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Development of a sustainable biorefinery process from microalgae

A case study on lipid, lutein and protein recovery from *C. prototheco*ides grown on wastewater

Iris Houthoff

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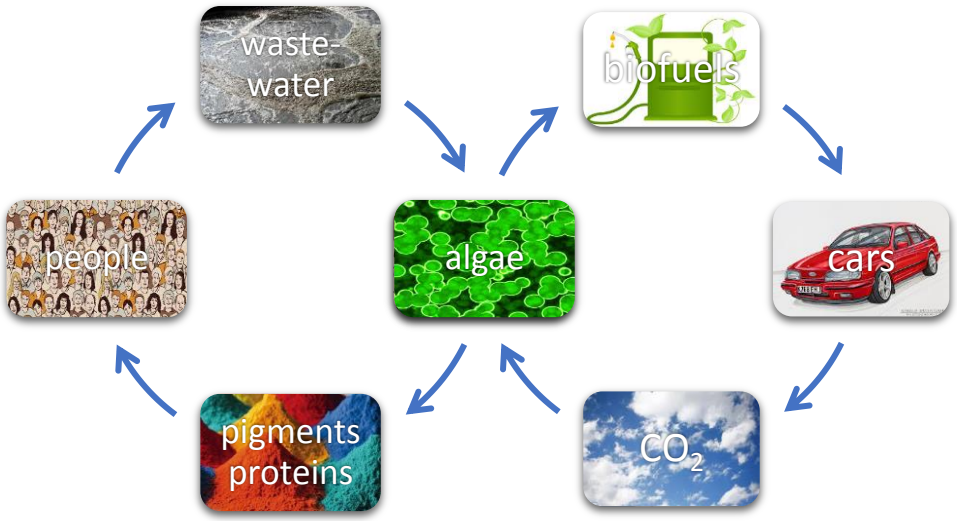

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ABSTRACT

Due to fossil depletion, microalgae biorefinery is explored as replacement of petrol-based production processes of fuel and chemicals. To be competitive a biorefinery process has to make use of wastewater as cultivation medium and yield multiple end products. This research is aimed at developing sustainable biorefinery processing routes for *C. protothecoides* grown on centrate leading to the production of biodiesel, lutein and proteins. These routes are assessed on sustainability by comparing the performance in terms of energy consumption, waste production and chemical usage. A model was developed to minimise the relative energy consumption per obtained product and used to determine the sensitivity towards a changing biomass composition. Also the feasibility of routes aimed at multiple end products was assessed by comparing the complications which arise when designing routes aimed at multiple products. Results showed that separation of lipids and lutein requires the most energy for heating and distillation of the used solvents. A significant part of this energy can however be reduced through heat recovery. The route demanding the least energy per recovered products involves flocculation combined with centrifugation for harvesting and dewatering, a high pressure homogenizer for disruption, solvent extraction for separation of lipids and lutein and shifting of pH for protein recovery. A disadvantage of flocculation is that, depending on the type of flocculant used, different complications arise in further processing of the biomass. Whether transesterification is performed prior to or after lipid extraction, has also implications for protein and lutein recovery. Assessment of the environmental impact points out that biodiesel formation through transesterification is the most polluting step and that the residual biomass fraction can be made valuable through additional processing. It was concluded that the formation of a microalgae biorefinery process resulting in multiple end products is feasible and can be made sustainable by accepting increased energy consumption. The focus should however shift away from biodiesel production, towards the combined recovery of pigments, proteins and carbohydrates as this significantly reduces the energy needed for waste handling.

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1. INTRODUCTION

Due to fossil oil depletion, new resources are being explored that could replace our petrol-driven industries. This concerns both the search for new energy sources as the development of new chemical production pathways. The challenge is to form a sustainable society that works on a closed-cycle basis while making use of renewable resources. A potential renewable approach is to utilise microalgae.

