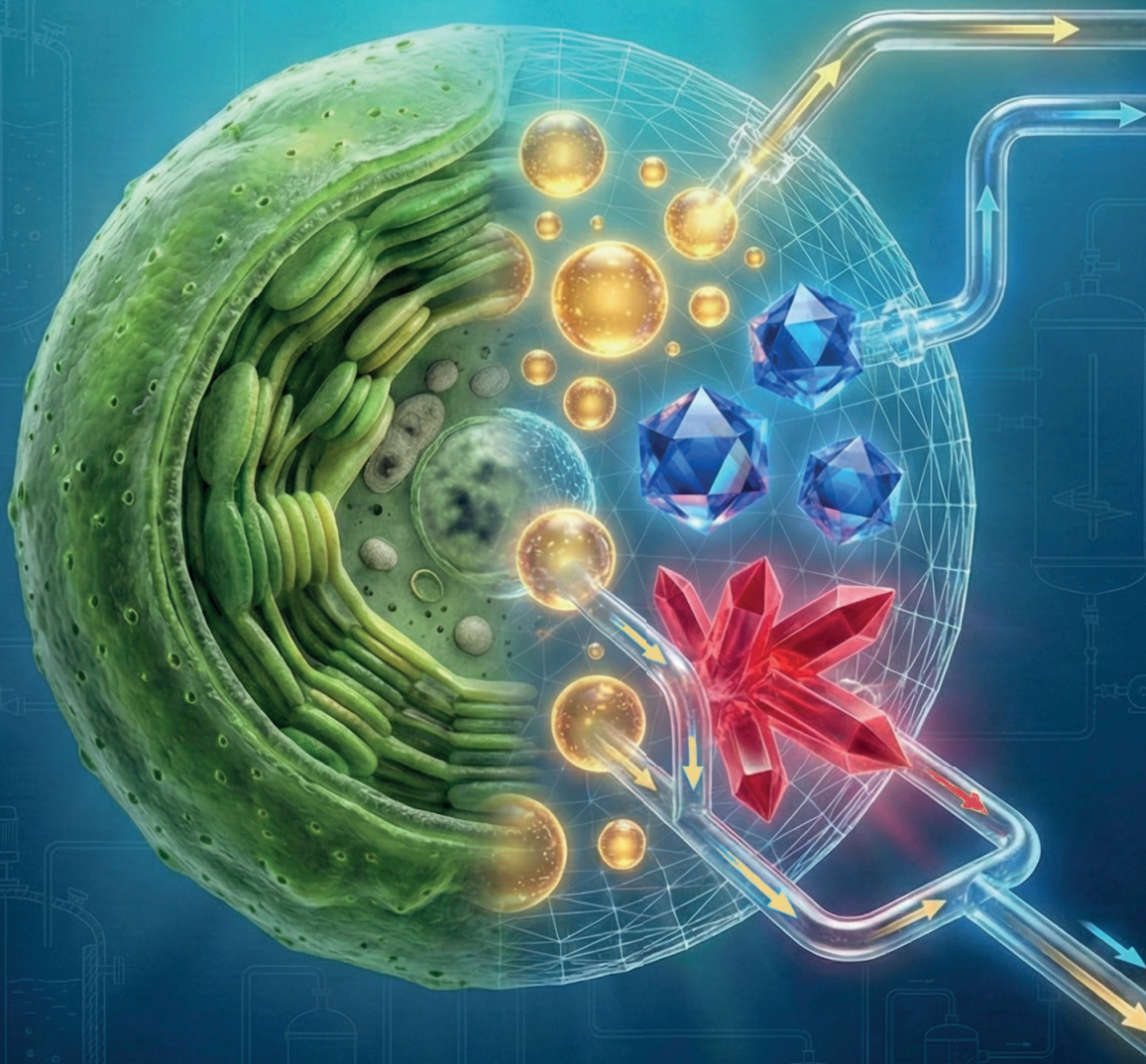
BREAKING CELLS WHILE KEEPING THE VALUE

MILD CELL DISRUPTION FOR MICROALGAE BIOREFINERIES



EMRE GÜNERKEN

Propositions

1. The biorefinery chain can only be optimized as a whole system instead of as a single unit operation.
2. Industrial entrepreneurs will be attracted if microalgal biorefineries are realized.
3. For humankind, becoming interstellar before breaking the barrier of native, biomarker free disease detection technologies equates to suicide.
4. Terraforming Earth is more important than terraforming any other planet.
5. Rights that are not actively defended become intellectual property of others.
6. The "next big thing" can be achieved when public relations and marketing are in agreement with science.

Propositions belonging to the thesis, entitled

Breaking Cells While Keeping the Value: Mild Cell Disruption for Microalgae Biorefineries

Emre Günerken

Wageningen, 22 April 2026

BREAKING CELLS WHILE KEEPING THE VALUE:
MILD CELL DISRUPTION FOR MICROALGAE BIOREFINERIES

EMRE GÜNERKEN

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BREAKING CELLS WHILE KEEPING THE VALUE:
MILD CELL DISRUPTION FOR MICROALGAE BIOREFINERIES

EMRE GÜNERKEN

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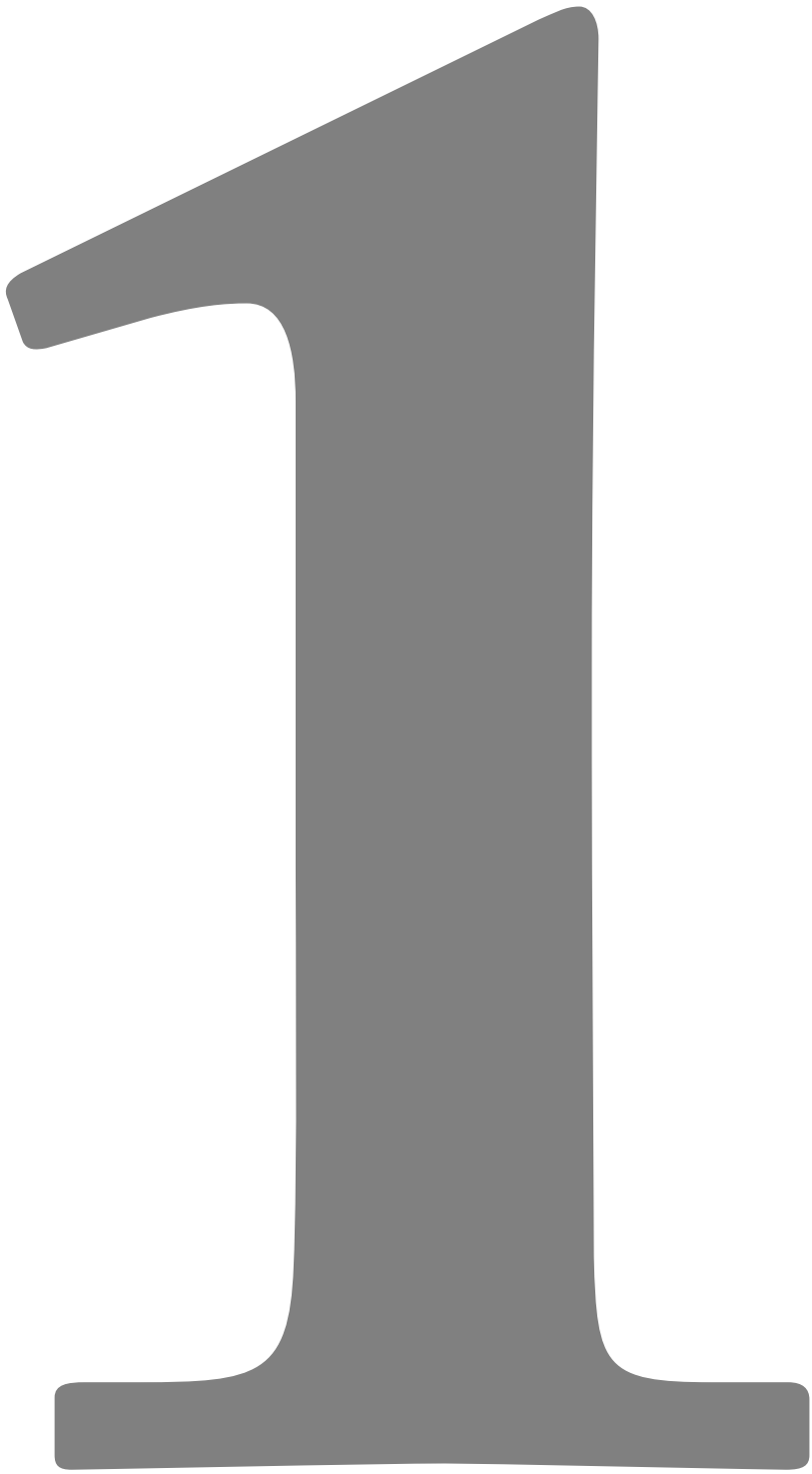

Chapter 1

GENERAL INTRODUCTION

The scarcity of fossil fuel-based resources and the urgent threat of climate change and global warming serve as critical drivers for the promotion of fuels and chemicals derived from renewable sources. The Intergovernmental Panel on Climate Change (IPCC) has documented that global greenhouse gas (GHG) emissions increased by 1.5% in 2022 relative to 2021, reaching 36.1 $GtCO_2$ and consuming 10-36% of the remaining carbon budget to limit warming to 1.5°C, with projections suggesting that permissible emissions could be depleted within 2-7 years at 67% likelihood (Friedlingstein et al., 2023). The IPCC continues to identify CO_2 as the most significant anthropogenic greenhouse gas contributing to climate change while energy sector is the biggest contributor of CO_2 gas emissions (IPCC, 2021). Energy consumption in the transport sector remains substantial, accounting for approximately 30% of final energy demand and approximately 25% greenhouse gas emissions in Europe (Abid et al., 2022). In response to these challenges, microalgae have emerged as a promising renewable biomass feedstock with significant potential for producing both biofuels and biochemicals, including high-value bioactive compounds, platform chemicals, and renewable materials through integrated biorefinery approaches (Eladl et al., 2024; Sharma et al., 2024). Recent advances demonstrate that microalgae-based systems can simultaneously address wastewater treatment, carbon dioxide fixation, and the production of lipids, proteins, and carbohydrates for conversion into biodiesel, bioethanol, biohydrogen, and various value-added chemicals (Shahid et al., 2021; Zhou et al., 2017). These developments position microalgae as a multifunctional solution aligned with Paris Agreement goals and the transition toward sustainable, low-carbon bioeconomy.

Microalgae are ubiquitous photosynthetic microorganisms found across terrestrial and aquatic ecosystems, playing a pivotal role in global carbon cycling by contributing over 40% of Earth's biological carbon fixation (Kleinegris et al., 2011). Recent advances in carbon sequestration research have demonstrated that microalgae possess 10–50 times higher carbon fixation capacity than terrestrial plants, with carbon content accounting for more than 50% of their dry weight biomass—approximately 1.83 tons of CO_2 are fixed to produce one ton of microalgal biomass (Vuppaladadiyam et al., 2023; Zhang et al., 2024a). As of November 2023, AlgaeBase has documented 50,589 living species and 10,556 fossil species of algae distributed across four kingdoms, 14 phyla, and 63 classes, representing a substantial increase from earlier estimates (Guiry, 2024). Advanced metabarcoding techniques have revealed significantly higher microalgal diversity than previously detected through conventional microscopic methods, with numerous nano-sized species in Chlorophyta, Haptophyta, and Chrysophyceae being newly documented (Wang et al., 2024).

Modern microalgae biotechnology began in the post-World War II era with the 1952 Stanford University symposium, which established the foundation for systematic investigation of mass cultivation techniques and identified fundamental bioprocess challenges including light limitation, gas-liquid mass transfer, contamination control, and reactor

design (Borowitzka, 1999; Borowitzka & Moheimani, 2013; Burlew, 1953; Coaldrake, 2021). This period witnessed pioneering contributions from William J. Oswald, who integrated microalgae cultivation with wastewater treatment through paddle-wheel driven High Rate Algal Ponds (HRAPs) that exploited symbiotic algae-bacteria relationships to provide photosynthetic oxygen for bacterial degradation while producing biomass, establishing operational systems with capacities ranging from 1 to 200 million liters per day (Abdel-Raouf et al., 2012; Oswald, 1988; Oswald & Gotaas, 1957; Van Wagenen, 2016).

Commercial production accelerated during the 1960s-1980s with establishment of large-scale Chlorella facilities in Japan achieving approximately 5,000 tons per year production by the 1970s, where spray-drying and cell wall disruption technologies increased digestibility from 47% to over 80% (Habib et al., 2008). Spirulina (Arthrospira) production commenced at Lake Texcoco in Mexico in 1967, with pilot facilities producing 150 tons annually by 1973 and global production exceeding 12,000 tons by the 2000s (García-López & Olguín 2020; Habib et al., 2008; Camacho et al., 2019). Dunaliella salina cultivation for β-carotene began in Australia at Hutt Lagoon and in Israel near Eilat during the 1980s, demonstrating that stress conditions in salt-saturated environments could induce β-carotene accumulation exceeding 10% dry weight, with current global production surpassing 3,000 tons annually (Ben-Amotz & Avron, 1990; Borowitzka et al., 1984; de Souza Celente et al., 2024; Garcia et al., 2017).

The U.S. Department of Energy's Aquatic Species Program (1978-1996) represented a transformative government initiative that isolated over 3,000 strains, advanced understanding of lipid accumulation mechanisms, and scaled outdoor raceway pond cultivation in New Mexico, though economic constraints during low fuel prices led to program discontinuation despite significant technical achievements (Sheehan et al., 1998; Van Wagenen, 2016). This era established two primary cultivation paradigms: open raceway ponds offering low construction costs ($10-15/m^2) and operational simplicity but exhibiting lower productivity (10-20 g/m^2/day) and limited environmental control versus closed photobioreactors providing superior contamination control, higher biomass concentrations (1-4 g/L versus 0.3-0.5 g/L), and enhanced areal productivity at substantially greater capital investment ($100-200/m^2) and operational costs (Borowitzka & Vonshak, 2017; Norsker et al., 2011; Sutherland et al., 2014; Wang et al., 2012).

The second, more dramatic initiative emerged in 2008–2009 when oil prices exceeded $120–147 per barrel. ExxonMobil announced a $600 million partnership with Synthetic Genomics, while Shell, BP, and Chevron invested heavily alongside venture capital start-ups including Solazyme and Sapphire Energy. The U.S. Department of Energy allocated $97 million for demonstration projects (Biofuel Watch, 2017).

However, by 2012, the National Research Council concluded that scaling was unsustainable with current technologies (Biofuel Watch, 2017). The European Enalgae project (2011–2015) similarly concluded that algae were unlikely to contribute significantly to energy needs. By 2023, ExxonMobil had abandoned its program after investing over $350 million (Bloomberg, 2023). Current global production remains 15,000–20,000 tons annually, serving only high-value niche markets (Koyande et al., 2019).

Recent decades have witnessed continued innovation in reactor design including thin-layer cascade systems, ARID ponds with enhanced temperature management, and hybrid two-stage configurations (Crowe et al., 2012; Narala et al., 2016). Downstream processing has advanced through improved harvesting methods achieving recovery efficiencies exceeding 95%, optimized cell disruption technologies, and extraction techniques utilizing supercritical CO_2 and switchable solvents (Borowitzka, 2013). Furthermore, genetic engineering applications including truncated light-harvesting antennae, CRISPR/Cas9 gene editing, overexpression of rate-limiting enzymes, and heterologous pathway expression, combined with systems biology approaches integrating multi-omics platforms, have enabled comprehensive metabolic characterization and rational strain optimization for enhanced growth, stress tolerance, and metabolite productivity (Ajjawi et al., 2017; Sreenikethanam et al., 2022; Grama et al., 2022; Guarnieri & Pienkos, 2015; Nymark et al., 2016; Wang et al., 2016, Naduthodi et al., 2018; Panahi et al., 2024). Artificial intelligence (AI) and machine learning (ML) algorithms have revolutionized microalgae bioprocess optimization (Fu et al., 2025; Pahariya et al., 2024).

These technological advances spanning AI&ML, reactor engineering, process optimization, downstream processing, and molecular biotechnology continue improving the technical feasibility and economic viability of microalgae bioprocesses for sustainable industrial-scale production of food, feed, nutraceuticals, biochemicals, and biofuels (Stephens et al., 2010; Campbell et al., 2011; Gamal & Shreadah, 2024).

The concept of microalgae biorefineries has gained substantial momentum as a promising platform for circular bioeconomy applications, enabling simultaneous production of multiple high-value products including above mentioned ones (Mutanda et al., 2020; Geng et al., 2025). Biorefinery refers to the concept of using biological matter to produce fuels, chemicals, heat and power to enhance the cost-effectiveness of biosynthetic platforms. This relatively complex framework briefly explained in Fig. 1. The complexity of this concept makes it harder to implement on an industrial scale successively. The main issue when building up a commercially feasible biorefinery is the right selection between the product and energy-based concepts. The mid-point choice is hybrid-integrated biorefineries which is most suitable for microalgae. Integration of other industrial processes with microalgae cultivation/biorefineries has emerged as a cost-effective strategy, where nutrient-rich industrial, agricultural, and municipal wastewater serves as a cultivation medium while facilitating carbon capture from

flue gases containing 10–99.5% CO_2 and recycling waste heat flow in especially drying steps as a approach for sustainable (carbon-negative, energy-positive) operations (Wang et al., 2022; Srimongkol et al., 2022, SundarRajan et al., 2020; Anyaoha et al., 2024). Advanced cultivation systems, including photobioreactors and hybrid configurations combining closed reactors with open systems, have demonstrated improved biomass productivity through precise control of temperature, pH, light intensity, and CO_2 concentration (Weise et al., 2020). Over 15,000 bioactive molecules produced by microalgae are now utilized across food, feed, pharmaceutical, cosmetic, and chemical industries, with emerging applications in bioplastics, biofertilizers, cosmetics, nutraceuticals and carbon credit markets (Sathasivam et al., 2021; Cardozo et al., 2007; Olabi et al., 2023).

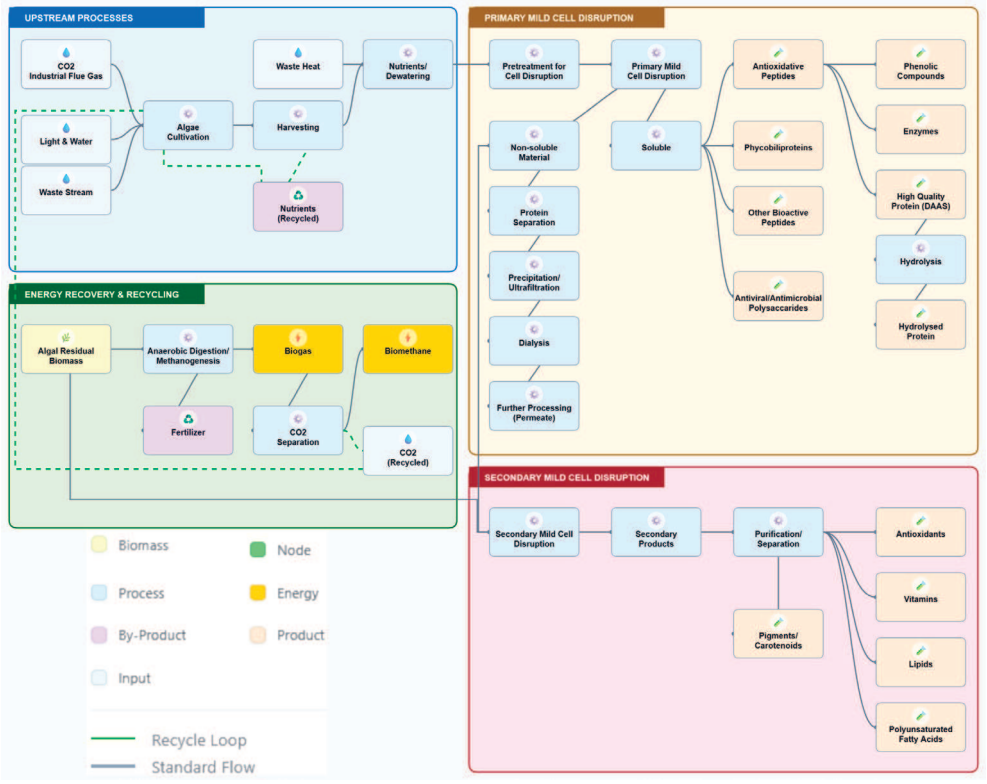

Fig. 1: The interconnected nature of a conceptual biorefinery system presenting upstream (upper-left), primary mild cell disruption (upper-right), energy recovery & recycling (bottom-left) and secondary mild disruption (bottom-right) unit operations in a conceptual design.

However, commercialization challenges persist, including high capital and operational costs, energy-intensive downstream processes, and the need for robust, contamination-resistant cultivation systems (Padi et al., 2022; Geng et al., 2025). Future research directions emphasize the development of multi-product biorefinery platforms focusing on downstream processes, standardized LCA/TEA protocols, supportive policy frameworks, and continued

advancement in genetic engineering, photobioreactor design, and AI-driven process optimization to enhance the economic viability and environmental sustainability of microalgae biotechnology (Kim et al., 2023; Tran et al., 2025).

Most of the target biomolecules of microalgae are located intercellularly. Usually, microalgae cells are covered with a thick elastic cell wall which enhances their resistance to downstream processes. Thus, a cell disruption step is needed before the products can be extracted and isolated.

Since microalgae cell physiology is highly variable in between species (Gerken et al., 2013; Scholz et al., 2014), it is challenging to find an appropriate, universal cell disruption method for microalgae. So far, although the mechanical cell disruption methods e.g. bead milling and high speed homogenization and other high yield methods e.g. high energy ultrasonication, microwave treatment and high pressure homogenization in use are giving high cell disruption yields (Chapter 2), they are not suitable for processes in which multiple products are targeted because local temperature and pressure differences have negative effects on the final products. Moreover, high energy consumption, scalability or economical concerns make most of the cell disruption methods unfavorable for industrial scale processes (Chapter 2). Thus, mild cell disruption methods, which are energy efficient, scalable and do not depend on high value equipment/chemicals and working under mild conditions, are gaining more interest.

To make microalgae based biochemical production economically more attractive, a feasible cell disruption method should be established to ensure a low operating cost, high product recovery, and high quality of the recovered products. Finding the most appropriate method of cell disruption for the microalgae in issue would maximize the product concentration and improve the quality of the extracted lipids and other biochemicals.

AIM OF PHD RESEARCH PROJECT AND RESEARCH QUESTIONS

Withstanding the persisting commercialization challenges and future research directions this PhD research project focused on improvements in cell disruption as the first step of downstream processes.

More specifically the aim of this PhD research project is "*The Development of a Feasible Technique for Disruption of Single Cell Microalgae Biomass as a First Step in Microalgae Biorefineries*". The cost effectiveness, cell disruption ratio, treatment time, extractability and recoverability of products are considered as key aspects.

The innovative aspects of this PhD research project are translated in the following Research Questions:

1. What are the fundamental technical and economic limitations of current cell disruption methods that prevent their effective implementation in mild microalgae biorefineries, and what evaluation framework is needed to assess alternative approaches?
2. Can cultivation strategy (nitrogen depletion) enhance the disruption efficiency and selectivity of mechanical cell disruption methods, and does this optimization pathway address the fundamental limitations?
3. How can cell disruption efficiency be rapidly and accurately quantified to enable systematic optimization and method comparison?
4. Can a novel continuous explosive decompression system address the fundamental limitations identified, incorporate the optimization insights gained, and be developed using the analytical methods established to create a mild, energy-efficient, industrially scalable cell disruption solution?

RESEARCH JOURNEY AND THESIS OUTLINE

To answer all research questions explosive decompression method was studied to develop a feasible mild cell disruption technique (Chapter 5) and compared with a conventional cell disruption method, i.e. bead milling (Chapter 3). First comprehensive literature research on cell disruption methods used to treat biomass was investigated (Chapter 2). *Neochloris oleoabundans* cultured under stressed and unstressed conditions was chosen as a biomass resource for the rest of the study (Chapter 3), starting with the creation of baseline data with selected microalgae strain and state of the art cell disruption method in Chapter 3. The cell disruption efficiency was being determined by evaluating the release of biochemicals from the pellet to the supernatant after cell disruption by using the data from dry biomass and biochemical analyses in Chapter 4. Finally, by integrating all information collected from literature and the experimental work done during this research project a new, mild cell disruption technique was developed and patented in Chapter 5.

Chapter 2

To address the 1st research question a literature review study was performed and published. For this purpose, over 400 scientific articles were investigated. The overlapping, contradicting information from research articles were eliminated by filtering them regarding impact factors and citation numbers. All remaining research data collected was investigated and compared from a statistical and mathematical point of view, resulting in a comprehensive literature overview to establish a scientific background for this PhD research project. Some of the key aspects of mechanical and non-mechanical cell disruption methods were investigated and compared in the review for energy consumption, effect on product quality, product recovery yield, scalability, effect of experimental and growth conditions of investigated methods.

This review study published in 2015 has over 800 citations and is still receiving over 50 citations per year as a useful addition to microalgae community.

Chapter 3

To address the 2nd research question first a literature review was done to investigate 5 different microalgae strains and *Neochloris oleoabundans* was chosen as the primary microalgae for this PhD research project. The main reasons for this choice are the thick cell wall, high lipid accumulation capability and the excessive experience at Bioprocess Engineering Department with this green microalga strain. *Scenedesmus sp.* was proposed as the secondary option for this project, to be used in case of experiencing any extreme problems with primary option, due to the biochemical and morphological differences from *N. oleoabundans*. *Dunaliella salina* and *Phaeodactylum tricornutum* were not chosen due to their fragile nature while the reason of not selecting *Nannochloropsis salina* was its morphological similarity to *N. oleoabundans*. Afterwards by using the literature collected from Chapter 2, bead-milling was selected as the state-of-the-art cell disruption method. Additionally, more in-depth investigations were carried out with nitrogen depleted and repleted production conditions of *N. oleoabundans* and its effect on cell disruption. The aim of the bead milling experiment was to calculate the release of biochemicals from pellet to the supernatant and determine the mass ratios at full disruption in terms of cell number reduction.

Chapter 4

To simplify the rather time-consuming analyses methods also used in Chapter 3, the answer to the 3rd research question was to develop a fast and easy to handle flow-cytometry method as an analysis method for screening experiments and potentially to find an unified method for all microalgae cell disruption research. As a result, an empirical formula was found to correlate the output of the flow-cytometer to biomass release and cell number concentration since the latter proved a valid approximation for the cell disruption efficiency. To develop this fast method, biomass release, manual counting and flow-cytometry data from bead milling of *Chlorella sp.* cultured at Bioprocess Engineering (BPE)and *Chlorella sorokiniana* cultured at PhyCom (Veenendaal, The Netherlands) were compared. The BPE culture was cultivated under photosynthetic conditions while the PhyCom culture was cultivated under mixotrophic conditions. Additionally, to validate the method also repeatability, manual cell counting and flow cytometry data of 15 runs of bead milling of *Chlorella sp.*, performed at BPE by Richard Postma (Another PhD Student), were compared.

Chapter 5

As the final step of this PhD research project, a novel, mild cell disruption method is developed by using explosive decompression as the basis addressing the 4th and last research question. First studies on development were carried out by using 100 ml Premex stirred tank high pressure autoclaves. Briefly, the effects of pressure, stirring, treatment time and EtOH addition were investigated. The treated soluble phase was enriched with lipids while

unfortunately a relatively low number of proteins and carbohydrates was transferred from insoluble to soluble phase.

The problems and the possible solutions for explosive decompression of microalgae were evaluated systematically through process development/optimization and intensification in several steps throughout 2013 till 2015. As the output of this long development study, a new explosive decompression method was developed and all the experiments made were evaluated regarding economic, technical and success parameters. Finally, also a Method and Device patent is filed (WO2016012613).

Chapter 6

Building upon the systematic progression through literature review (Chapter 2), cultivation-disruption integration studies (Chapter 3), analytical methodology development (Chapter 4), and process innovation through continuous explosive decompression (Chapter 5), Chapter 6 synthesizes these interconnected investigations into a cohesive framework. This general discussion evaluates cell disruption not as an isolated unit operation but as a critical part of biorefineries. By reconceptualizing the biorefinery as an integrated, adaptive system rather than a sequence of independently optimized stages, this chapter articulates how cultivation conditions, disruption mechanisms, analytical capabilities, and process intensification principles must be jointly optimized to advance microalgae production toward industrial-scale viability and meaningful impact on global challenges including protein security, water scarcity, and carbon emissions reduction.

2

CHAPTER 2

Cell disruption for microalgae biorefineries

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ABSTRACT

Keywords: Cell disruption, Bead milling, High pressure homogenization, High speed homogenization, Ultrasonication, Microwave treatment, Pulsed electric field treatment, Non-mechanical cell disruption, Microalgae, Biorefinery

Microalgae are a potential source for various valuable chemicals for commercial applications ranging from nutraceuticals to fuels. Objective in a biorefinery is to utilize biomass ingredients efficiently similarly to petroleum refineries in which oil is fractionated in fuels and a variety of products with higher value. Downstream processes in microalgae biorefineries consist of different steps whereof cell disruption is the most crucial part. To maintain the functionality of algae biochemicals during cell disruption while obtaining high disruption yields is an important challenge. Despite this need, studies on mild disruption of microalgae cells are limited. This review article focuses on the evaluation of conventional and emerging cell disruption technologies, and a comparison thereof with respect to their potential for the future microalgae biorefineries. The techniques discussed are bead milling, high pressure homogenization, high speed homogenization, ultrasonication, microwave treatment, pulsed electric field treatment, non-mechanical cell disruption and some emerging technologies.

1. GENERAL INTRODUCTION

Microalgae are considered as an industrially interesting source for the sustainable production of numerous products because of significantly higher growth rates, photosynthetic efficiencies and process optimization possibilities compared to conventional terrestrial plants and are already used in many commercial applications including food, animal feed, cosmetics, pollution abatement, and therapeutics (Brennan and Owende, 2010; Butler, 2006; Chisti, 2007; de Stefano et al., 2011; Harun et al., 2011; Hazar and Aydin, 2010; Huang et al., 2010; Li et al., 2008; Liu et al., 2008; Posten and Walter, 2012a,b; Rodolfi et al., 2009; Solovchenco et al., 2008; Spolaore et al., 2006; Thamsiriroj and Murphy, 2009; Tornabene et al., 1983; Usui and Ikenouchi, 1997). Moreover, microalgae can be cultured in marginal areas in brackish or saline water resulting in a lower water and land footprint (Tsukahara and Sawayama, 2005). Despite these advantages, microalgae also have their limitations. One of the major economic bottlenecks cited in the literature due to the high energy demand is downstream processing. The biorefinery concept, analogous to petroleum refineries, aims to fractionate biomass into fuels and multiple added-value co-products simultaneously by focusing on downstream processes (Chisti, 2007; Clark et al., 2009; DOE, 2010; IEA Bioenergy, 2009; Sánchez Mirón et al., 2003; Schmid Straiger, 2009). The main issue in designing a biorefinery is optimizing the balance between products and energy to obtain a maximum financial profit (Anastas and Zimmerman, 2003). Products cannot be recovered effectively from microalgae using methods designed for product extrusion from crops such as soybeans since the microalgae morphology is different from land crops. Microalgae cells are small, covered with a relatively thick cell wall and products are usually located in globules or bound to cell membranes, making extraction of intracellular products challenging. Additionally, the cell wall structure of microalgae is complex and poorly understood (Gerken et al., 2013; Scholz et al., 2014) and is known to have an important effect on the disruption efficiency. However, there are no broad studies investigating the relation between cell wall composition disruption efficiency and energy consumption. Thus, inter- and intra-species variations and variations observed from different cultivation conditions make predictions or extrapolations very difficult. Some microalgal cells are very easy to break so a mild or more energy efficient disruption technique can be chosen. However, calculating a universal energy consumption value for a given cell disruption method and therefore making a direct comparison of different techniques is impossible.

Despite these challenges, efficient cell disruption is an essential pretreatment step to maximize product recovery from microalgae biomass. A feasible energy-efficient cell disruption method should be established to ensure a low operating cost, high product recovery, and high quality of the extracted products.

This review article focuses on the evaluation of the fundamentals, physics, and case studies of conventional cell disruption techniques, already in use for microalgae, as well as

emerging mild disruption technologies. All techniques are evaluated and compared with respect to the potential for the future microalgae biorefineries.

2. CELL DISRUPTION

A variety of disruption methods is currently available for cell disruption. In general, these techniques are divided into two main groups based on the working mechanism of microalgal cellular disintegration, *i.e.*, (i) mechanical and (ii) non-mechanical methods (Fig. 2) (Agerkvist and Enfors, 1990; Chen et al., 2009; Chisti and Moo-Young, 1986; Lee et al., 1998, 2010; Middelberg, 1995; Mutanda et al., 2011). In this section, microalgae cell disruption methods and related case studies will be discussed, divided into mechanical and non-mechanical methods, and evaluated in terms of suitability for mild microalgae biorefinery. An overview of the parameters affecting the disruption yield for the studied cell disruption processes is provided in Table 1, the summary of the case studies from **Table 2** to Table 8, and the comparison of cell disruption methods in Table 9.

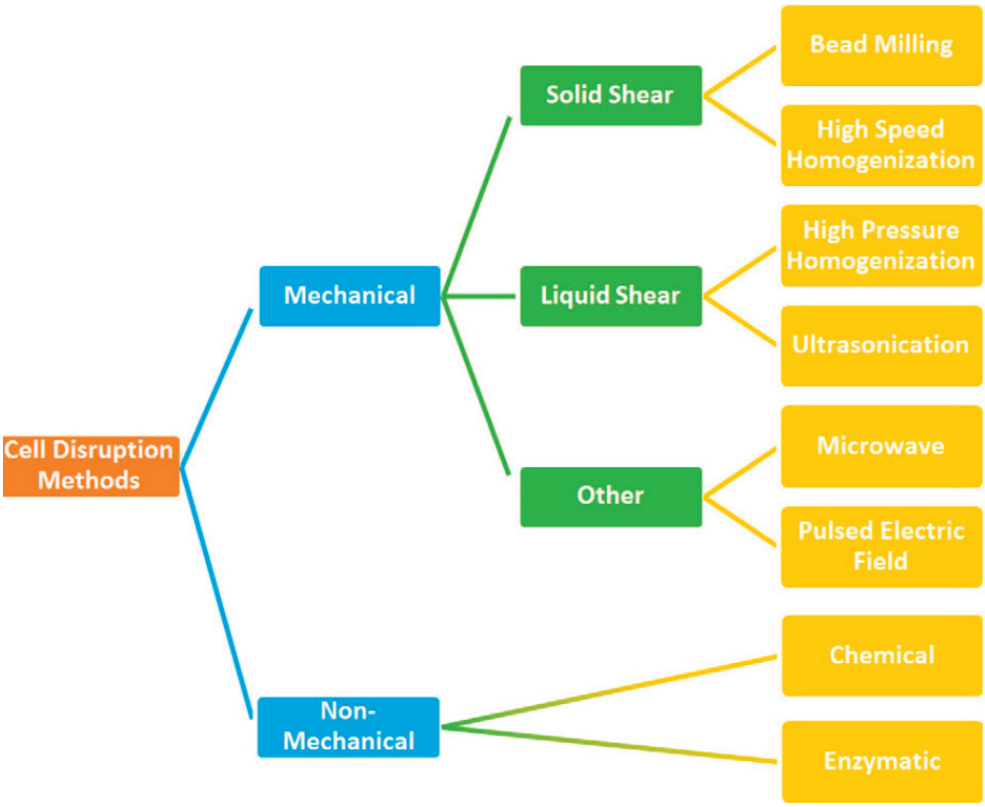

Fig. 2: Classification of the cell disruption methods.

2.1. Mechanical methods

Destruction of the cell wall in a non-specific manner is usually achieved by mechanical forces such as solid-shear forces (*e.g.*, bead mill, high speed homogenization), liquid-shear forces (*e.g.*, high pressure homogenization, microfluidization), energy transfer through waves (*e.g.*, ultrasonication, microwave), currents (*e.g.*, pulsed electric field) or heat (*e.g.*, thermolysis, autoclaving).

2.1.1. Bead milling

High disruption efficiency in single-pass operations, high throughput, high biomass loading, good temperature control, commercially available equipment, easy scale up procedures, and low labor intensity are the primary factors that make bead milling an interesting cell disruption method with high potential for industrial implementation (Bierau et al., 1999; Jahanshahi et al., 2002). The most common design for this system is shown in Fig. 3. The shaft may carry agitators of varied design (concentric or eccentric disks or rings) that export kinetic energy to small steel, glass or ceramic beads in the chamber resulting in multiple collisions (Chisti and Moo-Young, 1986). It is hypothesized that the suspended cells are disrupted in the bead collision zones by compaction or shear forces (Bunge et al., 1992; Melendres et al., 1992) with energy transfer from the beads to the cells (MacNeill et al., 1985).

Table 1: Process parameters of the cell disruption methods.

