae cells, are denatured in the presence of organic solvents (Desai et al., 2016). Ionic liquids (ILs) in recent years have been successfully used for the extraction of single components from microalgae (Orr and Rehmann, 2016; Desai et al., 2016; Desai et al., 2019). However the drawbacks like their high price, non-volatile features and subsequently restricted back extraction, and unknown environmental impacts have prevented the development at large scale (Chemat and Vian, 2014). More recently the solvent-free separation of lipids has been considered as an alternative method. This method is based on the insolubility of oil in water rather than on dissolution of oil (Rosenthal et al., 1996; Johnson and Lusas, 1983). In this method, oil and protein are extracted simultaneously and then the extraction mixture is centrifuged to form oil-rich layer and protein and sugar rich aqueous phase (Cater et al., 1974). An enzymatic hydrolysis is also assisted to facilitate the disruption bodies covered around lipids and releasing them in the aqueous phase. Much safer, environmentally friendly, and economical technology rather than solvent-based process (Liu et al., 2016) next to the simultaneous separation of protein and oil are some advantages of solvent-free systems (Latif and Anwar, 2011; Passos et al., 2009). Liu et al. (2016) reviewed some publications which implemented a solvent-free system to extract oil from oilseeds. In 2012 Liang et al. investigated the potential of solvent-free system to extract natural lipids anchored in *C. vulgaris*. They reported a 49.8% lipid recovery using this method, which was lower than that of oilseeds like soybean (about 90%). Moreover, they concluded that a higher amount of polar lipids e.g. glycolipids and phospholipids in microalgal cells than in oilseeds resulted in lower lipid recovery. Actually glycolipids and phospholipids, because of relatively dispersion properties in aqueous phase, are not extracted without organic solvents (Liang et al., 2012). There are studies, which have reported enzymatic treatment coupled with organic solvents as an approach for high yield extracting microalgal lipids compared to untreated biomass (Cho et al., 2013; Fu et al., 2010; Sierra et al., 2017; Zheng et al., 2011). From this point of view for a multi-product biorefinery, the selective separation of proteins before the extraction of lipids by organic solvents should be performed for complete biomass utilization ('t Lam et al., 2018). As far as we know, various researches have applied enzymatic hydrolysis to extract lipids or proteins separately (Cho et al., 2013; Liang et al., 2012; Safi et al., 2017) while extraction of two or more components with preserving the integrity of all of them have been poorly studied (Sierra et al., 2017).

The aim of this work was to develop a multi-product biorefinery based on *Chlorella vulgaris* as a reservoir of 35% carbohydrate, 27% lipid, and 21% protein. First, a benchmark bead mill was used as mechanical disruption to release water-soluble proteins in the native form with high-value properties. Then the enzymatic treatments were applied to fractionate remaining carbohydrates and proteins in the liquid phase and lipids in the solid phase without losing any products.

## 2. Material and methods

The experiments in this study were divided into three main parts, i.e., microalgae cultivation and harvesting, bead milling experiments, and enzymatic hydrolysis. In this section, the details of each part are presented.

### 2.1. Microalgae cultivation and harvesting

The green microalgae *C. vulgaris* UTEX 259 was obtained from the culture collection of algae, the University of Austin, USA. *C. vulgaris* was cultivated in *LGem* photobioreactor (GemTube™ MK2-750, The Netherlands) for 10 days in *M8a* medium (Mandalam and Palsson, 1998) to reach a concentration of 2.4 g $L^{-1}$. The used *LGem* photobioreactor with 1300 L volume was located in a greenhouse (AlgaePARC, The Netherlands). The pH value was adjusted to 6.7 by $CO_2$ injection on demand and the temperature was kept between 25 °C and 30 °C. Also, the light intensity was provided by natural light during the period of February 2019 (Wageningen, The Netherlands) and high-pressure mercury greenhouse lamps (18 h light, 8 h dark). After cultivation, the biomass was concentrated to reach 20% (w/w) using spiral plate centrifuge (Evodos 25, Evodos, The Netherlands) at 80 Hz, $\sim 3000 \times g$, and 0.75 $m^3$ $h^{-1}$. The concentrated biomass was sorted in small bags and stored at −20 °C in freezer for further using. Each time, one small bag containing biomass was taken out from the freezer to defrost slowly at room temperature. Then the concentrated defrosted biomass was stored at 4 °C for maximal 5 days until further use.

### 2.2. Bead milling

To disrupt the algae cell wall, a horizontal 75 mL bead mill (DynoMill Research Lab, Willy A. Bachofen AF Maschinenfabrik, Switzerland) was implemented. A 65% filling percentage by 0.4 mm $Y_2O_3$ stabilized $ZrO_2$ beads was used. Biomass suspensions with a 25 $g_{DW}$ $L^{-1}$ concentration as feed for bead mill were prepared in distilled water. The bead mill ran in batch recirculation mode with a constant agitation speed of 2039 rpm and at constant temperature 25 °C which was controlled by an external cooler (FP40-HE, Julabo® GmbH).

### 2.3. Sample collection

Samples were taken from the feed chamber every 30 s to analyze the yield of released components from biomass during bead milling. Samples were centrifuged at $10000 \times g$ and 4 °C for 10 min to separate disrupted cells and supernatant. Each phase was analyzed separately.

### 2.4. Enzymatic hydrolysis

After the disruption of the cells by bead milling and releasing water-soluble proteins into the liquid phase, the suspension was centrifuged at 4000 × *g* and 4 °C for 45 min to separate the broken cells as much as possible from the supernatant. Subsequently, 2.0 g harvested pellets were suspended in 50 mL tris-HCl buffer (pH 7.4) to perform the enzymatic hydrolysis by different enzymes such as cellulase, lipase, phospholipase A1, and protease. The dosage of each enzyme in each experiment was adjusted to 2% (v/w). All of enzymes were provided by Sigma-Aldrich company. The information of all enzymes used in the experiments are summarized in Table 1. The enzymatic hydrolysis by the cellulase and protease was performed at 45 °C whereas the experiments with the lipase and phospholipase A1 were done at 37 °C. The total time of all experiments was 24 h.

### 2.5. Biomass characterization

To measure the dry weight and total ash percentage, the biomass was dried in an oven at 100 °C for 24 h and burned in a furnace at 575 °C respectively. The calculation was done by the following equation (Eq. (1)):

$$dry\ weight\ (\%) = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

$W_1 = weight\ of\ biomass\ (g)$

$W_2 = weight\ of\ biomass\ after\ drying\ or\ burning\ (g)$

Proteins were measured with the Lowry method (Lowry et al., 1951). In short, 1 mL of 0.4 M NaOH was added to 20 mg freeze dried biomass. The samples were incubated for 30 min at 100 °C. Then, modified Lowry protein assay kits (BioRad) were used and total protein content was measured based on the absorbance at 750 nm. Bovine serum albumin was used as a protein standard.