Microalgae have already many applications in the food and feed industry and are explored as feedstock for biodiesel production (Chisti 2007; Schenk et al. 2008). They are referred to as the third generation of biofuel, replacing the second generation that is based on the conversion of higher plants. Compared to plants, microalgae have a higher photosynthetic efficiency and higher biomass production. They require less land and have the advantage that they can be grown on arid land, preventing a negative impact on food availability (Huang et al. 2010; Yen et al. 2013). They do require a lot of water and substrate for cultivation, but that does not necessarily have to lead to a competition for freshwater. Seawater or wastewater are suitable sources for growth medium as well (Pittman et al. 2011).

An advantage of using wastewater is that it would serve a dual purpose; remediation of the waste flow while supplying nutrients to the algae. The use of microalgae for wastewater treatment is not a new concept. Wastewater can contain high concentrations of nutrients and often traces of toxic metals. Microalgae are able to purify many wastewater flows by accumulation of nutrients and toxins into biomass (de la Noüe et al. 1992). The algae cultivation thereby contributes to the recovery of fresh water, lowers the water footprint of the algal production chain and prevents eutrophication. The ability of algae to grow on wastewater depends on the source, since the wastewater composition is highly variable among different waste streams. Selection of a suitable wastewater flow as feedstock for algae is therefore important.

Within the treatment process of municipal wastewater, the centrate flow has characteristics which are very beneficial for microalgae production systems (Wang et al. 2010). It is produced from dewatering wastewater sludge before anaerobic digestion. Centrate is currently recycled to the head of the treatment plant. Discharge of centrate causes environmental pollution due to the high concentrations of carbon and nutrients and the presence of metals. On the other hand, recycling of the centrate increases the loading rate for the activated sludge process, which makes it difficult to reach the required effluent concentrations. Therefore, treatment of centrate by microalgae combined with a biorefinery process is considered as an advantageous alternative.

Multiple studies point out that *Chlorella* sp. shows the best growth rates and biomass productivity when grown on untreated centrate (Hu et al. 2012; Li, Zhou, et al. 2011; Zhou et al. 2011). In particular *Chlorella protothecoides* is a promising candidate as this microorganism is also capable of accumulating large lipid fractions which are suitable for biodiesel production. *C. protothecoides* is also known for a high lutein content; a carotenoid that is considered to be healthy due to the anti-oxidant activities and application as a food colorant (Lin et al. 2014). The production of high valuable compounds, like lutein, is necessary to cover the majority of the processing costs (Wijffels et al. 2010). A biorefinery process has to utilize all components inside the biomass in order to be sustainable and economically feasible. This means that after recovery of the desired products, applications should be found for the waste flows. Examples of such waste flows are the glycerol produced during the conversion of lipids to biodiesel and the residual biomass containing ash and the insoluble proteins and carbohydrates.

The concept of microalgae biorefinery originates from the urge for renewable resources. The petrol-based production processes of fuel and chemicals need replacement. To be competitive a biorefinery process has to yield multiple end products. For implementation of such biorefineries it is necessary to identify which complications are

encountered when designing processing routes for multiple end products. Another interesting question to study is how green biorefinery processes are in terms of chemical usage and waste production. Within this research, these two research questions will be answered for a promising case study that combines wastewater treatment with biorefinery. The case study involves the growth of *C. protothecoides* on centrate and the production of biodiesel, lutein and proteins via different processing routes. For each route the energy consumption, use of chemicals and production of waste are discussed. The energy requirements are determined by means of modelling and the produced waste flows are analysed for alternative uses and treatment. This will be done in two scenario studies: the extraction of lipids and proteins with or without lutein as additional end product. By comparing these scenarios the impact of a high valuable compound on the production process is evaluated. As the microalgae are grown on wastewater, varying nutrient and chemical concentrations are expected that influence the composition of the algae biomass. Therefore, the sensitivity in energy input rate of the processing routes will be tested for varying fractions of lipid, lutein and protein. Finally a conclusion can be made on the feasibility and sustainability of a biorefinery process resulting in three end products.