Disruption method	Mechanism of cell disruption	Process parameters	References
Bead milling	Mechanical compaction and shear stress	Agitation disk design, speed Bead filling, size, material Dry weight Feed rate Growth phase and conditions Microalgae type Time Cooling	Engler (1985), Kula and Schütte (1987)
High pressure homogenization	Cavitation and shear stress	Cycle number Dry weight Flow rate Growth phase and conditions Homogenizator design Microalgae type Pressure	Schütte et al. (1983), Kula and Schütte (1987)
High speed homogenization	Cavitation and shear stress	Blade design, speed Dry weight Growth phase and conditions Microalgae type Time	Shirgaonkar et al. (1998), Kumar and Pandit (1999)
Ultrasonication	Cavitation and free radical formation	Cycle number and time Dry weight Growth phase and conditions Microalgae type Power of ultrasound	Chandler et al. (2001), Taylor et al. (2001), Onyeche et al. (2002), Fykse et al. (2003), Borthwick et al. (2005), Gogate and Pandit (2008)
Microwave treatment	Temperature increase, molecular energy increase	Agitation Dry weight Growth phase and conditions Microalgae type Power of microwave Time	Pan et al. (2002), Terigar et al. (2010), Balasubramanian et al. (2011)
Pulsed electric field treatment	Proliferation due to electricity	Conductivity (electrolyte concentration) Current Dry weight Growth phase and conditions Microalgae type Oscillation Time	Brown et al. (1992), Muraji et al. (1993), Ganeva et al. (1995), Qin et al. (1995), Ho and Mittal (1996), Muraji et al. (1998), Muraji et al., 1999; Ade Omowaye et al. (2003)
Enzymatic lysis	Enzyme substrate interaction	Agitation Dry weight Enzyme concentration Enzyme type Growth phase and conditions Microalgae type Oxygen level Type and amount of buffer Temperature Pressure Time	Andrews and Asenjo (1987), Harrison (1991)
Chemical treatment	Chemical substrate interaction	Agitation Chemical concentration Chemical type Dry weight Growth phase and conditions Microalgae type Temperature Time	Middelberg (1995), Mendes Pinto et al. (2001), Harun and Danquah (2011b), Harun et al. (2011), Halim et al. (2012b), Miranda et al. (2012)

Table 2: Summary and comparison of case studies on bead milling.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
Bead milling						
Scenedesmus quadricauda (*fresh*)	Disrupted biomass	Ballotini beads, 33% bead filling, 2800 rpm agitator speed, 5 min, 5% DCW	1 l grinding chamber,	55% cell disintegration	Cell count	Hedenskog et al. (1969)
Scenedesmus quadricauda (*spray dried*)				87% cell disintegration		
Scenedesmus quadricauda (*fresh*)		0.35–0.5 mm beads, 50% bead filling, 1450 rpm agitator speed, 40 l/h flow rate, 5% DCW	5 liter grinding chamber	90% cell disintegration		
Chlorella sp.	Disrupted biomass	7.5 kW, 0.5 mm ZrO_2 beads, 70% beads filling, 15.8% DCW, 62 kg/h feed rate, 90 min	1.5 l grinding chamber	98.5% cell disintegration of *Chlorella*	Cell count, dry weight	Doucha and Lívanský (2008)
		3.3 kW, 0.42–0.58 mm glass beads, 82% beads filling, 10.7% DCW, 3 kg/h feed rate	1.4 l grinding chamber	99.9% cell disintegration of *Chlorella* and 90.2% cell disintegration of bacteria		
		25 kW, 0.6–0.8 mm ZrO_2 beads, 85% beads filling, 12.4% DCW, 35 kg/h Feed rate	18.3 l grinding chamber	85.29% cell disintegration of *Chlorella* and 81.2% cell disintegration of bacteria		
		3 kW, 0.3–0.4 mm glass beads, 85% beads filling, 6.9% DCW, 10 kg/h Feed rate, 3000 rpm agitator speed, 2 cycles	0.6 l grinding chamber,	98–99% cell disintegration of *Chlorella* and 99.5% cell disintegration of bacteria		
Tetraselmis sp.	Protein	3.3–4 kW, 0.3–0.4–0.6 mm ceramic beads, 65% bead filling, 12% DCW, 1.5 l/min flow rate, 30 min	0.3 l grinding chamber	21% of proteins transferred to algae juice after treatment	Total protein	Schwenzfeier et al. (2011)

Based on the results of the case studies, summarized in Table 2, it is concluded that increasing the bead diameter has a positive effect when the beads are smaller than 0.5 mm and has a negative effect above 0.5 mm (Doucha and Lívanský, 2008). Additionally, high density beads (*e.g.*, zirconium) are more effective in media with high viscosity while low density beads (*e.g.*, glass) are preferred in low viscous media (Schütte and Kula, 1990; Doucha and Lívanský, 2008; Hedenskog et al., 1969). Increasing the treatment time, agitator tip speed (peripheral velocity 5–10 $m \cdot s^{-1}$), number of cycles and bead filling up to 85% of grinding chamber volume have a positive effect on the disruption process (Doucha and Lívanský, 2008; Hedenskog et al., 1969; Postma et al., 2015). Increasing dry cell weight (DCW; 0.5–8% w/w) and biomass flow rate (kg DCW/h) negatively affect the cell disruption efficiency. However, increasing these parameters positively affect the cost of the cell disintegration process by reducing the specific energy consumption (Doucha and Lívanský, 2008; Postma et al., 2015). The effect of biomass flow rate on specific energy consumption shown in Fig. 7. The biomass flow rate is given as DCW influent (kg/h) and the specific energy consumption (kWh/kg) is calculated based on total energy consumed (kWh) to disrupt per kg (in dry basis) of microalgae biomass. Doucha and Lívanský (2008) recorded, for an increasing retention time from 1.3 to 2.3 min, an increase of 70% in biomass disruption efficiency and a decrease of 44% in the specific energy consumption. Oppositely, at significantly larger retention times (16 and 28 min), the specific energy consumption increased with 32% because of a lower throughput. The energy consumption of single pass bead milling operation of *Chlorella sp.* by using a Netzsch, Labstar LS1 recorded as 0.85 kWh/kg dry weight by Doucha and Lívanský (2008). Similarly, Postma et al. (2015) calculated the energy consumption for disrupting *Chlorella sp.* by a semi continiously operated Dyno-Mill Research Lab as 0.81 kWh/kg dry weight. The recorded values are just 13%- 14% of the caloric value of microalgae biomass calculated by Weyer et al. (2010) (6.083 kWh/kg). In practice, however, the specific energy consumption highly depends on DCW concentration/load, the species and the growth conditions of biomass.

Despite many positive characteristics, the high energy demand of bead milling make it less favorable for microalgae biorefineries. The inefficient energy transfer from the rotating shaft to the individual cells (Schütte and Kula, 1990) and energy conversion into heat (Doucha and Lívanský, 2008) require intensive, energy demanding cooling to allow the recovery of functional fragile products (e.g., RuBisCO). Additionally, the formation of very fine cell debris and non-selective distribution of biochemicals over the soluble and solid phase (Günerken et al., 2013) result in increased downstream processing costs. Although protein extractability and digestibility are increased after treatment (Hedenskog et al., 1969; Schwenzfeier et al., 2011) and the method is effective against microbial and fungal infestations present in the microalgae culture (Doucha and Lívanský, 2008; Hedenskog et al., 1969), it is not an ideal disruption method for mild microalgae biorefineries.

2.1.2. High pressure homogenization

High pressure homogenizers (HPHs) are especially suitable for emulsification processes. Various valve-seat configurations are available for HPHs to optimize the disruption efficiency (Masucci, 1985; Pandolfe, 1993). The cell suspension flows radially across a valve, strikes an impact ring, exits the valve and flows either to a second valve or to a discharge (Fig. 4). Cell disruption is thus achieved through high-pressure impact (shear forces) of the accelerated fluid jet on the stationary valve surface as well as hydrodynamic cavitation from the pressure drop induced shear stress (Chisti and Moo-Young, 1986; Halim et al., 2012a,b). Cavitation is defined as a 3-step phenomenon taking place in short time intervals (micro to milliseconds) that starts with the formation of bubbles, followed by growth and ends with the collapse of microbubbles. This causes the release of large amounts of energy into a very small volume. Very high energy densities (energy released per unit volume) are obtained locally which leads to cell disruption (Petrier et al., 1998). An overview of the case studies is given in Table 3 and discussed below.

The literature on HPH shows that a high working pressure followed by the cycle number have the most positive effect on cell disruption efficiency. Lower DCW concentrations and culture stress levels (N-depletion) were significant but to a minor extent and the nozzle diameter was determined as not effective (Halim et al., 2012a,b). The specific energy consumption of HPH is highly dependent on DCW concentration, algae species and the growth conditions of biomass. In different studies, the specific energy consumption varies from 0.25 kWh/kg (1% DCW, N-depleted) to 147 kWh/kg (0.85% DCW, no stress) (Grimi et al., 2014; Halim et al., 2012b; Lee et al., 2012). The lowest recorded specific energy consumption is approximately 4.1% of the microalgae biomass' caloric value (6.083 kWh/kg).

Table 3: Summary and comparison of case studies on high pressure homogenization.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
High pressure homogenization						
Nannochloropsis salina	Anaerobic digestion and biogas from treated biomass	100 bar, 2 passes, 0.875% DCW	35 ml	32.6% increase in biogas production in comparison with untreated biomass	Biogas production	Schwede et al. (2011)
Chlorococcum sp.	Disrupted biomass	850 bar, 0.85% DCW, 4 passes	200 ml	Over 90% cell disintegration, 83% of colony diameter reduction after first pass	Intact cell count, average colony diameter measurement	Halim et al. (2012b)
				146.94 kWh/kg dry biomass energy consumption	Energy calculations by using the data from Halim et al. (2012b)	Lee et al. (2012)
Nannochloropsis oculata	Disrupted biomass, lipid	2760 bars, 0.1% (wet w/w) approx. 0.023–0.035% DCW cell concentration, 4 passes, nitrogen depleted culture	15 ml	67% cell disintegration, 8.5 times more oil extraction than undisrupted algae	Intact cell count, total lipid	Samarasinghe et al. (2012a)
Nannochloropsis oculata	Disrupted biomass	2100 bar, 0.15% (wet w/w) approx. 0.015–0.023% DCW, cell concentration, 100 µm Nozzle, 3 passes	15 ml	≈100% cell disintegration	Intact cell count	Samarasinghe et al. (2012b)
Nannochloropsis sp.	Protein	1500 bar, 1% DCW cell concentration, 6 passes, nitrogen depleted culture	250 ml	≈91% Protein extraction	Bradford protein analysis	Grimi et al. (2014)

Although HPH is, together with bead milling, the most preferred method for the industrial scale cell disruption of microalgae, there are some disadvantages. The main drawback of using HPH in the mild microalgae biorefinery is the use of low dry cell weight concentrations

(0.01–0.85% w/w). This increases the energy demand of downstream processing and water footprint due to isolation of products from dilute streams. Also the non-selective intracellular compound release, difficulties to break hard cell walls and the generation of very fine cell debris are among main problems of HPH. Finally, the reduced digestibility of proteins after treatment (Janczyk et al., 2005; Komaki et al., 1998) can indicate that HPH is not a mild technique and thus not suitable for the isolation of fragile functional compounds.

2.1.3. High speed homogenization

A high-speed homogenizer (HSH) is a stirring device at high rpm and usually consists of a stator–rotor assembly, preferably made of stainless steel, with a variety in designs of stators and rotors. The effective cell disruption mechanisms are hydrodynamic cavitation, generated by stirring at high rpm, and shear forces at the solid–liquid interphase. When the impeller tip speed reaches a critical value (8500 rpm), hydrodynamic cavitation occurs due to a local pressure decreases nearly down to the vapor pressure of the liquid (Kumar and Pandit, 1999; Shirgaonkar et al., 1998). Subsequently, as the liquid moves away from the impeller, the liquid pressure restores proportional to the decrease in velocity and the distance from impeller tip and causes the collapse of the cavities (Gogate, 2011). An overview of HSH case studies is given in Table 4 and the main characteristics for the mild microalgae biorefinery are discussed below.

High speed homogenization is the most simple, very effective, but aggressive cell disruption method. Advantages are short contact times and the potential to process suspensions with relatively high dry cell weight concentration (2–6% w/w) thus reducing the water footprint and downstream process costs. Additionally, with HSH increased extraction yields of different biochemicals were observed (Balasubramanian et al., 2013; González-Delgado and Kafarov, 2012; Guedes et al., 2013; Khoo et al., 2011; Wang and Wang, 2011). Unfortunately, the lowest energy consumption is 156.4% of the microalgae biomass' caloric value and protein denaturation due to shear induced local and bulk temperature increase make this method less favorable for mild microalgae biorefinery.

Table 4: Summary and comparison of case studies on high speed homogenization.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
High speed homogenization						
Nannochloropsis sp.	Lipid	10,000 rpm for 1 min, %6 DCW	≈16 ml	Wet extraction yield with high speed homogenization reached 75.8–78% of dry extraction yield	Total lipid analysis	Wang and Wang (2011)
Nannochloropsis sp.	Lipid	12,000 rpm, 1:50 (g/ml) biomass:solvent, 2% DCW	50 ml	%38 ± 2 (w/w) lipid extraction	Total lipid analysis	Balasubramanian et al. (2013)
Phaeodactylum tricornutum	Antioxidant	14,000 rpm, 30 s, 1:1 (v/v) EtOH (or MetOH)/water solvent, approx. 0.12% DCW	5 ml	EtOH: ≈30 mg equivalent ascorbic acid/l antioxidant activity MetOH: ≈22.5 mg equivalent ascorbic acid/l antioxidant activity	Total intracellular antioxidant determination (ascorbic acid equivalent)	Guedes et al. (2013)
Pavlova lutheri		14,000 rpm, 30 s, 1:1 (v/v) EtOH (or MetOH)/water solvent, approx. 0.36% DCW		EtOH: ≈22.5 mg equivalent ascorbic acid/l antioxidant activity MetOH: ≈20 mg equivalent ascorbic acid/l antioxidant activity		

2.1.4. Ultrasonication

During an ultrasonic treatment, the energy of high frequency acoustic waves initiates a cavitation process and a propagating shock wave forms jet streams in the surrounding medium causing cell disruption by high shear forces (Chisti and Moo-Young, 1986; Mendes Pinto et al., 2001). Numerous designs for ultrasonic systems (Fig. 5) are available for different purposes such as micro/nano emulsion production, cell disruption and product extraction. For bacterial cell disruption, ultrasonic disrupters operating at 20, 40 kHz and 1 MHz are proposed (Chandler et al., 2001; Fykse et al., 2003; Taylor et al., 2001), but nowadays only large scale 18, 20, 24, and 30 kHz ultrasonication devices are in use due to energy consumption concerns. In literature the specific energy consumption ranges from efficient disruption with 0.06 kWh/kg (Hielscher, 2011) over inefficient disruption with 36.67 kWh/kg (Halim et al., 2012b) to efficient disruption with 100 kWh/kg (Lee et al., 2010). The lowest specific energy demand found in literature was provided by a device manufacturer and the only shared parameter of the process was the 15% DCW concentration of microalgae feedstock. To reduce the amount of energy needed for cell disruption, ultrasonic vibration is frequently combined with chemical cell disruption methods (Nanu et al., 2011; Prabakaran and Ravindran, 2011; Priego Capote and de Castro, 2007; Sheng et al., 2012; Sherrit et al., 1999; Thoe et al., 1998). In literature the direct effect of ultrasonication on solubilization and conversion of biochemicals is also studied. The positive effect on soluble chemical oxygen demand, and nutritional value, the insignificant effect of lipid solubilization and conversion to fermentable sugars and negative effect on monodigestion determined by different studies (Janczyk et al., 2005; Lee et al., 2010; Miranda et al., 2012; Schwede et al., 2011; Sheng et al., 2012). An overview of case studies is given in Table 5.

Several forces are behind the mechanism of ultrasonic cell disruption. Ultrasonic vibrations from the emitting tip result in acoustic cavitation that can disrupt cells as discussed in the High pressure homogenization section, but cavitation also results in thermolysis of water around the bubbles forming highly reactive free radicals (H•, HO•, and HOO•) (Riesz et al., 1985) that react with the substances in water. Bubble implosion and fragmentation during acoustic cavitation produce micro-regions of extreme conditions with estimated temperatures as high as 5000 °C and pressures up to 100 MPa. During treatment, the sample temperature can increase significantly with 50 to 90 °C (Borthwick et al., 2005; Chandler et al., 2001; Taylor et al., 2001; Zhang et al., 2007) and destroy proteins and other intracellular metabolites (Borthwick et al., 2005; Gogate and Pandit, 2008; Sartory and Grobbelaar, 1984; Schumann et al., 2005; Suslick, 1990). According to Gogate and Pandit (2008) the mechanical mechanisms resulting from the intense turbulence associated with liquid circulation currents (Luche, 1999; Mason and Lorimer, 1988; Mason and Lorimer, 2002) are more effective on the ultrasonic cell disruption yield than the chemical changes such as the formation of free radicals. The formation of free radicals, however, is the main cause according to Zhang and Hua (2000) and Zhang et al. (2007). The major drawback of ultrasonication of microalgae biomass is the relatively low cell disruption efficiency for some microalgae species

and the local and overall heat production. Temperature control during treatment can improve product quality, however, the effectiveness of cell disruption decreases significantly (Sheng et al., 2012). The possibility of combining ultrasonication with different solvent systems or other disruption methods to increase the efficiency and decrease the energy demand, remains interesting for the mild microalgae biorefinery concept.

Table 5: Summary and comparison of case studies on ultrasonication.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
Ultrasonication						
Stichococcus sp. *Chlorella* sp.	Chlorophyll a	70 W, 90 s 3 cycles with 5 min breaks	3 ml	Local heat caused degradation of chlorophyll a	Chlorophyll a	Schumann et al. (2005)
Scenedesmus dimorphus *Chlorella protothecoides*	Lipid	100 W, 2 min, 2 cycles	15 ml	Lipid recovery 21 wt.% No considerable difference in comparison with methods Lipid recovery 10.7 wt.% Considerable difference in comparison with methods	Total lipids, dry weight	Shen et al. (2009)
Botryococcus sp. *Chlorella vulgaris* *Scenedesmus* sp.	Lipid	10 kHz, 5 min, 0.5% DCW	100 ml	Lipid recovery 8.8 wt.% Considerable difference in comparison with methods Lipid recovery 8 wt.% No considerable difference in comparison with methods Lipid recovery 9 wt.% No considerable difference in comparison with methods	Total lipid, fatty acid composition	Lee et al. (2010)
Nannochloropsis salina	Anaerobic digestion and Biogas from treated Biomass	200 W, 45 s, 30 kHz	Analytical, volume not given	21% decrease in biogas production in comparison with untreated biomass	Biogas production	Schwede et al. (2011)
Chlorella vulgaris	Lipid	600 W, 30 s 34 cycles with 5 second breaks	Laboratory (>50 ml), volume not given	5.11 fold more extraction than untreated cells	Total lipid	Zheng et al. (2011)
Chlorella sp. *Nostoc* sp. *Tolypothrix* sp.	Lipid	50 kHz, 15 min, 0.5% DCW	100 ml	2.625 fold more extraction than untreated cells 2.57 fold more extraction than untreated cells 3.625 fold more extraction than untreated cells	Total lipid	Prabakaran and Ravindran (2011)
Chlorococcum sp.	Disrupted biomass	130 W, 5 min, 5 cycles, 0.85% DCW	200 ml	Nearly no cell disruption, ≈70% of colony diameter reduction after 3rd cycle 36.67 kWh/kg dry biomass energy consumption	Intact cell count, average colony diameter Energy calculations by using the data from Halim et al. (2012b)	Halim et al. (2012b) Lee et al. (2012)
Scenedesmus obliquus	Fermentable sugars	200 W, 30 s 5 cycles with 10 min breaks, approx. 7–10% DWC	5 ml	Complex sugars were converted to fermentable sugars, yield: 0.025 equal gram of glucose/gram biomass	Total sugars, monosaccharides	Miranda et al. (2012)
Synechocystis PCC 6803	Lipid	2 kW, 3 min, 52 °C outflow temperature, approx. 0.2% DCW 2 kW, 30 s 15 cycles with 30 s breaks, 26 °C outflow temperature, approx. 0.2% DCW	Analytical, volume not given	27.8% (w/w) Lipid release, SCOD increase as much as 29.8% of total COD of biomass 14.77% (w/w) lipid release, SCOD increase as much as 6.7% of total COD of biomass	Total lipid, SCOD analysis	Sheng et al. (2012)

2.1.5. Microwave treatment

Microwave treatment at 2450 MHz is known as the optimal value for heating, drying and cell disruption (Vasavada, 1986). When a suspension is exposed to microwaves, the microwaves interact selectively with the dielectric or polar molecules (*e.g.*, water) and cause local heating as a result of frictional forces from inter- and intramolecular movements (Amarni and Kadi, 2010). The free water concentration in cells contributes to the microwave efficiency for cell disruption. Water exposed to microwaves reaches the boiling point fast resulting in expansion within the cell and an increase in the internal pressure (Chuanbin et al., 1998). The local heat and pressure combined with the microwave induced damage to the cell membrane/

wall, facilitates the recovery of intracellular metabolites (Choi et al., 2006; Rosenberg and Bogl, 1987). To distinguish the effect of microwaves from microwave induced temperature increase, the yield of microwave treatment compared to a regular heat treatment at the same temperature and 37.5–44.4% of total yield determined as related to microwaves (Balasubramanian et al., 2011). However, since only a fraction of the water is held inside the cells, the majority of the radiation energy is absorbed by the surrounding medium and lost as heat (Lee et al., 2012) causing protein aggregation and denaturation (Woo et al., 2000).

As shown in Table 6, the variations in species and the DCW (0.16– 7.6%) concentrations make a direct comparison of the specific energy consumption impossible. The potential of using high DCW concentrations compared to some other techniques is beneficial for the specific energy consumption. However, since the disruptive effect is mainly based on the absorption of microwave energy by water molecules and subsequently the formation of heat and radicals (Amarni and Kadi, 2010; Chuanbin et al., 1998), it can be derived that the effect of microwave treatment is higher on diluted suspensions in comparison with concentrated suspensions. Advantages of microwave treatment are effectiveness, even for robustness, and easy scaled-up (Balasubramanian et al., 2011) because of the simplicity of the technique (Lee et al., 2010). The temperature increase is more homogeneous compared to conventional heating, thus heat related denaturation occurs less readily (Pasquet et al., 2011). Depending on the microalgae species microwave treatment is even more efficient than both ultrasonication and bead milling (Prabakaran and Ravindran, 2011; Zheng et al., 2011). Additionally, disruption can be combined with selective extraction (microwave assisted extraction, MAE) which is superior to ultrasonication and microwave heating in terms of speed, efficiency and protection against thermal denaturation (Balasubramanian et al., 2011; Pasquet et al., 2011).

Even though microwave assisted (extraction) processes have the potential to increase the extraction yield and decrease the amount of solvent, there are also numerous problems. The technique is limited to polar solvents and not suitable for volatile target compounds (Zheng et al., 2011). The formation of free radicals, temperature increase and chemical conversion could interfere with the recuperation of fragile functional compounds making microwave treatment less favorable for mild microalgae biorefinery as a cell disruption method.

Table 6: Summary and comparison of case studies on microwave treatment.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
Microwave treatment						
Chlorella sp.	Lipid	2450 MHz, 100 °C, 5 min, 0.5% DCW	100 ml	2.25 fold more extraction than untreated cells	Total lipid	Prabakaran and Ravindran (2011)
Nostoc sp.				2.21 fold more extraction than untreated cells		
Tolypothrix sp.				5.33 fold more extraction than untreated cells		
Chlorella vulgaris	Lipid	2450 MHz, 100 °C, 5 min	Laboratory (>250 ml), Volume not given	3.875 fold more extraction than untreated cells	Total lipid	Zheng et al. (2011)
Synechocystis PCC 6803	Lipid	1.4 kW, 57 °C, 1 min	Analytical, Volume not given	1.13 fold more extraction than untreated cells, SCOD increase as much as 14.5% of total COD of biomass	Total lipid, SCOD analysis	Sheng et al. (2012)
		1.4 kW, 26 °C, 30 s treatment 30 s pause		1.05 fold more extraction than untreated cells, SCOD increase as much as 4.4% of total COD of biomass		
Scenedesmus obliquus	Lipid	1.2 kW, 2450 MHz, 7.6% DCW, 95 °C, 30 min	Laboratory (>50 ml), Volume not given	77% of recoverable oil (1.64 fold of only heating method) extracted	Total lipid, lipid composition	Balasubramanian et al. (2011)
Botryococcus sp.	Lipid	100 °C, 2450 MHz, 5 min, 0.5% DCW	100 ml	28.6% (w/w) Lipid extraction	Total lipid	Lee et al. (2010)
Chlorella vulgaris				10% (w/w) Lipid extraction		
Scenedesmus sp.				10.4% (w/w) Lipid extraction		
Dunaliella tertiolecta	Pigments	56 °C, 50 W, 5 min, 0.16% DCW in acetone	30 ml	≈4.5 µg/l Chlorophyll-a, ≈1.4 Chlorophyll-b, ≈1.3 β,β-carotene extraction	Pigment analysis	Pasquet et al. (2011)
Cylindrotheca closterium				≈4.9 µg/l Chlorophyll-a, ≈3.8 fucoxanthin extraction		

2.1.6. Pulsed electric field treatment

Pulsed electric field (PEF) or high intensity electric field pulse (HELP) uses an external electric field to induce a critical electrical potential across the cell membrane/wall. Cell disruption by PEF is caused by electromechanical compression and electric field-induced tension inducing pore formation in the membrane/wall (electroporation) (Barbosa Cánovas et al., 1999; Ho and Mittal, 1996; Tsong, 1990; Weaver and Chizmadzhev, 1996; Zimmermann et al., 1985). The size and number of the pores is directly related to the electric field strength and pulses. It has been demonstrated that pore formation can be reversible or irreversible (Rols et al., 1990; Tsong, 1990; Weaver et al., 1988). Reversible cell membrane/wall damage occurs if the total area of induced pores is small in comparison to the total surface area of the membrane/wall. On the other hand, if the ratio of total pore area to total membrane/wall area exceeds a certain limit as a result of a process at relatively higher field strength, the membrane/wall is no longer able to repair itself and is irreversibly damaged.

PEF does not only destroy the cell wall, but also affects the molecules inside the cells. Though temperature increase is not the mechanism of cell disruption, the increase in bulk temperature during treatment leads to a reduced nutritional value and protein digestibility (Janczyk et al., 2005), the decomposition of fragile compounds (Sheng et al., 2011) and an increased extraction of lipids (Eing et al., 2013; Sheng et al., 2012; Zbinden et al., 2013) and proteins (Coustets et al., 2013). The specific energy demand, calculated with literature data, strongly depends on the concentration of the suspension and ranges from 0.42 kWh/kg for 10% DCW (Eing et al., 2013) to 239 kWh/kg for 0.03% DCW (Sheng et al., 2011, 2012). An overview of the case studies is given in Table 7.

Table 7: Summary and comparison of case studies on pulsed electric field.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
Pulsed electric field						
Synechocystis PCC 6803	Lipid	59.67–239 kWh/kg, 36–54 °C outflow temperature, 0.03% DCW	Analytical, volume not given	DW loss % 1.37–% 9.54, Reduced solvent need for lipid extraction	Cell viability, Total lipids, Lipid composition	Sheng et al. (2011)
Synechocystis PCC 6803	Lipid	120 kWh/kg, 46 °C outflow temperature, 0.037% DCW 120 kWh/kg, 36 °C outflow temperature, 0.037% DCW	Analytical, volume not given	Extraction similar to untreated cells, SCOD increase 4.9% 1.09 fold more extraction than untreated cells, SCOD 1.4%	Total lipid, SCOD analysis	Sheng et al. (2012)
Nannochloropsis salina	Protein	15.44–30.89 kWh/kg, 37 °C outflow temperature, 0.0545–0.109% DCW	1.08 ml	4 fold more extraction with water than methanol extraction of untreated cells	Bradford total protein, SDS-PAGE	Coustets et al. (2013)
Chlorella vulgaris		2.3 kWh/kg, 37 °C outflow temperature, 0.73% DCW				
Auxenochlorella protothecoides	Lipid	0.42–0.63 kWh/kg, 10% DCW	2.112 ml	Over 3 fold more extraction with ethanol	Water soluble dry dontents, Carbohydrate, Lipids	Eing et al. (2013)
Ankistrodesmus falcatus	Lipid	5.8 kWh/kg, 0.19% DCW	4 ml	Over 2 fold more extraction with ethyl acetate-methanol	Microscopic investigation, Total lipids, FAME analysis	Zbinden et al. (2013)

Pulsed electric field can be scaled-up easily and combined with different biomass treatment methods. However, the solution, which will be treated, must be free of ions, *i.e.*, electrically non-conductive, thus limiting the use of this cell disruption method in mild microalgae biorefineries. PEF treatment of marine microalgae would require prewashing and deionization to increase the electrical resistance of the medium surrounding the cells. Additionally, the energy consumption and cell disruption yield vary dramatically related to the medium composition. For example, the increased conductivity associated with the release of compounds from disrupted microalgal cells causes local temperature increases and subsequently a decrease in cell disruption efficiency. The decrease in disruption efficiency due to the release of intercellular compounds makes this technique less suitable for the mild microalgae biorefinery.

2.2. Non-mechanical methods

Non-mechanical methods often involve cell lysis with chemical agents, enzymes or osmotic shock (Agerkvist and Enfors, 1990; Chisti and Moo-Young, 1986; Lee et al., 1998; Lee et al., 2010; Middelberg, 1995; Mutanda et al., 2011). These methods are perceived as more benign than mechanical processes since cells are often only perforated or permeabilized rather than being shredded. For example, chemical and enzymatic methods rely on selective interaction with the cell wall or membrane components that modifies the cell boundary layer and allows products to leach (Middelberg, 1995; Vogels and Kula, 1992). An overview of case studies for non-mechanical methods is given in Table 8.

2.2.1. Enzymatic cell lysis

Enzymatic lysis is an excessively studied cell disruption method due to its biological specificity, mild operating conditions, low energy requirements, low capital investment, and the prevention of aggressive physical conditions such as high shear stress (Andrews and Asenjo, 1987; Harrison, 1991). Since the discovery of lysozyme, many researchers have contributed to the understanding of the mechanisms and other basic aspects of lytic enzymes (Salazar and Asenjo, 2007). Glycosidases, glucanases, peptidases and lipases are the main enzyme classes that have been investigated for cell lysis of different microorganisms. During lysis, enzymes bind to specific molecules in the cell membrane/wall to hydrolyze the bonds resulting in cell membrane/wall degradation (McKenzie and White, 1991).

Enzymatic treatments on microalgae have been tested in function of lipid extraction (Zheng et al., 2011) and the conversion of biomass into biogas through the hydrolysis of polysaccharides, *i.e.*, hemicellulose and saccharides of cell wall, and subsequent fermentation (Choi et al., 2010; Fu et al., 2010; Harun and Danquah, 2011a). The main parameter influencing the disruption yield in enzymatic processes is the type of enzyme (Zheng et al., 2011) because of the specificy of the mechanism. The type of enzyme also largerly determines process costs and therefore enzyme immobilization (Fu et al., 2010) could be a solution to allow the implemention of large scale processes. In contrast to the specificy, other high value products can be converted resulting in the loss of valuable end products such as astaxanthin due to oxidation (Mendes Pinto et al., 2001). Working in anaerobic conditions would require process adaptations, but could be a solution to prevent product degradation. Because of a strong dependancy between the concentrations of the reagents, *i.e.*, reactive bonds in the biomass and the activity and amount of enzymes, finding a good balance between the amount of biomass and (immobilized) enzymes is needed for an economically interesting process. Increasing DCW concentration up to 1% enhances the cell disruption efficiency (Harun and Danquah, 2011a) but further increase between 2 and 6 % results in a decreased efficiency (Fu et al., 2010; Harun and Danquah, 2011a). Depending on the type of enzyme, other parameters like temperature, pH, salt concentration and the biomass lipid content can cause significant changes in the disruption efficiency (Fu et al., 2010; Harun and Danquah, 2011a).

Table 8: Summary and comparison of case studies on enzymatic lysis and chemical treatment.

Micro-algae	Product	Conditions	Scale	Outcome	Analyses	Reference
Enzymatic treatment						
Chlamydomonas reinhardtii UTEX 90	Dextrin	Thermostable α-amylase 0.005%, 90 °C, 30 min	Laboratory, volume not given	25.21 g/l dextrin	Dextrin	Choi et al. (2010)
Haematococcus pluvialis	Astaxanthin	0.1% protease K and 0.5% driselase, 1 h, pH 5.8, 30 °C	Laboratory, volume not given	1.65 fold more extraction than untreated cells	Astaxanthin, total carotenoids	Mendes Pinto et al. (2001)
Chlorella vulgaris	Lipid	Cellulase, 10 h, pH 4.8, 55 °C, 5 mg/l enzyme Lysozyme, 10 h, 55 °C, 5 mg/l enzyme Snailase, 2 h, 37 °C, 5 mg/l enzyme	Analytical, volume not given	8.1 fold more extraction than untreated cells 7.46 fold more extraction than untreated cells 2.366 fold more extraction than untreated cells	Total lipid	Zheng et al. (2011)
Chlorella pyrenoidosa	Carbohydrates from cellulose, lipids	Cellulase, 24 h, pH 4.6, 50 °C, 140 mg/m^2 enzyme, 2% DCW	15 ml	62% cellulose hydrolysis, 75% increaset in lipid extraction	Total carbohydrates, reducing sugar, immobilized enzyme content, FAME analysis	Fu et al. (2010)
Chemical treatment						
Haematococcus pluvialis	Astaxanthin	0.1 M HCl, 15–30 min 0.1 M NaOH, 15–30 min	Laboratory, volume not given Laboratory, volume not given	2.65 fold more extraction than untreated cells 1.8–2.2 fold more extraction than untreated cells	Astaxanthin, total carotenoids	Mendes Pinto et al. (2001)
Chlorococcum humicola	Fermentable sugars	0.56 M (v/v) H_2SO_4, 160 °C, 15 min	Laboratory, volume not given	Complex sugars were converted to fermentable sugars, 0.52 g ethanol fermentation from treated microalgae biomass	Carbohydrates, ethanol	Harun and Danquah (2011b)
Chlorococcum infusionum	Fermentable sugars	0.3 M NaOH, 120 °C, 60 min, 5% DCW	Laboratory, 100 ml	Complex sugars were converted to fermentable sugars, 0.26 g ethanol fermentation for per gram treated microalgae biomass	Ethanol, glucose, cell size	Harun et al. (2011)
Chlorococcum sp.	Fermentable sugars	1.51 M H_2SO_4, 160 °C, 45 min, 0.85% DCW	Laboratory, volume not given	Proteins and pigments were destroyed. Complex sugars were converted to fermentable sugars	Intact cell count, average colony diameter	Halim et al. (2012b)
Scenedesmus obliquus	Fermentable sugars	1 M H_2SO_4, 120 °C, 30 min, 10% DCW	Laboratory, 5 ml	Complex sugars were converted to fermentable sugars, yield: 0.286 equal g of glucose/g biomass	Total sugars, monosaccharides	Miranda et al. (2012)

Generally, an enzymatic treatment is gentle, has a high selectivity and scale-up is relatively easy. Compared to microwave and ultrasonication, an enzymatic treatment can even result in a better lipid extraction yield (Zheng et al., 2011). However, some drawbacks affecting the efficiency of a disruption process, are long process times, thus a low production capacity compared to mechanical or chemical disruption, and product inhibition (Harun and Danquah, 2011a). Today the main limitations of using enzymes in the biorefinery are their high cost and the fact that not many enzymes are suitable for algae disruption. Enzyme immobilization could lower the needed amount of enzymes and additionally reduce the downstream process costs since separation of the enzymes from the products would be avoided. Since the effectiveness of the same enzyme differs for different microalgae and reaction times are generally long, the technique could potentially be improved by combining specific (immobilized) enzymes with other mechanical techniques.

2.2.2. Chemical cell disruption

Cell disruption can be caused by a large variety of chemical compounds such as antibiotics, chelating agents, chaotropes, detergents, solvents, hypochlorites, acids and alkali. The selectivity, suitability and efficiency of these compounds are dependent on the cell wall composition of the microorganism (Middelberg, 1995). As all of the chemical substances disrupt the cells differently, there are several mechanisms of chemical cell disruption. Antibiotics usually inhibit the production of cell membrane components, while chelating agents bind the cations that cross-bridge adjacent cell membrane molecules and chaotropes make the

surrounding medium less hydrophilic. Detergents form micelles together with membrane molecules while solvents dissolve or perforate cell membrane/wall. Bases saponify the membrane lipids while acids lead to poration of the cell membrane/wall (Halim et al., 2012b; Harun and Danquah, 2011b; Harun et al., 2011; Mendes Pinto et al., 2001; Middelberg, 1995; Miranda et al., 2012).

Chemicals such as surfactants and oxidizing chemicals (*e.g.*, surfactant, ozone, chlorine, UV-B) are already used to inhibit eutrophication. There are several studies on cell disruption of microalgae with these agents (Cheng et al., 2010; Ebenezer et al., 2012; Glembin et al., 2013; Huang and Kim, 2013; Hung and Liu, 2006; Miao and Tao, 2009; Pavlić et al., 2005; Ulloa et al., 2012), however, the product quality is highly affected by either oxidation (Phe et al., 2005; Saby et al., 1997; Virto et al., 2005) or disruption agent contamination. Some emerging technologies related to these chemicals, such as pressure-assisted ozonation (PAO) or advanced oxidation processes (AOP) and peroxone treatment, exist (Huang et al., 2014; Nguyen et al., 2013), however, they are not discussed in detail in this review because they are not considered as mild for use in the future biorefinery because of their effect on product quality.

2.2.1.1. Solvent induced cell disruption. The use of solvents in literature on the microalgae biorefinery is mainly focused on the extraction of specific biochemicals, *e.g.*, astaxanthin and c-phycocyanin. Some research however combines extraction with disruption (Benavides and Rito-Palomares, 2006; Benavides et al., 2008; Cisneros et al., 2004; Kang and Sim, 2007, 2008) or with cultivation in aqueous two phase systems (Hejazi and Wijffels, 2004; Hejazi et al., 2004; Jin Young et al., 2004; Kleinegris et al., 2011b; León, 2003; Mojaat et al., 2008). Kleinegris et al. (2011a) showed that cell death was the mechanism of this extraction process which can affect the cell growth rate in recultivation of treated batches (Jin Young et al., 2004). Additionally, direct contact of biomass with the organic phase led to aggregation and the high concentration of cell fragments resulted in a difficult phase separation and contamination in recultivation (Jin Young et al., 2004).

The data of solvent induced disruption is not displayed in Table 8 due to the lack of specific cell disruption data. Despite this, it can be concluded that cell disruption with aqueous two-phase systems has potential for the biorefinery as a relatively mild and selective extraction/cell disruption step. However, there are still some problems concerning efficiency, toxicity and economic feasibility, and knowledge on the cell disruption characteristics is incomplete.