Total lipids, fatty acids content and characterization of these lipids into polar and non-polar lipid were performed according to Breuer et al. (2013). Briefly, about 10 mg freeze-dried biomass was bead beated in 4 mL solution containing 1.78 mL chloroform and 2.22 mL methanol to extract all of total lipids in liquid phase. After bead beating, the solution was separated in two phases by adding 2.5 mL tris-HCl (pH 7.5). The bottom phase was collected, evaporated under nitrogen gas stream, and the weight of extracted lipids was measured gravimetrically. To fractionate the lipids into polar and non-polar lipids, a SEP column (Sep-Pak Vak Silica cartridge 6 cc/1000 mg, 125 A, 55–105 μm, Waters, 186004617) was used. In this method, SEP column was initially equilibrated with 10 mL hexane and followed by applying 0.5 mL hexan:diethyl ether (7:1 v/v) containing extracted lipids. Then, non-polar lipids were collected by eluting with 10 mL hexan:diethyl ether (7:1 v/v) and polar lipids by eluting with 10 mL methanol: acetone: hexane (2:2:1 v/v/v). Also, the total lipids released in the aqueous phase were measured according to the method by Liang et al. (2012). In short, 1 mL hexane was added to each mL of the aqueous phase and mixed at room temperature for 1 h. Then, hexane phase (upper layer) was separated, evaporated, and the weight of extracted lipids was measured gravimetrically. Finally, extracted lipids were converted into fatty acid methyl esters (FAME) in presence of methanol containing 5% (v/v) $H_2SO_4$ at 70 °C for 3 h and then determined by gas chromatography. Glyceryl tripentadecanoate (C15:0 TAG) was used as the internal standard of non-polar fatty acids and 1,2-didecanoyl-sn-glycero-3-phospho-soduim salt (C10:0) was used as the internal standard of polar fatty acids.

For total carbohydrates, the method of DuBois (1956) was used. Briefly, 0.5 mL of 2.5 M HCl was added into 1 mg freeze dried biomass and placed at 100 °C for 3 h. Once the sample was cooled to room temperature, 0.5 mL of 2.5 M NaOH was added for neutralization. After applying 0.5 mL of 5% (w/w) phenol/ water and 2.5 mL of concentrated sulfuric acid, total sugars were measured based on the absorbance at 430 nm. Pure glucose was used as a sugar standard. The characterization of carbohydrates into corresponding polymeric sugars like glucan and galactan was performed according to Van Wychen and Laurens (2013). In this method, 25.0 mg freeze-dried biomass was mixed with 250 μL of 72% (w/w) sulfuric acid in a 10 mL glass tube and placed in a 30 °C water bath for 1 h. After that, the sulfuric acid concentration of the sample was brought to 4% (w/w) by adding 7 mL Milli-Q water. Then the sample was placed in an autoclave to complete the dilute acid hydrolysis at 121 °C for 1 h. Once the sample was cooled to room temperature, the monomeric sugars like glucose and galactose were analyzed by high performance chromatograghy (HPLC).

### 2.6. Analytical methods

Glucose and galactose were measured by high performance liquid chromatography (HPLC, Shimadzu, Nexera X2, Japan) equipped with a Phenomenex organic acid H+ column (Rezex, CA, USA) and a refractive index detector. The eluent was 0.008 M H2SO4 in MilliQ water at 60 °C with a flow rate of 0.6 mL $min^{-1}$.

FAMEs were determined by gas chromatography (Agilent 7890, CA, USA) equipped with a Supleco Nucol 25357 (30 m × 530 μm × 1 μm) column. Helium (He) was used as carrier gas at flow rate of 20 mL $min^{-1}$. Split flow and split ratio were adjusted at 1.6 mL $min^{-1}$, and a of 0.1:1 respectively. Also, the injector temperature was 250 °C and detector temperature was 270 °C.

### 2.7. Mass yield

The calculation of mass yields (Yi) was performed according to Eq. (2):

$$Y_i\left(\% \frac{g}{g}\right) = \frac{m_{ij}}{m_{ib}} \times 100; \quad (2)$$

which $m_i$ refers to the weight of each component (g) such as proteins, lipids, etc and subscripts j and b correspond to each fraction estimated such as liquid and solid phase respectively. Also, this equation was applied for calculating the recovery yield with the difference that denominator refers to the initial amount of each component in the biomass.

### 2.8. SDS-PAGE analysis

The identification of released protein was performed using reducing SDS-PAGE analysis described by Postma et al. (2017). In addition, the gels were scanned with ImageScanner (GE-Healthcare) and Labscan 6.0 software and analyzed by ImageQuant TL 7.0 software.

### 2.9. Mass balance

The analysis of the mass balance was performed using the data of each component e.g. proteins, carbohydrates, and lipids after bead milling and enzymatic hydrolysis of *C. vulgaris* biomass. The solid recovery was used to calculate mass balance over the process. The calculation of solid recovery was performed according to the following equation (Eq. (3)):

$$\text{Solid recovery}\left(\frac{g}{g}\right) = \frac{W_f}{W_i} \quad (3)$$

where $W_f$ refers to the weight of biomass (g) after bead milling or enzymatic hydrolysis and $W_i$ is related to the initial weight of biomass (g).

**Table 1**
The information of enzymes used in enzymatic hydrolysis of *C. vulgaris*.

| Enzyme type | Activity | Source | Substrate | What enzyme degrade |
|---|---|---|---|---|
| Cellulase | ≥1000 U $g^{-1}$ | *Aspergillus species* | Carbohydrates | Cellolosic materials |
| Protease | > 2.4 U $g^{-1}$ | *Bacillus licheniformis* | Proteins | Peptide bonds |
| Lipase | 100–500 U $mg^{-1}$ | *Procine pancre* | Lipids such as triglycerides, phospholipids, and cholesteryl esters | Ester bonds |
| Phospholipase A1 | 1000 U $g^{-1}$ | *Aspergillus oryzae* | Phospholipids | Carboxy- and phospho-diester bonds |