2. BACKGROUND INFORMATION

2.1. BIOREFINERY PRODUCTS

When designing a microalgae biorefinery process, it is important to first collect information on the characteristics of the desired end products, the existing production processes and commercial uses. For this reason, a short introduction is given on the applications and location inside a microalgae cell of lipids, lutein and proteins. Also carbohydrates are addressed since this will be the major component inside the residual biomass fraction. Finally an indication is given on how these components are separated after cell disruption in current practice.

One of the most extensively researched products derived from lipids is biodiesel. Biodiesel is a mixture of esterified fatty acids that has to meet certain standards before it can be used as a fuel. To accomplish this, the chain length and level of unsaturation of fatty acids is crucial (Daroch et al. 2013). Lipids are converted to biodiesel by means of transesterification as shown in Figure 1a. During this reaction a short chain alcohol, e.g. methanol, with the help of a catalyst forms fatty acid esters with glycerol as a side product. There are different types of catalyst suitable for this reaction, but the use of a base catalyst is most preferred due to the high efficiency (Daroch et al. 2013). The fraction of lipids considered to be suitable for biodiesel production is triacylglycerides (TAG). These neutral lipids are present in the shape of oil droplets inside the cell and are formed as storage product under N-deprived conditions. Cell disruption methods do not break these droplets, but will still succeed in making them available for extraction. Polar lipids such as phospholipids, glycolipids and cholesterols can be found inside the cell membrane and chloroplast membrane. These lipids are strongly bound to proteins via hydrogen or electrostatic bonds (Halim et al. 2011). The disruption methods are not able to fully break apart these lipid-protein interactions, but will partly tear the cell wall apart so that the intracellular content becomes exposed.

Lutein is a yellow carotenoid with strong antioxidant properties. It is the only carotenoid that is absorbed into the bloodstream after ingestion (Fernández-Sevilla et al. 2010). Consumption of lutein is confirmed to be healthy and therefore there are many applications in the pharmaceutical, feed and food industry. For growth on wastewater, the suitability of the obtained lutein for human consumption should be studied further. Otherwise lutein can be added to skin care products. Marigold flowers are the conventional source of lutein, but lutein-accumulating microalgae are gaining interest (Lin et al. 2014). Carotenoids are in general photosynthetic pigments situated inside the chloroplast. The hydroxyl groups at both ends of lutein can react with for example fatty acids to form a lutein ester. This bond can be broken by addition of a base to form free lutein again. This reaction is also referred to as saponification and shown in Figure 1b. Both lutein esters and free lutein are present inside a cell and the partition depends on culture conditions and strain. There is no evidence yet that one form of lutein is more beneficial and both forms have a comparable bioavailability (Wu et al. 2009). After cell disruption the lutein ester will most likely stay attached to cell debris, while the free lutein dissolves into the TAG droplets.

Proteins are often used as food or feed additive due to the nutritional value. However, since the algae are grown on wastewater, the non-food applications are considered. Proteins can be used within adhesives in case they show good adhesion and bond strength, in coatings when they are resistant to water and in plastic materials if they show strength. Surfactants are present in paint, coatings and adhesives as pigment disperser, wetting agent and defoamer. Other possibilities are to produce films based on proteins and glycerol and protein-based microspheres containing β-carotene or lutein for cosmetic purposes (De Graaf et al. 2001). Proteins can also serve as basis for chemical industry in case they are hydrolysed to obtain amino acids. An example is to form nitriles by selective oxidative decarboxylation (But et al. 2012). Nitriles can serve as basis for various different plastic products. Biobased production of nitriles could replace the fossil based production process that is considered energy demanding and produces toxic side products. Apart from nitriles, many other chemicals could theoretically be