2.2.1.2. Acid & alkali treatment. Acid treatment has been applied to various microalgae biomasses. Acid treatments at high temperatures (≈160 °C) generally lead to a higher degree of cell disruption than the same treatments at lower temperatures (≈120 °C) (Halim et al., 2012b; Harun and Danquah, 2011b; Mendes Pinto et al., 2001; Miranda et al., 2012).

Harun et al. (2011) and Mendes Pinto et al. (2001) showed that the average particle size in alkali-treated samples is decreased. Many small sized cell fragments were formed and microscopic studies revealed a less distinct cell wall structure indicating an effective cell disruption. However, high temperatures (120 °C) are needed and alkali induced protein denaturation (Molina-Grima et al., 2003) making this technique less favorable for mild microalgae biorefinery.

For the recovery of unstable or fragile molecules, mild temperatures and relatively low concentrations of chemicals are preferred. If the chemical treatment could be combined with other techniques for cell disruption, the resulting mild process would be more suitable for the microalgae biorefinery. However, the effect of acid and alkali on cell constitutes, such as denaturation effect of alkali on proteins and degradation of pigments by acid, are the problems that should be overcome before applying acid and alkali treatment in mild microalgae biorefineries.

2.3. New developments

New developments as well as new technologies in microalgae cell disruption are emerging rapidly including explosive decompression, atomic force microscopy, laser treatment, microfluidizer, pulsed arc, high frequency focused ultrasonication and cationic polymer coated membranes. Studies related to the aforementioned methods are described below.

Dierkes et al. (2012) studied explosive decompression with CO_2, propane or butane of a 18.11% DCW suspension of *Haematococcus pluvialis* for simultaneous cell disruption and lipid and astaxanthin extraction. As a result, 72.3–92.6% of astaxanthin and 80–100% of lipid was extracted. Because of the high DCW concentration in the influent thus lowering the energy consumption per kg of disrupted biomass, high extraction yields due to the effectiveness of the technique, mild temperature and the use of gaseous products instead of liquid, often aggressive chemicals, explosive decompression is a promising technique for the mild microalgae biorefinery. McMillan et al. (2013) used laser technologies to disrupt 30 μl of cell suspensions and showed that the method in analytical scale results in rapid cell disruption in comparison with other cell disruption techniques. The disruption is induced by high temperature and high shear and because this technique is not scalable, laser treatment in this form is not a potential disruption method for mild microalgae biorefinery.

A microfluidizer is a high shear fluid processors that functions as a high pressure homogenizer in which large pressure differences accelerate cell suspension. The difference with the high pressure homogenizers is the generation of hydrodynamic impact by branching the flow and reconnecting the branches instead of using impact walls. This reduces cavitation making the method more mild than high pressure homogenization (Microfluidics Corp., 2012). The cells are disrupted by shear and by explosive decompression of the gas entrapped in the sample liquid. Overall, the microfluidizer needs a higher pressure thus more energy

to disrupt the cells in comparison to explosive decompression. Because of the possibility of treating high DCW concentration samples, no solvent usage, mild temperature and the potential for up-scaling, microfluidization is a promising technique for mild microalgae biorefinery.

Boussetta et al. (2013) studied pulsed arch technology (*i.e.*, pulsed electric discharge) to disrupt grape seeds. Although this technique was not studied on microalgae, it is included as an emerging technology. By using high amplitude electricity discharges for short time courses (in μs level), big cavities are produced causing extreme pressure and temperature differences as well as extreme shear stress. The energy requirement was calculated as only 1/47 of the energy needed to disrupt grape seeds with PEF. Although this technology is one of the most aggressive cell disruption technologies studied, it has the potential to be modified to a milder cell disruption method, since it has low sample-electric charge interaction time and temperature/shear effects could be reduced by modifying the vessel design.

Wang et al. (2014) compared high frequency focused ultrasonication (3.2 MHz, 40 W) with the conventional ultrasonication (20 kHz, 100 W) with *Scenedesmus dimorphus* (UTEX 417) and *Nannochloropsis oculata* (UTEX 2164) as model microalgae. The data showed that for the same cell disruption efficiency, high frequency focused ultrasonication had a higher energy efficiency. Additionally, both techniques can be used in series to acquire a better cell disruption efficiency.

Yoo et al. (2014) used a very gentle method wherein a membrane with a coated cationic polymer disturbs the local electrostatic equilibrium of the amphiphilic microalgae cell membrane caused by direct contact with the tertiary-amine cations. The microalgal culture was simply shaken together with the membrane resulting in the bursting of the cells.

3. COMPARISON

The methods of cell disruption were discussed individually per method in the 2. Cell disruption section. To evaluate the differences, a comparison of specific parameters is discussed in the following paragraphs. The methods are compared qualitatively based on their working mechanism, efficiency, product quality, process parameters, energy demand, and scalability. The main characteristics with a focus on industrial applicability are summarized in Table 9.

3.1. Mechanism of cell disruption

When the methods are evaluated based on the mechanism of cell disruption, it is clear that the best known mechanical cell disruption techniques such as bead milling and high speed homogenization are using solid–liquid interfacial shear forces which have a high cell disruption efficiency. The cell disruption during bead milling, high pressure homogeniza-

tion and high speed homogenization is explained by fluid and solid–liquid interfacial shear forces while energy transfer through waves or currents causes the effects in ultrasound, pulsed electric field (PEF) and microwave treatments. Other techniques apply energy beams directly such as laser or pulsed arc induced cell disruption. Some energy transfer types such as microwave, laser, PEF or heat allow longer transfer distances in comparison with techniques based on cavitation, such as bead milling, high pressure homogenization, high speed homogenization, ultrasonic treatment and pulsed arc. Nonmechanical methods are based on chemical interactions of organic (*e.g.*, enzymes, organic solvents) or inorganic (alkaline, acid treatment) molecules with the molecules of the cell membrane/wall with or without thermal energy transfer.

Table 9: Comparison of cell disruption methods in terms of key aspects.

Disruption method	Mildness	Selective product recovery	Optimum DCW concentration	Energy consumption	Practical scalability	Repeatability
Bead milling	Yes/no	No	Concentrated	High/medium	Yes	High
High pressure homogenization	Yes/no	No	Diluted/concentrated	High/medium	Yes	High
High speed homogenizer	No	No	Diluted	High/medium	Yes	High/medium
Ultrasound	Yes/no	No	Diluted	Medium/low	Yes/no	Medium
Microwave	Yes/no	No	Diluted	High/medium	Yes/no	Medium
Enzymatic lysis	Yes	Yes	Diluted	Low	Yes	High
Chemical treatment	Yes/no	Yes	Diluted/concentrated	Medium/low	Yes	High
Pulsed electric field	Yes/no	No	Very diluted/diluted	High/medium/low	Yes/no	Medium

3.2. Effect on product quality

During cell disruption, the aforementioned forces cause phenomena such as cavitation, temperature and pressure changes, molecular energy variations, production of free radicals, solid shear, interfacial shear and/or hydrodynamic shear as mentioned in the 2. Cell disruption section. These phenomena can occur individually or together and can affect the final product quality through degradation of the algal constituents and/or the formation of impurities. In cavitational methods, all the phenomena occur together and cause extreme temperature, pressure variation, high shear rate and formation of free radicals, which highly effect product quality and extraction efficiency. Thomas and Geer (2010) reviewed the effect of shear on proteins and concluded that hydrodynamic shear alone is not causing proteins denaturation, but interfacial shear stress (air–liquid or solid–liquid) was identified as the effective and predominant mechanism for disruption and protein denaturation. Thus cell disruption methods that depend on interfacial shear stress such as HSH, HPH and US can cause damage to the proteins (Thomas and Geer, 2010). Additionally, cavitation during HPH, HSH and ultrasonication can lead to the formation of free radicals causing oxidation which highly effects product quality (Luche, 1999; Mason and Lorimer, 1988; Mason and Lorimer, 2002; Riesz et al., 1985; Zhang and Hua, 2000; Zhang et al., 2007). Product quality, however, is mainly measured indirectly via for example digestibility (Komaki et al. (1998) for HPH and Janczyk et al. (2005) for PEF).

In contrast to single cavitation source methods such as HSH, HPH and ultrasonication, cavity formation is more uniform in bead mills resulting in more homogeneous cell disruption. During cell disruption with energy waves, the molecular energy and temperature rise. For

example, PEF causes energy variation in adherent cell membrane/wall molecules, resulting in electroporation of the cell membrane/wall and microwave treatment increases the energy of intra- and extracellular water molecules resulting in cell disruption through the expansion of intracellular water. Molecular energy variations may cause radical formation and unwanted reactions such as oxidation which might reduce product quality. Chemical and enzymatic cell disruption may form unwanted side products that can be present in the end product together with the reagents requiring specific downstream processing steps. The effect of cell disruption on product quality is also related to the morphology and biochemical composition of microalgae. Komaki et al. (1998) investigated the effect of cell disruption on digestibility with 3 different strains of *Chlorella vulgaris* and showed that the digestibility of one strain remained unchanged while for the two others a significant decrease was observed. Overall, the effect of cell disruption on product quality can be a direct indicator of the mildness of the cell disruption process. Next to product quality, the selectivity of a disruption process has an impact on product quality and the downstream process steps. Selectivity in the context of cell disruption is referred to as targeting specific cell compounds. With respect to algae cell disruption, only chemical treatment and enzymatic lysis are described as selective in the state of the art.

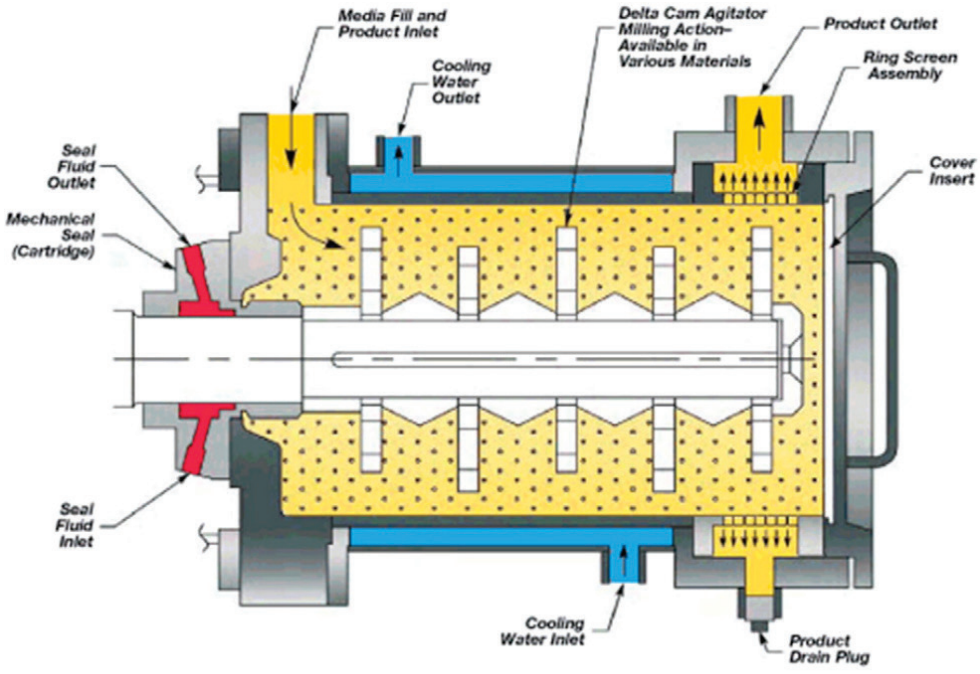

Fig. 3: Bead mill. Available from: AGT-Mining [Internet], 2014 http://www.agt.cl/mining/doc/6_dmq_mill.pdf.

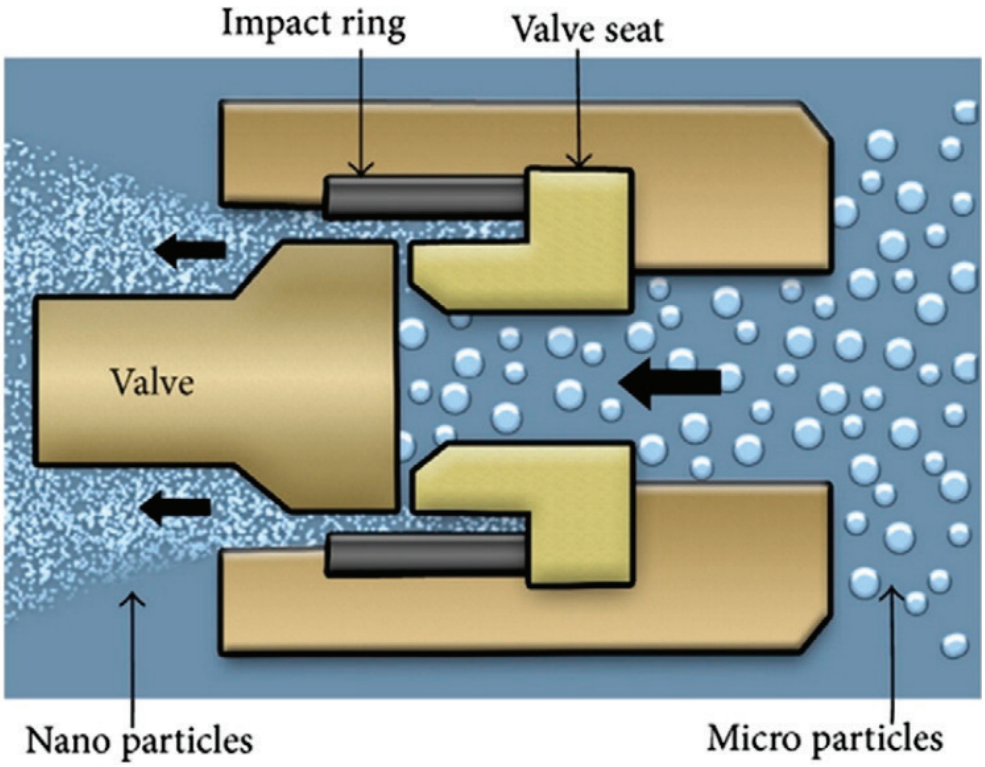

Fig. 4: High pressure homogenizator (Sutradhar et al., 2013).

3.3. Specific energy consumption (kWh/kg dry biomass)

The evaluation of literature data on energy consumption and cost effectiveness of the different cell disruption techniques is economically relevant and more straightforward and accurate than comparing cell disruption efficiencies or the increase of biomass utilization (*e.g.*, extraction efficiency, digestibility, mono-digestibility). To allow comparison between the different techniques, in this review the specific energy consumption (kWh/kg) was calculated as total energy consumed (kWh) to disrupt 1 kg of dry microalgae biomass (= consumed energy / (treated biomass ∗ cell disruption yield)). However, the comparison is only valid if similar conditions are being used (microalgae strain, stage of growth, fermentation system, DCW concentration etc.). Therefore, the comparison is more indicative than quantitative.

For the non-mechanical methods, energy consumption is changing proportionally to treatment time, temperature and stirring. The settings of these parameters, and therefore the energy consumption, are related to the type of disruption agent, cell properties, morphology, fermentation conditions (*e.g.*, open pond, photobioreactor, temperature, medium) and stage of growth. Generally, for mechanical methods, the energy consumption and cost effectiveness are influenced by process parameters, DCW concentration, the scale, type of microalgae, fermentation conditions (*e.g.*, open pond or photobioreactor) and stage of growth, whereof the dry cell weight concentration has a strong influence. To allow comparison between continuous and batch mechanical processes, the biomass treatment rate, kg of dry biomass disrupted (effluent) or treated (influent) per hour (kg/h) together with cell disruption yield, can be used which depend on dry cell weight concentration of the processed mixture (kg/L), flow rate (L/h) or batch volume and treatment time (h) as important parameters for industrial cell disruption. As shown from **Table 2** to Table 8, DCW concentrations between 0.015 and 15.8%, flow rates (bead milling) between 1.5 and 282.25 L/h and treatment times from milliseconds to hours were used in different cell disruption methods. PEF and ultrasonic treatment need relatively short treatment times (milliseconds to minutes), but use more diluted DCW (PEF = 0.03–0.2%; ultrasonic treatment = 0.2–0.85% DCW) while others, *i.e.*, enzymatic lysis and bead milling, need treatment times of minutes to days with higher DCW concentrations (enzymatic lysis = 1–2%; bead milling = 5–15% DCW). Additionally, the disruption efficiencies for bead mill, high pressure homogenizer and high speed homogenizer are positively affected by an increasing dry cell weight until a certain level (15–25%; see Bead milling, High pressure homogenization and High speed homogenization sections and references therein), while other methods such as microwave treatment (see the 2.1.5. Microwave treatment section) are negatively affected. Aggressive mechanical techniques, such as bead mill, high pressure homogenizer and high speed homogenizer, consume per method nearly the same amount of energy to process a unit of volume, independent whether the feed is diluted or concentrated. Hence, for these methods, processing higher DCW concentrations per unit of time is more cost effective. For example, Doucha and Lívanský (2008) reported that the energy consumption of bead milling can be reduced from 10.3 to 0.86 kWh/kg by changing the process parameters.

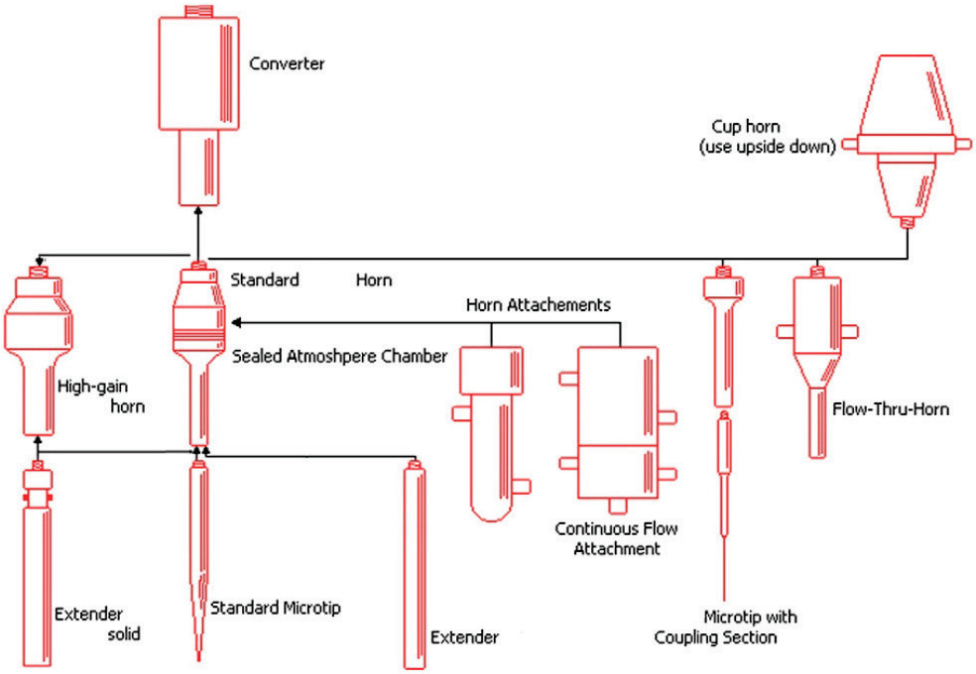

Fig. 5: Different designs of ultrasonic systems. Available from: Apollo Ultrasonics [Internet], 2014 http://www.apolloultrasonics.co.uk/sonifier.html.

Several authors studied the energy consumption of cell disruption techniques. An overview of the data is given in Table 10. The literature discussed below will show that energy consumption is highly related to both process and design parameters. This makes a universal comparison based on all studies and articles not possible since the data does not show any trends. Several authors compared different methods at low DCW concentrations, i.e., ultrasonication (Lee et al., 2012) and HPH (Halim et al., 2012b; Lee et al., 2012), bead milling and microwave treatment (Lee et al., 2010). According to these studies, high pressure homogenization has the highest energy consumption, followed by microwave treatment and ultrasonication. McMillan et al. (2013) studied the disruption of Nannochloropsis oculata cells without dewatering and compared microwave treatment, water bath, laser treatment and high speed homogenization. They concluded that microwave treatment is the most energy consuming method, followed by water bath, laser treatment, and high speed homogenization. Boer et al. (2012) used data from GEA (2011), Doucha and Lívanský (2008), Hielscher (2011) and Diversified Technologies (2010) and calculated the energy consumption of different commercial cell disruption methods at higher DCW concentrations. They concluded that bead milling consumes the most energy followed by high pressure homogenization, PEF and ultrasound. It must be noted that the data from the company websites (Diversified Technologies, 2010; GEA, 2011; Hielscher, 2011) is not verified by literature data. Balasubramanian et al. (2011) used comparable DCW concentrations as Boer

et al. (2012) and obtained an energy consumption of 10 times the value of HPH for solvent assisted microwave treatment.

In an attempt to evaluate trends in specific energy consumption (kWh/kg), some literature data on PEF and bead milling were plotted in Fig. 6 and Fig. 7 respectively. For continuous methods, the biomass treatment rate (kg/h), *i.e.*, biomass disrupted per unit of time, has a strong effect on the specific energy consumption. Unfortunately, this parameter could not be used for PEF due to lack of literature data on disruption efficiencies. However, PEF data was plotted in a logarithmic scale and a correlation was found with DCW concentration (Fig. 6). Since these data points originate from different types of microalgae, it appears that the energy consumption for PEF is less dependent on the type of microalgae. On the other hand, the specific energy consumption for ultrasonication showed no correlation with DCW concentration and was therefore not shown in Fig. 6. Apparently, the type of microalgae and growth conditions have a more profound effect on the energy efficiency of this disruption process. As shown in Fig. 7, the biomass treatment rate correlates well to the specific energy consumption of different laboratory/pilot scale bead milling equipment. More specific, two correlation curves are found that correspond to the installed power (kW) of the specific equipment used. Therefore, the energy consumption for bead milling is highly related to a design parameter, *i.e.*, installed power (kW), and the biomass treatment rate (kg/h). However, it should be noted that each equipment has its own optimal feed flow rate/biomass flow rate and DCW limitations since higher feed flow rate and DCW lead to a lower cell disruption efficiency. For example, a 3-fold increase of the flow rate of a 12.5% DCW feed to Fryma Koruma MS 18 led to a 42% decrease in cell disruption yield. For Dyno-Mill KDL-Pilot-A, on the other hand, a 12-fold flow rate increase led to a 10% the cell disruption yield decrease. Two DCW concentrations were tested for Netzsch, Labstar LS1. A 2-fold increase in biomass treatment rate resulted in a 22.9% decrease in cell disruption efficiency when the DCW concentration was 14.17%. For a 6.94% DCW microalgae suspension, a 3-fold increase in biomass treatment rate resulted in a decrease of only 3.7% in cell disruption yield.

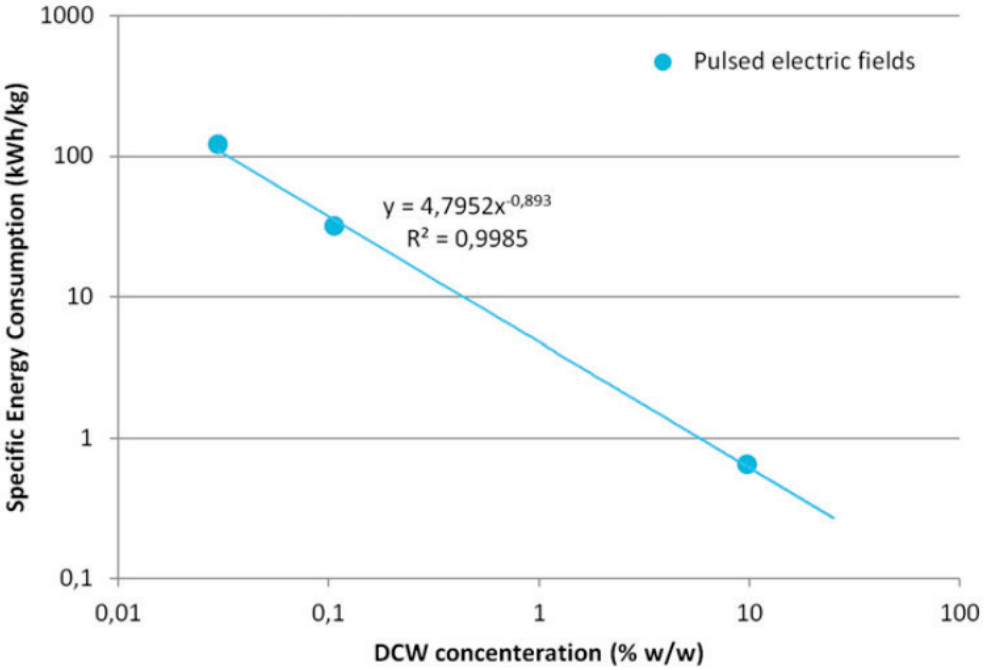

Fig. 6: The effect of DCW concentration on specific energy consumption of pulsed electric fields. Both the horizontal and vertical axis are in a logarithmic scale. Data from Sheng et al. (2011), Coustets et al. (2013) and Eing et al. (2013).

A recent study on the disruption of *Tetraselmis suecica* through atomic force microscopy by Lee et al. (2013) measured an energy consumption of 0.000187 kWh/kg on analytical scale. These experiments were performed to measure the actual energy needed for single cell disruption. The large difference between the energy need to disrupt cells directly on analytical and preparative scale demonstrates the influence of energy transfer. The energy consumption of non-mechanical methods is therefore relatively low compared to mechanical methods since disruption is not directly related to a mechanical or physical energy transfer. As mentioned before, comparing the energy consumption of cell disruption methods can be ambiguous. Nevertheless, different methods for one microalgae or one method for several microalgae are comparable. Additionally, numerous LCA studies on microalgae production highlight cultivation and dewatering/drying before extraction as major energy consumers within the overall process (*e.g.*, Razon and Tan, 2011; Xu et al., 2011; Soratana et al., 2014). Generally, energy and labor are the most predominant costs, thus cost effectiveness of a cell disruption method does not only depend on the energy consumption of the cell disruption process itself but also is the resultant of the overall energy demand, supplementary chemicals, labor, and other operational and capital expenditures. For example, an energy efficient cell disruption method that needs an extremely skilled person to be operated may not be cost effective because of the high labor costs.

Table 10: Comparison of cell disruption methods in terms of specific energy consumption.

Microalgae	DCW (%)	Cell disruption method	Specific energy consumption (kWh/kg disrupted biomass)	Reference
Chlorococcum sp.	0.85%	HPH	146.94	Lee et al. (2012), Halim et al. (2012b)
		Ultrasonication	36.67	Lee et al. (2012)
Scenedesmus sp.	7.5%	Solvent assisted microwave treatment	2.67	Balasubramanian et al. (2011)
Botryococcus sp., *Chlorella* sp., *Scenedesmus* sp.	0.5%	Microwave treatment	116.67	Lee et al. (2010)
Nannochloropsis oculata	0.14%	Microwave treatment	17.26	McMillan et al. (2013)
		Water Bath	4.67	
		Laser treatment	3.7	
		HSH	0.125	
Chlorella sp.	3.5%	Bead milling	10	Boer et al. (2012), Doucha and Lívanský (2008)
	15%	HPH	0.25	Boer et al. (2012), GEA, (2011)
Isocrysis sp.	25%	PEF	0.07	Boer et al. (2012), Hielscher (2011)
Species not given	15%	Ultrasound	0.06	Boer et al. (2012), Diversified Technologies (2010)

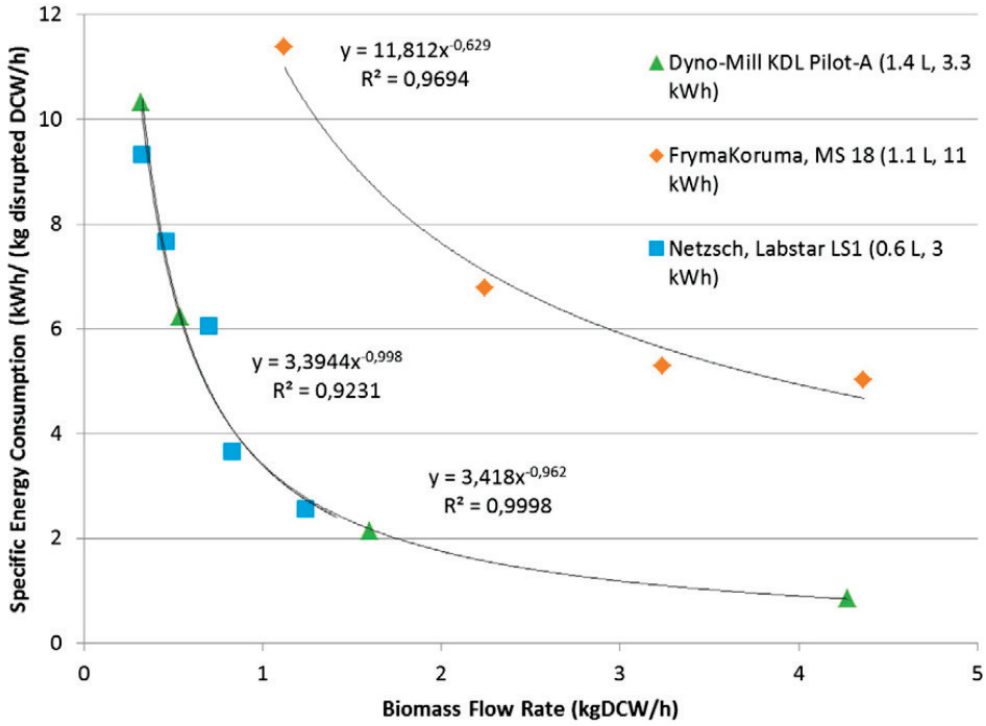

Fig. 7: The effect of biomass flow rate on specific energy consumption of bead milling with different equipment with similar grinding chamber volume and different dry cell weight concentrations (Doucha and Lívanský, 2008).

3.4. Practical scalability

Practical scalability of cell disruption methods is related to many characteristics, but cost effectiveness and product quality are the most important for implementation on large scale. Bead milling, highpressure homogenization, high speed, and ultrasonication treatments are the most frequently used mechanical methods on laboratory-scale for microalgal cell disruption. For industrial-scale applications, bead milling, HPH and HSH are considered as the most feasible methods (Chisti and Moo-Young, 1986; Harrison et al., 2003). Nowadays, other industrial scale cell disruption equipment is available on the market such as ultrasoni-

cation with 100 m^3/h (Hielscher, 2011) or PEF units with 10 m3/h (Diversified Technologies, 2010) capacities.

3.5. Gaps in data comparison

To build up a knowledge database to allow the selection of a suitable, scalable cell disruption method for mild microalgae biorefineries, comparable data is needed. As concluded from the comparisons, all the parameters (*e.g.*, microalgae type, growth conditions, process conditions) influence cell disruption efficiency and energy consumption. Considering the diversified data available in literature and from providers, it is currently not possible to select the best universal disruption technique for the future mild microalgae biorefinery. For example, LCA studies that calculate Capital Expenditures (CAPEX) and Operational Expenditures (OPEX), usually rely on literature data and tend to choose organisms or process types they consider to be similar to their studied system.

In the following, several examples emphasize the importance or effect of these parameters on overall LCA calculations. Several authors used energy consumption data from literature wherein other types of microalgae were used while the effect of microalgae was proven to be severe (see the 3.3. Specific energy consumption (kWh/kg dry biomass) section) (Razon and Tan, 2011; Xu et al., 2011; Yuan et al., 2014). Similarly, several authors used energy data from the GEA company webpage (0.25 kWh/kg on dry basis) which claims that energy consumption is independent from the type of algae used (Adesanya et al., 2014; Handler et al., 2012; Stephenson et al., 2010; Yanfen et al., 2012). Other studies, however, already proved a significantly higher energy consumption (146.94 kWh/kg on dry basis) with the same technique (Halim et al., 2012a,b; Lee et al., 2012). Sometimes, the reference for the energy consumption is not mentioned and thus not traceable (Mata et al., 2014) or data on the technique is not found in the reference (Soratana et al., 2014). Based on these studies, it can be stated the LCA studies in this field are not straightforward.

4. FUTURE NEEDS

To develop a large scale energy-efficient cell disruption method for microalgae biorefineries, a considerable amount of research is still needed. New methods should consider overall cost effectiveness including pre-processing and downstream processing, controllability, energy consumption at least below the caloric value of algae (≈21 kJ/g), mildness, adaptability and extractability/recoverability of products as key aspects. To achieve an economically feasible mild microalgae biorefinery, it is also necessary to fully utilize the biomass and to minimize total process cost by applying process intensification approaches.

Within process intensification approaches for cell disruption, it is important to design devices with improved process control and reduced specific energy demand (kWh/kg; see the 2.1.1.

Bead milling section). This improvement could be achieved by focusing to equipment designs generating specific pulses (*e.g.*, electric, steam, ultrasonic). Alternatively, the combination of conventional techniques, *e.g.*, ultrasonication with chemical/enzymatic pre-treatment, could reduce the energy demand of mechanical disruption methods. To reduce the amount of unit operations, two phase aqueous systems could be promising since disruption and functional product separation can be achieved in one step. Explosive decompression also has the potential to provide cell disruption and hydrophilic/hydrophobic compounds separation in one step as well as simultaneous reagent separation. This method has been excessively studied for bacteria and yeast, but was until recent generally ineffective on microalgae cells. Process intensification for explosive decompression in a continuous operating mode should be studied in more detail. Another aspect of process intensification is to design the cell disruption process to empower the subsequent downstream process. An example is the emerging method of cationic polymer coated membrane treatment since the reagent is already separated from the products through immobilization. In summary, methods combining cell disruption and product separation in one unit operation should be developed with improved process control, reduced specific energy demand and zero disruption agent contamination.

A considerable amount of research is still needed for some promising emerging methods, such as cationic polymer coated membrane treatment and explosive decompression, to achieve conventional cell disruption method efficiencies. To support future research and to be able to compare cell disruption efficiencies, a generic method for data collection should be used systematically to produce comparable data. The data in literature should allow the calculation of at least specific energy consumption for disrupting a unit of microalgae biomass (see the 2.1.1. Bead milling section). This could result in a higher level of understanding which would accelerate the development of new mild methods. This research area could also benefit from a better understanding in the relationship between cell wall characteristics and disruption efficiencies. Additionally, the cell wall characteristics are influenced by the microalgae type, growth conditions, growth phase and the existence of stress factors which could be studies more thoroughly. To summarize the latter, a structured methodology is needed to build up a knowledge database to allow discrimination in the selection of a suitable full scale installation.

5. CONCLUSIONS

Industrial microalgae biorefineries are not yet feasible due to high operational costs. Cell disruption, because of the high energy demand and effect on the efficiency of subsequent steps, is the most crucial step in biorefinery. In this review several methods have been described for microalgae cell disruption. The main aspects of an industrially interesting microalgae cell disruption method include energy efficiency, mildness, selectivity, controllability and universality. Recent studies indicate that mechanical methods are optimal for industrial scale

cell disruption, but have high specific energy consumption. Non-mechanical methods might affect product quality to a minor extent, have lower energy needs, might obtain a higher selectivity and may provide uniform cell disruption. However, treatment time is longer and at large scale relatively large treatment vessels are required, the demand for chemicals is costly, a waste stream is generated and side products can be formed due to the increased temperatures. Additionally, the process itself is more difficult to control compared to mechanical methods. As a result, energy demanding mechanical methods such as high-pressure homogenizers or bead mills are generally preferred for large-scale applications. However, these conventional mechanical methods require energy intensive cooling for the isolation of fragile compounds. On the other hand, the efficiency of mild cell disruption methods dramatically changes from one microalgae species to another related to their cell membrane/wall composition and morphology. Among the new developments on microalgae cell disruption, laser and pulsed electric arc technologies are not suitable for scale-up, microfluidizer is aggressive with high shear and ultrasonication is known as not universal and strongly dependent on type of microalgae and cultivation conditions. Other emerging technologies such as cationic polymer coated membrane treatment and explosive decompression are promising, but a considerable amount of research is needed to obtain acceptable disruption efficiencies.



Chapter 3

Influence of nitrogen depletion in the growth of *N. oleoabundans* on the release of cellular components after beadmilling

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HIGHLIGHTS

- Pellet and supernatant were analyzed to evaluate algae cell disruption.
- Energy consumption per kg of released product was calculated.
- Nitrogen depletion results in a higher release ratio of biochemical components.
- Nitrogen depletion results in a lower energy consumption per kg of released product.

ABSTRACT

Keywords: Microalgae, Cell disruption, Nitrogen depletion, Biochemical release

Cell disruption by bead milling of *Neochloris oleoabundans* grown under nitrogen repleted (NR) and nitrogen depleted (ND) conditions was evaluated based on the release of biochemicals and biomass to the supernatant. Additionally, energy consumption for cell disruption was calculated per kg of released component. Bead milling of NR cells resulted on average in 34% (w/w) release of biochemicals into the supernatant, with a similar composition as the untreated microalgal biomass. With ND cells, the release was higher and more selective, *i.e.* 57%, 59%, 68% and 56% (w/w) of respectively biomass, proteins, carbohydrates and lipids whereof 92%, 57% and 46% (w/w) respectively of phospho-, glyco- and neutral lipids.

1. INTRODUCTION

Microalgal biomass and its constituents are considered as a future feedstock for fuels and chemicals (Harun et al., 2011; Günerken et al., 2015). However, the production of most microalgal biochemicals is not yet economically feasible since the energy consumption in production and downstream processing is high. It is therefore important to reduce the energy demand and to achieve higher yields in downstream processes. For efficient extraction, the cell wall is preferably broken to facilitate the release of the compounds into the medium. The disruption of cells however is often challenging since the cells are small, some algae have relatively thick cell walls and products are located in organelles or bound to cell membranes. Bead milling systems, originally designed for cosmetic, paint, pharmaceutics and food industries (Hopkins et al., 1991; Bunge et al., 1992), provide a simple and effective solution for microalgae cell disruption at industrial scale due to its high cost-efficiency. It has many attractive characteristics including high disruption efficiency in single-pass operations, high throughput and biomass loading, good temperature control and commercially available equipment from laboratory to industrial scale (Doucha and Lívanský, 2008; Postma et al., 2015; Günerken et al., 2015). In addition, the single-pass and continuous operating modus makes the bead mill an ideal feedstock generator for immediate and direct sequestration of released products in a fluidized bed (Jahanshahi et al., 2002). Because of all these features, including the current most effective cell disruption method (Günerken et al., 2015), bead milling was chosen as the benchmark cell disruption method in this study. Additionally, the industrial proven microalgae Neochloris oleoabundans was selected as the preferred organism. In this study, bead milling of nitrogen deplete (ND) N. oleoabundans and nitrogen replete (NR) *N. oleoabundans* are compared.