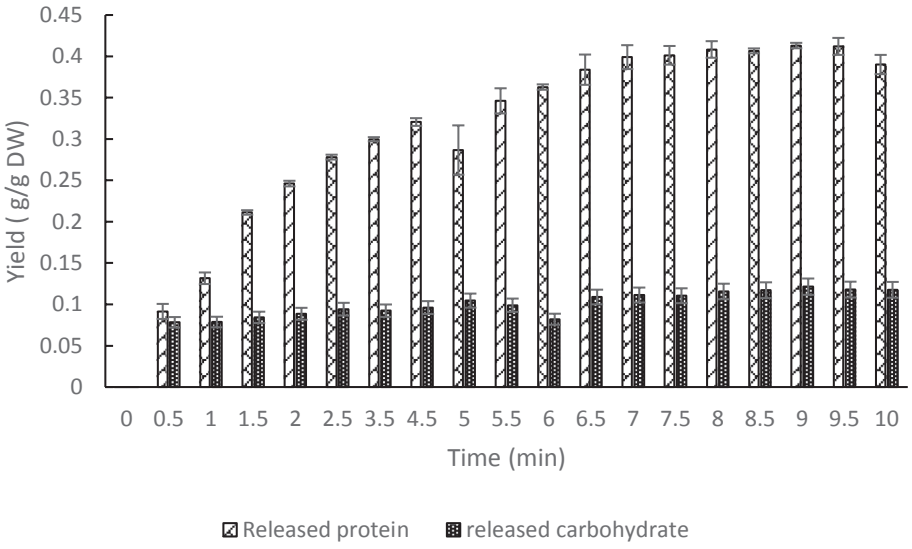


**Fig. 1.** The yield of released protein (▨) and carbohydrate (▩) during bead milling. All experiments were performed in duplicate.

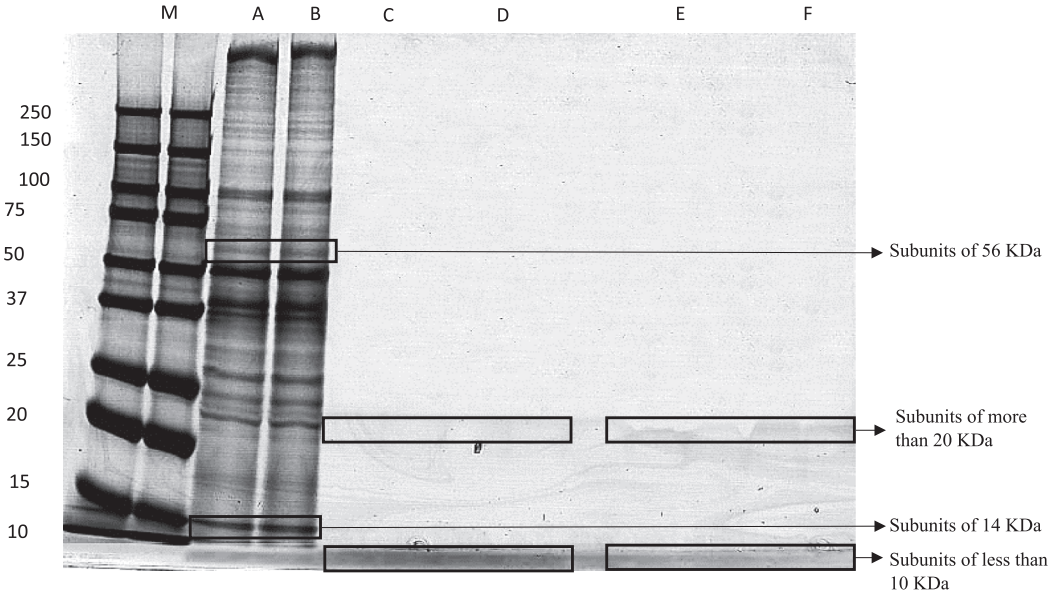


**Fig. 2.** SDS-PAGE gel after (A): 5 min bead milling, (B): 10 min bead milling, (C): 24 h enzymatic hydrolysis by mixture of protease and cellulase at 45 °C and pH 7.4, (D): 24 h enzymatic hydrolysis by protease at 45 °C and pH 7.4, (E): 24 h enzymatic hydrolysis by phospholipase A1 at 37 °C and pH 7.4, (F): 24 h enzymatic hydrolysis by lipase at 45 °C and pH 7.4, and (M): marker.

**Table 2**
Composition (% dry weight) and its class distribution (% total composition) of *C. vulgaris* biomass before and after bead milling.

| | Lipid | | | Carbohydrate | | | Protein | Ash |
|---|---|---|---|---|---|---|---|---|
| | Total lipid | TAG | Polar lipid | Total carbohydrate | Glucan | Galactan | | |
| Untreated biomass | 25.1 ± 0.0 | 1.4 ± 0.1 | 98.5 ± 0.3 | 35.0 ± 0.1 | 60.1 ± 0.2 | 39.9 ± 0.0 | 20.0 ± 0.4 | 2.0 ± 0.3 |
| Bead milled biomass | 28.0 ± 0.0 | 4.2 ± 0.0 | 95.7 ± 0.1 | 29.0 ± 0.6 | 63.6 ± 0.6 | 37.4 ± 0.0 | 18.0 ± 0.7 | 1.4 ± 0.5 |

**Table 3**
Solid recovery and composition of solid and liquid phase after bead milling and enzymatic hydrolysis of *C. vulgaris*. Data are expressed as mean ± 1–5% standard deviation.

| Sample | Treatment | Solid recovery (g/g) | Solid phase composition (g/g biomass) | | | Liquid phase composition (g/g biomass) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Lipid | carbohydrate | protein | lipid | Carbohydrate | Protein* |
| Algae | – | – | 0.25 | 0.35 | 0.20 | – | – | – |
| Bead milled algae | – | 0.83 | 0.28 | 0.29 | 0.18 | – | 0.10 | 0.39 |
| Bead milled algae | Incubating at 45 °C, 24 h | 0.64 | 0.30 | 0.27 | 0.18 | – | 0.15 | 0.16 |
| Bead milled algae | Lipase (2% v/w), 37° C, 24 h | 0.51 | 0.49 | 0.19 | 0.14 | 0.06 | 0.20 | 0.43 |
| Bead milled algae | Phospholipase A1 (2% v/w), 37° C, 24 h | 0.69 | 0.36 | 0.24 | 0.18 | 0.03 | 0.15 | 0.19 |
| Bead milled algae | Protease (2% v/w), 37° C, 24 h | 0.59 | 0.48 | 0.25 | 0.15 | 0.05 | 0.17 | 0.42 |
| Bead milled algae | Cellulase (2% v/w), 37° C, 24 h | 0.77 | 0.34 | 0.27 | 0.15 | 0.01 | 0.11 | 0.24 |
| Bead milled algae | Phospholipase, protease, and cellulase (2% v/w, 1:1:1), 37° C, 24 h | 0.59 | 0.42 | 0.20 | 0.14 | 0.03 | 0.20 | 0.42 |
| Bead milled algae | Protease (2% v/w), 45° C, 24 h | 0.50 | 0.58 | 0.17 | 0.24 | – | 0.23 | 0.25 |
| Bead milled algae | Cellulase (2% v/w), 45° C, 24 h | 0.88 | 0.41 | 0.19 | 0.24 | – | 0.14 | 0.18 |
| Bead milled algae | protease, and cellulase (2% v/w, 1:1), 45° C, 24 h | 0.50 | 0.63 | 0.18 | 0.25 | – | 0.23 | 0.24 |
| Bead milled algae | protease, and cellulase (2% v/w), 45° C, 24 h** | 0.43 | 0.64 | 0.20 | 0.23 | – | 0.23 at 12 h reaction with protease and 0.02 at reaction with cellulase | 0.19 at 12 h reaction with protease and 0.17 at reaction with cellulase |