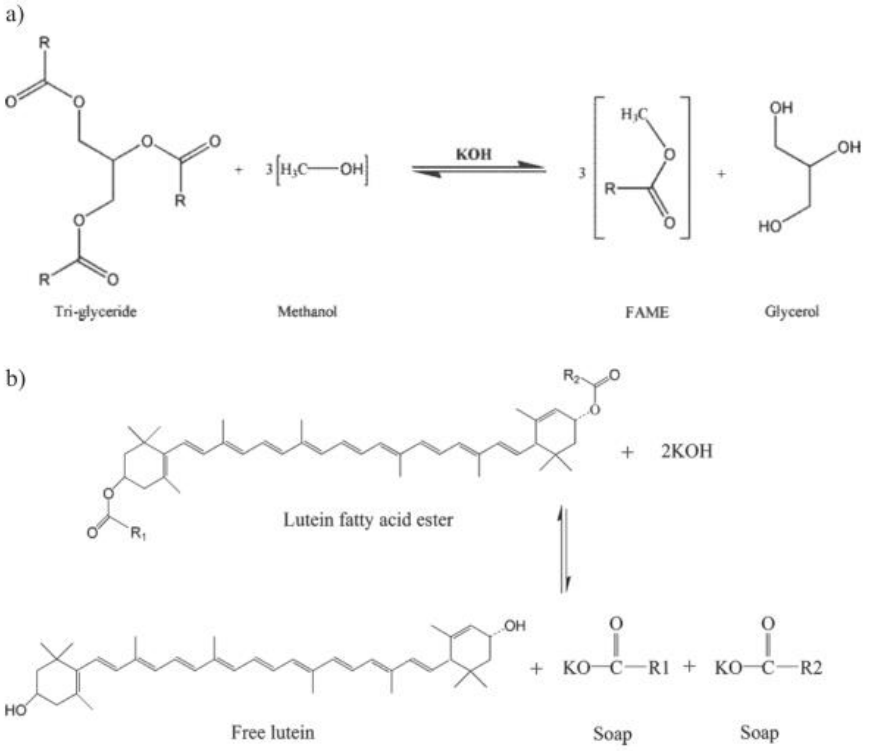

FIGURE 1 (PROMMUAK ET AL. 2013)

A) TRANSESTERIFICATION OF TRIGLYCERIDE RESULTING IN FATTY ACID METHYL ESTER (FAME) AND GLYCEROL,

B) SAPONIFICATION OF LUTEIN FATTY ACID ESTER FORMING FREE LUTEIN AND SOAP.

formed from amino acids as starting product. Though the specific properties of *C. protothecoides* proteins are not yet known, there is enough potential to find a suitable application. There is a high variety of proteins present within microalgae biomass. This high diversity can be explained by the fact that microalgae do not accumulate storage proteins as N-source (Schwenzfeier et al. 2011). Two types can be distinguished, namely soluble and insoluble proteins. The soluble protein fraction is expected to consist of functional proteins, such as enzymes involved in photosynthesis or other essential activities for survival and growth. The insoluble proteins most likely have structural functions and can be found inside the cell walls (Schwenzfeier et al. 2011; Gerde et al. 2013). At neutral pH conditions about 70% of the proteins present in *Chlorella* sp. is soluble (Ursu et al. 2014). Often the fraction of soluble proteins is first enlarged by addition of a base prior to removal of the insoluble fraction. This will however result in saponification of TAG which makes them unsuitable for biodiesel production. As this biorefinery case study is about extraction of multiple components, including lipids, it is decided to operate at neutral pH conditions. The proteins extracted at pH 7 also show better functional properties in terms of emulsifying capacity and emulsion stability (Ursu et al. 2014). This implies that these proteins are very useful as surfactants.

Carbohydrates are next to lipids and proteins also a main component of microalgae. Some polysaccharides are considered high valuable compounds for the food, cosmetic and textile industry or as emulsifier or thickening agent, the small sugars and starch are useful for biofuel production, such as bioethanol or hydrogen (Yen et al. 2013). Carbohydrates are present inside the cell wall to provide structure, are involved in cellular communication and can serve as storage products by the formation of starch droplets. Carbohydrates differ therefore greatly in composition; varying from large polysaccharides such as cellulose and hemicellulose to small monosaccharides like glucose and xylose. Within the microalgae *C. protothecoides* the fraction of starch is expected to be rather small as it uses lipids as storage product (Guccione et al. 2014). Due to lack of available data on the carbohydrate composition within microalgae in general, it is assumed that the division between soluble and insoluble carbohydrates is 50:50.