In literature, the cell disruption yield is generally evaluated by the extraction yield of a product or via a direct cell/particle count (Doucha and Lívanský, 2008; Postma et al., 2015; Günerken et al., 2015). The average extraction yield of a product, for example lipids, provides indirect information on the synergistic effect of particle atomization and product solubilization/dissolution. On the other hand, direct cell or particle count only gives information on the ratio of the broken cells. In the microalgae biorefinery, however, multiple products are targeted. Therefore, the release of biomass and biochemicals (lipids, carbohydrates and proteins) to the supernatant at 100% cell number based cell disruption was measured in this study. Well-established analytical techniques from literature to determine lipids, carbohydrates and proteins were compared and the most suitable methods were selected. The main parameters for this selection were the effects of interferences/impurities and the accuracy of determining the total amount of biomass/biochemicals. Evaluating mass transfer to the medium is generally interesting for the biorefinery as additional extraction steps may become unnecessary provided that sufficient biochemicals are directly released into the medium.

To our knowledge, this is the first study on microalgae cell disruption that focuses on the variation of mass distribution between the supernatant and pellet as function of different classes and types of biochemicals simultaneously under ND and NR conditions.

2. MATERIALS AND METHODS

All chemicals were purchased from either Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA) if not stated otherwise. *N. oleoabundans* UTEX 1185 was cultivated at the Bioprocess Engineering Department of Wageningen University Research Centre (see below). Fresh biomass was used for all studies.

2.1. Biomass production

The same equipment and process parameters were used for both ND (N-depleted) and NR (N-repleted) cultures. The composition of media used for NR and ND cultivations is the same for both cultures with exception of KNO_3, respectively 25.53 and 5 mM. The composition of salts in both media is 2.3 mM K_2SO_4, 0.7 mM KH_2PO_4•H2O, 1.365 mM K_2HPO_4•H2O, 0.3 mM $MgCl_2$•$6H_2O$, 0.17 mM $CaCl_2$•$2H_2O$, 0.43 mM NaCl, 0.054 mM NaFeEDTA, 0.3594 mM Na_2EDTA•$2H_2O$, 0.0921 mM $ZnSO_4$•$7H_2O$, 0.0219 mM $MnCl_2$•$4H_2O$, 0.0147 mM Na_2MoO_4•$2H_2O$, 0.0051 mM $CoCl_2$•$6H_2O$, 0.0189 mM $CuSO_4$•$5H_2O$. A 2% (w/w) aqueous solution of antifoam-B (J.T. Baker, Avantor Performance Materials, U.S.A.) was added to a 20-L jacketed baffled stirred tank reactor (Applikon Biotechnology B. V., Netherlands). The tank was agitated by two 6-blade impellers rotating at 300 rpm, the pH is controlled by adding CO_2 (99.99%, gas) or 1M NaOH solution and the air flow was 6 l/min. The reactor surface light intensity was increased gradually from 200 to 1000 μmol s^{-1} m^{-2} during the first 5 days (for NR) or from 100 to 540 μmol s^{-1} m^{-2} during the first 10 days of cultivation (for ND). The total cultivation time amounted 12 days (NR) and 21 days (ND) respectively.

The algae were harvested via centrifugation at 3600 g for 5 min. The supernatant was discharged and the pellet resuspended in deionized water for a washing procedure. The suspension was again centrifuged at 3600 g for 5 min. The supernatant was discharged and the pellet was re-suspended in deionized water to obtain a dry weight (DW) concentration of 3% (w/w).

2.2. Cell disruption by bead milling

Bead milling was carried out according to Schwenzfeier et al. (2011). In preliminary studies, a treatment time of 45 min resulted in 100% cell disruption in terms of cell number reduction for a NR (N-replete) suspension of approximately 3% (w/w) dry weight (DW) concentration. The same treatment time and similar dry weight concentrations as for NR suspensions were used for bead milling ND (N-deplete) in order to have the same energy

consumption per volume unit of algae suspension. In general, the algae suspension was re-circulated through the grinding chamber of DYNOMill Type MULTI LAB (Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland) for 45 min at 1.5 L/min with a mill rotation of 2000 rpm. The 0.3 L grinding chamber was filled with Zirconia beads, Yttria stabilized 0.4–0.6 mm, at maximum bead filling level. The jacket of the bead mill and the microalgae suspension vessel were cooled to 5 °C. During the process, the temperature of the algae suspension at the mill outlet never exceeded 30 °C. All studies were carried out in triplicates.

2.3. Biochemical analyses

Samples from the 'untreated phase' were collected before cell disruption. The untreated samples were separated via centrifugation at 20,000 g for 20 min and the pellet, as well as the supernatant were freeze dried and grinded before biochemical analysis (dry biomass, protein, lipid and carbohydrate). The analysis results of the untreated solid phase are the benchmark (given as UN) and considered as 100% in calculations for biochemical release to the supernatant. The analysis results from the untreated supernatant is the blank, thus considered as 0% and subtracted from treated supernatant values. The analysis results of the blank supernatants for NR and ND were on average respectively 0.029 and 0.018 g release of biomass per gram of dry algae with composition 14.38%, 40.92% and 44.86% of carbohydrates, proteins and lipids for NR and 23.94%, 15.37% and 60.68%, respectively for ND. After cell disruption, the supernatant and pellet were separated by centrifugation at 20,000 g for 20 min and this step was repeated 5 times. After every centrifugation step, the supernatant was separated from the pellet and centrifuged again in the next step. The pellets obtained in the subsequent centrifugation steps were collected, mixed and labeled as 'treated pellet'. The supernatant obtained after every step were collected, mixed and were filtered through 0.22 µm filter to obtain the 'treated supernatant'. The treated supernatant and treated pellet were freeze dried and analyzed to determine the total biochemical compositions. The flow chart of biomass treatment and analyses is shown in Fig. 8. All analyses were performed in triplicate.

Freeze drying, Dubois, BCA and modified Folch methods were applied on all samples to determine the release of biomass and biochemicals. Additionally, solid-phase extraction was applied to obtain information on the lipid composition, and SDS–PAGE to determine differences in the peptide/protein distribution among samples. Additionally, the specific energy consumption for biomass and biochemical release was calculated.

2.4. Dry weight and biomass release ratio

The mass of each sample was determined before and after drying. Before drying, the samples were stored overnight at -20 °C and subsequently transferred to -80 °C for 6–8 h to obtain frozen samples. The frozen samples were freeze dried under vacuum for 72 h. The dried samples were stored at -20 °C in sealed recipients before the analyses. The mass of the untreated pellet, thus before disruption and after phase separation, refers to 100% of the mass and the

mass of the untreated supernatant is the blank to correct the dry weight of the supernatant samples after disruption. After disruption and phase separation, the corrected dry masses of the fractions were used to calculate the biomass release ratio (see Eq. 1).

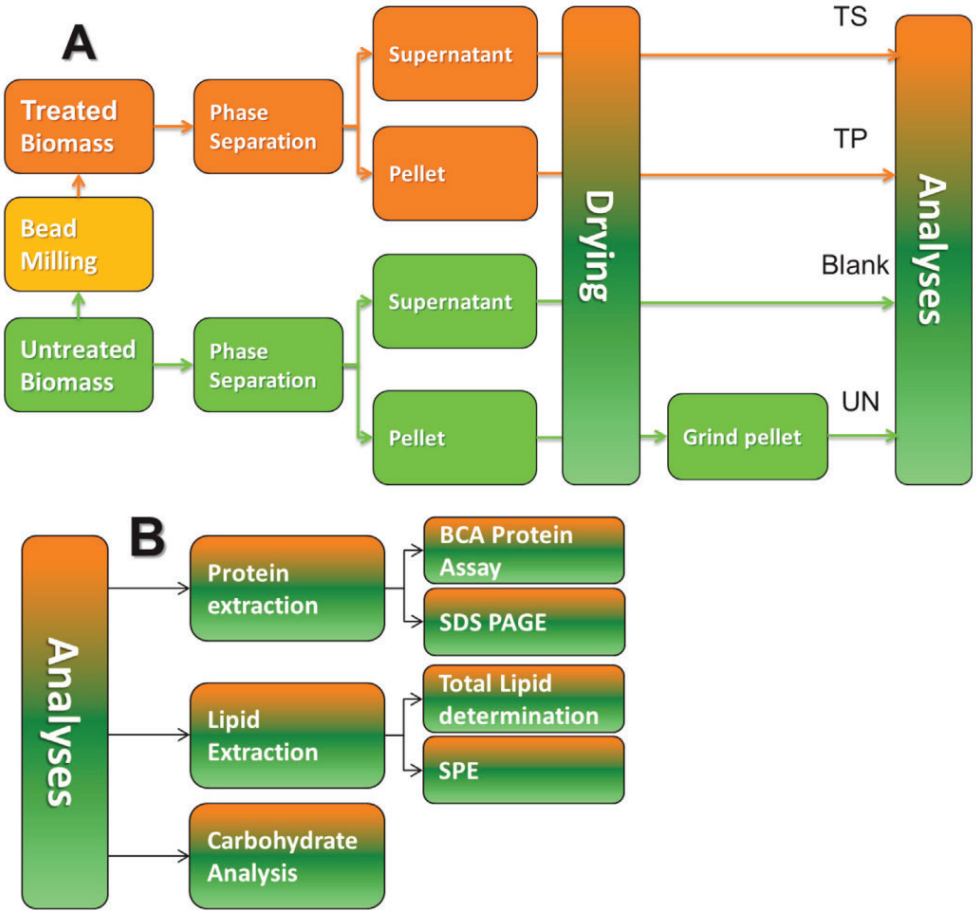

Fig. 8: The flow chart of biomass treatment (A) and analyses (B): TS = treated supernatant; TP = treated pellet; UN = untreated Neochloris oleoabundans biomass.

2.5. Carbohydrate analysis

To quantify carbohydrate release, the phenol–sulfuric acid reaction was used (Dubois et al., 1956). Briefly, 1 mg of freeze-dried sample was suspended in 250 µl of milli-Q water and mixed with 125 µl of 5% (w/w) phenol reagent. Afterwards, 625 µl pure H2SO4 was added to form the final mixture. The sample was kept at room temperature under dark conditions for 40 min prior to UV–VIS spectroscopy measurement at 492 nm.

2.6. Protein analyses

A protein extraction solution (150 mM NaCl, 1 mM EDTA, 0.5% (w/w) sodium deoxycholate, 70 mM NaF, 2 μM of PMSF, 0.1% (w/ w) sodium dodecylsulphate) was prepared and the dried samples were grinded prior to extraction. 20 mg of sample, 10 mL protein extraction solution and 1 g of glass balls (2–4 mm diameter) were mixed vigorously with a vortex mixer for 3 min in a 50 mL plastic centrifuge tube. The samples were kept at room temperature in the dark for 30 min and centrifuged at 20,000 g for 5 min. The supernatant was used to determine the protein concentration with the Pierce BCA protein assay according to the producer's instructions (Pierce, USA). Bovine serum albumin was used as standard.

The protein compositions of the algae extracts were visualized using SDS–PAGE under reducing conditions (10 mM 2-mercaptoethanol) on a Mini-protean II system (Bio-Rad Laboratories, USA) according to the instructions of the manufacturer. Gels were stained with Bio-Coomassie brilliant blue (BioRad Laboratories, USA).

2.7. Lipid analyses

The Folch (Folch et al., 1957) method with some slight modifications was used for total lipid analysis. Briefly, 10 mL of $CHCl_3$ was added to 1 g of freeze-dried sample, the solid particles were grinded with a glass stick. Subsequently another 10 mL of CH_3OH was added to the mixture and vortexed for 3 min and repeated with another 10 mL of $CHCl_3$. 8 mL of phosphate buffer saline (PBS) (8 g/l of NaCl; 0.2 g/l of KCl; 1.44 g/l of Na_2HPO_4; 0.24 g/l of KH_2PO_4) was added to the final mixture. The suspension was mixed in an orbital shaker for 45 min at 150 rpm and centrifuged at 1000 g for 1 min to accelerate the phase separation. The bottom layer was collected, and the middle and upper layer were extracted 2 times more with the same method. The bottom $CHCl_3$ layers from the 4 extractions runs were collected and dried under N2 flow at room temperature overnight.

Lipids were separated into neutral lipids (NL), glycolipids (GL) and phospholipids (PL) by solid-phase extraction (Christie, 2003; Servaes et al., 2015). A 500 mg Sep-PakTM cartridge of silica gel (Waters, USA) was first conditioned by elution with 5 mL of chloroform. Then 0.5 mL of chloroform containing about 50 mg of lipids was applied to the column. Elution was obtained with 10 mL of solvent in the following order: chloroform, acetone and methanol to yield respectively neutral lipids, glycolipids and phospholipids. Each fraction was dried under a stream of nitrogen gas and weighted.

2.8. Calculation of energy consumption

To evaluate the effect of NR and ND on the specific energy consumption, values from this study and information of the equipment provider were used in calculations.

The specific energy consumption for bead milling (SEC-BM, kWh/kg DW algae) is the total energy used (kWh) to treat 1 kg of dry biomass loaded into the bead mill. Based on

information from the provider (Willy A. Bachofen AG), the experimental specific energy consumption for 45 min of bead milling in this study was 7.5 kWh/kg DW (SEC-BM). The specific energy consumption for biomass or biochemical release to the supernatant (SEC-released, see Eq. 2) was calculated by dividing SEC-BM by the release ratio. The release ratio (Eq. 1) is the amount of biomass or biochemical released into the supernatant relative to the amount of biomass loaded into the bead mill. The experimental SECreleased values for ND and NR are given in Table 11.

$$Release\ ratio\left(\frac{kg\ DW\ released}{kg\ DW\ algae}\right) = \frac{Biomass\ or\ biochemical\ released\ (kg\ DW\ released)}{Algae\ biomass\ (kg\ DW)} \quad \text{Eq. 1}$$

$$SEC_{released}\left(\frac{kWh}{kg\ DW\ released}\right) = \frac{SEC_{BM}\left(\frac{kWh}{kg\ DW}\right)}{Release\ ratio\left(\frac{kg\ DW\ released}{kg\ DW}\right)} \quad \text{Eq. 2}$$

To compare the energy request of the bead milling step with the energy content of algae, SEC-BM and the release factor can be divided by the energy content of the algae biomass, *i.e.*, 5.42 kWh/kg DW algae (Illman et al., 2000).

3. RESULTS AND DISCUSSION

Nitrogen repleted (NR) and nitrogen depleted (ND) cultures of *N. oleoabundans* UTEX 1185 were grown in a photobioreactor and 3% DCW suspensions thereof were bead milled to evaluate the effect of N-depletion and N-repletion on biomass and biochemicals release (see Fig. 9 and Fig. 10). The difference in release of proteins and lipids is discussed below in detail. The specific energy consumption for the release of biomass and specific biochemicals during bead milling was also calculated.

3.1. Release of biomass and biochemicals

The release of biomass during bead milling was determined by measuring the dry weight of the untreated sample, the treated pellet and treated supernatant. Phase separation after bead milling 3% (w/w) DW algae suspensions of ND and NR resulted in treated supernatants suspensions with respectively 1.71% and 1.14% (w/ w) DW concentration. The results for the blank supernatants, thus prior to bead milling, were subtracted from these supernatant results, e.g. 0.054% and 0.085% (w/w) for ND and NR respectively. Thus, after bead milling ND released significantly more biomass compared to NR with release ratios of respectively 0.57 and 0.38. The supernatant of ND was not transparent while NR resulted in a transparent supernatant.

Cell disruption was evaluated via the analyses of total protein, total lipids, total carbohydrates, SDS–PAGE and solid-phase extraction. The results in Fig. 9 show that with N-replete (NR) *N. oleoabundans*, all biochemicals as well as total biomass were released for 34±3.6% (w/w) thus with no discrimination between the different types of biochemicals. The distribution

of biochemicals is therefore proportional to the transfer of biomass and is considered as non-selective. These results validate the hypothesis of Balasundaram et al. (2009) for a non-selective release of biochemicals during bead milling. With N-deplete (ND) *N. oleoabundans* however, the total biomass release was 57% (w/w) whereas the release of proteins, carbohydrates and lipids was not proportional to the biomass release and mounted up to 59%, 68% and 56% (w/w) respectively. In other words, all biochemicals of ND had higher transfer ratios towards the supernatant than NR and the release of biochemicals was found to be more selective.

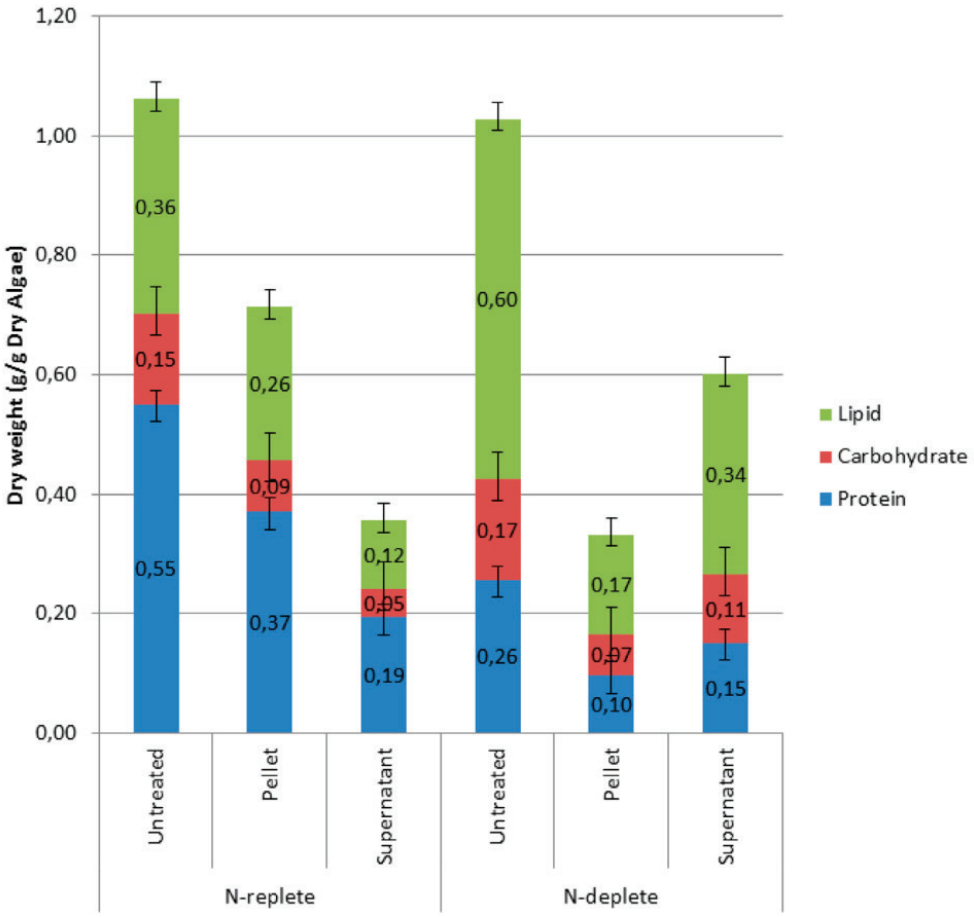

Fig. 9: The composition of untreated biomass, treated pellet and treated supernatant in terms of dry weight of biomass and biochemical per dry weight of nitrogen depleted (ND) and nitrogen repleted (NR) cultures of Neochloris oleoabundans.

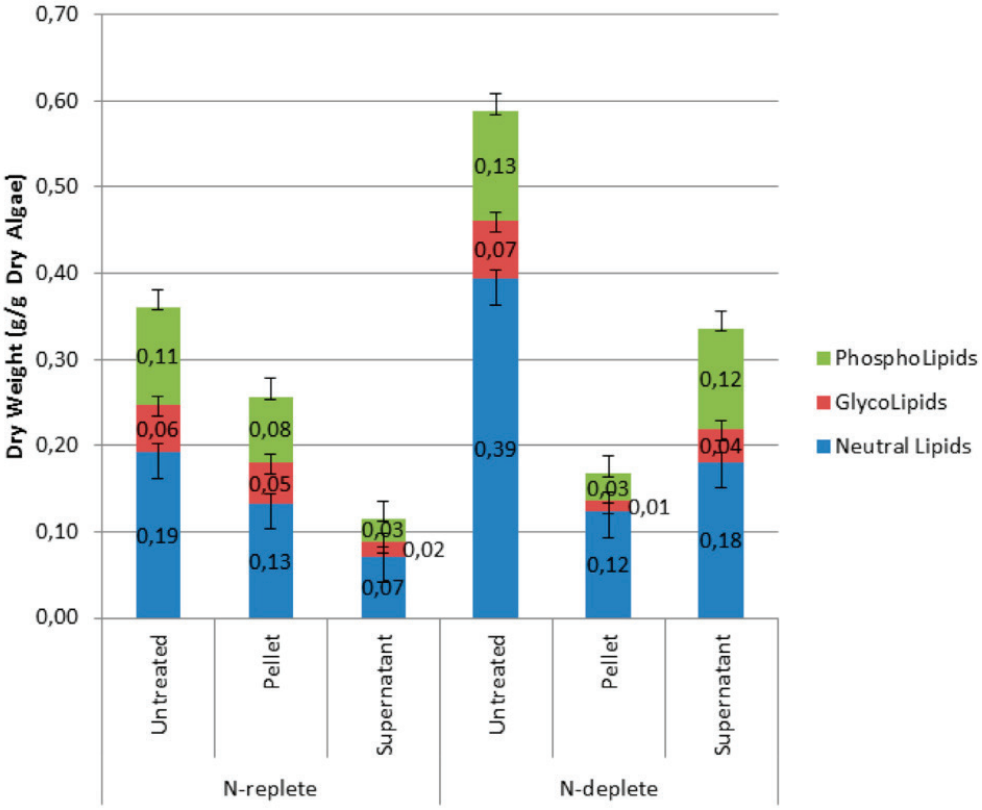

Fig. 10: The lipid composition of untreated biomass, treated pellet and treated supernatant in terms of dry weight per dry weight of nitrogen depleted (ND) and nitrogen repleted (NR) cultures of *Neochloris oleoabundans.*

Fig. 9 displays the dry weight of biochemicals in the untreated biomass, treated supernatant and treated pellet expressed per dry weight of ND and NR algae. Taking into account the error bars, the sum of pellet and supernatant biochemicals should therefore be the same as in the untreated biomass. The figure indicates that the untreated biomasses of ND and NR have significant differences in lipid and protein concentration. The supernatant of ND contained significantly more lipids and carbohydrates, but less proteins than the supernatant of NR. The decrease in absolute mass of released proteins is related to the lower total and soluble protein content in ND cultures.

Overall, after bead milling, more biomass and biochemicals are found in the treated supernatant for ND while for NR more biomass and biochemical remain in the treated pellet. The overall increased biomass release during bead milling of ND can be explained by the higher lipid content in N-deplete microalgae biomass and/or is related to a different physiology (discussed below).

3.2. Release of lipids

Fig. 10 shows the concentrations of the different lipid fractions in dry weight, *i.e.* neutral lipids (NL), glycolipids (GL) and phospholipids (PL). In the untreated biomass, the amounts of glycolipids and phospholipids are similar for ND and NR (GL = resp. 0.07, 0.06 g/g DW and PL = resp. 0.13, 0.11 g/g DW) while the amount of neutral lipids in ND is about two times the amount present in NR (resp. 0.39 and 0.19 g/g DW). After bead milling, the release of glycolipids and phospholipids is remarkably increased with ND (resp. 57% and 92%) compared to NR (resp. 33% and 27%) and the release of neutral lipids is slightly increased (from 37% to 46%). The higher absolute amount of neutral lipids in ND could potentially enhance the transfer of the polar lipids to the supernatant and vice versa. Additionally, N-depletion may cause changes in cell wall composition (Gerken et al., 2013), which can be the reason of higher release ratios for lipids in ND cultures.

During the bead milling process, the cell membrane (containing phospholipids and glycolipids) disintegrate and liposomes and micelles/lipid droplets with nano-/micrometer diameter are spontaneously formed (Hedenskog et al., 1969). Additionally, microalgae can accumulate neutral lipids as cytoplasmic lipids droplets in result of stress such as N-depletion (Yang et al., 2013). These microalgal lipid droplets are generally composed of 90% (w/w) triacylglycerol, 10% (w/w) free fatty acids with minor levels of charged galactolipids and trace amounts of proteins (Davidi et al., 2014). The lipid droplets, which are capable of fusing into bigger droplets in vivo, are stable in vitro because of a galactolipid monolayer (Tauchi-Sato et al., 2002; Wang et al., 2009; Murphy, 2012). The disruption of these lipid droplets can lead to formation of smaller lipid droplets.

The overall mass transfer to the supernatant during bead milling can be explained by two mechanisms. The first phenomenon involves the dissolution of water soluble chemicals as a result of disruption and release to surrounding medium. The second mechanism involves micro-/nano-meter diameter liposomes, micelles/lipid droplets. Liposomes are composed of a lipid bilayer surrounding a polar vessel while micelles and lipid droplets have a polar lipid monolayer with an apolar interior. During bead milling, phospholipids and glycolipids are transferred to the supernatant through the formation of liposomes and/or micelles. The higher concentration of neutral lipids in ND biomass can be the driving force for the increased transfer of polar lipids to the supernatant. The high amount of neutral lipids requires more polar lipids to act as surfactants to lower the energy at the apolar–polar interphase through the formation of micelles and/or liposomes. On the other hand, N-depletion leads to more fragile cells, thus during disruption potentially more organelles and internal membranes are being atomized leading to more phospholipids and glycolipids in the supernatant. As a result of the formation of many micelles and/or liposomes, the treated supernatant of ND is not transparent. The higher mass transfer ratio of proteins in ND compared to NR is probably caused by the same phenomenon and the difference in mass transfer of carbohydrates is

potentially related to the altered physiology due to N-depletion. However, no data is found in literature on the effect of N-depletion on carbohydrate metabolism of *N. oleoabundans*.

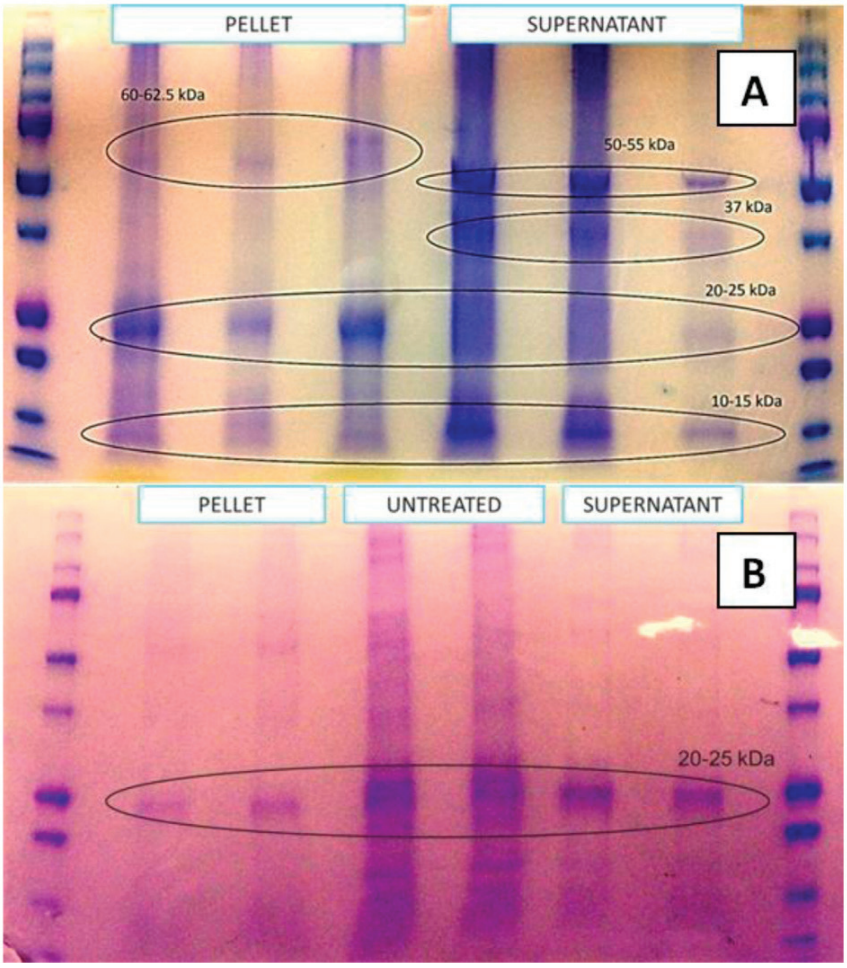

Fig. 11: SDS–PAGE results of NR (A) and ND (B) samples.

Table 11: Specific energy consumption of nitrogen depleted and nitrogen repleted *Neochloris oleoabundans* for release of biomass & biochemicals during bead milling (SECreleased).

SEC-released	Nitrogen depleted	Nitrogen repleted
kWh/kg DW supernatant biomass	13.23	19.98
kWh/kg DW supernatant protein	54.84	36.80
kWh/kg DW supernatant carbohydrate	86.48	165.59
kWh/kg DW supernatant lipid	21.83	59.44

3.3. Release of proteins

RuBisCO (Ribulose-1,5-biphosphate Carboxylase/Oxygenase), the most abundant protein in nature responsible for fixating CO2, is normally stored inside pyrenoids or bodies inside the chloroplasts of microalgae (Yellowlees and Leggat, 1997). The presence of RuBisCO in the supernatant can therefore be a good indicator for thorough cell disruption.

The RuBisCO enzyme consists of 8 identical heavy (55–56 kDa) and 8 identical light (13 kDa) chain and can be seen with reduced SDS–PAGE as separated bands or as one integrated 66 kDa (1 heavy + 1 light chain) band (Wang and Kolattukudy, 1996), whereas with Native-PAGE one band is visible at approx. 500 kDa (Desai et al., 2014 (Green Chemistry)). Additionally, Ishida et al. (1997) showed that the large subunit of RuBisCO degraded by active oxygen results in a 37 kDa fragment containing the Nterminal region and a 16 kDa fragment containing the C-terminal region. The bands near to 25 kDa usually refer to proteins of light harvesting complexes such as Chlorophyll a/b binding proteins, PsaI and PsaL in the thylakoid membranes (Smith et al., 1990; Funk et al., 1994; Chen et al., 2004).

All bands described above with exception of 37 kDa were observed in the untreated samples of NR (Fig. 11 A). For the NR suspension, the distribution of proteins between the treated supernatant and treated pellet showed a level of selectivity. Two major (55 and 13 kDa) and two minor (37 and 25 kDa) bands were found in the treated supernatant. For the treated pellet, one major (25 kDa) and two minor (66 and 14 kDa) bands were determined. In other words, the major part of RuBisCo was transferred to the treated supernatant. The only visible band for ND biomass was 25 kDa for the untreated, treated supernatant and treated pellet fractions (Fig. 11 B). Thus significant signals for RuBisCo were not observed supporting the statement that protein production could be strongly inhibited.

The data of NR clearly shows that the cell disruption yield can be associated with the transfer of RuBisCO protein to the supernatant. This mass transfer (55 kDa) suggests that approximately all pyrenoids might be broken while the thylakoid membranes (carrier of 25 kDa) are only partially disrupted. To break the pyrenoids, first the cell and then the chloroplast should be broken. After evaluating the data, it is assumed that 100% cell number based cell disruption was obtained.

Additionally, oxidation of proteins (*e.g.*, RuBisCO) could occur. After disruption, biochemicals are no longer protected by the reducing intracellular environment. After release, they interact with oxygen from the air which is enhanced at higher temperature and in presence of free radicals. Bead milling increases the temperature and causes free radical formation (Günerken et al., 2015), thus bead milling could promote oxidation of sensitive components, such as proteins or anti-oxidants.

3.4. Specific energy consumption

The overall effect of beadmilling on OPEX when using ND or NR cultures in the biorefinery is elaborated in a simplified example. The 'SEC-released' values in Table 11 represent the specific energy consumption for the release of a single product during bead milling if only one product is targeted. Thus, all bead milling energy is attributed to a single product, for example algae cultivation in function of lipids. The specific energy consumption for biomass/biochemical release during bead milling (SEC-released) was calculated via Eq. 1 and Eq. 2, thus based on bead milling energy consumption (SEC-BM) and release ratios. The release factors reflect the effects of biomass composition and algae physiology on disruption and release.

As expected, differences in release of biomass and/or biochemicals are seen between the two cultivation methods. From an energetic point of view, nitrogen depleted (ND) cultivation is to be preferred when targeting the full biomass and lipids, especially phospholipids, released to the supernatant. NR cultivation, on the other hand, provides a better compromise when targeting proteins. Carbohydrates for both cultivation methods should not be targeted due to a low release which is related to the low concentration in the biomass itself. Since energy consumption demands are high when targeting only one single product, the need to produce multiple products in a biorefinery concept or the use of the whole disrupted biomass seems economically more feasible (Vanthoor-Koopmans et al., 2013).

4. CONCLUSIONS

During bead milling, the nitrogen depleted culture of *N. oleoabundans* shows a higher and more selective release of biomass and biochemicals, with exception of proteins. The largest difference is found for phospholipids which are almost entirely transferred to the supernatant compared to only 27% with the nitrogen repleted culture. Nitrogen depleted cultivation gives a better energetic compromise when targeting the full biomass or lipids while nitrogen repleted cultivation provides a better compromise for proteins. Nevertheless, the overall energy input exceeds the energy content of algae, therefore optimization of cultivation, harvesting and downstream processing remains essential.



Chapter 4

Flow cytometry to estimate the cell disruption yield and biomass release of Chlorella sp. during bead milling

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ABSTRACT

Keywords: Microalgae cell disruption yield, Efficient analytical method, Flow cytometry, Biomass release

A number of visual, chemical and fluorescence-based methods are generally employed for monitoring of algae cell growth, culture health and biomass concentration. These methods are often time-consuming, demand destructive and high volume sampling. Rapid, efficient, cost-effective and automated methods which facilitate high-throughput and non-destructive sampling would highly benefit microalgae biotechnology. It is known in literature that with flow cytometry it is possible to monitor microalgae growth and microalgae culture health. Flow cytometry, however, has not been used to estimate biomass release and cell disruption yield. In this study with representative cultures of *Chlorella sp.*, flow cytometry data were generated by a long pass filter (> 670 nm) and proved to be a promising technique to rapidly evaluate these parameters during cell disruption by bead milling. Both a laboratory and a commercial culture of Chlorella were bead milled. Prior to and during bead milling, biomass release was evaluated gravimetrically and the cell count was determined via hemocytometer manual counting and 3 flow cytometry methods: 1) direct event count of a certain population, 2) quadrant-upper right event count of a long pass filter (> 670 nm) and 3) a calculation based on the long pass filter data. The data of all methods were compared and correlated to gravimetric biomass release data. Manual counting resulted in an underestimation of the cell disruption yield as one of the *Chlorella* cultures manual counts did not agree with biomass release data. Good correlations for both cultures were found for cell disruption yield calculations based on flow cytometry data and gravimetric biomass release data. Flow cytometry is therefore an efficient analytical method to rapidly screen disruption yields during cell disruption in microalgae and can be a substitute for the time-consuming direct methods (*e.g.*, gravimetric, manual counting) for estimating biomass release and cell disruption yield.

1. INTRODUCTION

Microalgae have been employed as substrate for industrial biosynthesis of fuel, food, pharmaceuticals, nutraceuticals, cosmetics and a variety of other bioactive molecules (Greenwell et al., 2010). The ease of metabolite recovery and thus cell disruption efficiency are critical for the economic viability of microalgae biorefineries (Mata et al., 2010). Evaluation of the cell disruption yield is therefore of great importance.

Most laboratory settings employ direct counting via a standard hemocytometer to determine the cell disruption yield. While direct counting methods are precise and sensitive, they are time-consuming and labor-intensive. Microalgae biomass growth is conventionally monitored by a combination of dry weight-, optical- and fluorescencebased methods for microalgae biomass concentration estimation and culture health monitoring. These analyses are also used for monitoring the yield of downstream processing steps. Estimation of dry weight concentration is the most direct method, but requires milligram/gram dry weight of sample amounts and is time consuming since the result is determined after the sample is fully dried. Additionally, dry weight analysis does not provide specific information. For example, dry weight analysis will result in an overestimation of biomass growth or biomass release during cell disruption due to salt accumulation in the suspension.

Coulter counter, dynamic light scattering or optical density monitors are optical methods that estimate cell particle diameter and correlate the results with biomass volume and dry weight (Chioccioli et al., 2014). In general, optical methods are rapid, precise, sensitive and suitable for non-destructive sampling. However, these methods are not suitable to distinguish biologic contaminants, abiotic particles and microalgae cells and are often hampered by morphological and physiological heterogeneity of microalgae cultures and the presence of cellular debris (Butterwick et al., 1982; Chioccioli et al., 2014). Nephelometry and fluorometric estimation of chlorophyll-α have also been employed (Butterwick et al., 1982). Photomicrography followed by automated image analysis is another useful approach, but might not accurately estimate dry mass from the wet volume (Alvarez et al., 2012).

Chioccioli et al. (2014) reported that OD750 values and cell concentrations did not correlate reliably with biomass dry weight in *Chlorella vulgaris* cultures. The authors, however, observed a good correlation between flow cytometer and hemocytometer based measurements. Chlorophyll fluorescence from the microalgae cells was measured in order to monitor microalgae biomass accumulation. It showed a good correlation with the cell counts determined via a hemocytometer, biomass concentration determined by freeze drying and counting data from the flow cytometer. Additionally, Premazzi et al. (1989) reported a good correlation between microalgae cell number via manual counting and size estimation by flow cytometry to determine the dry weight concentration. In another study, Wood and Horan (1985) observed that

flow cytometry, compared to OD750, exhibits a greater accuracy in estimation of the specific growth rate when cell numbers were low.