* This yield was calculated as g/g total protein.
** First 12 h hydrolysis by protease (2% v/w) at 45 °C, then the liquid was removed and finally 12 h hydrolysis by cellulase (2% v/w) at the same temperature

## 3. Results and discussion

In this section, the results of released components such as carbohydrates and proteins by bead milling as physical disruption are presented first. Afterwards, the effect of enzymatic hydrolysis by different enzymes on fractionation of proteins, carbohydrates, and lipids are evaluated.

### 3.1. Bead milling and release of proteins and carbohydrates

The release of proteins and carbohydrates during the bead milling of *C. vulgaris* cells was investigated by Postma et al. (2017). The optimum bead size and agitation speed, reported by Postma et al. (2017), was used in our study. Fig. 1 shows the yield of the released intracellular components during bead milling of *C. vulgaris*. According to these results, after just half a minute, about 10% of soluble proteins were released. By increasing the contact time, the high contact surface between beads and microalgae cells increased and consequently more cells were broken. As can be seen from Fig. 1 after 8 min the yield of released soluble proteins reached 40% and did not change until 10 min significantly. A similar trend in protein yield was observed by Safi et al. (2015) and Postma et al. (2017). To investigate the size of released proteins and the effect of bead milling on degradation or aggregation of them, SDS-PAGE was performed (Fig. 2-A, B). Rubisco (Ribulose-1,5-biphosphate carboxylase oxygenase), which is mainly located in an intracellular organelle (Meyer et al., 2012) consists of 8 small subunits (~14 kDa) and 8 large subunits (~56 kDa). As can be observed from Fig. 2-A, B, both large and small subunits of Rubisco are released during bead milling of microalgal cells. The release of Rubisco during bead milling was also reported by Postma et al. (2017).

Also, the carbohydrate concentration was analyzed during bead milling which can be seen in Fig. 1. From the results, after half a minute, 7% of carbohydrates were released as free sugars and after 10 min 10% was reached. According to the primary analysis of *C. vulgaris* biomass, 35% of dry weight biomass consists of carbohydrates such as glucan, and galactan (Table 2). The low concentration of released carbohydrates during bead milling referred to the mild conditions of this mechanical treatment. Bead milling was performed at 25 °C and at this temperature dextrinization of the storage starch and cellulosic structure of the cell wall did not happen. However, we know that part of the carbohydrates resides in the cytoplasm as free sugars and will be released during the bead milling when the cells are disrupted. Further release of glucose from starch may take more time during interaction of the Zirconium beads with the starch during bead milling by breaking the polysaccharide chains of starch to monomeric sugars, due to the mechanical forces of beads (Postma et al., 2017, Suarez Garcia et al., 2018).

### 3.2. Enzymatic hydrolysis

The composition of biomass before and after bead milling is reported in Table 2. Also, the lipid class distribution of *C. vulgaris* before and after bead milling showed that the total amount of lipid in *C. vulgaris* consisted of polar lipids (Table 2).

The density of phospholipids is 1.09 g $L^{-1}$ which is equal to the density of the aqueous phase. Also due to the phosphate group, they are polar hydrophilic molecules relatively soluble in the aqueous phase. Therefore they will not be simply recovered by using centrifugation and the separation of layers which is a common process in solvent-free systems (Liang et al., 2012). On the other hand, the separation of them by organic solvents denatures aqueous soluble proteins as other high-value components that are not able to withstand organic solvents. So the separation of proteins and carbohydrates by enzymatic-assisted methods prior to the extraction of lipids with organic solvents is essential. Accordingly, the fractionation of remaining components in the biomass after bead milling was performed selectively by different

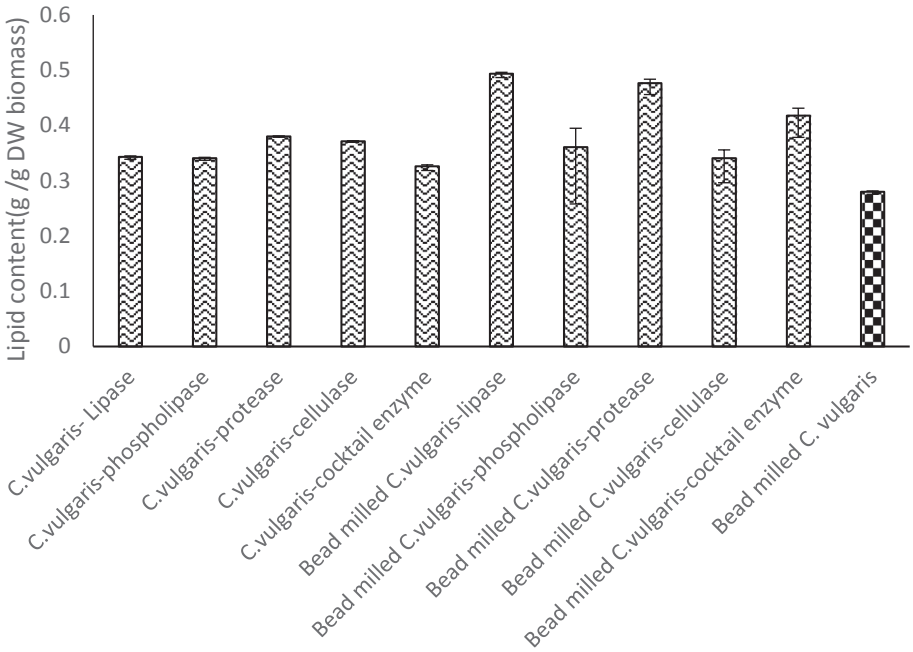


**Fig. 3.** lipid content *C. vulgaris* after enzymatic hydrolysis with and without bead milling. All enzymatic hydrolysis experiments were performed at pH 7.4 and temperature 37 °C for 24 h.

enzymes. Different enzymes degrade different ingredients of biomass, thus affecting the fractionation yield. For example lipase and phospholipase A1 were selected to fractionate lipids. Lipase is an enzyme that hydrolyzes the triglyceride into monoglyceride and two fatty acids (Svendsen, 2000). Phospholipase A1 is an enzyme that catalyzes the hydrolysis of phospholipids and converts them into fatty acids and other lipophilic substances (Richmond and Smith, 2011). For these reasons, in this study two enzymes were evaluated as extractors of fatty acids.