Separation of cellular components is generally achieved by differences in solubility potential. The first step in a biorefinery process is simple; separating the water soluble from the water insoluble fractions by means of centrifugation. The insoluble fraction will mainly consist of the cell wall, chloroplasts and oil droplets, while the water phase contains soluble proteins and carbohydrates. The challenge is to separate the desired components which are located within these two fractions. As lipids and lutein both have hydrophilic properties, the separation of these components based on polarity does not result in pure products. The developed extraction processes therefore lead to the formation of two lipid fractions; each containing a low and a high concentration of lutein respectively. The differences in lutein content can be achieved by changing the polarity of the extraction phase. The most commonly used solvents for extraction of lipids and lutein are hexane (Soxlet) and a mixture of chloroform and ethanol (Bligh and Dyer). Although chloroform is most efficient, hexane is often preferred as this solvent is less toxic, extracts less contaminants and has a higher selectivity towards neutral lipid fractions (Pragya et al. 2013). For lutein extraction particularly, ethanol is also considered as a harmless solvent with a high solubility towards carotenoids due to the more polar character (Mäki-Arvela et al. 2014). The soluble protein fraction can be separated from the soluble carbohydrates by the addition of an acid. Proteins precipitate in an acidic environment (Ursu et al. 2014), while carbohydrates are partially hydrolysed to sugars and remain soluble (H. Wang et al. 2014).

2.2. SUPERSTRUCTURE AND PROCESSING UNITS

Figure 2 shows the superstructure of this specific biorefinery case study. Different routes are possible to get from the algae solution towards the products biodiesel, lutein and proteins. Three routes are considered for the harvesting and dewatering step and twelve routes for obtaining the end products from the concentration algae flow. The processing units are grouped according to the general steps involved in biorefinery of microalgae: harvesting, dewatering, disruption and separation. The initial characteristics of the microalgae solution are determined by the cultivation process. After harvesting and dewatering, the flow and concentration of the algae slurry is fixed. Within this chapter the unit operations which are assessed in this study are shortly described. Extensive descriptions and additional formulas used within the models can be found in Appendix A.