Flow cytometry can yield valuable information on microalgae replicative cycles, and can determine cell concentration and cell size to evaluate biomass production. However, such estimations are based on the assumption that the entire population has the same cell size which may cause an over- or under-estimation. Environmental microalgae cultures exhibit significantly different physiological and biochemical characteristics compared to unialgal cultures employed in industrial bioprocesses. Several authors have reported the use of specialized flow cytometers that accommodate the great variation in the cellular concentrations, chlorophyll α content, and DNA content between algal species (Veldhuis & Kraay, 2000). Flow cytometry could thus yield reliable estimates of biomass for either type of microalgae populations (Franklin et al., 2002; Stauber et al., 2002; Wang et al., 2010) since biomass concentration can be estimated by correlating known data on carbon content to forward scatter pulse area (FSC-A), or species-specific chlorophyll fluorescence or DNA content of the cultured algae. It can also yield data on several indicators of microalgae growth and physiology, such as the cell size, DNA, protein and lipid content and offers the possibility to monitor microalgae metabolites by fluorescent staining. Dominguez Teles et al. (2014) reported a method based on flow cytometry to screen and sort lipid accumulating microalgae. Petrescu et al. (2016) reported the use of flow cytometry to evaluate physiological alterations induced in *Chlorella fusca* by heavy metal exposure. Most of the earlier works have used the FSC-A and side scatter pulse area (SSCA) data for these purposes (Collier, 2000; Franklin et al., 2001; Franklin et al., 2001; Green et al., 2003; Toepel et al., 2005). Chioccioli et al. (2014) reported for the first time the use of pulse width data in estimating the biomass dry weight of Chlamydomonas reinhardtii and Chlorella vulgaris cultures. Moreover, Green et al. (2003) and Toepel et al. (2005) studied flow cytometry to estimate the biomass concentration via data from cell diameters using forward scatter and corrected chlorophyll fluorescence signal data.

All the above mentioned techniques are used to differentiate strains or monitor algae cultivation. The current study, however, is focused on monitoring the cell disruption efficiency since cell wall and cytoplasmic membrane disruption are essential to recover intracellular biomolecules. A number of mechanical and non-mechanical methods are employed for cell disruption of which bead milling is the chosen technique in this study (Günerken et al., 2015). Conventionally, cell disruption is evaluated by monitoring cell counts, particle size and release of metabolites (*e.g.*, protein, lipids) (Günerken et al., 2016). Cell counts are used to estimate the intact microalgae population after the disruption process, but suffer from some drawbacks. In general, manual counting assays tend to cause an under- or overestimation of the cell disruption yield because the disrupted cells that have secreted constituents but remain intact can be counted as healthy cells. Dyes that color living and death cells differently, can tackle this problem, but this is usually labor intensive and requires experience. Particle size

estimation yields similar data, but is time-consuming and prone to misinterpretation due to clumping of the biomass (Joyce, 2003; Samarasinghe et al., 2012). Nephelometric monitoring is also used, but may not be suitable for all types of algae (Foster, 1995). In comparison, methods for monitoring metabolite release (*e.g.*, protein estimation by Bradford method, UV absorptiometry for metabolites) are simple and yield quicker results. However, the underlying analytical procedures frequently yield inconsistent results and the inter-species and inter-strain variations in microalgae productivity further compromise data accuracy (Laurens et al., 2012). Metabolite denaturation at high levels of cell rupture is another concern which may cause an underestimation of cell disruption (Van Gaver & Huyghebaert, 1991). Similarly, at low levels of cell rupture (> 30% of cells remaining), metabolite release indicators may estimate higher cell rupture rates than by cell counting. Spiden et al. (Spiden et al., 2013) have proposed a model of continuum of cell rupture, in which cells become leaky to metabolites prior to their fragmentation. This applies in particular to *Chlorella* in which the cell rupture may be underestimated through cell counting.

Flow cytometry represents a promising approach in this context since the benefits include direct processing of samples, fast generation of results (2 min vs approximately 20 min for manual counting), feasibility for counting larger number of cells (5000–10,000 vs about 400 cells in manual counting), automation and low sample requirements (Hall & Cumming, 2003). In this study, the possibility of using a flow cytometer with standard configurations to monitor the cell disruption of two *Chlorella* cultures was evaluated. Flow cytometer data was compared with other methods, *e.g.* number reduction through direct cell count and gravimetric measurements to validate the relevance of this fast method.

2. MATERIALS AND METHODS

2.1. Microalgae biomass

The study was performed with culture specimens of two species of *Chlorella*. *Chlorella sp.* cultured under photoautotrophic conditions was obtained from Wageningen University Research Center Bioprocess Engineering department (Wageningen, The Netherlands). The microalgae cultured according to Postma et al. (2015). The second specimen consisted of commercially grown *Chlorella sorokiniana* biomass obtained from Phycom (Ochten, The Netherlands). In order to minimize variations induced by growth and nutrient conditions, samples of the microalgae cultures were collected during the late logarithmic phase of growth. In the following, the culture specimens are referred to as WUR and PHY, respectively.

2.2. Dry weight determination of the untreated algae cultures

15 mL of culture samples were transferred to pre-weighed 50 mL tubes and were frozen at −20 °C overnight and subsequently at −80 °C for 6–8 h. This was followed by freeze-drying for 72 h under vacuum conditions, and subsequent storage in a desiccator until they attained

room temperature. Finally, the dry samples were weighed at room temperature, and stored in sealed sample tubes at −20 °C until further analysis. The ash-free dry weight of the culture samples was not determined since the reported ash content of the used strains was < 5% (Agwa et al., 2013). The result was used to prepare the 6% (w/w) microalgae suspension for bead milling.

2.3. Cell disruption by bead milling

The protocol of Schwenzfeier et al. (2011) was used for cell disruption by bead milling. Briefly, the 6% (w/w) microalgae suspension was recirculated through the grinding chamber of the DYNO®-MILL Model MULTI LAB RL (Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland) for 50 min at 1.5 L/min with mill rotation of 2000 rpm. The 0.6 L grinding chamber contained Yttria-stabilized zirconia beads (0.4–0.6 mm) at maximum level of bead filling. Subsequently, the jacket of the bead mill and the microalgae suspension vessel were cooled to 5 °C with the intention to maintain the temperature of the microalgae suspension at the mill outlet below 30 °C. All experiments were performed in duplicate with intermediate cleaning of the bead

2.4. Processing of samples and determination of biomass release

Prior to bead milling, 2 samples of both microalgae cultures were collected in pre-weighed tubes as reference for disruption time zero. During bead milling, samples were taken at certain treatment times. From every sample, treated and untreated, 2 small volumes (≥100 μL) were used for manual counting and flow cytometry. The remaining samples were weighed to obtain net sample wet weight and centrifuged at 20,000g for 20 min at room temperature. After centrifugation, the supernatants and pellets were collected separately. The dry weight of the pellets (net pellet dry weight) and dry weight of the supernatant (net supernatant dry weight) were measured after freeze drying according to the procedure described above. The sum of net pellet dry weight and net supernatant dry weight is the net sample dry weight. The measurements were performed in duplicate. The dry weight concentration for pellet samples and biomass release were calculated according to formulas Eq. 3 and Eq. 4.

$$Pellet\ dry\ weight\ concentration\ (g/g) = net\ pellet\ dryweight/netsample\ dry\ weight \quad \text{Eq. 3}$$

$$Biomass\ release\ (\%) = 100 - 100 * net\ pellet\ dryweight/netsample\ dry\ weight \quad \text{Eq. 4}$$

2.5. Manual cell counting

A Neubauer hemocytometer (Wertheim, Germany) was used to determine the cell number concentration of WUR and PHY samples before, during and after bead milling. The 100 μL samples were diluted 100 or 1000 times before counting. The cell number reduction of the samples was calculated as the ratio of their event number to the event number of the untreated sample.

2.6. Flow cytometry analyses

Samples for flow cytometry (FC) were diluted with MilliQ water until their OD750nm was < 0.075. The threshold for forward scattering was set as FSC-H 8000 and a sample flow rate of approximately 130 µL over 2 min was used. The event number and signals of the long pass filter (> 670 nm, fluorescent red signal), FL3, were evaluated by a BD Accuri™ C6 flow cytometer software (BD Biosciences). The height based FL3 signal, FL3-H, was plotted as function of the integral fluorescent red signal, FL3-A, and graphs for each sample were obtained as shown in Fig. 12.

Three methods were investigated to correlate cell disruption efficiency by flow cytometry:

1) In the first method FC direct cell counting data is generated by the software after manually drawing a border around the healthy population in the FL3 graph and is one of the FC methods used to calculate the cell disruption efficiency (FC - direct). Alternatively, the graphs were split into quadrants, i.e. Q-UL (Quadrant Upper Left), Q-UR (Quadrant Upper Right), Q-LL (Quadrant Lower Left) and Q-LR (Quadrant Lower Right). The borders of the upper right quadrant (QUR) were determined by the location of the healthy microalgae population of the untreated samples.
2) The event count in the upper right quadrant is the second FC method to calculate the cell disruption efficiency by dividing the event number of the treated sample by that of the untreated sample (FC-QR).
3) Since FL3-H is proportional to the radius of spherical cells (\~r) and FL3-A to the area (\~r2), the multiplication of FL3-Hmean with FL3- Amean values from the FL3 signal in upper right quadrant (Q-UR) of treated samples is proportional to the volume of the spherical cell (\~r3). The result of this multiplication of treated samples versus untreated is a third method to calculate cell disruption yields (FC-FL3).

3. RESULTS

3.1. Effect of bead milling time on biomass release and cell number reduction via manual counting

Fig. 13 shows the effect of the bead milling time on the biomass release and cell number reduction via manual counting for both WUR and PHY microalgae culture specimens. The values are the averages of 2 runs per algae species. Within 3 min after the start of the bead milling procedure, a relatively high biomass release of around 23% is observed for both cultures. The release progresses steadily for PHY and reaches a maximum of about 48% after 50 min of bead milling. The WUR culture, on the other hand, reaches a plateau after 30 min of bead milling with a value of 36% biomass release. The cell number of both microalgae culture specimens reduces exponentially with increasing milling time until the cell number is reduced up to 1% for WUR and 4% for PHY.

3.2. Correlation of biomass release with cell disruption yields estimated by manual counting

As a benchmark for conventional techniques, Fig. 14 shows manual counting data plotted against biomass release for the two bead milling runs of each Chlorella species, WUR1 & 2 and PHY1 & 2. For WUR, cell disruption yields calculated via manual counting data are linearly correlated to the biomass release ratio, but the two runs show a slightly different slope. For PHY, run 1 is linearly correlated to biomass release, but run 2 fits less accurately (R^2 = 0,8123). Both slopes for PHY are quite similar. At the start of the second run of both WUR and PHY, a higher biomass release is observed at similar disruption degree calculated from manual counting data.

3.3. Correlation of biomass release with cell disruption yields estimated by flow cytometry

The correlation of the biomass release and the cell disruption yields based on flow cytometric data (FC-direct, FC-QR and FC-FL3) is presented in Fig. 15. As a reference, manual counting data (manual) was also added to the figure. The slopes of the trend lines for WUR (full lines) have a significantly different inclination than the trend lines of PHY (dashed lines), similar as observed in Fig. 14 with manual counting. In contrast to the PHY culture, the trend lines of WUR are rather parallel and show a similar correlation with biomass release. For PHY, the curves based on flow cytometry data show a good linear correlation with biomass release while the curve based on manual counting has a lower fit (see R^2 in Table 12).

4. DISCUSSION

Microscopic counting of cell numbers and dry weighing are the most used laboratory-scale methods to estimate dry weight concentration and cell number concentration of microalgae biomass. Fig. 13 and Fig. 14 display these two conventional methods applied to bead milled samples.

The progressively decreasing cell number during bead milling of both culture specimens in Fig. 13 indicates that the bead mill conditions were appropriate to obtain the goal of a near complete cell disruption yield. The slight variations in cell number reduction between both cultures, *i.e.*, WUR and PHY, support earlier observations of differential susceptibility of microalgae strains to bead milling (Günerken et al., 2015). Additionally, the plateau in WUR biomass release at high disruption yield and steadily increase for PHY (Fig. 13) also indicate a culture dependent behavior resulting from differences in physical and nutrient conditions employed during their growth (Günerken et al., 2016). Both methods, i.e. gravimetric analysis and manual counting, are compared in Fig. 14 to evaluate their applicability to determine cell disruption yields.

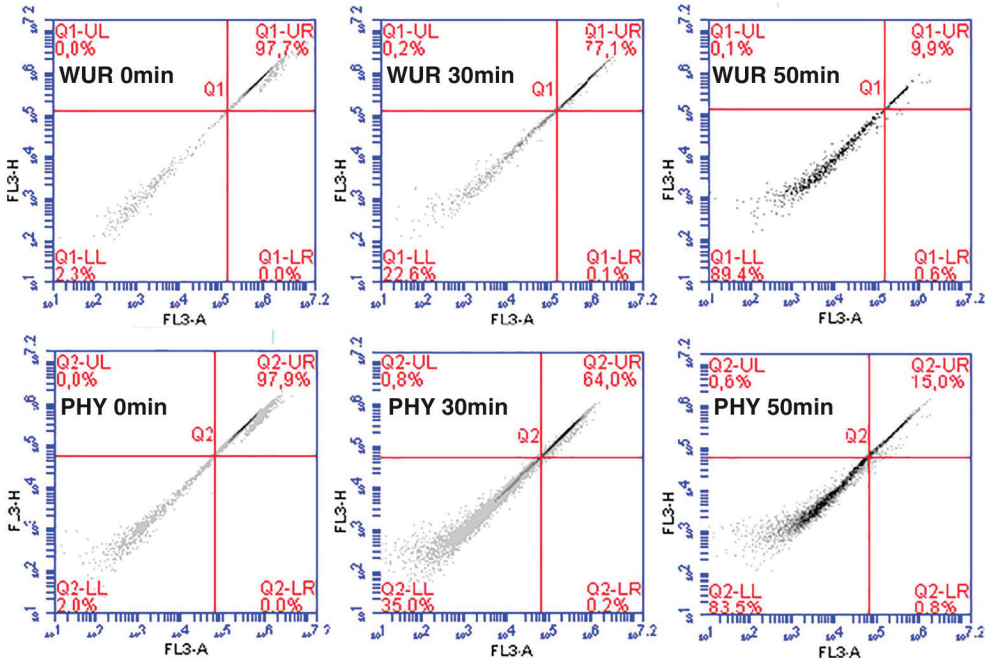

Fig. 12: Flow cytometer long pass filter (> 670 nm) data, FL3, after 0; 30 and 50 min of bead milling two Chlorella species (WUR and PHY).

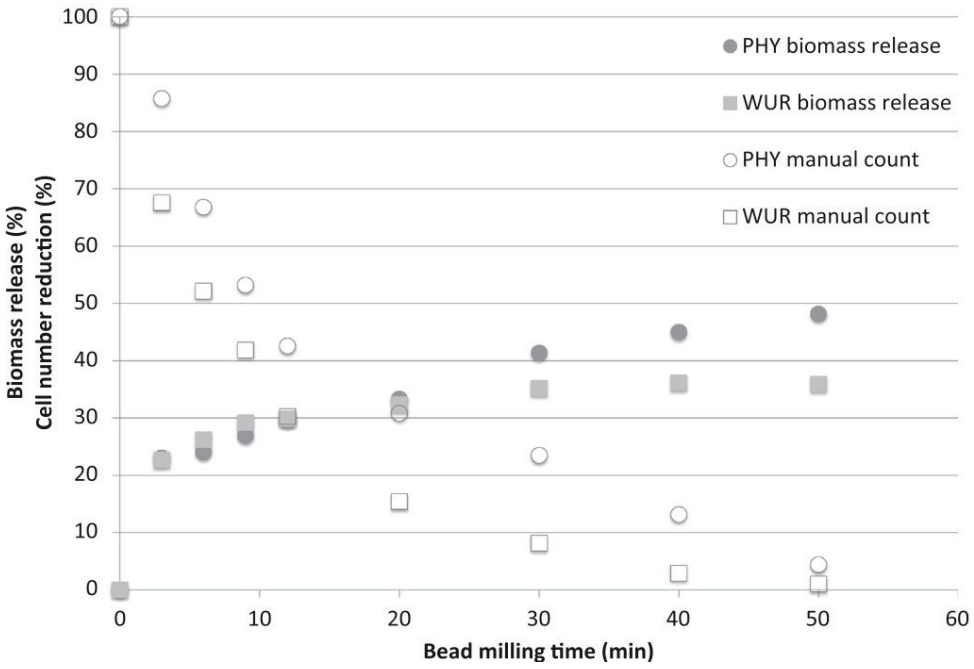

Fig. 13: Average values of gravimetric biomass release (■, ●) and cell number reduction based on manual counting (□, ○) for two Chlorella species (PHY, WUR) in function of bead milling time.

Fig. 13 shows that for the WUR Chlorella culture, cell disruption yields calculated via manual counting data correlate linearly with biomass release although the fit is not very good (see R^2 Table 12).

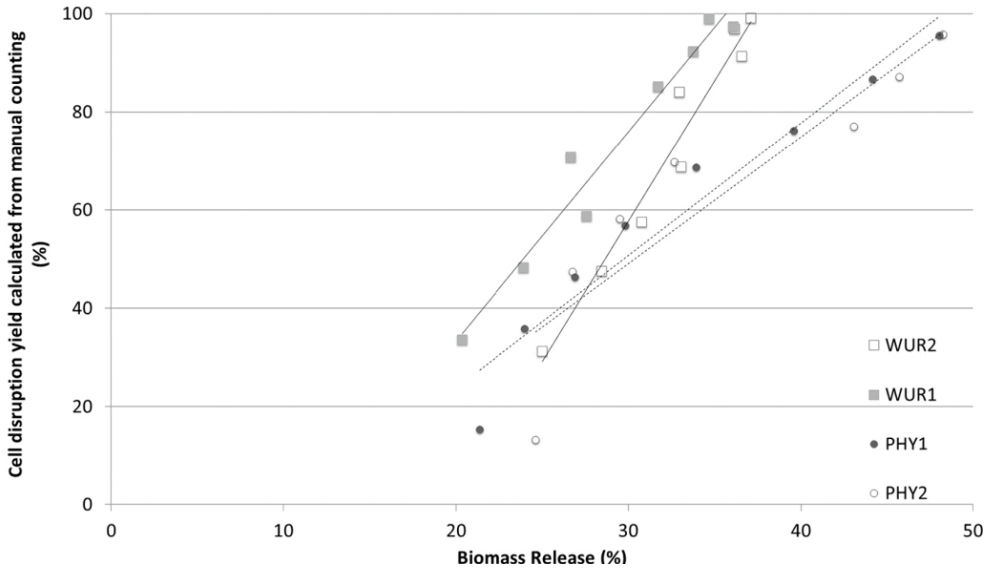

Fig. 14: Correlation of cell disruption yields calculated from manual counting data and biomass release during 4 runs of bead milling WUR and PHY Chlorella species.

Additionally, both WUR runs show a significantly different trend line equation. For PHY1, a similar R as for the WUR trend lines is observed, but PHY2 shows a poor linear fit. In contrast to the WUR samples, both PHY runs have a similar trend line equation.
Although manual counting at discrete time intervals gives similar results for both runs of each culture, initial biomass release during bead milling for both cultures is higher in the second run. Differences are thus observed in biomass release, but manual counting does not reveal these differences. This could imply that manual counting cannot observe changes in the algae that result in a higher release of biomass. If this is the case, manual counting resulted in an underestimation of the cell disruption yield due to the presence of intact, but leaching cells. This observation is in line with Laurens et al. (Laurens et al., 2012) stating that the estimation of the cell disruption yield and biomass release entirely based on manual counting and dry weight determination is likely to yield erroneous information and might incur economic losses due to improper downstream processing.

According to literature, with optical methods it might be possible to overcome the limitations of gravimetric analysis and manual counting, but have not yet been explored to determine cell disruption yields in downstream processing. Several authors reported the use of specialized flow cytometers, but this study used a flow cytometer with standard configurations. Fig.

15 shows the experimental relation between biomass release and disruption yield calculated from manual counting data and 3 different methods based on flow cytometer data.

In Fig. 15 the trend lines of each culture are easily distinguished via their inclination indicating a culture dependent behavior. For WUR, all curves show a good linear fit. For PHY, all curves are sufficiently linearly correlated to biomass release with exception of 'PHY-Manual' (see R^2 in Table 12). Chioccioli et al. (2014) have earlier reported that *Chlorella vulgaris* cultures grown in regular and supplemented media exhibit variations in cell size distribution and OD750nm values, which resulted in different calibration lines for the estimation of the biomass concentration. This could explain why 'manual' counting leads to formation of a trend line with lower R^2 value. When comparing the results of both cultures, the calculations based on flow cytometer data and especially the 'FC-FL3' curves, fit well for both cultures. The correlation between the cell number reduction and biomass release, however, is only valid for assessing high disruption yields and a biomass release higher than 23% since disruption rate was already high after 3 min of bead milling. Flow cytometry data, and more specific the FL3 signal, could probably be used industrially via the 'FCFL3' calculation to estimate biomass release and cell disruption yield during or after disruption since this method is faster than manual counting and gravimetric biomass release. If the 'FC-FL3' calculation will be used by others as a fast method to determine biomass release during disruption, the correlation between the cell disruption yield and gravimetric analysis must be known since the trend lines show a culture dependent behavior. The properties of all described methods are summarized in Table 13. The evaluation of the industrial applicability is mainly based on the correlation of the calculated disruption yield with biomass release and the time efficiency of the method. Additionally, it must be noted that in an industrial setting, gravimetric analysis to calculate biomass release must take the salt content of the medium in consideration.

Table 12: Trend line equations and R2 values from Fig. 14 and Fig. 15.

RUN	Trend line equation	R^2
WUR 1 manual	4,2668*x-52,062	0,9599
WUR 2 manual	5,7141*x-113,77	0,9558
WUR average manual	5,0146*x-82,516	0,9826
WUR FC QR	5,9796*x-125,68	0,9771
WUR FC direct	5,1812*x-88,735	0,9869
WUR FC FL3	5,0545*x-98,094	0,9810
PHY 1 manual	2,7040*x-30,418	0,9453
PHY 2 manual	2,5872*x-28,625	0,8123
PHY average manual	2,6895*x-31,216	0,9016
PHY FC QR	2,5483*x-27,466	0,9411
PHY FC direct	2,5780*x-24,708	0,9413
PHY FC FL3	2,8639*x-40,453	0,9924

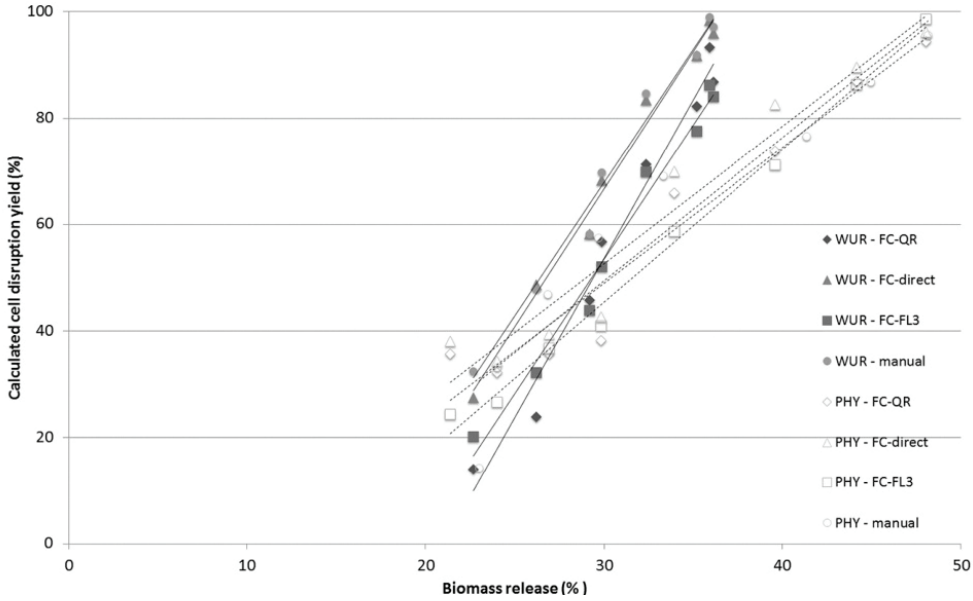

Fig. 15: Correlation of biomass release during bead milling of WUR and PHY Chlorella species and cell disruption yields calculated with flow cytometry (FC) data (QR = quadrant count; direct = direct count; FL3 = calculated via FL3-Hmean*FL3-Amean) and manual counting data.

Table 13: Comparison of 4 methods to evaluate algae cell disruption yield.

Method → Parameter ↓	Gravimetric analysis	Hemocytometer manual count	FC-direct count	FC-QR count	FC-FL3 count
Time efficiency	Low	Low	High	High	High
Sample volume	High	Low	Low	Low	Low
Sample biomass concentration	High	Low	Low	Low	Low
Disruption yield	Ok at intermediate yields	Underestimation	Ok	Ok	Ok
Relation with biomass release		–	+	+	+ + +
Industrial applicability	+	+	+ +	+ +	+ + +

5. CONCLUSION

This study is a proof of principle that a flow cytometer with standard configurations can be used to evaluate biomass release and cell disruption yields to evaluate downstream processing efficiency. Moreover, the results based on flow cytometer data proved to be an efficient alternative for conventional hemocytometer-based cell counting (manual counting). It could provide real time disruption data since the analysis is fast, non-destructive and requires low sample volumes, and even works with highly diluted samples. Additionally, since flow cytometer data correlated with biomass release of both Chlorella species, the method might be universal and applicable to other species. To validate the extend of this technique, it would be interesting to use other algae cultures and species and perform measurements in an industrial algae production facility.

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CHAPTER 5

Disruption of microalgae with a novel continuous explosive decompression device

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ABSTRACT

Keywords: Microalgae, Mild cell disruption, Continuous process, Explosive decompression, Techno-economic assessment

Most microalgae cells are very resistant to mechanical and chemical stresses. Because target products are mainly located inside the cells, disruption of the cell wall is an essential step in downstream processing to recover the microalgae ingredients. This study is focused on the development of a mild cell disruption method based on explosive CO2 decompression to allow the recovery of multiple sensitive products.

The principle behind cell rupture via explosive decompression is the uptake of a gas under elevated pressure into the microalgae cells, where after a sudden depressurization gas expansion causes rupture of the cells. Conventional explosive decompression uses stirred tank reactors wherein, because of inefficient mixing, the gas/liquid interfacial area is minimal, resulting in long contact times, inefficient gas uptake and inefficient cell rupture. To increase the mixing yield and the formation of a gas-liquid micro-emulsion, a new continuous apparatus and a method were developed to process microalgae biomass by CO2 based explosive decompression. The final design consisted of 2 pumps, a tubing system, a sparger, a hydrodynamic agitation zone and a heated relief valve. Experimental comparison of the conventional batch and the new continuous devices showed that process time was reduced 3.2–9.6 fold, the biomass and biochemical release efficiency increased more than 2-fold, and CO2 consumption reduced 2–4 fold. The method can be considered as mild, since it can operate at room temperature without addition of chemicals. Moreover, the calculated shear rate on the algae cells is only 0.33% of the shear rate recorded during bead milling, while depending on the process parameters releasing a similar amount of biomass components from the cells into the supernatant. The article describes the development of the explosive decompression systems supported by computational fluid dynamics simulations and experimental data on algae cell disruption, complemented with a first techno-economic assessment.

1. INTRODUCTION

Microalgal target products are mostly located intracellularly, but microalgal cells are usually covered with a thick cell wall. This makes downstream processing in the algae biorefinery complex, and a cell disruption step is required for product extraction and isolation. Since microalgal cell physiology is cultivation dependent and highly variable between species (Gerken et al., 2013; Scholz et al., 2014), it is challenging to find an appropriate, universal cell disruption method for microalgae. Although the used mechanical cell disruption methods have a high cell disruption yield (Günerken et al., 2016), they are not suitable for processes in which heat and stress sensitive products are targeted, because local temperature and pressure differences negatively affect the stability of the final products (*e.g.*, proteins). Moreover, high energy consumption makes them less favourable for industrial scale processes (Günerken et al., 2015). Cell disruption methods, which are energy efficient and work under mild conditions, are gaining interest.

Cell disruption via explosive decompression was first reported for bacteria by Fraser in 1951 (Fraser, 1951). The application of this method on various microorganisms was studied by several researchers (Debs-Louka et al., 1999; Garcia-Gonzalez et al., 2007). Various methods have been proposed to determine the necessary process parameters and describe cell disruption phenomena during explosive decompression process (Garcia-Gonzalez et al., 2007). The most important process parameters are batch time, pressure and moisture content to enhance the penetration of gas or liquid into cells. The main phenomenon in cell disruption by explosive CO_2 decompression was attributed only to the acidifying effect of carbonic acid by dissolving CO_2 in water. This was postulated to disturb some metabolic pathways and units, such as the cytoplasm, membranes and enzymes within the cells. As the main target of this research was to achieve microbial inactivation, typically the loss in cell viability was considered as a main parameter to determine the yield of explosive decompression processes (Debs-Louka et al., 1999; Garcia-Gonzalez et al., 2007; Lin et al., 1992a; Lin et al., 1992b; Nakamura et al., 1994). There are no recent studies in the literature on explosive decompression of microalgal biomass. However, in some studies, the use of explosive decompression to release intracellular compounds from bacteria was also studied, and the influence of the diverse process parameters was assessed (Enomoto & Nakamura, 1997; Ginty et al., 2006; Khosravi-Darani et al., 2004; Lin et al., 1992a; Lin et al., 1992b; Nakamura et al., 1994; Rivard & Nagle Pretreatment of High Solid Microbial Sludges, 1998). Lin et al. (Lin et al., 1992a; Lin et al., 1992b) have indicated that supercritical/subcritical carbon dioxide penetrates into cells and ruptures them when pressure is suddenly released, and the process yield can be increased by repeatedly releasing the pressure and re-pressurizing the system. Fraser (Fraser, 1951) investigated the same subject and observed that the best cell disruption results were obtained when the CO_2 pressure was released as rapidly as possible. In their study (Fraser, 1951), not cell viability, but the release of biomass to the supernatant was used to evaluate the rupture efficiency.

Both dissolution of CO_2 in water to form carbonic acid and penetration of CO_2 into cells are strongly correlated to mass transfer characteristics of the system. The systems used for conventional explosive decompression processes are based on stirred tank systems with several drawbacks related to their design, such as poor heat transfer, low average mixing intensity, and inhomogeneous distributions of reactants, temperature and mixing energy. This results in yield reduction, energy efficiency, profitability, and requires long contact times.

To overcome these drawbacks, a process intensification approach was used in this study to gradually improve the design of a continuous disruption installation. Process intensification is defined as "the development of innovative apparatus and techniques that offer drastic improvements in manufacturing and processing, substantially decreasing equipment volume, energy consumption, or waste formation, and ultimately leading to cheaper, safer, sustainable technologies" (Boodhoo & Harvey, 2013; Stankiewicz & Moulijn, 2000). The approach first aimed to intensify fluid dynamics to lower mass and heat transfer resistance by reducing path lengths of the diffusion/conduction interfaces. Additionally, CO_2 injection was optimized, mixing was maximized, CO_2 consumption minimized, and the processing times were reduced from hours in batch to the order of minutes with the continuous designs. Subsequently, a statistical study via linear regression analysis was performed to further investigate the effects and statistical importance of the parameters with the newly developed explosive decompression method and equipment.

To our knowledge, there is no record in the literature of successful explosive decompression of microalgal cells. In this paper, the problems of the conventional batch decompression method are analysed, and the development of a continuous explosive decompression method is discussed in detail. Finally, a solution is presented with the patented final design (D'Hondt et al., 2014) and industrially relevant aspects for all designs are compared.

2. MATERIAL AND METHODS

2.1. Preparation of algae suspensions

Neochloris oleoabundans UTEX 1185 was used for all studies. The cultivation and harvesting was performed according to Günerken et al. (Günerken et al., 2016). After cultivation and harvesting, the microalgae suspension was washed by centrifuging at 3600g for 5 min. The pellet was collected and re-suspended in water after every centrifugation step. Additionally, the supernatant was centrifuged again to recover the remaining suspended algae cells in pellet form. This washing procedure was repeated 5 times, and after the last centrifugation step, all the obtained pellets were mixed together to form the final pellet sample. This final pellet

sample was re-suspended in the process liquid (*i.e.*, water, water/ethanol mixture, water/isopropyl alcohol mixture) to obtain the final sample with a specific dry cell weight (DCW) concentration for the different experiments (Table 14: DCW and co-solvent column).

2.2. Cell disruption efficiency

The cell disruption efficiency was evaluated via the biomass released from the algae to the supernatant, by measuring the wet weight of the whole samples, the dry weight of the supernatants and the dry weight of the pellets, where the supernatant weights were corrected by blank measurements (see below). This approach allowed the construction of two mass balances, i.e., (1) based on the dry pellet and (2) based on the dry supernatant weight, to confirm the accuracy of the results.

Samples coded as "UN" (untreated/blank) in Fig. 1 represent the characteristics of the raw material used in the experiments (*i.e.*, untreated suspension - not treated with CO_2 explosion). To discriminate the influence of the CO_2 explosion itself, the algae suspension obtained after washing and re-suspension was passed through the system (*i.e.*, Conventional design, 1st or 2nd design) without CO_2 addition or pressurization to investigate the effects of the physical design on the cell disruption efficiency, such as shear stress at the tip of the impeller blade in the resource vessel or orifices and valves in the explosive decompression unit. The supernatants of these samples were analysed for their biochemical composition, and the results were used to correct the supernatant analyses of the actual samples, via subtraction.

The samples coded as "Run x" in Table 14 and Fig. 16were collected after cell disruption with CO_2 under specific processing conditions (Table 14). After the cell disruption, the whole wet sample was weighed ("Whole wet sample weight" in formulas), and the supernatant and the pellet were separated by cascade centrifugation at 20,000g for 20 min in 5 cycles. After every centrifugation step, the supernatant was separated from the pellet and centrifuged again in the next step. The pellets obtained in each subsequent centrifugation steps were collected and merged. The supernatants obtained after every step were collected, merged and filtered through 0.22 μm filter to obtain the final supernatant samples. Prior to the drying process, the samples were frozen overnight at −20 °C and subsequently transferred to −80 °C for 6–8 h to obtain frozen samples. The frozen samples were freeze dried under vacuum for 72 h. The dried samples were weighed and stored at −20 °C in sealed recipients before the analyses.

The weights of the samples (untreated, supernatant and pellet) were determined before and after drying. The weight of the untreated suspension, before and after drying, was used to calculate the dry cell weight concentration (DCW g/g). As explained above, the weight of the supernatant from untreated samples were used as a blank to correct for the compounds already dissolved in the untreated suspension. More specifically, the weight was subtracted from the supernatant dry weight of the actual samples before calculating

the biomass release ratio. All calculations were made by assuming that the whole sample dry weight values and related untreated samples are the same.

The biomass release ratio (BRR), *i.e.*, the amount of biomass released into the supernatant relative to the amount of initial biomass, was calculated in two ways. The first approach was based on the dry weight of the supernatant and is cited as BRR (SN) throughout the manuscript.

Additionally, to be able to calculate the overall mass balance, the biomass release ratio was also determined based on the reduction in dry pellet (P) weight, cited as BRR(P) in the manuscript.

To evaluate the results and confirm the mass balances based on the pellet and the supernatant samples, the mass balance error (MBE) was calculated. MBE refers to the absolute difference expressed in percentage between both (SN and P) calculated biomass release ratios.

2.3. Biomass composition

All dried samples, supernatants and pellets, were analysed to determine their biochemical composition, i.e., lipids, proteins, carbohydrates, via Eq. 5. The biochemical analysis results of the untreated pellet are the benchmark (given as UN) and considered as the starting composition of the algal biomass.

$$Biochemical\ Content\ (\%) = \frac{DW_{BC(sample)}(g)}{DW_{WS(sample)}(g)}\ x\ 100 \qquad \text{Eq. 5}$$

DW = dry weight; BC = biochemical; WS = whole sample.

Table 14 Overview of the process parameters of the experiments performed in the different explosive decompression devices, i.e., two batch and two continuous designs. DCW = Dry Cell Weight concentration.

Run	Stirring (rpm)	Pressure (bar)	Batch time (min)	CO_2 to liquid ratio (vol%)	Relief valve T (°C)	Oven T (°C)	Flow rate (ml/min)	DCW (wt%)	Co-solvent (vol%)
Conventional batch design (Fig. 1 Batch)									
1	300	200	15	60	Room T	35	–	6.4	–
2	100	200	15	60	Room T	35	–	6.4	–
3	100	100	15	45	Room T	35	–	6.4	–
4	100	200	45	60	Room T	35	–	6.4	–
5	300	100	45	45	Room T	35	–	6.4	–
Conventional batch design (Fig. 1 Batch)									
6	300	200	45	45	Room T	35	–	6.4	–
7	300	200	45	45	Room T	35	–	6.4	100 vol% EtOH
8	300	200	45	45	Room T	35	–	6.4	10 vol% EtOH
1st design (Fig. 1 Design 1)									
1	–	125	–	241	70	35	4.15	3.2	–
2	–	200	–	169.5	70	35	2.95	3.2	–
3	–	125	–	208	70	35	2.40	3.2	–
2nd design (Fig. 1 Design 2)									
1	–	250	–	20.6	35	4	15	4.6	8 vol% IPA
2	–	250	–	20.6	17	4	15	4.6	–
3	–	162.5	–	20.1	20	35	19,44	2.5	–
4	–	250	–	20.6	20	20	15	2.5	–
5	–	75	–	4.8	20	35	19,17	2.5	–
6	–	75	–	19.32	20	4	4,8	2.5	8 vol% IPA
7	–	250	–	5.18	35	4	15	2.5	–
8	–	162.5	–	5.02	20	4	15	4.6	–
9	–	162.5	–	20.1	35	20	10	4.6	–
10	–	75	–	4.8	35	20	11.32	2.5	–
11	–	162.5	–	5.02	35	35	15	2.5	8 vol% IPA
12	–	250	–	5.18	20	35	10	4.6	8 vol% IPA
13	–	250	–	20.6	35	20	15	4.6	8 vol% IPA
14	–	162.5	–	5.02	20	20	10	4.6	8 vol% IPA

rpm = rotations per minute; min = minute; ml = millilitres; vol% = volume ratio; wt% = weight ratio; °C = degrees Celsius; T = temperature; EtOH = ethanol; IPA = isopropyl alcohol.