Cellulases break down the cellulose molecule into monosaccharides. The biomass after bead milling containing 29% carbohydrate could not be dissolved in the aqueous phase and remained part of the biomass. Thus, to fractionate this amount of carbohydrates, cellulase was used. Protease was another choice because of its ability to solubilize and hydrolyze the peptide bonds and facilitate the fractionation of components. The potential of individual enzymes and also the mixture of them were investigated and summarized in Table 3. The biomass concentration, enzyme dosage, and incubation time were selected based on previous reports (Cho et al., 2013; Liang et al., 2012).

First, to evaluate the effect of incubation temperature on the release of the components, bead milled biomass was treated in the same buffer without any enzymes at 45 °C next to the other enzymatic treatments. According to the composition of the solid and liquid phase after each enzymatic treatment, only 15% of carbohydrates and 16% of soluble proteins was released to the liquid phase due to incubation at 45 °C. Lipase could concentrate the lipids from 28% to 49% in solid phase and 43% of soluble proteins were released to liquid phase. According to a little amount of lipids released to the liquid phase compared with that of concentrated in solid phase, it could be concluded that the lipase performance was not on breaking glycerol backbone and releasing fatty acids. However, it could act on the release of proteins and carbohydrates into liquid phase and subsequently purified lipids in the solid phase by 1.9-fold compared with nonhydrolyzed biomass. Similar results were reported by Sander et al. (Sander and Murthy, 2009). They reported higher concentrations of glucose in the liquid phase after enzymatic treatment by lipase compared with untreated algae. This phenomenon may be due to the potential of lipase/ phospholipase in digesting the monolayer of polar lipids or membrane proteins that cover around the lipid droplets (Dixon and Wilken, 2018). Although the obtaining results of phospholipase A1 were lower than that of lipase. It is might be related to *C. vulgaris* cell wall which is protected by algaenan, an aliphatic and nonhydrolyzed polymer (Gelin et al., 1999). The construction of long aliphatic chains of algaenan is similar to the actual substrate of phospholipase A1 (Gerken et al., 2013) and subsequently affect the yield of phospholipase A1 to release expected components. Also, the combination of phospholipase A1 with cellulase and protease at 37 °C for obtaining a higher yield of lipids was not successful and changed the fraction of lipids in the solid phase slightly more than that of phospholipase A1 alone.

Protease at 45 °C released 25% soluble proteins in the liquid phase and concentrated 58% lipid in the solid phase. In this case, about 50% of carbohydrates was hydrolyzed as monomeric sugars to the liquid phase. For cellulase as the main enzyme, there were not high carbohydrate yields as well as for protease. Using a combination of these two enzymes at their optimum temperature, cellulase as cellulose digester and protease as protein digester, had the highest yield to concentrate high-value fatty acids in biomass (63%). In the biorefinery approach, the fractionation of all components separately is strongly proposed. For this reason, the enzymatic hydrolysis was done with this strategy: first hydrolysis by protease, then followed by hydrolysis using cellulase. The advantage of this enzymatic hydrolysis strategy was the solubilization of proteins in the first part of hydrolysis and the separation of carbohydrates subsequently. The results showed that the biomass after hydrolyzing by this plan containing 64% lipid which was 2.4-fold higher than nonhydrolyzed biomass.

Also, the effect of bead milling prior to enzymatic hydrolysis on lipid fractionation was investigated. Additionally, the intact biomass was treated by different enzymes (Fig. 3). The results show that breaking the cell wall before enzymatic hydrolysis had a positive effect on the fractionation of lipid. The positive effect may be interpreted by reduction in cell size and facilitating the accessibility of enzymes to digest corresponding substrates.

### 3.3. Fatty acid distribution

In order to investigate the effect of enzymatic hydrolysis on the distribution of fatty acids content of the microalgal lipid composition, the remaining lipids in the biomass after enzymatic treatment was

analyzed by gas chromatography. Fig. 4 shows the fatty acid composition after each enzymatic treatment. Based on these results, all of the tested enzymes and also bead milling as mechanical disruption had no significant effect on the distribution of fatty acids of microalgal lipid.

### 3.4. Recovery yield

In this study, to evaluate the ability of each enzyme to fractionate valuable components efficiently, the parameter recovery yield was introduced. The recovery yield was calculated based on mass balance analysis. These calculations were done by considering the initial fraction of each component in biomass before any treatments and its fraction in solid and liquid phase after bead milling and each enzymatic hydrolysis. Table 4 shows the recovery yield of lipids in the solid phase and recovery yield of proteins and carbohydrates in liquid phase. According to these results, without enzymatic hydrolysis the recovery yield of lipids, carbohydrates, and proteins for bead milled biomass were 75%, 31% and 40% respectively while these results were improved significantly after enzymatic hydrolysis. For example, the enzyme cocktail of protease and cellulase at 45 °C had the highest lipid recovery yield. It means 93% of initial lipid content in dry biomass was recovered in the solid phase and about 80% of the initial carbohydrate content and 54% of the initial protein content were recovered in the liquid phase. Also enzymatic hydrolysis by lipase had higher recovery yields after the protease/cellulose mixture. Fig. 2-(C-F) shows SDS-PAGE analysis of proteins released during enzymatic hydrolysis. As can be seen in Fig. 2- (C-D) the treatment in presence of protease released short fragment of proteins with molecular weight lower than 10 kDa. Protease is an enzyme which digests proteins and converts them to shorter proteins, peptides, and amino acids (Ali et al., 2019). Also, its optimum temperature is 45 °C and at this temperature proteins become prone to losing functionality ('t Lam et al., 2018). On the other hand enzymatic hydrolysis by lipase did not release proteins which have the molecular weight lower than 10 kDa (Fig. 2-F). Additionally, the optimum temperature of lipase was 37 °C and at this temperature, the probability of losing the protein functionality is low. By taking into account the recovery yield of proteins, lipids and carbohydrates, it seems that lipase treatment after bead milling is the best choice for fractionation of valuable components in *C. vulgaris*.

The extraction/recovery yield of lipids and other valuable components like proteins and carbohydrates after enzymatic hydrolysis also have been reported in other publications. Although a lot of studies which used enzymatic hydrolysis to disrupt the cell walls of microalgae, only focused on the extraction of one component. For example, Fu et al.