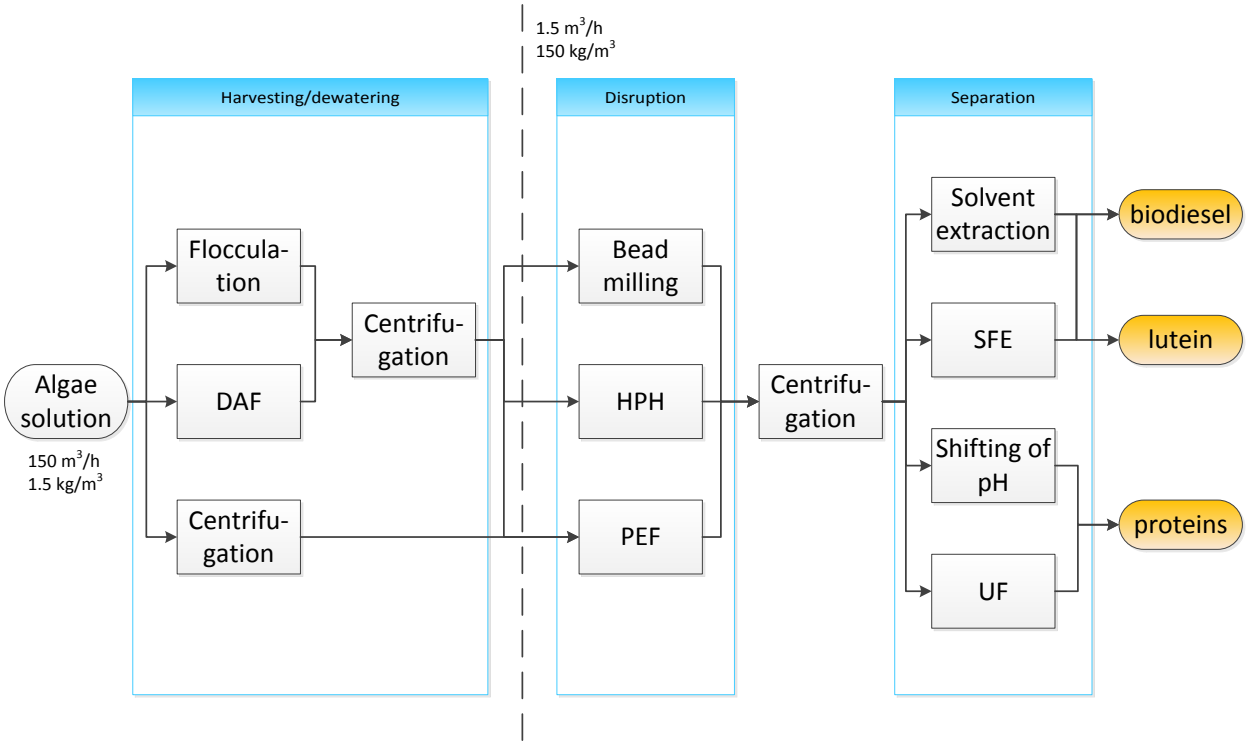

FIGURE 2, SUPERSTRUCTURE OF PROCESSING UNITS USED WITHIN THIS STUDY FOR DOWNSTREAM PROCESSING OF THE MICROALGAE BIOMASS. THE PROCESSING UNITS ARE REPRESENTED BY SQUARES AND THE STARTING ALGAE SOLUTION AND FINAL PRODUCTS BY OVALS. THE STRIPED LINE REPRESENTS THE CONSTRAINT IN TERMS OF FLOW AND ALGAE CONCENTRATION PRIOR TO DISRUPTION.

2.2.1. CULTIVATION

The centrate from wastewater sludge is rich phosphorus, ammonium and organic carbon (Wang et al. 2010). For cultivation of *C. protothecoides* the hetero-photoautotrophic two-stage cultivation process is selected as described by Zhou et al. (Zhou, Min, et al. 2012). During the first heterotrophic growth phase most of the nutrients and carbon sources are consumed resulting in high growth rates. After three days the carbon sources become limiting and the algae biomass is harvested. The nitrogen and carbon depleted centrate is reused as a medium during the second stage where under addition of CO_2 photoautotrophic growth is stimulated. During this stage the nitrogen limitation favours the formation of lipids and lutein (Zhou, Li, et al. 2012; Shi et al. 2002). This cultivation mode yields a final biomass production of *C. protothecoides* of 1.5 $kg\ m^{-3}\ day^{-1}$ (Zhou, Min, et al. 2012). Considering an average centrate flow of 3500 m^3/day (Zhou, Li, et al. 2012), this results in a daily potential production of 5250

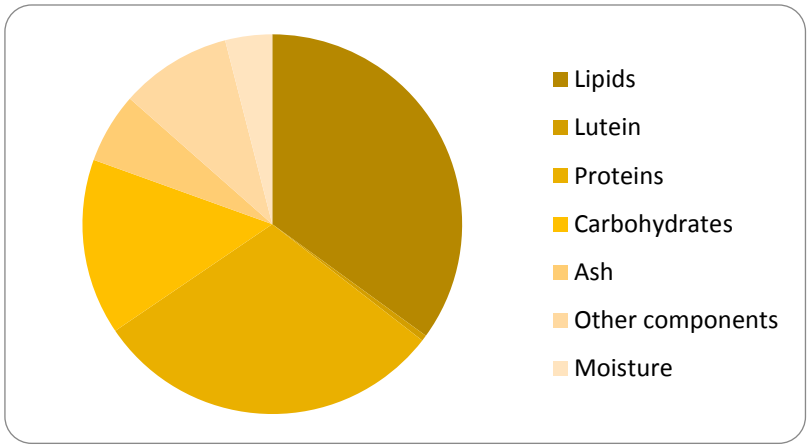

FIGURE 3, BIOMASS COMPOSITION OF *C. PROTOTHECOIDES* CULTIVATED ON CENTRATE

kg *C. protothecoides.* The fractions of components within the average *C. protothecoides* cell are based on literature and are shown in Figure 3 (Cerón-García et al. 2013; Miao & Wu 2006; Sforza et al. 2012; Shi et al. 2002).