Dubois (carbohydrate measurement), BCA (protein measurement) and modified Folch (lipid measurement) methods were applied to all samples to determine their composition (Günerken et al., 2016). All chemicals were purchased from either Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA), if not stated otherwise.

To quantify carbohydrate release, the phenol-sulphuric acid reaction was used (Dubois method) (Günerken et al., 2016). Briefly, 1 mg of freeze dried sample was re-suspended in 250 µl of milli-Q water and mixed with 125 µl of 5 (wt%) phenol reagent. Afterwards, 625 µl pure H_2SO_4 was added to form the final mixture. Glucose was used as standard to obtain a standard curve and calculate glucose equivalent mass of carbohydrates in related samples. The samples were kept at room temperature under dark conditions for 40 min prior to UV–VIS spectroscopy measurement at 492 nm (Günerken et al., 2016).

To determine the protein content via BCA protein measurement method (Günerken et al., 2016), first dried samples were ground and a protein extraction solution (150 mM NaCl, 1 mM EDTA, 0.5 (wt%) sodium deoxycholate, 70 mM NaF, 2 µM of PMSF, 0.1 (wt%) sodium dodecylsulphate) was prepared. Subsequently, the sample (20 mg), the protein extraction solution (10 ml) and 1 g of glass balls (2–4 mm diameter) were mixed vigorously with a vortex mixer for 3 min in a 50 ml plastic centrifuge tube. Afterwards, the samples were kept at room temperature under dark conditions for 30 min and centrifuged at 20,000g for 5 min. As the last step, the supernatant was used to determine the protein concentration

with the Pierce BCA protein assay according to the producer's instructions (Pierce, USA). Bovine serum albumin was used as a standard (Günerken et al., 2016).

A modification of the Folch method (Günerken et al., 2016) was used for total lipid analysis. Briefly, 10 ml of CH_3OH was added to 1 g of freeze dried sample, the solid particles were ground with a glass stick. Subsequently, another 10 ml of $CHCl_3$ was added to the mixture and vortexed for 3 min and repeated with another 10 ml of $CHCl_3$ and 8 ml of saline phosphate buffer (8 g/l of NaCl; 0.2 g/l of KCl; 1.44 g/l of Na_2HPO_4; 0.24 g/l of KH_2PO_4) was added to the final mixture. The suspension was mixed in an orbital shaker for 45 min at 150 rpm and centrifuged at 1000g for 1 min to accelerate the phase separation. The bottom layer was collected, and the upper layer was discarded. The middle layer was extracted 2 more times with the same method. The bottom $CHCl_3$ layers from the 3 extractions runs were collected and dried under N_2 flow at room temperature overnight (Günerken et al., 2016).

2.4. Conventional batch explosive decompression

The conventional batch method was tested with 2 different reactor setups (Run 1 to 8 in Table 14 and Fig. 16 Batch). In Run 1 to 5 in Table 14 and Fig. 16 Batch, depressurization occurs via an exit in the headspace, thus, releasing only gaseous products, while in Run 6 to 8 in Table 14 and Fig. 16 Batch depressurization is via a submerged tube in the suspension, thus, releasing the whole biomass suspension together with the gas.

The same procedure was used for both batch designs (Run 1 to 8 in Table 14 and Fig. 16 Batch). All experiments were performed at 35 °C, to prevent protein degradation (Edebo & Magnusson, 1973). Several process parameters were evaluated, i.e., stirring velocity, pressure, batch time and CO2 to liquid ratio (Table 14). After harvesting according to the method explained in Section 2.1. Preparation of algae suspensions, the microalgae pellet is resuspended to obtain an adequate volume of microalgae suspension to be used in conventional batch explosive decompression studies. A microalgae suspension with 6.4 ± 0.2 % DCW was loaded in the high pressure stirred tank reactors (with 32 mm tank and 18 mm stirrer diameters; Premex Solutions GmbH, CH) with 100 ml working volume to reach 60% or 45% CO2/Water (Vol%) ratio and heated in a water bath. After the suspension reached 35 °C, the reactors were loaded with CO_2 and the process continued until the pre-determined batch time was reached.

The same procedure without CO_2 or pressure was performed to obtain untreated samples. Because of low biomass release ratios, only pellet samples were analysed on biochemical composition in these experiments. The conditions for the 8 runs are given in Table 14 and the results in Fig. 16.

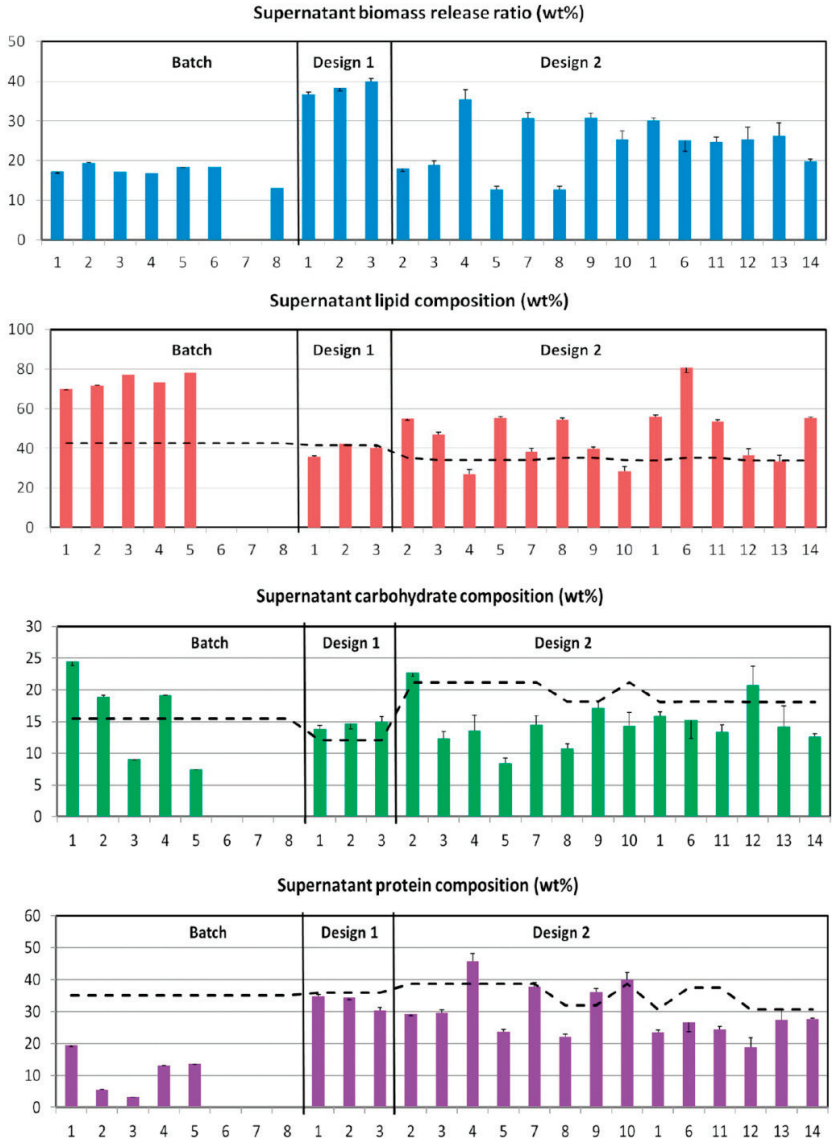

Fig. 16 Results from all the experimental runs described in Table 1.

Batch = First studies with conventional system.
Design 1 = Tests made with first continuous explosive decompression system design.
Design 2 = Tests made with second continuous explosive decompression system design.
The top figure shows for each run the biomass fraction released into the supernatant (in wt%). The three other figures provide compositional information of the biomass fraction released in the supernatant. Different conditions led to different efficiencies and also differences in biomass fractions in the supernatant. The horizontal dashed line is given for comparative reasons and represents the biochemical content of the untreated sample that was used in the run (expressed as wt%).
A biochemical release above the dashed line indicates a selectivity of the process towards this biochemical. The error bars are based on the mass balance error. Wt% represents the weight ratio in percentage.

2.5. Continuous explosive decompression

Two different continuous apparatus designs were constructed, *i.e.*, 1st and 2nd design. The different designs are elaborated in detail in Section 3. Results and discussion together with the results. Every design was developed by modifying the latest version of the apparatus to maximize the disruption yield, minimize the energy and CO_2 consumption, and to overcome practical problems. In general, the continuous systems consisted of: (1) two pressure pumps for the algae suspension and CO_2; (2) CO_2 injection via T-junction or sparger; (3) a mixing zone with different configurations depending on the design with tubes either at room temperature, in an oven, or in an ice bath; (4) a heated relief valve to release the suspension up to atmospheric pressure; and (5) a sample collection recipient (2nd design shown in Fig. 18). Before collecting samples for each run, a waste sample was collected to ensure a steady state regime of the applied conditions in the system. The process parameters for a selection of experiments with the different designs are given in Table 14. The pellet and supernatants for both treated and untreated samples were analysed according to the methods of Sections 2.2. Cell disruption efficiency and 2.3. Biomass composition and the results are displayed in Fig. 16.

2.6. Computational fluid dynamics simulations

To improve the equipment, computational fluid dynamics (CFD) simulations were executed with Multiphysics software of Comsol Inc., Palo Alto, CA, to develop, evaluate and choose the designs. The goal of the simulations was to reduce the $scCO_2$ droplet size by increasing the mixing efficiency to maximize CO_2 transfer into the cells. The results of the simulation are displayed in Fig. 19 and Table 15 and are discussed in Section 3. Results and discussion together with the experimental results.

In all simulations, the system pressure and temperature were set to 250 bars and 35 ˚C as constant values. The CO_2 flow to liquid flow ratio was set at 29%. In an ideal well mixed system, the volume fraction of CO_2 should be equal to the CO_2 flow to liquid flow ratio of 29% in the whole system and in every sample volume. The mixing efficiency of each system was, therefore, evaluated by the deviation of the actual CO_2 volume fraction value, as calculated by simulation after the system reached steady state to the "ideal" CO_2 volume fraction value of 29% of the "well mixed" system (cfr. variation % in Table 15). Another output of the simulation was the shear rate value. High shear rates generally facilitate cell disruption, but also cause local temperature increases and pressure differences that can negatively affect the quality of the biomolecules.

Table 15: Overview of the various designs used in the Comsol simulations and the corresponding results. They are grouped in two sections related to their basic design features (T-junction or in house manufactured sparger).

Case	Design: CO_2 injection diameter – mixing zone	CO_2 volume fraction value in case of well mixing	Simulated CO_2 volume fraction in given part of the system	CO_2 fraction variation (%)	Simulated shear rate (1/s)
Continuous design with T-junction sparger					
1	T-junction - orifice	0.29	0 or 1	- or 244.83	22
2	T-junction - no orifice (1st design)	0.29	0 or 1	- or 244.83	7
Continuous design with in house manufactured sparger					
3	100 µm - orifice (2nd design)	0.29	0.62	113.79	4902
4	100 µm - no orifice	0.29	0.74	155.17	2388
5	50 µm - orifice	0.29	0.39	34.48	5653
6	50 µm - no orifice	0.29	0.55	89.66	2758
7	25 µm - orifice	0.29	0.31	6.90	6129
8	25 µm - no orifice	0.29	0.44	51.72	2990

1st and 2nd design indicated in brackets refer to the designs as used in experiments and discussed in this article. The other designs in the table were approached solely theoretically.

Given its complex nature, several approximations were taken to reduce the complexity of the fluid. The fluid consisted of a compressible (CO_2) and an uncompressible (water) liquid with a high surface tension at the interface, and a solid phase (cells) with a surface viscosity similar to the uncompressible liquid. The rheological characteristics of CO_2 were assumed as constant, because of the stable temperature inside the system. The effect of pressure fluctuations on the CO_2 characteristics in the hydrodynamic agitation zone was not taken into account to reduce the complexity of the simulation. Therefore, the fluid flow was assumed to be an incompressible fluid, since the pressure inside the system was approximated as constant. The solid particles were assumed to be part of the buffer solution, since they have a spherical shape and their surface viscosity and density resembles that of water. In conclusion, the system was simplified to a two phase liquid system, *i.e.*, an emulsion of supercritical CO_2 in the uncompressible liquid. Therefore, the Laminar Two-Phase Flow module of Comsol was used for this study, with Phase field and phase initialization options.

The system was simulated as non-conservative and first order discretisation for velocity and pressure was used together with the consistent stabilization tools of Comsol. Water and CO_2 characteristics were taken from the Comsol database, the characteristics were validated by the Peace Software database (http://www.peacesoftware.de), and the missing parameters were completed. The surface tension between water and CO_2 at 250 bars was taken from the literature, 0.028 N/m (Nielsen et al., 2012). The walls of the system were set as wetted walls with a $\prod/2$ contact angle. As an initial condition, the system was assumed to be filled with water with a flow value considering just the sample flow rate. The initial conditions for the CO_2 inlet of the sparger or T-junction were taken similarly filled with CO_2 and with a flow value considering just the CO_2 flow rate. The inlets were taken as standard flow rates (with standard pressure and temperature option) to be able to add more parameter values and increase the precision of the simulation results. The outlet boundary condition was taken as pressure without any stress against the flow, by suppressing the backflow.

The time dependent simulation was carried out by taking the result of the phase initialization as the initial expression. The results were calculated via a general finite element solution method, which provided by the CFD program, used as a base calculation method, *i.e.*, the constant Newton method with the Jacobian update on every iteration and the Anderson acceleration was used to decrease the calculation time. The results are discussed in Section 3. Results and discussion.

3. RESULTS AND DISCUSSION

The development and optimization of a device for algae cell disruption via explosive decompression is elaborated in this section. Experimental disruption data is accompanied by the results of the simulation for the continuous installations, and the benefits and drawbacks are discussed for each design. Since the overall development is the resultant of many series of experiments, only a selection of experiments is discussed to illustrate the developments up to the final design. All experimental conditions are given in Table 14 and the results thereof in Fig. 16. Section 3. Results and discussion ends with a comparison of all designs, discusses mechanisms of rupture and biomass release and evaluates parameters relevant for industrial implementation.

3.1. Conventional batch explosive decompression

The experiments in the reactor setup shown in Runs 1 to 5 in Table 14 and Fig. 16 Batch were carried out to evaluate the effect of the most important process parameters, i.e., stirring power, CO_2 pressure and batch time. All tests had a similar and relatively low biomass release ratio (17–19%) and the small differences in the mass balance based on supernatant and pellet (MBE, see section 2.2) confirmed that the samples were representative (error bars in Fig. 16). Different conditions led to different efficiencies and also differences in biomass fractions in the supernatant, *i.e.*, ranging from 70% to 78% for lipids, 3% to 20% for proteins and 8% to 24% for carbohydrates. Based on Table 14 and Fig. 16, a short contact time in combination with a low stirring speed resulted in a significant decrease in released proteins (Runs 2&3 vs. Runs 1&4). The results of these runs suggest that the contact between CO2 and the cells was not sufficient to cause extended cell rupture.

A lower pressure on the other hand, significantly inhibited the release of carbohydrates (Runs 3&5 versus Runs 1&2&4) and the release of lipids was relatively unaffected by the varying process parameters. These observations suggest that the mechanism of biomass release in this set-up is probably mainly attributed to supercritical CO_2 extraction without significant cell rupture.

At the end of the studies with the 1st reactor setup (Run 1 to 5 in Table 14 and Fig. 16 Batch), three problems were observed:

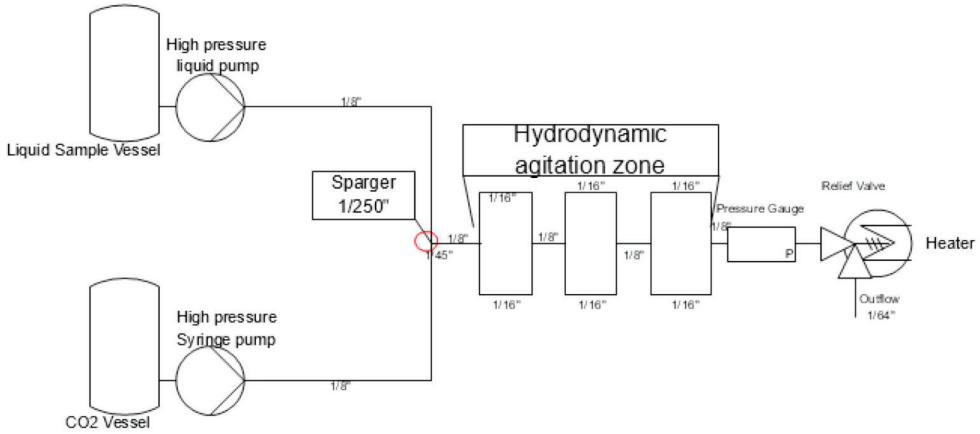

Fig. 17: Design of the continuous explosive decompression system where pressurized CO2 and algae suspension are mixed in a sparger followed by a hydrodynamic agitation zone and an outlet via a heated back pressure regulator to induce explosive decompression of algae cells. 1/250″ = 100 µm, 1/64″ = 0.4 mm, 1/ 16″ = 1.6 mm, 1/8″ = 3.2 mm.

To overcome these problems, the design was modified by submerging the outlet-tube in the algae suspension (Run 6 to 8 in Table 14 and Fig. 16 Batch). This modification allowed fast depressurization by evacuating the entire suspension through the exit valve. Additionally, a mixture of ethanol (EtOH)/water with varying volumetric ratios (Run 6 = 0%, Run 7 = 100% and Run 8 = 10%) was added to the pellet to obtain a sample suspension less prone to freezing, and to prevent freezing and clogging. To determine the effects of EtOH on the cell rupture, all tests (Runs 6, 7 and 8 in Table 14 and Fig. 16) were performed at high stirring speed, high pressure and a long residence time.

The biomass release of Runs 6, 7 and 8 was 18%, 0% and 13% respectively. The result of Run 6 shows that the modification of the reactor design had no effect on the cell disruption efficiency. Alternatively, the increasing negative influence of EtOH/Water (Vol%) ratio on biomass release can be seen in the results of the Runs 7 and 8. Since ethanol can act as a disruption agent, release of biomass constituents prior to explosive decompression resulted in high blank values and subsequently less biomass release by explosive decompression process itself. Additionally, protein denaturation may occur due to the EtOH addition and this could induce clogging of the system. Therefore, the EtOH addition may not be feasible for industrial scale applications.

Three main problems were defined for the batch explosive decompression set-up of Run 6 to 8 in Table 14 and Fig. 16 Batch:

1. Freezing due to cryogenic expansion still causes clogging at the outlet
2. Long contact time
3. Biomass release via explosive decompression in batch remains inefficient

The first problem can be easily prevented by providing heat at the outlet to prevent clogging as a result of cryogenic expansion. The second and third problem are more profound and probably caused by the mass transfer resistance at the CO_2 – water interface preventing the apolar supercritical CO_2 to diffuse efficiently into the aqueous suspension and algae cells. This resistance could be reduced by replacing water by an apolar medium, thus, by drying and re-suspending the algae in an apolar solvent. This is, however, out of scope for this research and not favourable for industrial applications since this increases the operational expenditures and may denature the ingredients (*e.g.* proteins). Therefore, in this study, new designs were developed based on the idea that the mass transfer could be increased by reducing the mass transfer distance (Δx) and Kolmogorov eddy diameter. The mass transfer area and probability of direct contact between CO_2 and the cells can be enhanced by increasing the mixing yield. Increasing the mixing yield between CO_2 and the algae suspension was, therefore, the first step towards a more optimal and functional design.

3.2. Development of the new apparatus

There are two common ways to maximize mass transfer inside systems, *i.e.*, scale-down with the same or increased throughput or introducing a turbulent regime. The first is the most effective and easy way to increase the mass transfer. Building up a similar system by using smaller diameter tubes or vessels will increase the surface area to volume ratio, which will increase the mass transfer. Alternatively, mass transfer can be increased by introducing a turbulent flow regime. This can be obtained by increasing the flow rate or altering the stirring regime but can be more complex if a low shear stress is important. Therefore, a laminar flow with a high number of small diameter Kolmogorov eddy seems ideal for this application. In this flow regime, the bulk flow is laminar while local eddies mix the two phases inside the laminar flow.

Based on the last batch design (Run 6 to 8 in Table 14 and Fig. 16 Batch), where depressurization was done in a continuous liquid flow through the outlet, the reactor and the next designs were constructed as continuous explosive decompression devices. Micro-reactors seemed the most suitable at first. However, when evaluating the financial and mechanical (suitability for the scale up) aspects needed to keep the throughput the same or higher than the reactors used in conventional method, this approach was considered not to be feasible. Instead, millireactors (reactors consisting of small diameter tubes) were chosen for the next step of development. As a function of optimisation and process intensification, the designs were simulated via computational fluid dynamics prior to the construction of the lab scale devices. In Sections 3.2.1. Novel continuous explosive decompression devices and 3.2.2. Experiments with new apparatus designs, first the continuous experimental set-up is elaborated and followed by a discussion of the simulations and experimental results for each design.

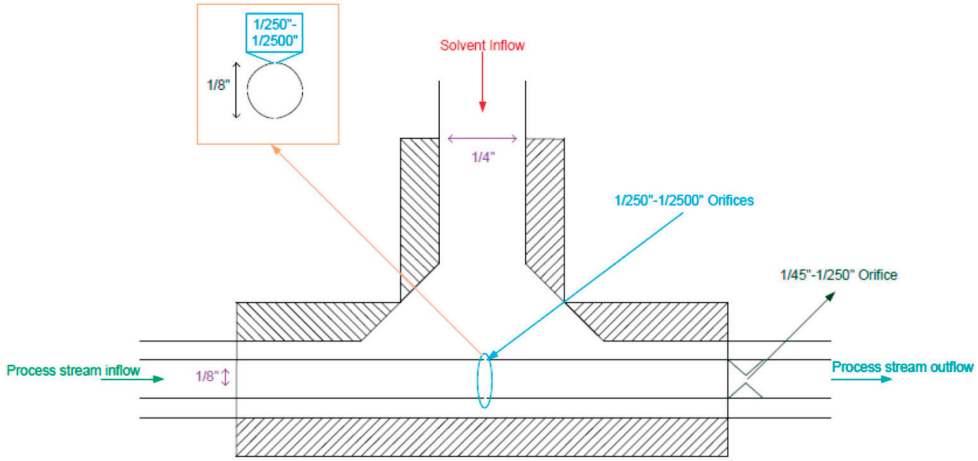

Fig. 18: Optimized sparger of the continuous explosive decompression system used in the 2nd design where CO2 (=solvent fow) is injected into the algae suspension (=process fow) via four laser drilled 100 µm holes and the mixed fow exits the sparger via an orifce of 250 µm. 1/250″ = 100 µm, 1/64″ = 0.4 mm, 1/ 16″ = 1.6 mm, 1/8″ = 3.2 mm, 1/4″ = 6.4 mm.

3.2.1. Novel continuous explosive decompression devices

Two designs for continuous explosive decompressions are discussed, whereof the second design is an improvement of the first based on simulations and experimental data. The design of the continuous set-up is displayed in Fig. 17. This figure shows that the system is fed by a high-pressure syringe pump and a high-pressure liquid pump for, respectively, CO2 and the microalgae suspension. Both fluids flow through 3.2 mm tubes and are mixed together with a sparger. This is achieved either by a simple 6.4 mm T-junction (called T-junction sparger) in the 1st design or by an in house manufactured sparger (called optimized sparger) in the 2nd design.

The in house manufactured sparger of the 2nd design is shown in Fig. 18 and described in detail in the next paragraph. After CO2 injection, the mixed fluids continue in 3.2 mm tubes to the "hydrodynamic agitation zone". The latter is a static mixer to prevent de-mixing of the CO_2 and algae suspension and reduce coalescence of bubbles. It is constructed by splitting the 3.2 mm tube into two 1.6 mm tubes and rejoining the split-flow in one 3.2 mm tube. This size of branching tubes was chosen to prevent a pressure drop by splitting the flow and to avoid problems like clogging. There are 3 static mixers in series in the hydrodynamic agitation zone. At the end of the set-up the mixed fluid passes through a pressure gauge and a heated relief valve which is set to a certain working pressure and temperature. Here, the mixture is decompressed to atmospheric pressure to induce cell rupture and samples are collected in a glass recipient.

The T-junction sparger of the 1st design was a simple 6.4 mm T-junction. The optimized sparger of the 2nd design is shown in Fig. 18 and consisted of a 3.2 mm tube that was

laser welded inside a 6.4 mm T-junction. The inlet and exit of the T-junction were used as welding points for the 3.2 mm tube while the 3rd connection was used as CO2 inlet ("solvent inflow" in Fig. 18). The welded 3.2 mm tube inside the T-junction contained 4 laser drilled holes of 100 μm diameter positioned in angels of 90° in the wall of the 3.2 mm tube. As a result, CO2 is sparged via the laser drilled holes into the 3.2 mm tube containing the algae suspension. The mixed fluid exits the optimized sparger via an orifice of 250 μm before entering the "hydrodynamic agitation zone".

The design of the sparger and its effect on the mixing efficiency were simulated via Comsol prior to manufacturing the design.

3.2.2. Experiments with new apparatus designs

Eight different computational fluid dynamics simulations were performed on the system with the T-junction or optimized sparger with different hole diameters and with or without orifice at the end of sparger system (Table 15 and Fig. 19) to obtain preliminary data to compare different designs. To simplify the simulations and reduce the calculation time, the sparger and the orifice were considered as the zone where the mixing yield had the largest effect on the total mixing yield of the system. The results of the eight simulated cases are discussed below in the sections of each design and are displayed in Table 15. Fig. 19 demonstrates the mixing efficiency and flow regime of the two designs (A, B) as function of time (1, 2, and 3). In Fig. 19, the colour pattern from blue (0% CO_2 volume fraction (Vol%)) to red (100% CO_2 volume fraction (Vol%)) represents the CO_2 volume fraction and white contours represent the streamlines of the fluid flow.

3.2.2.1. 1st design. Fig. 19 A1 to A3 shows the simulated flow regime in the 1st design (Case 2 in Table 15). CO_2 bubbles with diameters as large as the tube are formed and no CO_2/water mixing is observed. Therefore, the simulated volume fraction of CO_2 oscillates between 0 and 100% (Table 15, Cases 1&2). The interfacial surface area between water and CO_2 and consequently the mass transfer rate are minimal. Additionally, Table 15 shows that even the T-junction sparger with an orifice at its exit results in the same, low mixing efficiency since both phases pass subsequently through the orifice. The only observed difference of installing an orifice was the slight, but not significant, increase in shear rate. Therefore, as no advantages were noted and to keep the design as simple as possible, the actual experiments of design 1 were performed on the T-junction sparger without extra orifice.

Three experimental runs were performed with the first design. The process parameters are given in Table 14 and the results in Fig. 16 (Design 1). A similar biomass and biochemical release was observed as the 100% cell disruption point (maximal disruption) of bead milling studies (Günerken et al., 2016). This system, therefore, shows good potential as a cell disruption method with high yields. The residence time of the microalgae suspension under CO_2 and high pressure was reduced from 15–45 min in batch to 0.5–1 min in the continuous set-up. Moreover, the decompression time was minimized by using a relief valve. Clogging by freezing due to cryogenic expansion was minimized by using a heated relief valve at the end of the system. However, a high CO_2 flow rate compared to the liquid flow rate was necessary to achieve high cell disruption yields in this version of new apparatus design (CO_2 to liquid ratio of 170–241% continuously versus 45–60% in batch), which results in higher operational expenditures. Additionally, freezing was still observed at relief valve temperatures below 70 °C, thus, energy input for relief valve heating is necessary, which also increases operational expenditures. Problems related to high CO_2 consumption (freezing, clogging and high operational expenditures) are in line with the results obtained from CFD studies. Since the maximum shear stress is low, it results in small total interfacial area between the suspension and CO_2, the cell disruption can only be achieved with high CO_2 consumption. Additionally, since the CO_2 bubbles are relatively big, they cause freezing in the pressure release point of the system. Because of the excessive CO_2 consumption and aforementioned practical inconveniences (freezing and clogging based on freezing), the 1st design was modified according to the results from CFD simulations to develop the 2nd design.

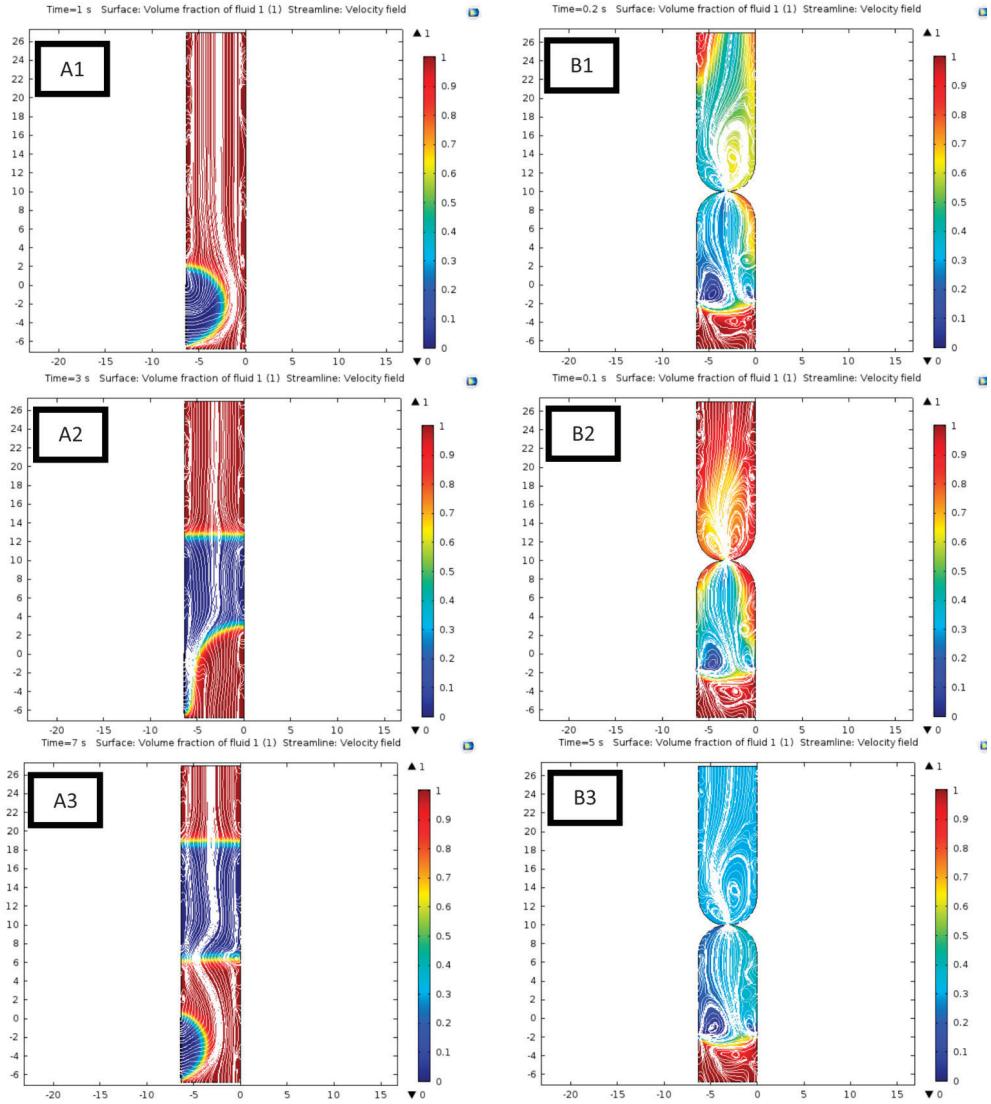

Fig. 19: The results of computational fluid dynamic simulations for A: 1st design, B: 2nd design; and from left to right (1, 2, 3) the evolution of the flow regime as function of time. The colour pattern from blue (100 vol% CO2) to red (0 vol% CO2) represents the CO2 volume fraction and white contours represent the streamlines of the fluid flow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 16: The Linear Regression results of the parameter screening study with the continuous explosive decompression system using the 2nd design. DCW (wt%) = Dry Cell Weight concentration (gdry/gwet × 100). IPA = isopropyl alcohol. The parameters with a p-value lower than 0.05 are significant to the response and are underlined.

Parameters	Coefficient estimates for parameters in the linear models for the release of				p values of parameters in the linear models for the release of			
	Biomass	Protein	Carbohydrate	Lipid	Biomass	Protein	Carbohydrate	Lipid
Intercept	22.53	29.48	17.70	45.77	0.0050	0.047	0.018	0.0050
CO_2/water ratio (vol%)	4.55	5.94	4.77	8.71	0.091	0.29	0.090	0.11
Relief valve temperature (°C)	14.24	16.93	10.35	2.21	0.0080	0.086	0.035	0.78
IPA (vol%)	−2.31	−11.561	−3.95	12.20	0.29	0.041	0.10	0.022
DCW (wt%)	−17.60	−28.977	−8.61	−25.04	0.0094	0.034	0.13	0.039
Oven temperature (°C)	5.92	8.39	6.35	−8.40	0.062	0.20	0.057	0.16
Sample flow rate (ml/min)	−26.083	−39.47	−31.42	−15.32	0.0031	0.019	0.0015	0.22
Pressure (bar)	20.84	29.028	22.17	2.37	0.0022	0.020	0.0020	0.78
R-squared	0.92	0.82	0.92	0.84				
p-Value (model)	0.0071	0.062	0.0074	0.039				

3.2.2.2. 2nd design. To obtain the 2nd design, a CO_2 sparger with 100 µm holes and a 250 µm orifice at the sparger exit replaced the T-junction of the 1st design. This was expected to increase the CO_2/water mixing efficiency and, consequently, to reduce the mass transfer distance. The overall goal was to reduce the CO_2 consumption and the temperature drop due to cryogenic expansion.

Fig. 19 B1 to B3 shows the result of the simulation with the 2nd design (Case 3 in Table 15), where a heterogeneous CO_2/water mixture is formed. The mixing efficiency of CO2 and water, however, is enhanced significantly compared to the first design (Cases 3 versus 2 in Table 15). The shear rate in the 2nd design, calculated as 4902 s–1 during CFD simulations (Table 15), is only 0.33% of the highest shear rate recorded in bead mill studies (Lane, 1999). The best CFD simulation result for the 2nd design was obtained with a sparger with 25 µm hole diameters (Case 8 in Table 15). However, laboratory experiments failed because of a high-pressure drop and fouling, when using spargers with 25 µm or 50 µm hole diameters. The experiments described below were, therefore, performed with the 100 µm hole sparger (Case 3, Table 15). Alternatively, the calculated shear rate did not vary significantly by reducing the sparger hole diameter from 100 to 25 µm but was increased significantly by replacing the T-junction with the optimized sparger for CO_2 injection (Table 15, Case 2 versus 3).

3.2.2.3. Results for studies with 2nd design. The conditions of the runs with the 2nd design are displayed in Table 14 and the results in Fig. 16 (Design 2). A selection of runs with the most diverting results is discussed first, followed by a statistical analysis of all runs.

The highest biomass release ratio was recorded in Run 4. The biomass release ratio was 35%, of which 45% were proteins. The untreated biomass only contained 39% of proteins and, therefore, proteins are enriched in the supernatant. The highest lipid releases were obtained in Runs 1 and 6. Since the mass balance error of Run 6 is too high because of an extremely high lipid release, only Run 1 is discussed. The biomass release ratio was 30%, of which 56% was composed of lipids. The untreated biomass only contained 34% of lipids, so in this run the lipids were enriched in the supernatant. In Run 7, high energy efficiency was observed

because of a low CO_2 consumption and high release ratios. In this run, the release ratio for biomass was 30%, which was composed of respectively 38%, 14% and 39% of proteins, carbohydrates and lipids. The lowest CO_2 consumption per released biomass was observed in Run 12, where 25% of biomass release was recorded with a composition of 18% protein, 20% carbohydrate and 36% lipids. The overall results showed that 30% to 35% of biomass release was achieved by using CO_2 in the level of 5% to 20% of the liquid sample flow rate. In other words, the CO_2 consumption was reduced 8.5 to 48.2 fold by adding this sparger to the system. As shown in Table 15 and Fig. 19 (as the results of CFD simulations), no CO_2 water mixing can be achieved in 1st design, while there is a heterogeneous CO_2 water mixture formed in 2nd design. This development resulted in relatively big decrease (8.5 to 48.2 fold) of CO_2 demand for high yield cell disruption. Moreover, the system showed some selectivity by favouring proteins in Run 4, and protein release was decreased when IPA was added as a co-solvent in Run 12.

3.2.2.4. Statistical analysis of the studies with 2nd design. To analyse the effects of the parameters (CO_2/Water ratio (Vol%), relief valve temperature (°C), isopropyl alcohol (Vol%), DCW (wt%), oven temperature (°C), sample flow rate (ml/min) and pressure (bar)) on the release of biomass and biochemicals (responses) as a percentage of the starting value, a statistical study was performed. Briefly, four simple linear regression lines were calculated to predict the responses, *i.e.*, release of biomass, proteins, carbohydrates and lipids. To obtain a more explanatory output from coefficient estimates, all the parameters were normalized by simply dividing related parameter values to the maximum value of related parameter value array, prior to the linear regression. The results of linear regression studies are given in Table 16. Coefficient estimates represents the weights of the parameters in linear regression equation, which calculates related release ratios (biomass, protein, carbohydrates, and lipid) from given parameter values. The effects of each parameter on the responses and the interpreted effect mechanisms are explained in following paragraphs.