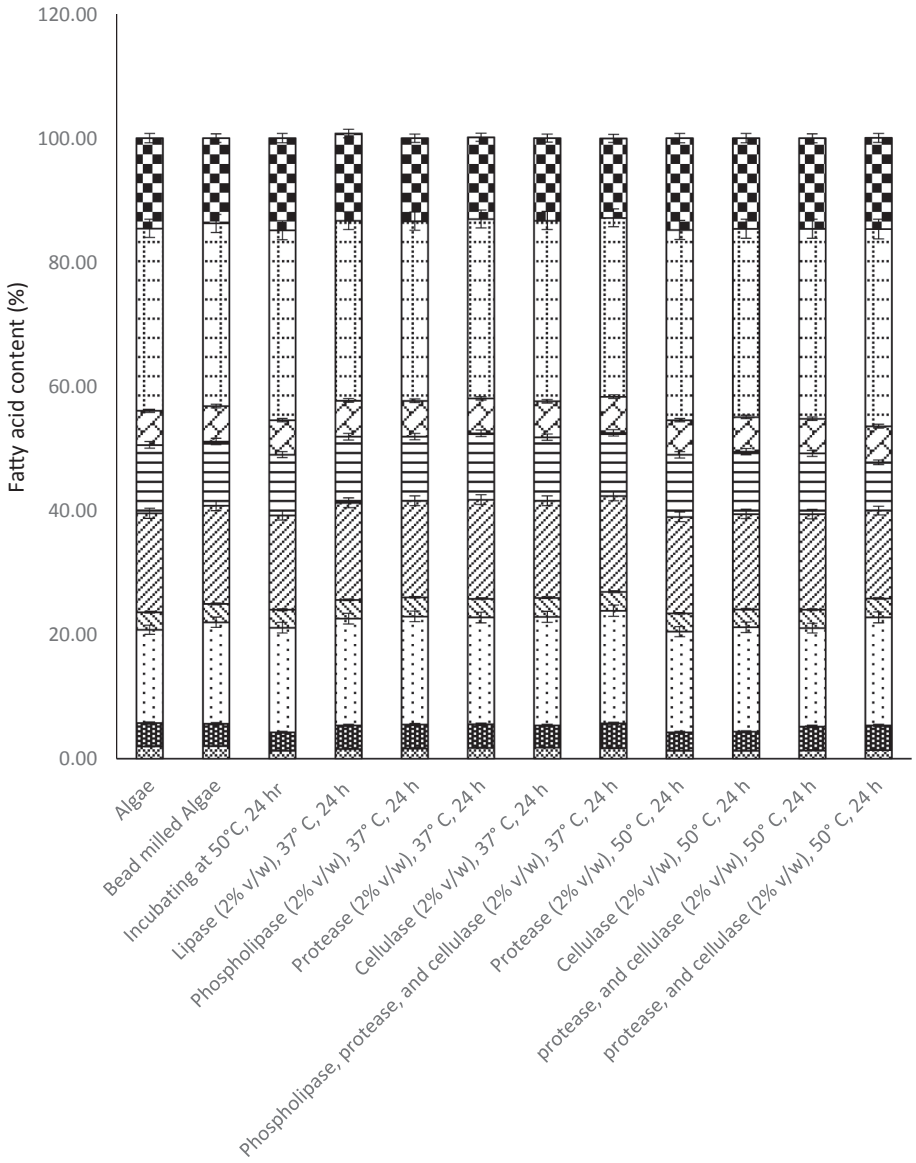


**Fig. 4.** Fatty acid distribution in *C. vulgaris* biomass, bead milled, and enzymatic treated biomass. (▩) C13:0, (■) C14:0, (⬚) C16:0, (▧) C16:1, (▨) C16:2, (▤) C16:3, (▨) C18:1, (⬚) C18:2, (▩) C18:3.

**Table 4**
Recovery yield of proteins and carbohydrates in the liquid phase and recovery yield of lipids in solid phase.

| Sample | % Recovery yield (g component in final product/g component in dry biomass) | | |
|---|---|---|---|
| | Protein (liquid phase) | Carbohydrate (liquid phase) | Lipid (solid phase) |
| Bead milled Algae | 40 | 31 | 75 |
| Incubating at 45 °C, 24 h | 50 | 63 | 58 |
| Lipase (2% v/w), 37° C, 24 h | 68 | 74 | 88 |
| Phospholipase (2% v/w), 37 °C, 24 h | 51 | 63 | 75 |
| Protease (2% v/w), 37° C, 24 h | 67 | 69 | 85 |
| Cellulase (2% v/w), 37° C, 24 h | 55 | 54 | 79 |
| Phospholipase, protease, and cellulase (2% v/w), 37° C, 24 h | 68 | 74 | 75 |
| Protease (2% v/w), 45° C, 24 h | 56 | 82 | 87 |
| Cellulase (2% v/w), 45° C, 24 h | 50 | 63 | 87 |
| Protease, and cellulase (2% v/w), 45° C, 24 h | 54 | 81 | 93 |
| Protease, and cellulase (2% v/w), 45° C, 24 h* | 63 | 82 | 81 |

* First 12 h hydrolysis by protease (2% v/w) at 45 °C, then the liquid was removed and finally 12 h hydrolysis by cellulase (2% v/w) at the same temperature.

(2010) applied immobilized cellulase to increase the extraction yield of lipids containing in *chlorella vulgaris* 14% more than unhydrolyzed biomass. In another study, Liang et al. (2012) used the combination of snailase and trypsin to separate lipids containing in chlorella vulgaris by 35% while they executed the enzymatic aqueous extraction method. Sierra et al. (2017) reported the fractionation yield of lipids in the solvent phase by 95% and proteins in aqueous phase by 20% during enzymatic hydrolysis of *Chlamydomonas reinhardtii* with autolysin. Also Maffei et al. (2018) developed an enzymatic paocess for *Nannochloropsis* sp. with cellulase and mannanase to fractionate proteins and carbohydrates in aqueous phase by 20% and 20% respectively prior to the extraction of lipids by 70% in solvent phase. However, from the biorefinery point of view, it is essential that all major fractions are valorized to achieve an economically large scale algae biorefinery (Eppink et al., 2019). The proposed procedure in this study not only recovered almost all of the valuable components in maximum yield without losing any products (68% proteins, 74% carbohydrates, and 88% lipids) and using any auxiliary solvents but also separated proteins as the most valuable fraction in food and feed market with keeping full functionality.