2.2.2. HARVESTING AND DEWATERING

Harvesting is the first step in concentrating the biomass by removal of water. It is often followed by a dewatering step to further concentrate the algae flow. Harvesting and dewatering are together responsible for 20-30% of the total costs associated with processing of microalgae (Molina Grima et al. 2003). Single-step centrifugation is the most applied method for harvesting microalgae, but is known to consume a lot of energy. Multi-step approaches are thus designed where a harvesting step first concentrates 10-20 times before a dewatering unit takes over (Pahl et al. 2013). Since the suitability of a harvesting method is algal species specific (Milledge & Heaven 2012), many different techniques are being developed. Within this study the performance of single-step centrifugation is compared to a two-step approach in which either flocculation or Dissolved Air Flotation (DAF) precede centrifugation. These two harvesting methods have been selected for the favourable ratio of energy consumption to biomass recovery (Hagen 2014; Slegers et al. 2014).

Flocculation is a simple harvesting technique that involves the addition of a flocculant that attaches to the cells to form aggregates. As these aggregates start to increase in weight they tend to settle, forming a dense biomass layer at the bottom of a settling tank. The chitosan concentration determines to a large extend the recovery efficiency together with the speed of mixing (Riaño et al. 2012). The purpose of mixing is to ensure that the flocculant is spread homogeneously among the algae solution. It is the only energy input required for this process. Although such low energy requirements are beneficial, the sustainability of the process should also be considered and this is influenced by the choice of flocculant. Metal salts are cheap and often added in large quantities, due to the low recovery efficiency (Granados et al. 2012; Beach et al. 2012). This results in contamination of the biomass and affects further processing (Pragya et al. 2013). The use of polymeric flocculants increases the harvesting efficiency due to the high charge density and large surface area. They are used in water treatment systems and have a low toxicity (Bolto & Gregory 2007). Two examples of promising organic polymers are cationic starch and chitosan. Within this study chitosan is chosen as flocculant, which is produced from crustacean shell waste of fishing industries (Beach et al. 2012). It is a very safe polymer with many applications, even in the pharmaceutical industry (Ravi Kumar 2000). Flocculation has thus a very low energy demand, but also results in low biomass recovery efficiencies and needs addition of chemicals.

During DAF treatment an aqueous flow saturated with air is kept under high pressure before release into the algae solution. The resulting air bubbles rise to the surface while attaching particles via hydrophobic interactions. The addition of a flocculant is essential for high harvesting efficiencies, since a reduction in the number of particles and

an increase in flock size both increase the chance of capture by bubbles. The study that focussed on harvesting of 1.5 g/l of a *Chlorella* species by dissolved air flotation is used as reference point (Zhang et al. 2014). Here the harvesting efficiency reached 90% when 50 mg/g aluminium sulfate was added as flocculant. Since a much lower dose of flocculant is required during DAF treatment compared to flocculation, the toxicity effect of using a metal salt is less severe. The water flow saturated with air enters the system under pressure. This flow is produced by recycling 20% of the outgoing flow followed by addition of air. The energy balance thus involves energy consumption for pressurizing, mixing and for pumping of the main, waste and recycle stream. DAF has higher energy requirements compared to flocculation, but also leads to higher biomass recovery efficiency while using less chemicals.

The mechanical harvesting technique centrifugation has the highest energy demand but does not require the addition of chemicals. The centrifuge separates particles based on density difference by generating a centrifugal force. This can be accomplished very quickly and a centrifuge has therefore a much lower footprint compared to settling processes relying on gravity such as flocculation. The energy consumption of a centrifuge depends on the incoming algae concentration and flow rate and on the desired concentration factor.