Relief valve temperature (°C) has a positive effect on all responses. The heat input at the pressure release border of the system results in faster decompression and, thus, the total energy of expansion is transferred more quickly. This is reflected in the positive correlation with the intensity of decompression and negative correlation with total decompression time. Higher energy input and lower time result in an increased energy transfer rate during expansion and the intensity of decompression increases in parallel. This increases the mechanical cell disruption power of the system and affects all responses in a positive way.

Isopropyl alcohol (Vol%) has a strong negative effect on protein release, a weak negative effect on biomass and carbohydrate release and a strong positive effect on lipid release (highest positive effect on lipid release). These results are in line with literature indicating that isopropyl alcohol has a negative effect on protein and carbohydrate release and a positive effect on lipid (fatty acid) release (Abdolshahi et al., 2015; Alves et al., 2007; Pace et al., 2004).

DCW (wt%) has a negative effect on all responses. Additionally, DCW (wt%) has the highest negative effect on lipid release. This is represented in the negative correlation with CO_2 mass per unit biomass and total energy input per biomass. The reduced energy input per unit biomass leads to lower cell disruption yields. The DCW % was the parameter with the highest negative effect on lipid release. Released lipids form micro-/nano-meter diameter liposomes or micelles/lipid droplets (Günerken et al., 2016). Milder cell disruption methods lead to the formation of cell debris with bigger dimensions since the mechanical effects such as shear rate are lower in comparison to conventional cell disruption methods, such as bead milling. Based on the hydrophobic behaviour of lipids, the affinity of lipids for cell debris is higher than the affinity for water. Therefore, the micro-/nano-meter diameter liposomes and micelles/lipid droplets formed during cell disruption can interact with cell debris and transfer to the pellet phase during centrifugation after the cell disruption process. Higher DCW values lead to formation of more cell debris, which increases the possibility of liposome/micelles/lipid droplet debris interaction and reduces the total lipid release.

Sample flow rate (ml/min) has the highest negative effect on every response except lipid release. This can be attributed to its negative correlation with the CO_2 mass input per unit biomass and total energy input per biomass. Sample flow rate is also negatively correlated with total processing time. Shorter processing time, shorter mass transfer and not-well mixed fluid behaviour inside of the 2nd design can lead to lower CO_2 transfer into cells. Reduced CO_2 and energy input together with shorter processing time can lead to a low cell disruption yield, which negatively affects all the investigated responses.

Pressure (bar) has the highest positive effect on every response except lipid release. This can be explained by the positive correlation with energy input per unit biomass and the intensity of the rapid decompression at the exit of the system. The increased energy input positively affects the cell disruption yield and thus the related responses. Additionally, as suggested in the literature, the dissolved CO_2 in water (carbonic acid) correlates positively with cell disruption (Debs-Louka et al., 1999; Enomoto & Nakamura, 1997; Fraser, 1951; Garcia-Gonzalez et al., 2007; Ginty et al., 2006; Khosravi-Darani et al., 2004; Lin et al., 1992a; Lin et al., 1992b; Nakamura et al., 1994; Rivard & Nagle Pretreatment of High Solid Microbial Sludges, 1998) and it is positively correlated with pressure (bar) values.

Both CO_2/Water ratio (Vol%) and Oven temperature (°C) have p values higher than 0.05 for all linear regression studies, which indicates that these parameters are statistically not important at the given parameter value ranges.

As a brief overview of aforementioned results carried out with the 2nd design, sample flow rate (ml/min) and pressure (bar) were found to be the most important parameters within the tested parameter ranges. The interpretation of the results is discussed in more

detailed in Section 3.3. Overall discussion and includes the results of all designs and process parameters.

3.3. Overall discussion

Table 17 gives an overview of the industrially most relevant aspects of all explosive decompression designs of this article with bead milling as benchmark technology and is discussed below in relation to the conditions and results displayed in Table 14 and Fig. 16.

In general, the release of biomass and more specific biochemicals from the algae to the supernatant can be linked to two phenomena, *i.e.*, secretion and scattering. Secretion is the release and/or extraction of biochemicals from whole cells with a perforated cell membrane/wall. Scattering, on the other hand, is the rupture of cells that results in formation of cell debris and/or release and/or scattering of organelles, such as chloroplasts. Secretion/extraction from whole cells would result in a released biomass composition that differs from the resource composition. Additionally, a low protein content of the released biomass is expected when there is no cell rupture or scattering.

Observations with the conventional batch method indicate that none or only a limited amount of cells were ruptured and the biomass release was mainly the result of lipid extraction by supercritical CO_2. Especially at low stirring speed in combination with a short contact time (15 min processing time), intracellular proteins were not released indicating that cell rupture did not occur. Under those conditions, where the contact between CO_2 and the cells is limited, inefficient cell disruption is to be expected. On the other hand, biomass compositions of the dry supernatant and the overall higher biomass release with the continuous installations suggest that cells were ruptured in both continuous designs. Because of the low calculated shear rate in the 1st design, cell disruption must have been the result of the explosive decompression. This hypothesis is confirmed by the amount and composition of the released biomass that gives similar results as cell disruption by bead milling. Bead milling is known to result in high scattering and the formation of small size cell debris. Alternatively, biomass release and composition was the same in all discussed experiments with the first design, and even lower pressure (approx. 75 bars) was effective. This could indicate that the speed of decompression, compared to the batch set-up, is a major factor in the effectiveness of the installation. Since the mixing efficiency was low in the first design, a large amount of CO_2 was needed to obtain cell disruption. Additionally, problems such as the high CO_2/water ratio (Vol%), the low sample flow rate (biomass load) and freezing in the pressure relief point, the 1st design was economically less interesting in comparison to 2nd design.

The in-house sparger was introduced to replace the T-junction for CO_2 injection to decrease the needed amount of CO_2, resulting in the 2nd design. Although a slightly lower to similar biomass release was recorded as with the first design, the amount of CO_2 was reduced 8.5- to 48.2-fold compared to the first design. In Run 7 with the second design, for example, the

released biomass amount and composition was similar to results with the first design, but with only 5% CO2 to liquid ratio instead of 169.5%–241%. Another observation with the second design was a reduced protein release when isopropyl alcohol was present in the algae suspension. Isopropyl alcohol was added as anti-freeze, but the released amount of biomass and proteins was reduced, likely due to denaturation and precipitation of proteins.

Cell disruption was achieved by fast decompression in the 1st design and the overall operational expenditure was enhanced by introducing the in house sparger and an orifice in the 2nd design, since better mixing enhances CO_2 mass transfer into the cells. Next to biochemical release, several other parameters are also relevant for industrial implementation. With the batch explosive decompression, process time is relatively long, especially because of a long residence and decompression time (15 to 45 min to process 70 ml in batch versus 15 ml/min in continuous lab scale processing). Additionally, the CO_2 to liquid ratio was high, between 45% and 60%, and the process led to some practical problems, like clogging because of cryogenic expansion. These observations render this device less favourable for industrial implementation. The design and optimization of a continuous process eliminated the above-mentioned problems. In more detail, in the 2nd design the need for a high CO_2 to liquid ratio and, consequently, high relief valve temperature to prevent freezing, were reduced by adding the optimized sparger resulting in overall decreased energy consumption per kg of dry weight algae.
A preliminary study was made to compare capital (CAPEX) and operational (OPEX) expenditures of the novel explosive decompression device against bead milling (see supplementary information). The first analysis indicates that the new device provides a more cost and energy effective solution than bead milling. In brief, in terms of CAPEX, for a throughput

Table 17: Overall comparison of cell disruption methods. CAPEX = capital expenditures and OPEX = operational expenditures.

	Bead milling	Conventional batch	1st design	2nd design
Industrial potential	High	Low	Medium	High
Operation modus	Batch	Batch	Continuous	Continuous
Problems	Batch time, high shear	Batch time, low release, clogging	High CO_2 consumption	Less efficient at high liquid flow rate
CAPEX	High	Highest	Medium (larger CO_2 demands)	Medium to low
OPEX	High (cooling to counter high shear)	Highest (long process time, no efficient disruption)	Medium (heating to counter cryogenic expansion)	Low
CO_2 consumption	/	Medium	High	Low
Mass transfer	/	Low	Low to medium	High
Biomass release	High	Low	High (similar to bead mill)	Medium to high
Biomass release mechanism	Scattering & secretion	No scattering, only secretion	Scattering & secretion	Scattering & secretion

between 4 and 12 kg DCW/day, the explosive decompression device is estimated at 45 k EUR as compared to 77 k EUR for bead milling. In terms of OPEX, bead milling consumes 2.8 kWh/kg dry biomass when 12% DCW (in wt%) biomass is processed at its maximum efficiency (37% Biomass release) (Doucha & Lívanský, 2008; Günerken et al., 2015). The explosive decompression device on the other hand is estimated to consume only 0.3 kWh/kg dry biomass when 10% DCW (in wt%) biomass is processed at its average efficiency (24%

Biomass release). This means that, even at an average efficiency the explosive decompression device demands less energy than a bead-milling device working at maximum efficiency. Both cell disruption techniques require trained personal. However, since the explosive decompression system doesn't contain moving parts, maintenance costs are expected to be lower in comparison to bead milling system. Additionally, as far as fixed operational expenses are concerned, explosive decompression also seems favourable. In the case of bead milling these are estimated at approximately 2.7 EUR/kg DCW related to the use of zirconia beads, while explosive decompression is expected to only cost roughly 0.03 EUR/kg DCW when CO_2 is recycled. In conclusion, at the conditions where explosive decompression has the highest biomass release efficiency (Run numbers 1–2, 8–9 and 12–14 of the 2nd design in Fig. 16), explosive decompression is expected to provide a more energy and cost friendly solution as bead milling. Moreover, the new technique allows to work at lower shear stress. A more detailed explanation of CAPEX and OPEX calculations can be found in the supplementary document.

4. CONCLUSION

High operational expenditure is the most important bottleneck of microalgae biotechnology, and more specific high energy consuming unit operations. Additionally, cell disruption has a crucial effect on operational cost, since the energy demand is generally high and it strongly affects the downstream processing efficiency. Efficiency and mildness are the most important characteristics of an industrially favourable cell disruption process. In this study, a new apparatus was designed to successfully perform microalgae cell disruption via explosive decompression. Moreover, it was shown that by modifying the design and optimizing the process parameters, suspensions can be processed with a higher dry cell weight concentration and at a higher sample flow rate, with relatively lower CO_2 consumption. In result, the new explosive decompression device for cell disruption allows to treat more biomass reducing the need of scaling-up while consuming less energy. The study demonstrated the effectiveness of this new continuous processing technique even at low CO_2 consumption. Nevertheless, in order to understand the technique in a more profound way and to demonstrate its broader industrial potential, further studies are still needed to compare its performance against conventional techniques. This includes, among others, the optimization of the design, assessment of its effectiveness for a variety of algae strains in comparison to reference cell disruption techniques as well as determination of its boundaries in terms of energy efficiency, biomass concentration and liquid flow.

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Statement of Informed Consent, Human/Animal Rights, if any, for the work described. No conflicts, informed consent, human or animal rights applicable.

Declaration of authors agreement to authorship and submission of the manuscript for peer review. The authors agree to their contribution, authorship and submission of the manuscript for peer review.

AUTHORS' CONTRIBUTIONS

Emre Günerken made a proposal for set-up, performed all the experiments and associated analyses, and elaborated the CFD simulations. He also prepared the first draft of the article. Els D'hondt was deeply involved in the set-up of the various experimental designs and performed a critical analysis of the data obtained. Michel Eppink coordinated the production of the algal biomass used for the experiments and provided input on production and analytical methods. Moreover, he critically assessed the experimental designs and the results obtained also in the light of more conventional disruption approaches. Rene Wijfels provided input on algae production the analytical methods used. Moreover, he critically assessed the experimental designs and the results obtained. He helped to position the work in a broader framework on algae valorisation. Kathy Elst provided input related to the possible effects of CO_2 and the reactor concepts applied. Moreover, she critically assessed the experimental designs and the results obtained. She helped to position the work in the broader frame of CO_2-based processes as well as other disruption processes. All authors did a critical reread of the article and approved the manuscript that was submitted.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

APPENDIX A. SUPPLEMENTARY DATA: FIRST CAPEX/OPEX ANALYSIS OF THE CO2 EXPLOSIVE DECOMPRESSION SYSTEM COMPARED TO BEADMILL

The CAPEX and OPEX of the new explosive CO_2 disruption system was estimated based on its performance for *Neochloris oleoabundans* and compared with bead mill based on literature and suppliers information. An overview is given in Table 18 and the assumptions are detailed in the next sections. This is to be considered as a first estimate of the CAPEX/OPEX of the new system. A more detailed comparison will have to be performed by comparing the performance of the new system against bead mill on identical biomass, as well as performing a more extended CAPEX/OPEX analysis.

Table 18: Comparison of bead mill and the explosive decompression device as drop-in replacement. DCW = dry cell weight of the biomass

	Bead mill	Explosive decompression	Remarks
CAPEX			
Approx. CAPEX (€)	77000	45000	Equipment, pumps, cooler and utilities (see Supplementary Table 2SS)
Throughput (kg dry biomass/day)	3.8-11.5[1]	8.6[2]	[1] For grinding chamber volume = 0.6 L; [2] For biomass flow rate of 60 mL/min
Energy consumption: scenario 1: minimal energy consumption during cultivation			
Energy for disruption at ~10% DCW (kWh/kg DCW)	2.8[3]	0.3[4]	[3] Bead milling specific energy consumption for 0.6 L grinding chamber (based on Fig. 6 in (Günerken et al., 2015)) [4] See calculations in OPEX section
Minimal energy for cultivation + harvesting (kWh/kg DCW)	1.6	1.6	Energy consumption for production of Non N depleted microalgae based on (Meyer, 2012)
Energy% of disruption against total (cultivation/harvesting/disruption)	64%	16%	
%reduction in total energy consumption against bead mill	-	57%	Based on the reduction in the total energy consumption for cultivation + harvesting + disruption when using explosive decompression compared to bead mill.
Energy consumption: scenario 2: maximal energy consumption during cultivation			
Energy for disruption at ~10% DCW (kWh/kg DCW)	2.8[3]	0.3[4]	[3] Bead milling specific energy consumption for 0.6 L grinding chamber (based on Fig. 6 in (Günerken et al., 2015)) [4] See calculation OPEX section
Maximal energy for cultivation + harvesting (kWh/kg DCW)	8.3	8.3	Energy consumption for production of Non N depleted microalgae based on (Park and Lee, 2016)
Energy% of disruption against total (cultivation/harvesting/disruption)	23.6%	3.5%	
%reduction in total energy consumption against bead mill	-	22%	Based on the reduction in the total energy consumption for cultivation + harvesting + disruption when using explosive decompression compared to bead mill.
Cost CO2 (€ per kg DCW)	-	2.9[5]	[5] 4 kg CO2 per kg DCW, usage at purchase price of 28 €/50 L liquid CO2

Table 18: Comparison of bead mill and the explosive decompression device as drop-in replacement. DCW = dry cell weight of the biomass (*continued*)

	Bead mill	**Explosive decompression**	**Remarks**
Cost of reusing CO2 (€ per kg DCW)	-	0.03[6]	[6] CO2 can be recollected and pressurized by spending 0.05 - 0.27 kWh/kg DW (0.16 €/kWh) (See calculation OPEX section)
Cost ZrO2 beads (EUR per kg DCW)	2.7[7]	-	[7] ZrO2 beads at ~230 €/kg (see suppliers information in (Glenmills, n.d.)) on 1500 hours lifetime.
Low maintenance	no	Yes[8]	[8] Less moving parts and cleaning without deconstruction

CAPEX info for bead mill and explosive decompression

The capital cost was based on suppliers information as explained in Table 2SS.

Table 19: Cost break down of the capital Expenditures (in €) for the systems described in Table 18.

	Main	Automation	Pumps	Cooler	Other	Total
Bead mill	62000[9]			10000[10]	5000[11]	**77000**
Explosive Decompression	10000	7500[12]	15000[13]	5000[14]	7500[15]	**45000**

[9] Complete bead mill, including automation, estimated based on suppliers information for lab-scale bead mill with milling chamber of 0.6 L.

[10] Cooler for cooling the milling chamber, including pump and automation, estimated based on suppliers information for cooling capacity needed for lab scale bead mill of 0.6 L.

[11] Periphery equipment, including storage tank, estimated based on suppliers information.

[12] Flowmeter, valve and controller compatible with the pressurized system: (1) Swagelok RVS 316 Variable flowmeter ~800 €, (2) back pressure valve suitable for flowmeter (digital controlled from brooks) ~3500 €, (3) Brooks Controller ~2500 €.

[13] Pumps: (1) CO2 pump ~10000€ (example NanJing YaLong Technology CO., LTD 3CQ,5CQ,3RC,5RC series), (2) Liquid pump ~5000 € (example Vem Motors Thurm GmbH)

[14] Small volume cooler for CO2 pump

[15] Pressure leakage test, electricity panel etc.

OPEX info for explosive decompression

Energy consumption for the explosive decompression

For the calculation of the total energy consumption for the explosive decompression, two major sources of energy consumption were considered: (1) a liquid pump pressurizing and pumping the biomass to 250 bar at 60 mL/min; (2) a CO2 pump, pressurizing and pumping the CO2 to 250 bar at 40 mL/min. In addition, as an alternative to (3), also the energy consumption for repressurizing the recycled CO2 was calculated.

1. Biomass pump

The energy consumption of the biomass pump was calculated using the calculator of https://www.engineeringtoolbox.com/pumps-power-d_505.html, assuming water and using a pump efficiency of 62%. For 60 mL/min this corresponded to an energy consumption of 0.09 kW, or 0.25 kWh/kg DCW at 10% DCW, or 1.25 kWh/kg DCW at 2% DCW.

2. CO2 pump

For the calculation of the energy consumption of the CO2 pump, the methodology described by McCollum and Ogden (2006) was completely followed. The power consumption was split in two parts, the first for pressurizing to the critical point (73.8 bar) and the second for subsequently pressurizing till the final 250 bar. For the first part, the same formulas were used assuming also 4 stages for pressurizing to the critical point. In the last stage (stage 5),

the formula was adapted to our end pressure of 250 bar (instead of the 150 bar used in the reference). This resulted in a total energy consumption of 0.02 kW for 250 bar at 40 mL/min CO2 flow, corresponding to an energy consumption of 0.05 kWh/kg DCW at 10% DCW, or 0.27 kWh/kg DCW at 2% DCW.

3. Total energy consumption

For 2% DCW à 1.25+0.27 = 1.52 kWh/kg DCW of microalgae

For 10% DCW à 0.25+0.05 = 0.30 kWh/kg DCW of microalgae

6

CHAPTER 6

GENERAL DISCUSSION

HOLISTIC VIEW ON THE THESIS: CONNECTING THE CHAPTERS

This thesis delineates and evaluates the role of single-cell disruption within downstream processing of microalgal biomass. The study is framed by the biorefinery system, but it is only an attempt to optimize cell-disruption process itself, not the entire system. Emphasis is placed on disruption efficiency, treatment time, and the extractability and recovery of target products, with the understanding that cell-disruption constitutes one of multiple interventions capable of advancing economic and environmental performance. The work integrates technological advancement, analytical method development, and process optimization to address one of the critical bottlenecks in achieving industrial scale and economically feasible microalgal production: efficient and selective release of intracellular products without sacrificing their quality. The project also shows that sustainable microalgae biorefineries require simultaneous optimization of multiple process parameters across cultivation and downstream processing stages.

Chapter 2 established the foundation by providing a systematic review of conventional and emerging cell disruption technologies while also identifying critical gaps in the field. In this comprehensive analysis mechanical methods (e.g. bead milling, high pressure homogenization, high speed homogenization, ultrasonication, microwave treatment, pulsed electric field treatment) and non-mechanical approaches (e.g. enzymatic lysis, chemical treatment) with respect to their suitability for future microalgae biorefineries were evaluated and key parameters including disruption mechanism, energy consumption, product quality, and scalability were identified. The review revealed that conventional mechanical methods, particularly bead milling and high-pressure homogenization, dominate industrial applications due to their scalability and high disruption efficiency. However, these methods consume substantial energy (0.81-147 kWh/kg dry biomass depending on conditions) and generate high shear forces that can degrade sensitive biomolecules. The analysis identified three interconnected challenges:
(1) High specific energy consumption limiting economic feasibility,
(2) Quality degradation of fragile functional compounds due to harsh conditions,
(3) Insufficient understanding of how cultivation conditions affect disruption efficiency.
These findings established the research questions addressed in subsequent chapters.

The review concluded that bead milling represents the most effective state-of-the-art technology despite high energy demands and harsh nature. One of the main conclusions of this chapter is that “cell membrane/wall composition and morphology are highly effecting the cell disruption efficiency “ became crucial for understanding the experimental results in Chapter 3, where cultivation strategies were shown to influence not only biomass composition but also disruption efficiency. Critically, this chapter highlights the need for mild disruption methods that maintain the functionality of sensitive bio-

molecules while achieving high disruption yields that presents a challenge that directly motivates the subsequent experimental investigations.

Chapter 3 fundamentally challenged the conventional separation between upstream (cultivation) and downstream (cell disruption) process optimization by demonstrating that nitrogen depletion during cultivation significantly influences both disruption efficiency and product selectivity during bead milling. This chapter bridged the gap identified in Chapter 2 by showing that cultivation conditions are active parameters in cell disruption process design since they are determinants of cell membrane/wall composition and morphology.

The results demonstrated that nitrogen depleted cells exhibit significantly higher and more selective biomass release (57% versus 34% for nitrogen repleted cells), with particularly enhanced transfer of phospholipids (92% versus 27% for nitrogen repleted cells) and glycolipids (57% versus 33% for nitrogen repleted cells). This selectivity represented a change in assumed non-selective release pattern in bead milling, where all biochemicals (*i.e.*, total proteins, total carbohydrates, and total lipids) are released proportionally. The mechanism underlying this differential behavior was attributed to altered cell physiology and potentially increased lipid droplet formation under nitrogen stress, which facilitates the formation of liposomes and micelles that remain suspended in the aqueous phase.

Furthermore, the energy consumption per kilogram of released product was substantially reduced for nitrogen depleted biomass, particularly for lipid recovery (21.83 versus 59.44 kWh/kg for nitrogen depleted biomass, a 63% reduction). However, this improvement must be contextualized within the broader biorefinery framework. While nitrogen depleted cultivation favors lipid recovery, protein release efficiency decreases in absolute terms due to lower total protein content in nitrogen-depleted biomass. This trade-off exemplifies the need for cultivation strategy selection based on target product priorities within an integrated biorefinery approach.

The mechanistic insights from Chapter 3 align with emerging understanding in microalgae biotechnology that stress-induced physiological changes extend beyond compositional shifts to affect structural cell wall properties. The hypothesis that nitrogen depletion creates more fragile cells with altered cell wall polysaccharide composition provides a mechanistic foundation for the observed enhanced disruption efficiency. This understanding connects upstream with downstream process design, demonstrating that biorefinery optimization requires transdisciplinary integration.

Chapter 4 addressed a critical methodological bottleneck that constrained both fundamental research and industrial implementation: the absence of rapid, accurate, high-throughput methods for cell disruption yield assessment. Traditional methods, such as manual hemocytometer counting requiring 20 minutes per sample and gravimetric analysis demanding 72-

hour freeze-drying, were fundamentally incompatible with real-time process optimization or high-throughput screening applications.

The developed and validated flow cytometry method, utilizing long-pass filter (>670 nm) fluorescence data (FL3-Hmean × FL3-Amean proportional to cell volume), correlates strongly with biomass release (R^2=0.9867±0.0057) and reduces analysis time to approximately 2 minutes per sample. Importantly, the method successfully differentiates between intact cells and disrupted/leaching cells, overcoming the limitation of manual counting which underestimates disruption yields when cells remain morphologically intact but release intracellular contents. This analytical advancement enables high-throughput screening of disruption conditions and real-time process optimization which are capabilities essential for industrial implementation.

Critically, the decrease in measurement time and sampling volume proved that the developed and validated Flow-cytometry method is a valuable addition to microalgae community. However, still regarding the nature of this method (needs approx. 100 µL sample and dilution, additionally 2 min of measurement time) make it a challenge to integrate to photobioreactors as an inline measurement system, while non-disruptive and native (no chemicals or biochemicals used) nature of the method keeps the possibility open. Finally, it can be concluded that individual native, high throughput method focusing on cell volume and granularity at the same time with zero sampling processes for every step of the biorefinery as well as for cell disruption step is necessary. Newly emerging methods utilizing machine learning methods and visual measurement methods such as Digital Holographic Microscopy and Full Field Optical Coherence Tomography and new optical/impedance flow-cytometry methods are promising candidates to develop a truly inline method to both follow cultivation and cell disruption (Kang et al., 2025; Briel et al., 2025; Dambruin et al., 2025).

Chapter 5 represented the culmination of insights gained from previous chapters by developing and validating a novel continuous explosive decompression device that addressed the fundamental limitations of conventional disruption methods. The chapter exemplified process intensification principles by addressing mass transfer limitations through engineering innovation rather than increased energy input.

Conventional batch explosive decompression suffered from three fundamental limitations identified through initial experiments:
(1) Poor CO_2 -water mixing due to stirred tank reactor geometry,
(2) Long contact times (15-45 minutes),
(3) High CO_2 consumption (45-60% CO_2/liquid ratio).

Computational fluid dynamics simulations revealed that T-junction CO_2 injection created large bubbles with minimal interfacial surface area, resulting in oscillating CO_2 volume fractions between 0% and 100% rather than uniform dispersion.

The solution integrated multiple design innovations:
(1) An optimized sparger with four laser-drilled 100 μm holes positioned at 90° angles enabling fine bubble generation,
(2) A hydrodynamic agitation zone comprising three sequential static mixers preventing bubble coalescence,
(3) A heated relief valve preventing cryogenic freezing during explosive decompression.

These modifications transformed system performance and with the experiments done with nitrogen repleted biomass of *N. oleoabundans*:
(1) CO_2 consumption decreased 8.5-48.2 fold (to 5-20% ratios),
(2) Processing time reduced 3.2-9.6 fold,
(3) Biomass release achieved 24-36% with more than 2-fold increase and became comparable to bead milling (34%).

Moreover, the calculated shear rate (4902 s^{-1}) represents only 0.33% of that experienced during bead milling, confirming the mild nature of the technique. Techno-economic analysis indicates that the explosive decompression device consumes only 0.3 kWh/kg dry biomass (at 10% DCW and 24% biomass release efficiency) compared to 2.8 kWh/kg for bead milling (at 12% DCW and 34% biomass release efficiency). This represents an approximately 89% reduction in energy consumption while maintaining an acceptable biomass release, positioning explosive decompression as a more sustainable and cost-effective alternative.

The statistical analysis of process parameters revealed that pressure (bar) and sample flow rate (mL/min) were the most significant factors influencing biomass and biochemical release. Relief valve temperature showed positive effects across all responses through enhanced decompression intensity, while isopropyl alcohol addition negatively affected protein release but enhanced lipid recovery, demonstrating selectivity control through co-solvent manipulation. These findings provide a parametric foundation for future studies.

Techno-economic assessment indicated favorable economics compared to bead milling: capital expenditures estimated at 45,000 EUR versus 77,000 EUR for equivalent capacity (4-12 kg DCW/day), operational expenditures of 0.3 kWh/kg versus 2.8 kWh/kg, and fixed costs of 0.03 EUR/kg (with CO_2 recycling) versus 2.7 EUR/kg for consumable zirconia beads. The absence of moving parts suggests reduced maintenance costs, further improving operational economics.

A KEY INSIGHT INTERCONNECTING CHAPTERS

A conceptual framework emerged across chapters distinguishing two biomass release phenomena being secretion (extraction from perforated but intact cells) and scattering (cellular rupture generating debris). While some cell disruption methods such as high pressure homogenization and bead milling are more likely to produce only scattering for some other cell disruption methods, especially for mild cell disruption methods, like PEF and low frequency ultrasound lead to secretion. This phenomenon is first proposed in Chapter 4 and explained in Chapter 5 through developed explosive decompression method. In Chapter 5, batch explosive decompression primarily induced secretion with minimal scattering, evidenced by low protein release despite high lipid extraction, consistent with supercritical CO_2 extraction without significant cellular rupture. The continuous designs, particularly the second iteration with optimized sparger, achieved substantial scattering evidenced by protein release patterns similar to bead milling.

This mechanistic distinction connects to the selectivity observations in Chapter 3, where lipid droplet-rich nitrogen depleted biomass exhibited different release patterns than nitrogen repleted biomass. The hypothesis that scattering intensity influences biochemical distribution through altered debris surface area and adsorption characteristics provides a unifying explanation for observations across both mechanical (bead milling) and mild (explosive decompression) disruption methods.

Flow cytometry data from Chapter 4 supports this framework by distinguishing intact cells, leaking cells, and debris populations based on fluorescence signatures. The observation that manual counting underestimated disruption for certain *Chlorella sp.* by not detecting leaking-but-intact cells validates the secretion phenomenon and highlights why conventional assessment methods inadequately capture disruption complexity.

MAIN CONCLUSIONS

This thesis proposes a reconceptualization of microalgae biorefinery design by presenting cell disruption as a fundamental step and hypothesizing that it cannot be optimized in isolation but must be integrated within a holistic framework spanning cultivation through downstream processing. The key conclusions are:

1. **Biorefinery optimization requires a fundamental methodological change from sequential unit operation optimization to integrated system design.** The traditional approach of independently optimizing cultivation for biomass productivity and then separately optimizing downstream processing is fundamentally flawed. This work demonstrates that cultivation conditions actively determine cell disruption efficiency through

their effects on cell structure and composition, necessitating co-optimization strategies that consider the entire value chain simultaneously.

2. **The dichotomy between "harsh but effective" and "mild but inefficient" disruption methods is false.** Through process intensification principles focusing on mass/energy transfer enhancement rather than brute force application, this thesis demonstrates that mild conditions can achieve industrially relevant disruption efficiencies. This finding challenges the prevailing assumption that energy-intensive mechanical methods are necessary for commercial viability and opens new pathways for sustainable biorefinery development.
3. **Analytical methodology development is not merely supportive but transformative for bioprocess optimization.** The flow cytometry method developed here does more than accelerate measurements. It changes what questions can be asked and answered about cell disruption mechanisms and it can be used to understand changes occurring during cultivation. This methodological advance enables the transition from empirical trial-and-error to systematic, mechanistic understanding of disruption phenomena.
4. **Selective product release represents a critical but underexploited opportunity for biorefinery economics.** Rather than pursuing complete cell disruption followed by complex separation, this work demonstrates that controlled, selective release through manipulation of both cultivation conditions and cell-disruption parameters can dramatically simplify downstream processing. This selectivity emerges from understanding the interplay between cell physiology, disruption mechanisms, and product localization.
5. **Process intensification through engineering innovation offers greater sustainability improvements than incremental efficiency gains in existing technologies.** The continuous explosive decompression system exemplifies how rethinking fundamental process design, in this PhD project study case CO_2-liquid mass transfer and shear rate, can achieve order-of-magnitude improvements in resource efficiency while maintaining product quality.
6. **The future of microalgae biorefineries lies in adaptive, product-specific processing strategies rather than one-size-fits-all approaches.** This thesis establishes that optimal processing pathways depend on target product priorities, with cultivation strategies and disruption methods selected synergistically to maximize value extraction while minimizing environmental impact. The framework developed here provides the base analytical tools and mechanistic understanding necessary for designing such adaptive systems.

ADDITIONAL INSIGHTS FROM UNPUBLISHED OBSERVATIONS

The following section addresses 3 advanced research hypotheses that emerged during the experimental progression of this PhD research project, providing a synthesized discussion rooted in comparative literature, experimental evidence, and considerations for future work.

1. Freeze Shock and Cold Storage Responses in Microalgae: Evidence for Metabolic and Apoptotic Adaptation?

Can freeze shock in microalgae, specifically in Neochloris oleoabundans, lead to activation of adaptive metabolic processes to make the cells more durable while cold storage induces apoptotic processes analogous to those observed in higher organisms such as fish?

There are lots of entries in literature regarding to the hypothesis presenting the idea: "Freeze shock in *Neochloris oleoabundans* activates adaptive metabolic processes that enhance cell durability, while cold storage (+4°C) induces apoptotic-like programmed cell death, as evidenced by differential cell disruption efficiencies" (Barreto Filho et al., 2021; Corredor et al., 2025; Zuppini et al., 2010).

Freeze shock related changes in microalgae studied extensively. Freeze shock triggers protective mechanisms including increased polyunsaturated fatty acid (PUFA) synthesis, production of cryoprotective compatible solutes (proline, glycerol, trehalose), and upregulation of antifreeze proteins (AFPs) that maintain membrane fluidity and prevent ice recrystallization (Huang & Cheung, 2021; Sharma et al., 2012; Kim et al., 2017). Cold shock proteins, heat shock proteins, and antioxidant enzymes activate rapidly to repair cold-induced protein misfolding and stabilize RNA structures (Liu & Huang, 2015; Song et al., 2024). Exopolysaccharide production and cell wall modifications provide additional structural protection, resulting in reduced bead milling susceptibility in frozen samples (approximately 40% reduction in disruption efficiency versus fresh biomass; Safi et al., 2020).

In contrast, cold storage at +4°C induces programmed cell death with apoptotic hallmarks (mechanisms homologous to animal apoptosis) including plasma membrane alterations, nuclear condensation, DNA fragmentation, and metacaspase activation (Zuppini et al., 2010; van Creveld et al., 2021). Recent discovery of apoptotic bodies in *Guillardia theta* confirms that microalgae possess conserved cell death machinery predating multicellularity (Corredor et al., 2025). Chronic mild stress at +4°C progressively depletes energy reserves, accumulates reactive oxygen species (ROS), and triggers mitochondrial dysfunction with cytochrome c release, leading to enhanced cell disruption yield (approximately 5% improvement; Corwin et al., 2009; Xiong et al., 2025).

The mechanistic distinction between freeze shock and cold storage reflects evolutionary adaptation: freeze shock represents acute severe stress activating pro-survival pathways, while cold storage represents chronic moderate stress activating pro-death pathways (Montuori et al., 2021; van Creveld et al., 2021). Approximately 50% of aquatic microalgae tolerate freeze-thaw cycles naturally, whereas sustained +4°C conditions lack adaptive mechanisms, explaining increased apoptosis vulnerability (Tesson et al., 2018). Therefore, freeze shock enhances cell durability through metabolic and structural adaptations persisting post-thaw, while cold storage compromises cell integrity through apoptotic processes. This identification presents 2 fundamentally distinct responses with profound implications for microalgal biotechnology and cultivation strategies (Corredor et al., 2025).

Unpublished experimental observations collected during this PhD research study demonstrated a marked difference in bead milling between +4°C and –20°C stored *N. oleoabundans* biomass. In comparison to no stored biomass, +4°C storage increased the cell disruption yield of 45 min bead milling process approx. 5% while –20°C decreased it approx. by 40%. The possible freeze shock induced defense mechanisms and cold storage induced apoptosis and apoptosis-like mechanisms mentioned above and the unpublished data collected during this PhD research study strongly indicates that the hypothesis presented in here is interesting.

Application of design of experiments (DOE) methodology could clarify the interactive effects of growth phase and storage conditions. Integration of targeted metabolomics and molecular markers for apoptosis (e.g., caspase activity, DNA fragmentation) as well as the freeze shock adaptation (PUFA accumulation, antioxidant compounds, antioxidant enzymes, DEAD-box proteins) will be essential for confirming the mechanistic hypothesis. The future potential for apoptosis and freeze adaptation in microalgae to inform improved preservation, extraction and large-scale not heat regulated production protocols represents a novel research and bioprocess opportunity.

2. Viability and Recultivation After Explosive Decompression: Can Microalgae be "Milked"?

What is the viable cell concentration after conventional explosive decompression, and can microalgae be repeatedly cultured post-treatment, supporting a continuous "milking" process?

Explosive decompression, especially with supercritical fluids (e.g., CO_2), has emerged as a gentle cell disruption approach capable of high-value product extraction with minimal use of chemical reagents. However, the recultivation of viable cells post-disruption represents a substantial challenge. Previously, the application of two-phase aqueous systems or solvent-based extraction led to distinct reductions in cell viability, often attributed to phase toxicity and mechanical stress. The literature indicates that while "milking" of certain strains (e.g.,

Dunaliella salina) is feasible via two-phase liquid extraction under mild enough conditions, efficiency and cell survival are highly variable and strain dependent (Hejazi & Wijffels, 2004; Tanguy et al., 2021).

Experimental work in this thesis supports the notion that, although mechanical stress and osmotic imbalance typically result in sublethal damage most of the microalgal cells may survive conventional explosive decompression process. This opens the possibility of milking *N. oleoabundans* through explosive decompression since the cells are not exploded but components are secreted. Key parameters influencing outcome include the decompression rate, pressure differential, and culture density, as well as intrinsic species resilience. An optimized flow cytometry protocol with dual staining (fluorescein diacetate and propidium iodide) enables real-time assessment of post-treatment viability and automated processing, providing an excellent tool for iterative process refinement (Pozzobon et al., 2020; Wang et al., 2023).

Notably, substantial amounts of lipids can be extracted from non-disrupted *Neochloris oleoabundans* (Du et al., 2017). This demonstrates the existence of naturally permeabilized cells and an indirect indication of reversible cell-membrane processes that permit extraction without mechanical cell-disruption. Similarly, *Dunaliella salina* has been successfully milked for β-carotene over extended periods (>47 days), with cells remaining viable in the presence of biocompatible organic phases during simultaneous production and extraction (Hejazi & Wijffels, 2004).

Downstream processing strategies further support this concept. Multistep fractionation using conventional supercritical CO_2 extraction-based separation methods can potentially be effective to obtain different fractions of apolar phases from *N. oleoabundans.* Additionally, other methods such as aqueous two-phase systems (ATPS) with ionic liquids can separate lipids, proteins, pigments, and carbohydrates from without requiring complete cell lysis, with lipids recoverable at the interface or in the polymer-rich phase (Suarez Ruiz et al., 2020). The promising findings by Yoo et al. (2014) support the use of polymeric membranes for mild, effective cell lysis and separation. These approaches may enable green separation of bioactive compounds while preserving biomass quality (Tzima et al., 2023).