### 3.5. Mass balance

Fig. 5 demonstrates the overall mass balance of *C. vulgaris* after bead milling and enzymatic hydrolysis by lipase. One hundred grams of dry *C. vulgaris* contains 25 g lipid, 35 g carbohydrates, 21 g protein, and 5.7 g ash. As can be seen in Fig. 5 each 100 g dry biomass can produce 11 g carbohydrates and 7.8 g proteins, which are released in the liquid phase after bead milling. Further treatment by lipase on solid residue obtained after bead milling lead to produce 44.9 g solid which half of it made by lipid (22 g). Functional proteins which is separated after bead milling has a selling price of 1,100 € $Ton^{-1}$ and the part of the proteins and carbohydrates, which are released to the liquid phase after enzymatic hydrolysis as source of bulk chemicals, have totally a selling price of 750 € $Ton^{-1}$(Wijffels et al., 2010). Also lipids concentrated in the solid residue as source for food/feed would be worth 950 € $Ton^{-1}$ (Postma et al., 2016b). Therefore the selective separation of *C. vulgaris* components by combined bead milling and enzymatic hydrolysis has potential to exploit maximum 455 € revenue per each ton of dry biomass.

## 4. Conclusions

This work evaluates the combination of bead milling and enzymatic hydrolysis with lipase for the fractionation of proteins, carbohydrates, and lipids of *C. vulgaris* at maximum recovery yield. The advantages of this process are the successful fractionation of components such as proteins, carbohydrates, and lipids without losing any products, short residence time, and performing without solvents or corrosive solutions. The phospholipid rich solid, which is of potential interest for food and

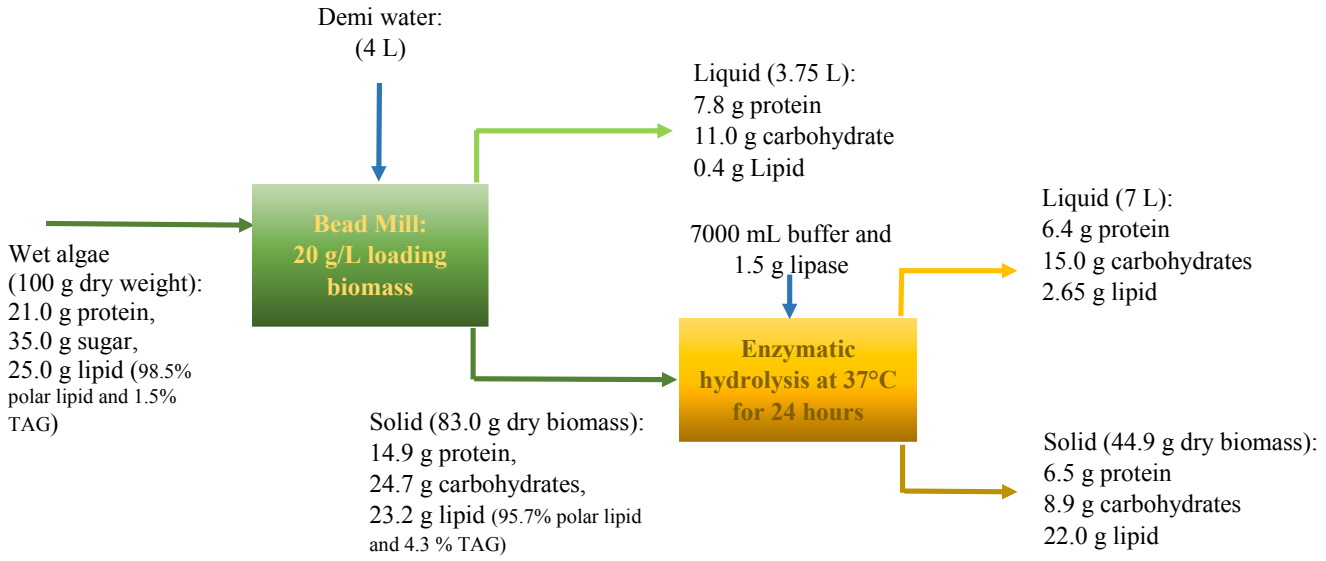


**Fig. 5.** Mass balance for fractionation of proteins, carbohydrates, and lipids from *C. vulgaris*.

cosmetics products, along with high value proteins obtained in this process could be justified the high cost of enzymes as major weakness of this process.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References


Ali, J., Sharma, D.C., Bano, A., Gupta, A., Sharma, S., Bajpai, P., Pathak, N. 2019. Exploiting Microbial Enzymes for Augmenting Crop Production. In: Enzymes in Food Biotechnology, Elsevier, pp. 503–519.

Breuer, G., Evers, W.A., de Vree, J.H., Kleinegris, D.M., Martens, D.E., Wijffels, R.H., Lamers, P.P., 2013. Analysis of fatty acid content and composition in microalgae. J. Vis. Exp. 80.

Cater, C.M., Rhee, K.C., Hagenmaier, R.D., Mattil, K.F., 1974. Aqueous extraction—an alternative oilseed milling process. J. Am. Oil Chem'. Soc. 51 (4), 137–141.

Chemat, F., Vian, M.A., 2014. Alternative Solvents for Natural Products Extraction. Springer.

Cho, H.-S., Oh, Y.-K., Park, S.-C., Lee, J.-W., Park, J.-Y., 2013. Effects of enzymatic hydrolysis on lipid extraction from Chlorella vulgaris. Renew. Energy 54, 156–160.

Desai, R.K., Streefland, M., Wijffels, R.H., Eppink, M.H., 2016. Extraction of proteins with ABS, Ionic-Liquid-Based Aqueous Biphasic Systems. Springer, pp. 123–134.

Dixon, C., Wilken, L.R., 2018. Green microalgae biomolecule separations and recovery. Bioresour. Bioprocess. 5, 14.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.t., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356.

Eppink, M.H., Olivieri, G., Reith, H., van den Berg, C., Barbosa, M.J., Wijffels, R.H., 2019. From current algae products to future biorefinery practices: a review. Adv. Biochem. Eng. Biotechnol. 166, 99–123.

Fu, C.-C., Hung, T.-C., Chen, J.-Y., Su, C.-H., Wu, W.-T., 2010. Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. Bioresour. Technol. 101 (22), 8750–8754.

Gelin, F., Volkman, J., Largeau, C., Derenne, S., Damsté, J.S., De Leeuw, J., 1999. Distribution of aliphatic, nonhydrolyzable biopolymers in marine microalgae. Organic Geochem. 30 (2–3), 147–159.

Gerken, H.G., Donohoe, B., Knoshaug, E.P., 2013. Enzymatic cell wall degradation of Chlorellavulgaris and other microalgae for biofuels production. Planta 237 (1), 239–253.