A continuous "milking" process for *N. oleoabundans* through controlled explosive decompression is hypothesized to be viable under the following integrated workflow (Fig. 20): Initial treatment with wet supercritical CO_2 at optimized pressure differentials and controlled slow decompression rates will induce selective permeabilization and component secretion while preserving cell membrane integrity and viability. While still in supercritical conditions, the separated CO_2 phase will go under a further separation phase to obtain apolar phases separated. The treated cell suspension will be centrifuged to separate viable biomass (pellet) from the released components (supernatant). The supernatant will undergo ATPS fractionation or any similar process to separate and isolate high-value lipids, carotenoids, and proteins,

while the pellet will be re-cultured with fresh medium to restore viability and biomass for subsequent cycles. Periodic cycling that alternating between extraction phases (high light, elevated pressure) and recovery phases (reduced light stress, organic solvent removal) may possibly enable long-term viability maintenance and biomass regeneration.

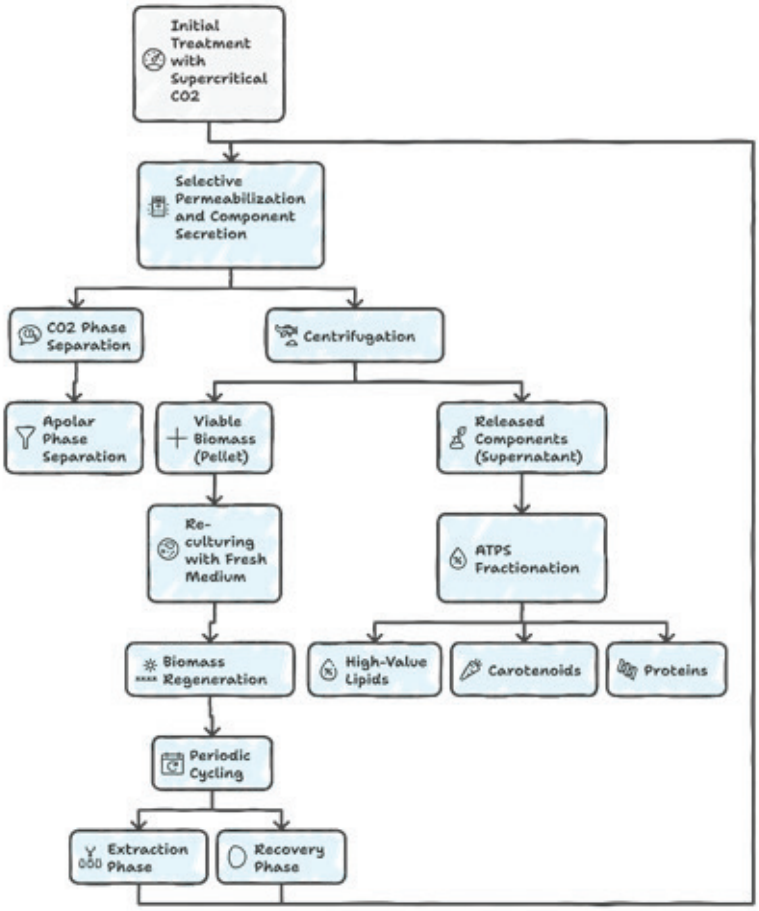

Fig. 20: A continuous "milking" process for N. oleoabundans through controlled explosive decompression

It is recommended to pursue integrated studies coupling mechanical stress markers (cell volume, membrane integrity via flow cytometry) with chemical stress indicators (reactive oxygen species production, osmotic state) and physiological recovery metrics (photosynthetic efficiency, growth rates) alongside standardized recultivation trials using industrially relevant continuous bioreactor systems. This systematic characterization will clarify the decompression rate thresholds, pressure cycling frequency, and culture recovery conditions required for sustained productivity, thereby advancing the feasibility repeated product harvesting and establishing *N. oleoabundans* as a model system for microalgae biorefinery models.

3. Coupling Explosive Decompression with Focused Ultrasonication/ PEF

Can continuous explosive decompression be effectively coupled with focused ultrasonication or pulsed electric field (PEF) from the sparger point to intensify microalgal disruption, as seen in CO_2-water dispersion and ultrasonic studies?

Process intensification via the coupling of different cell disruption methods is a focal point in recent literature. Explosive decompression targets intracellular pressure differentials, while ultrasonication and PEF induce membrane proration and localized cavitation effects. Ultrasonication and PEF induce membrane poration and localized cavitation effects through distinct yet complementary mechanisms (Carullo et al., 2020; Liu et al., 2022). Focused ultrasonication, operating at higher frequencies (1-3 MHz) with targeted energy delivery, has demonstrated synergistic effects when applied in series with mechanical disruption methods (Wang et al., 2014; de Farias Silva et al., 2018).

The mechanistic basis for coupling ultrasonication with explosive decompression centers on two primary enhancement pathways. First, ultrasonication significantly enhances CO_2 bubble dispersion and mass transfer in the liquid phase through acoustic streaming, microstreaming, and cavitation-induced microjets (Moreno Soto et al., 2020; Gao et al., 2021). Second, ultrasonication increases cell membrane permeability through both mechanical effects (acoustic cavitation, pressure waves) and chemical effects (reactive radical generation), thereby facilitating greater CO_2 penetration into cells prior to explosive decompression (Liu et al., 2022; Wu et al., 2025a).

Pulsed electric field treatment operates through electroporation wherein short, high-voltage pulses create transient or permanent pores in cell membranes (Carullo et al., 2020; Vaessen et al., 2019). PEF induces membrane permeabilization without complete cell disintegration, as demonstrated by scanning electron microscopy showing cell shrinkage but preservation of overall cell structure (Carullo et al., 2020; Bensalem et al., 2020). This controlled permeabilization would facilitate CO_2 penetration into algal cells during the pressurization phase of explosive decompression while maintaining cell structural integrity for subsequent mechanical disruption.

The concept of combining cell disruption methods has been successfully demonstrated in multiple contexts (Zhang et al., 2020; Perez et al., 2024 ;. Blanco-Llamero et al., 2021; Islam et al. 2017).For explosive decompression specifically, the integration of ultrasonication or PEF at the sparger point would leverage the existing continuous system architecture developed. The sparger design with laser-drilled 100 μm holes for CO_2 injection and 250 μm orifice exit created optimal conditions for gas-liquid mixing. Incorporating focused

ultrasound transducers or PEF electrodes at this critical mixing zone would amplify three key phenomena:

1. CO_2 bubble nucleation, growth, and dispersion enhancement through acoustic cavitation or electrophoretic effects;
2. cell membrane permeabilization to facilitate CO_2 uptake during the pressurization phase; and
3. weakening of cell wall structures to enhance susceptibility to explosive decompression forces.

During the studies completed and presented in Chapter 5 the data clearly indicated that the newly developed continuous explosive decompression method and equipment can achieve cell disruption yields similar to bead milling with a fraction of the energy consumed. However, for the equipment to treat higher biomass concentrations with cell high disruption yield, process intensification is still necessary. The addition of ultrasonication or PEF would incur additional energy costs but could potentially achieve even higher disruption efficiencies at lower CO_2 consumption and shorter processing times while treating more concentrated biomass slurries.

Effective integration will require precise engineering to control energy input, avoid excessive heating, and preserve sensitive biomolecules (Carullo et al., 2020). For ultrasonication, continuous versus pulsed application modes must be optimized to balance cavitation intensity with thermal management (Kerboua and Merabet, 2020).

The coupling of continuous explosive decompression with focused ultrasonication or pulsed electric field at the sparger point represents a promising strategy for process intensification in microalgal biorefineries. This hybrid approach leverages complementary mechanisms: explosive decompression exploits intracellular pressure differentials for mechanical cell rupture, ultrasonication enhances CO_2 bubble dispersion and mass transfer while increasing membrane permeability through cavitation effects, and PEF induces controlled membrane electroporation to facilitate CO_2 penetration without complete cell fragmentation. The integration builds upon the validated continuous explosive decompression platform which already demonstrated superior performance over batch systems and competitive energy efficiency versus bead milling.

The continuous explosive decompression platform provides an ideal foundation for process intensification, with the hydrodynamic agitation zone and heated relief valve already demonstrated to minimize CO_2 consumption while maintaining high biomass release ratios. Specific research priorities should include:

1. Determination of optimal ultrasonic frequency ranges (comparing HFFU at 1-3 MHz versus LFNFU at 20-50 kHz) and power intensities for CO_2 bubble dispersion enhancement at the sparger point;

2. Evaluation of PEF parameters (electric field strength 5-30 kV/cm, pulse duration 1-100 μs, total specific energy 20-100 kJ/kg) to achieve controlled membrane permeabilization without excessive energy input or cell lysis;
3. Investigation of sequential versus simultaneous application strategies, comparing pre-treatment approaches (PEF/ultrasonication before pressurization) versus in-situ coupling at the sparger mixing zone;
4. Assessment of strain-dependent responses across microalgae species with varying cell wall compositions and thicknesses;
5. Comprehensive energy balance analyses integrating additional energy inputs from PEF or ultrasonication against gains in disruption efficiency and CO_2 reduction to validate industrial feasibility.

Collaboration with equipment manufacturers and process engineers could be the 2nd step to translating laboratory findings into scalable industrial solutions. The development of specialized sparger designs incorporating ultrasonic transducers or PEF electrode arrays will require expertise in acoustic engineering and electrochemical reactor design to ensure proper energy distribution, avoid dead zones, and maintain structural integrity under high-pressure operating conditions.

FUTURE STUDIES

The research presented establishes foundations for mild, energy-efficient microalgal cell disruption through continuous explosive decompression integrated within biorefinery frameworks. However, several critical research directions warrant investigation to advance from laboratory-scale demonstration to industrial implementation across diverse microalgal systems and product portfolios.

Scale-Up and Pilot Studies

Transitioning from laboratory-scale (4-12 kg dry cell weight (DCW)/day) to industrial-scale (10,000-100,000 kg DCW/day) requires investigation of hydrodynamic scaling, pressure drop management, and corrosion-resistant materials. CO_2 recovery and recycling via pressure swing adsorption or membrane contactors is economically essential, potentially reducing costs substantially. Long-term pilot-scale trials (months to years) will reveal fouling rates, corrosion characteristics, and maintenance requirements critical for industrial viability (Chapter 5).

Application to Diverse Microalgae Strains and Stress Conditions

Industrial applications span diverse taxonomic groups (green algae, diatoms, cyanobacteria, eustigmatophytes) with distinct cell wall compositions affecting disruption of susceptibility. Systematic strain screening using design-of-experiments across critical parameters (pres-

sure 5-250 bar, temperature 4-70˚C, CO_2/liquid ratio 5-20%) will establish strain-specific optimization requirements and enable predictive modeling correlating cell wall composition with explosive decompression susceptibility.

Systematic Stress Screening (N Depletion, Salt Stress, Freeze Shock, Cold Storage)
Cultivation strategy actively modulates cell disruption efficiency through physiological modifications. Nitrogen depletion significantly enhances disruption efficiency in *N. oleoabundans* (57% vs. 34% biomass release), with selective phospholipid (92% vs. 27%) and glycolipid (57% vs. 33%) transfer through liposome and micelle formation (Chapter 3).

Salt stress in marine cultivation (30-35 g/L dissolved solids) decreases CO_2 solubility by 20-30%, requiring higher operating pressures, while osmotic stress induces compatible solute accumulation and membrane modifications that may enhance or inhibit disruption through altered permeability and cell wall mechanics.

Freeze shock activates pro-survival pathways including polyunsaturated fatty acid synthesis and cryoprotective compound production, reducing bead milling susceptibility by ~40%. Cold storage at 4˚C induces apoptotic-like cell death with plasma membrane alterations, DNA fragmentation, and metacaspase activation, enhancing disruption yields by ~5%, contrasting with 20˚C storage which decreases yields by ~40%. This mechanistic distinction identifies freeze shock as acute adaptive stress versus cold storage as chronic degenerative stress, represents a novel bioprocess opportunity (Zuppini et al., 2010; Xiong et al., 2025).

Future investigations should employ design-of-experiments clarifying interactive effects of growth phase and storage conditions, with targeted metabolomics and molecular apoptosis markers confirming mechanistic hypotheses.

Process Optimization and Control

Real-time flow cytometry integration (2 minutes analysis vs. 20 minutes hemocytometer or 72 hours gravimetric) supports automated online monitoring and closed-loop control. Model predictive control optimizes multivariable interactions between pressure, temperature, and CO_2/liquid ratio. Multi-objective optimization balances competing objectives (maximizing biomass release, minimizing energy consumption, maximizing product selectivity, ensuring quality), and identifying Pareto-optimal operating conditions. Adaptive control using recursive parameter estimation or machine learning-based reinforcement learning automatically adjusts parameters as biomass properties vary.

Product-Specific Applications

High-value protein recovery, pigment extraction (e.g. astaxanthin, phycocyanin), and lipid fractionation leverage explosive decompression's mild conditions. Astaxanthin extraction from *Haematococcus pluvialis* could employ mild explosive decompression coupled with

deep eutectic solvent-based aqueous two-phase systems, achieving near-100% recovery under organic-solvent-free conditions. Phycocyanin extraction requires optimization at low temperatures (4-20°C) to maintain protein stability. Selective lipid fractionation exploits differential polar versus neutral lipid recovery through liposome and micelle formation, simplifying downstream purification.

Integration with Biorefinery Concept

Disruption-extraction coupling simultaneously performs both operations within pressurized CO_2 systems with co-solvents. Sequential product recovery cascades extract highest-value products under mildest conditions (phospholipids, neutral lipids, proteins/carbohydrates, enzymatic hydrolysis). Residual biomass valorization through anaerobic digestion, hydrothermal liquefaction, or pyrolysis, with nutrient recycling to cultivation, achieves >95% biomass utilization necessary for economic viability.

Techno-Economic and Environmental Assessment

Capital expenditure for industrial-scale systems ($1-3 million for 100,000 kg DCW/day) shows 40-50% reduction versus bead milling equivalents. Operational expenditures favor explosive decompression: 0.3 kWh/kg versus 2.8 kWh/kg (89% reduction), with maintenance costs reduced due to absent moving parts and CO_2 recycling reducing consumable costs from 2.7 to 0.03 EUR/kg (Chapter 5). Life cycle assessment across multiple impact categories should incorporate CO_2 recycling, different electricity grids, and waste CO_2 integration scenarios. Business model development should identify market segments where mild processing justifies premium pricing.

Effect of Higher-Pressure Explosive Decompression

At elevated pressures (30-250 bar), CO_2 solubility increases proportionally, potentially enabling higher intracellular CO_2 and enhanced disruption efficiency. At supercritical pressures (>73.8 bar), CO_2 exhibits unique solvation properties. Investigation should characterize pressure-disruption relationships, failure mechanisms, product quality impacts, and energy trade-offs. Preliminary analysis suggests diminishing returns at very high pressures if mechanisms transition from controlled permeabilization to catastrophic fragmentation; optimization requires energy balance analysis identifying economically optimal pressures.

Conclusion

The continuous explosive decompression technology could represent a paradigm shift in microalgal biorefinery development in case of successful future development, offering substantial energy savings (89% reduction vs. bead milling), economic advantages (97% OPEX reduction through CO_2 recycling), and maintained product quality through mild processing conditions. Systematic investigation across diverse microalgal strains, cultivation stress conditions (nitrogen depletion, salt stress, freeze shock, cold storage), and optimized process control strategies will establish this technology's broad applicability. Integration within

multiproduct biorefinery cascades, coupled with rigorous techno-economic and life cycle assessments, will enable transition from proof-of-concept to industrial-scale implementation. The convergence of multiple research directions (scale-up engineering, strain-specific optimization, real-time control, product selectivity, and biorefinery integration) within a coherent framework may position continuous explosive decompression as a critical enabling technology for achieving economically viable and environmentally sustainable microalgal biorefineries at industrial scales. Future work should prioritize pilot-scale demonstrations validating key assumptions, business model development for market segments where mild processing commands premium value, and comprehensive assessment of system-level integration benefits that extend beyond individual unit operation performance.

EMERGING TECHNOLOGIES IN THE BIOREFINERY LANDSCAPE

The current landscape of microalgae biorefineries is being shaped by transformative advances that extend well beyond incremental improvements. Key developments center on the application of artificial intelligence and machine learning (AI/ML) for adaptive, real-time process control. Incorporating AI/ML with inline native high-throughput measurement (as detailed in Chapter 4) allows for dynamic system optimization across cultivation and disruption stages. Machine learning algorithms can adjust nutrient inputs and harvest timing based on flow cytometry and biomass quality data, resulting in substantially higher productivity and product yields (Wu et al., 2025b; Sá et al., 2022; Long et al., 2022; Imamoglu, 2024). The digital twin approach, utilizing CFD and thermodynamic models as developed in Chapter 5, enables predictive scenaristic control, further increasing process efficiency and yield, especially when applied to the explosive decompression system. Upstream and chain-wide optimization using these unified models have shown significant improvement over conventional strategies (Fabris et al., 2020; Tummawai et al., 2024; Moretta et al., 2022).

Process analytical technologies (PAT) are advancing with label-free, native, inline monitoring tools—such as flow cytometry (as also explained in Chapter 4), fluorescence/Raman spectroscopy, digital holographic microscopy, and hyperspectral imaging (Sá et al., 2022; Kang et al., 2025; Wu et al., 2025b). These enable high-throughput and nuanced evaluation, strengthening integration between monitoring and automated control. However, further analytical development is needed, especially for fluorescence/Raman spectroscopy, particularly for accurate discrimination in the presence of microalgal pigments.

Hybrid technology combinations, notably the integration of pulsed electric field (PEF) and ultrasound with explosive decompression (see Chapter 5 and the Additional Insights section), represent compelling pathways. PEF-explosive decompression coupling offers enhanced membrane permeability and CO_2 dispersion, while ultrasound can intensify

cavitation and cellular sensitization, both supporting more efficient and uniform disruption. These strategies hold the promise for lower energy inputs and increased target product yields when properly optimized (Shivakumar et al., 2024; Wang et al., 2015a-2015b).

Emerging membrane-based technologies facilitate integrated harvesting and fractionation, with innovations in antifouling, functionalized, and responsive materials (de Morais et al., 2023; Vatanpour et al., 2024). Strategic positioning of explosive decompression, either just after or before filtration, can maximize process flexibility (Chapter 5).

Addressing large-scale industrialization, modular and distributed biorefinery architectures that supported by blockchain, IoT, and plug-and-play standardization, enable risk sharing, local optimization, and better integration with external resource flows (Fabris et al., 2020; Ievina, 2024; Cheah et al., 2025). Integration of synthetic biology, such as cell wall and photosystem II engineering, continues to reshape upstream feasibility and downstream yield potential, though these innovations necessitate careful assessment regarding economic, technical, and regulatory dimensions (Shivakumar et al., 2024; Grama et al., 2022; Mandal et al., 2025).

Finally, integrating circular economy principles, particularly wastewater valorization, CO2 utilization, and residue upcycling, remains paramount for sustainable, zero-waste operation (Sakarika et al., 2024; Ievina, 2024; Srimongkol, 2022; Morais et al., 2023; Gaur et al., 2024). Overall, realizing the economic and industrial potential of microalgae biorefineries hinges upon the coordinated adoption of digitalization, hybridized disruption, emerging materials, modular designs, and circular paradigms, with explosive decompression emerging as a pivotal technology throughout the chain (Valdovinos-García et al., 2022; Nour et al., 2024; Zhang et al., 2024a-2024b).

CONCLUDING PERSPECTIVE

The research directions outlined in the Future Studies section address critical technical and operational challenges for advancing continuous explosive decompression and microalgae biorefinery integration. However, these technical innovations must be contextualized within the broader landscape of global challenges that microalgae biotechnology is uniquely positioned to address. Importantly, the conversation has fundamentally shifted: nowadays, scientists are not talking about the scarcity of fuel and fuel-based products. Instead, the number of reports on the scarcity of balanced protein sources and clean water for human consumption and their devastating effects on global populations are increasing at an alarming rate, especially for low-income countries.

The scale of these interconnected crises is staggering. Current data reveals that over 2.2 billion people worldwide lack access to safely managed drinking water, with nearly 50% of the global population experiencing severe water scarcity. The World Bank's 2025 Global Water Monitoring Report indicates the world is losing 324 billion cubic meters of freshwater annually driven by poor land and water management, deforestation, and excessive irrigation. By 2030, water demand could exceed supply by 40%, and water scarcity could displace over 700 million people. (WHO, 2024). At this point microalgae offer a unique opportunity to serve as a sustainable and renewable desalination source while producing biomass and bioproducts (Danaeifar et. al., 2023).

Simultaneously, protein malnutrition represents a devastating and underreported crisis, particularly affecting children in critical developmental stages. Global data from 2021-2024 shows that stunting and wasting affect an estimated 150.2 million and 42.8 million children under age five worldwide, respectively, with approximately 45% of child deaths in developing countries linked to malnutrition. Overall, malnutrition has decreased worldwide, but the rates vary. Between 2012 and 2024, the greatest relative increase in the number of children under age 5 years with stunted growth occurred in lower–income countries with increases of 13.7% (UNICEF, 2025). Favorable characteristics of microalgae proteins coupled with high yield concentrated protein production and biorefinery integration possibilities make microalgal productions an interesting option for the solution of scarcity of protein especially for the countries also dealing with clean water scarcity.

The biorefinery approach enables simultaneous production of proteins, lipids, pigments, and other high-value compounds while achieving >95% biomass utilization. In principle, microalgae biorefineries represent one of the most promising platforms for addressing global protein scarcity, water security through wastewater treatment and desalination, and climate change through carbon sequestration (Sivaramakrishnan, 2022).

Yet despite these compelling attributes and decades of research, industrial-scale implementation remains elusive. Understanding why requires examining the troubled history of microalgae commercialization.

The history of microalgae biotechnology is explained comprehensively in Chapter 1. Briefly since 1940s microalgae first considered as the new protein and nutritional sources till 1970s oil crisis and afterwards industrial scale efforts only taken by considering microalgae as the next biofuel source leading 2 cycles of industrial disappointment after industrial scale trials of producing microalgal biofuels without biorefinery approach. The 80 years of events graph of microalgae biotechnology shown in Fig. 21. Despite decades of scientific progress demonstrating that microalgae can produce numerous high-value compounds including proteins, pigments, and nutraceuticals, current global production remains limited. The scientific consensus strongly supports biorefinery approaches integrating multiple co-products

as essential for economic viability, as standalone biofuel production consistently results in negative economics (Hupfauf, 2016; Chew, 2017). Some research demonstrates that facilities producing high-value products alongside biofuels can achieve positive net present values, with one techno-economic analysis showing up to 92% return on investment for 100-hectare facilities (Beal et. al., 2018; Ruiz et. al., 2016). However, investor hesitancy persists due to high capital costs ($29-115 million for commercial facilities), production costs of €3.4-5/kg compared to commodity proteins at significantly lower prices, immature industrial know-how, complex biorefinery structure and past commercialization failures that created a fundamental reputational crisis (Beal et. al., 2018; Araújo et. al., 2021, Chekanov et. al., 2021). In result microalgal production continues to be regarded as successful only in niche markets targeting high value products. FAO data indicates total microalgae production reached only 56,456 tons in 2019 worldwide, representing merely 0.005% of only EU region biodiesel and 0.033% of worldwide baker's yeast production at the same year as comparison (FAO, 2019; EBB, 2024; FAO, 2023).

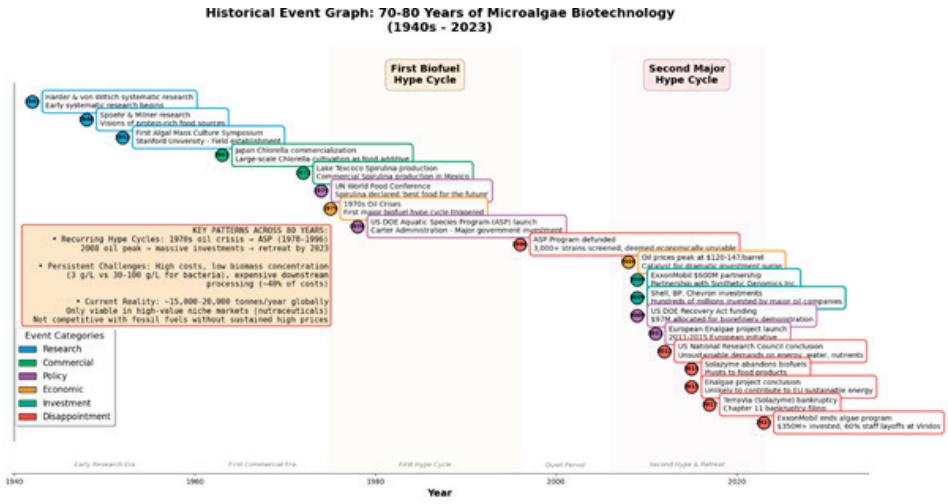

Fig. 21: Microalgae Biotechnology Historical Event Map

To this date, microalgae biotechnology faces an industrial-level reputation problem stemming from unsuccessful biofuel-only commercialization strategies, despite considerable scientific achievements demonstrating potential solutions to global challenges including climate change, malnutrition, CO_2 emissions, protein scarcity, and unsustainable agricultural practices in case of industrial scale production levels achieved similar to baker's yeast or 1% of biodiesel and also the industrial achievements reached especially in production of high-value compounds. Technology remains trapped in niche markets primarily due to investment followed by technical barriers, with researchers acknowledging that commercialization of microalgae biorefineries requires several billion dollars of funding and a decade or more of sustained commitment for know-how maturation. The path forward necessitates reframing

microalgae as a diversified biorefinery platform comparable to established fermentation products like baker's yeast, emphasizing successful commercial applications rather than failed biofuel ventures to attract the targeted investments needed for industrial-scale implementation (MDPI, 2022; Biotechnology for Biofuels, 2024; RSC Publishing, 2016).

The human cost of this disconnect between scientific potential and industrial reality is profound. A case study from Van, Turkey, illustrates the devastating consequences of protein malnutrition. Research on the effects of poverty on child physical, neurological, and cognitive development reveals that children aged 0-5 years in low-income areas experience 16-20% rates of stunting, wasting, and low BMI specifically attributable to insufficient protein intake. Compared with the general Turkish statistic of 7-8% protein-related malnutrition in this age group, the total number of affected children reaches approximately 1.2-1.5 million nationwide (Kızılyıldız, 2016).

While such malnutrition problems may currently seem less relevant for EU countries, the rising trends remain concerning indicators. Microalgae still possess the potential to solve scarcity of affordable, healthy protein sources for vulnerable populations—but only if a successful biorefinery approach is pursued. Critically, even to provide necessary protein to the children mentioned in this single case scenario through a biorefinery approach not primarily focused on protein production, approximately 300,000 tons/year production capacity would be necessary. This represents 5 to 6 times the 2019 worldwide microalgae production for all purposes regarding FAO data. The gap between need and capability is stark and growing.

To achieve production capacities that meaningfully address global issues such as protein and water security, microalgae biorefineries must discover "the next big thing": a catalyst that creates substantial industrial interest comparable to the biofuel enthusiasm of the 1970s and 2010s, but this time with a sustainable and economically viable biorefinery approach. Unfortunately, the next big thing will not be related to scarcity of protein or clean water since it targets consumers with lowest income in lowest income countries. However, the utilization of biorefinery approach can create a bridge at this point, where economic viability and the impact on global issues connect. Researchers acknowledge that commercialization of microalgae biorefineries requires several billion dollars of funding and a decade or more of sustained commitment for industrial know-how maturation. The path forward necessitates reframing microalgae as a diversified biorefinery platform comparable to established fermentation products like baker's yeast, emphasizing successful commercial applications rather than failed biofuel ventures to attract the targeted investments needed for industrial-scale implementation.

Here are 3 examples to the many fundamental pathways that microalgae biorefineries can be industrially interesting and pursued technologies again:

3) **Biological Discovery Pathway:** Exploring novel compounds or novel utilization methods of known compounds that can be produced industrially by microalgae in an economically feasible manner with strong market demand. This follows the traditional biotechnology model of discovering high-value compounds (pharmaceuticals, nutraceuticals, specialty chemicals) or high-impact utilization methods of known compounds that justify premium pricing and complex production processes. Recent investigations into biocontrol compounds (antifungal, antibacterial, pesticide) produced by microalgae in high-density cultures or newly found functions of siphonein and siphonaxanthin pigments represent one such direction. Success in this pathway could create anchor products that subsidize broader biorefinery operations.
3) **Economic Optimization Pathway:** Dramatically reducing industrial-scale microalgae biorefinery operational expenditures (OPEX) and capital expenditures (CAPEX) to levels competitive with or superior to established food and beverage industry investment options. This addresses investor risk tolerance directly: if returns are sufficiently attractive and costs sufficiently low, investors may accept the industrial know-how gap risks.
3) **Integration and Convergence Pathway:** Smart integration with emerging technologies both within and outside the microalgae field to create synergistic "next big thing" opportunities. This includes integration with AI-driven process control, inline spectroscopic monitoring, membrane-based fractionation, synthetic biology, and circular economy infrastructure (wastewater treatment, industrial CO_2 sources, aquaculture/aquaponics systems), other fields in life sciences. The value creation emerges not from microalgae alone but from microalgae as an enabling component within larger sustainable systems.

The continuous explosive decompression technology developed in this PhD project and presented in this thesis exemplifies a broader principle: **incremental innovation in individual unit operations, while valuable, is, by itself, insufficient to achieve transformative change in microalgae biorefineries**. The path forward to carry microalgae biorefineries in global level requires systems-level thinking that integrates cultivation, harvesting, disruption, and extraction as interconnected, co-optimized stages within intelligent, adaptive biorefinery frameworks.

Emerging technologies, being AI-driven process control, inline spectroscopic monitoring, membrane-based fractionation, hybrid disruption strategies, synthetic biology and economics shouldn't be studied as additive improvements but should be accepted as fundamentally new paradigms for how microalgae biorefineries can operate. When combined thoughtfully, these innovations could enable:

- **Adaptive biorefineries** that adjust processing strategies in real-time based on biomass composition, product demand, and resource availability
- **Distributed production networks** that localize cultivation while centralizing advanced processing, optimizing both biomass quality and economic efficiency

- **Circular biorefinery systems** that integrate with existing industrial infrastructure (wastewater treatment, CO_2-emitting facilities, aquaculture, aquaponics) to transform waste streams into valuable products
- **High-value, multi-product facilities (biorefineries)** that prioritize specialty biochemicals, pharmaceuticals, and nutraceuticals over commodity biofuels, achieving economic viability through product portfolio diversification while also targeting high volume productions to make impact on global issues

The new explosive decompression method, with its demonstrated energy efficiency, product selectivity, and mild processing conditions, is well-positioned to contribute to these integrated systems, as not a replacement for other technologies, but as a complementary tool deployed strategically where its unique attributes provide maximum value.

Realizing this vision requires continued collaboration across disciplines (bioprocess engineering, synthetic biology, computational sciences, industrial ecology), sustained investment in pilot-scale demonstrations that validate systems-level integration, and willingness to challenge conventional assumptions about how biorefineries should be designed and operated. The microalgae biorefinery of the future will possess four key attributes: intelligence, modularity, adaptability, and sustainability. The technologies developed in this thesis, along with those emerging across the broader field, are beginning to enable these transformative attributes.

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THESIS SUMMARY

This PhD thesis addresses key challenges in microalgae biorefineries by developing an efficient, mild cell disruption technique to enhance the recovery of intracellular biomolecules while minimizing energy consumption and product degradation. Driven by global imperatives to transition from fossil-based resources to sustainable alternatives amid escalating greenhouse gas emissions and resource scarcity, the research positions microalgae as versatile feedstocks for biofuels, biochemicals, nutraceuticals, and high-value compounds. Microalgae's superior carbon fixation capacity (10-50 times that of terrestrial plants) and ability to integrate wastewater treatment with CO_2 sequestration underscore their potential in circular bio-economies.

The work comprehensively evaluates mechanical and non-mechanical disruption methods, identifying limitations in energy efficiency, scalability, and product quality. Conventional approaches like bead milling and high-pressure homogenization achieve high yields but consume substantial energy (0.81-147 kWh/kg dry biomass) and generate shear forces that compromise sensitive biomolecules. To overcome these, the thesis explores cultivation strategies, such as nitrogen depletion, which alter cell physiology to improve disruption efficiency and selectivity, particularly for lipids (up to 92% phospholipid release).

A novel analytical method using flow cytometry enables rapid, accurate quantification of disruption yields, correlating strongly (R^2=0.9867) with biomass release while distinguishing intact, leaking, and disrupted cells. This advancement facilitates real-time process optimization, reducing analysis time from hours to minutes.

Culminating the research is the development of a patented continuous explosive decompression device using CO_2, which addresses mass transfer limitations in conventional explosive decompression method through innovative sparger design and hydrodynamic mixing. Compared to batch systems, it reduces processing time 3.2 - 9.6 fold, CO_2 consumption 8.5 - 48.2 fold, and achieves 24 - 36% biomass release at shear rates only 0.33% of bead milling. Techno-economic analysis reveals superior performance: 0.3 kWh/kg dry biomass versus 2.8 kWh/kg for bead milling (89% energy reduction), with capital costs 45,000 EUR versus 77,000 EUR for equivalent capacity.

Statistical modeling identifies pressure and flow rate as dominant parameters influencing release ratios, while co-solvents enable selectivity control. The technology demonstrates secretion (extraction without rupture) and scattering (debris formation) mechanisms, offering tunable product recovery.

Overall, the thesis argues that the field's usual trade-off ("harsh but effective" vs "mild but inefficient") is not inevitable; with integrated cultivation–disruption thinking, better analytics, and intensified continuous design, mild and scalable disruption can be engineered toward industrial relevance. This can be a step towards energy-efficient solution that could transform microalgae biotechnology into a viable industrial platform for addressing climate, energy, and resource challenges.



APPENDIX

CURRICULUM VITAE

MASPO Energy (Gürmen Group) – Technical Advisor / Expert
September 2025 - Present

- Developing strategic and technical frameworks for utilizing geothermal power plant resources in bioprocesses.
- Focusing on the production of high-value compounds using microalgae.
- Collaborating with cross-functional teams to enhance project infrastructure.
- Contributing to sustainable energy solutions at Maspo Energy, part of Gürmen Group.

AITechBio Freelancer Group - Co-Founder - CEO
October 2022 - Present

- Led the development of AI-Powered Pathogen Detection and Phonocardiology AI Assistant projects, revolutionizing microbial diagnosis and customer insights.
- Implemented Advanced Data Interpretation techniques to streamline data analysis processes, contributing to unparalleled support in the bio industry.
- Led the team in building new AI-based knowledge management systems for SaaS in the bio-industry.

FABLE Bio - Co-Founder, CTO
March 2020 - November 2021 (1 year 9 months)

- In my role as Co-Founder and CTO at FABLE Bio, I played a pivotal role in integrating various fields to create innovative products for the biopharma and medtech industries.
- Led the technical team in developing AI-supported business development strategies.
- Implemented cutting-edge technologies to streamline processes and enhance product offerings.
- Spearheaded projects that drove growth and efficiency within the organization.

SciCo - Founder
April 2017 - October 2019 (2 years 7 months)

- Provided consultancy on Scientific Data analysis, Population dynamics modelling, Statistics, (Q)SAR modelling, Bioprocess Design at SciCo.
- Developed innovative strategies to optimize data analysis processes and improve bioprocess design efficiency.
- Implemented statistical models leading to increased accuracy in population dynamics forecasting.
- Collaborated with cross-functional teams to drive successful projects and deliver high-quality results.

MK Teknoloji - CTO
May 2015 - May 2018 (3 years 1 month)

- Utilized skills in Scientific Data analysis and Analytic chemistry to lead a team in developing innovative solutions at MK Teknoloji.
- Managed team creation and training, fostering a collaborative environment for successful project execution.
- Implemented Machine Learning techniques to drive strategy development and project management, resulting in streamlined processes and improved outcomes.

Wageningen University - PHD Candidate January 2012 - July 2026 (14 years)
VITO- Flemish Institute for Technological Research / WUR- Wageningen University - Junior Researcher

March 2012 - September 2015 (3 years 7 months)

- PhD title: Mild Disruption of Single Cell Biomass as the First Step of Biorefineries

LIST OF PUBLICATIONS

Günerken E, Gonzalez LG, Elst K, Eppink M & Wijffels R. (2013). Disruption of *Neochloris oleoabundans* by bead milling as the first step in the recovery of intracellular metabolites. *Current Opinion in Biotechnology*, 24, S43. https://doi.org/10.1016/j.copbio.2013.05.093.

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OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities (courses, workshops, symposia, summer schools, conferences etc.)		
Name of the course/meeting	**Country**	**Year**
Biorefinery for Biomolecules 1st edition	NL	2012
1st international Symposium about Microalgae Biotechnology for Young Researchers	ES	2012
9th European Congress of Chemical Engineering / 2nd European Congress of Applied Biotechnology	NL	2013
European Biotechnology Congress 2013	SL	2013
Fundamentals and Practice of Process Intensification	NL	2013
Project meetings	BE & NL	2012-2016
EUBCE 2015	AT	2015
Biorefinery for Food & Fuel & Materials	NL	2013
General courses (e.g. PhD week, language courses, statistics, et.)		
Name of the course	**Country**	**Year**
VLAG PhD week	NL	2012
Project and time management	NL	2012
Scientific Publishing	NL	2012
Scientific Writing	NL	2013
Writing Grant Proposals	NL	2013
Mobilizing Your Network	NL	2013
Optionals (participation in discussion groups, PhD excursions, MSc courses, etc.)		
Name of the course	**Country**	**Year**
Preparation of research proposal	NL	2012
PhD study tour	NL	
Weekly group meetings	NL	2012-2016
PhD Days	NL	2012-2016

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