Ghasemi Naghdi, F., Thomas-Hall, S.R., Durairatnam, R., Pratt, S., Schenk, P.M., 2014. Comparative effects of biomass pre-treatments for direct and indirect transesterification to enhance microalgal lipid recovery. Front. Energy Res. 2, 57.

Günerken, E., d'Hondt, E., Eppink, M., Garcia-Gonzalez, L., Elst, K., Wijffels, R., 2015. Cell disruption for microalgae biorefineries. Biotechnol. Adv. 33 (2), 243–260.

Günerken, E., D'Hondt, E., Eppink, M., Elst, K., Wijffels, R., 2016. Influence of nitrogen depletion in the growth of N. oleoabundans on the release of cellular components after beadmilling. Bioresour. Technol. 214, 89–95.

Johnson, L.A., Lusas, E., 1983. Comparison of alternative solvents for oils extraction. J. Am. Oil Chem'. Soc. 60 (2Part1), 229–242.

't Lam, G., Vermuë, M., Eppink, M., Wijffels, R., Van Den Berg, C., 2018. Multi-product microalgae biorefineries: from concept towards reality. Trends Biotechnol. 36 (2), 216–227.

Latif, S., Anwar, F., 2011. Aqueous enzymatic sesame oil and protein extraction. Food Chem. 125 (2), 679–684.

Liang, K., Zhang, Q., Cong, W., 2012. Enzyme-assisted aqueous extraction of lipid from microalgae. J. Agr. Food Chem. 60 (47), 11771–11776.

Liu, J.-J., Gasmalla, M.A.A., Li, P., Yang, R., 2016. Enzyme-assisted extraction processing from oilseeds: Principle, processing and application. Innov. Food Sci. Emerg. Technol. 35, 184–193.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maffei, G., Bracciale, M.P., Broggi, A., Zuorro, A., Santarelli, M.L., Lavecchia, R., 2018. Effect of an enzymatic treatment with cellulase and mannanase on the structural properties of Nannochloropsis microalgae. Bioresour. Technol. 249, 592–598.

Mahmood, W.M.A.W., Theodoropoulos, C., Gonzalez-Miquel, M., 2017. Enhanced microalgal lipid extraction using bio-based solvents for sustainable biofuel production. Green Chem. 19 (23), 5723–5733.

Mandalam, R.K., Palsson, B., 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density Chlorella vulgaris cultures. Biotechnol. Bioeng. 59 (5), 605–611.

Meyer, M.T., Genkov, T., Skepper, J.N., Jouhet, J., Mitchell, M.C., Spreitzer, R.J., Griffiths, H., 2012. Rubisco small-subunit α-helices control pyrenoid formation in Chlamydomonas. Proc. Natl. Acad. Sci. 109 (47), 19474–19479.

Orr, V.C., Rehmann, L., 2016. Ionic liquids for the fractionation of microalgae biomass. Curr. Opin. Green Sustain. Chem. 2, 22–27.

Passos, C.P., Yilmaz, S., Silva, C.M., Coimbra, M.A., 2009. Enhancement of grape seed oil extraction using a cell wall degrading enzyme cocktail. Food Chem. 115 (1), 48–53.

Postma, P., Miron, T., Olivieri, G., Barbosa, M., Wijffels, R., Eppink, M., 2015. Mild disintegration of the green microalgae Chlorella vulgaris using bead milling. Bioresour. Technol. 184, 297–304.

Postma, P., Pataro, G., Capitoli, M., Barbosa, M., Wijffels, R.H., Eppink, M., Olivieri, G., Ferrari, G., 2016a. a. Selective extraction of intracellular components from the microalga Chlorella vulgaris by combined pulsed electric field–temperature treatment. Bioresour. Technol. 203, 80–88.

Postma, P., Barbosa, M., Wijffels, R., Eppink, M., Olivieri, G., 2016b. Microalgal biorefinery for bulk and high-value products: Product extraction within cell disintegration. in: Handbook of electroporation, pp. 1–20.

Postma, P., Suarez-Garcia, E., Safi, C., Yonathan, K., Olivieri, G., Barbosa, M., Wijffels, R.H., Eppink, M., 2017. Energy efficient bead milling of microalgae: effect of bead size on disintegration and release of proteins and carbohydrates. Bioresour. Technol. 224, 670–679.

Richmond, G.S., Smith, T.K., 2011. Phospholipases A1. Int. J. Mol. Sci. 12 (1), 588–612.

Rosenthal, A., Pyle, D., Niranjan, K., 1996. Aqueous and enzymatic processes for edible oil extraction. Enzyme Microb. 19 (6), 402–420.

Safi, C., Frances, C., Ursu, A.V., Laroche, C., Pouzet, C., Vaca-Garcia, C., Pontalier, P.-Y., 2015. Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins and pigments in the aqueous phase. Algal Res. 8, 61–68.

Safi, C., Olivieri, G., Campos, R.P., Engelen-Smit, N., Mulder, W., Van den Broek, L., Sijtsma, L., 2017. Biorefinery of microalgal soluble proteins by sequential processing and membrane filtration. Bioresour. Technol. 225, 151–158.

Sander, K.B., Murthy, G.S., 2009. Enzymatic degradation of microalgal cell walls. American Society of Agricultural and Biological Engineers Annual International Meeting 2009, ASABE 2009. 4.

Schwenzfeier, A., Wierenga, P.A., Gruppen, H., 2011. Isolation and characterization of soluble protein from the green microalgae Tetraselmis sp. Bioresour. Technol. 102 (19), 9121–9127.

Sierra, L.S., Dixon, C.K., Wilken, L.R., 2017. Enzymatic cell disruption of the microalgae Chlamydomonas reinhardtii for lipid and protein extraction. Algal Res. 25, 149–159.

Svendsen, A., 2000. Lipase protein engineering. Biochim. Biophys. Acta 1543 (2), 223–238.

Suarez Garcia, E., van Leeuwen, J., Safi, C., Sijtsma, L., Eppink, M.H.M., Wijffels, R.H., van den Berg, C., 2018. Selective and energy efficient extraction of functional proteins from microalgae for food applications. Bioresour. Technol. 268, 197–203.

Van Wychen, S., Laurens, L., 2013. Determination of total carbohydrates in algal biomass. Contract 303, 275–3000.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H., 2010. Microalgae for the production of bulk chemicals and biofuels. Biofpr 4 (3), 287–295.

William, P., Laurens, L., 2010. Microalgae as biodiesel and biomass feedstock: review and analysis of the biochemistry, energetic and economics. Energy Environ. Sci. 3, 554–590.

Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., Dou, C., 2011. Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl. Biochem. Biotechnol. 164 (7), 1215–1224.