2.2.3. DISRUPTION

Within the disruption step a mechanical force is applied causing cell damage which is beneficial for product separation. The incoming and outgoing mass flows remain therefore equal and no waste is produced. All methods used in this study work at conditions that cause permanent damage of the cells. This result in release of the intracellular components such as water soluble proteins. The free lipids and lutein present inside the cell wall become available for extraction. Three mechanical disruption options are evaluated: high pressure homogenizer (HPH), bead mill and pulse electric field (PEF). They have in common that they do not use any chemicals and therefore the products are not contaminated. The consequence is that cell disruption requires a much higher energy input. The energy requirement to disrupt one kilogram of dry microalgae biomass is theoretically only 763 J (Lee et al. 2013). Most mechanical treatment processes use at least 5 orders of magnitude more energy, meaning that over 99% of the energy input is converted to heat (Doucha & Lívanský 2008; Lee et al. 2012). This implies that just as much energy is needed for cooling, to prevent damage to the end products caused by high temperatures. The force causing disruption differs per processing unit and can therefore result in different degrees of disruption.

As the name implies, a high pressure homogenizer (HPH) disrupts algal biomass by placing it under high pressure, usually up to 1500 bar. The solution of algae collides against a valve seat and impact ring and encounters a sudden release of pressure when leaving the HPH (Lee et al. 2012). Multiple parameters influence the disruption efficiency, such as process temperature, medium flow rate, valve and orifice design, number of passes and applied pressure. The last mentioned has the highest influence and determines the release of cell contents for each pass. To be able to calculate the energy requirement for pressurizing, a relationship is determined between the applied pressure, number of passes and level of disruption (Yap et al. 2014).

A bead mill consists of a vessel that is filled with beads that collide with microalgae while the vessel is being shaken or rotated. Though this grinding causes the cells to disrupt, bead milling is considered a mild method as all products maintain functional. The efficiency of disruption is dependent on the size, density and amounts of the beads, the speed and design of the agitator and on the residence time and characteristics of the feed, such as temperature, viscosity and biomass concentration (Lee et al. 2012). Doucha et al. tested various types of bead mills and discovered that the most influential parameters are the biomass concentration and agitator speed (Doucha & Lívanský 2008). The model used for simulation of the bead mill is based upon the results obtained by

Postma et al. (Postma et al. 2014). The relationship between biomass concentration, agitator speed, time of operation and degree of disruption was determined. By means of this model both the specific energy consumption required for 87.5% release of water soluble proteins and for complete disruption of the cells is calculated.

Pulse electric field (PEF) is a disruption method that uses high intensity electric field pulses to disintegrate the cell membranes. Due to selective concentration of the electric field on membranes, the phospholipids start to change position which causes a membrane to become permeable (El Zakhem et al. 2006). The efficiency of electroporation is mainly dependent on the specific energy input and on the elapse time between recovery of components and PEF treatment. The specific energy input in turn depends on the biomass concentration, field strength and on the shape, duration and amount of pulses applied (Goettel et al. 2013). For release of intercellular components higher biomass concentrations seem to improve the extraction efficiency (Goettel et al. 2013). This implies that there is potential to lower the specific energy demand of PEF by increasing the biomass concentration. For lipid extraction however the relationship between biomass concentration and extraction efficiency has not yet been investigated. Eing et al. discovered that a minimal specific energy input of 1.5 MJ/kg_{dw} is required to obtain sufficient permeabilization for lipid extraction (Eing et al. 2013). As within this biorefinery study lipid and protein recovery is considered equally important, the specific energy input rate is fixed to this value. Next to the PEF treatment chamber, also a mixer is involved in order to keep the suspension well mixed after treatment. During resting intracellular substances continue to leak out of the microalgal cells, until the conductivity saturates after about two hours. Mixing is necessary to prevent the formation of equilibrium conditions near the cell membrane. The resting time is especially important to increase the availability of lutein, as lutein is situated inside the chloroplast and the chloroplast membrane takes some time to become permeable (Luengo et al. 2014).

2.2.4. Separation of lipids and lutein

After disruption of the cells, the biomass is separated from the water phase by means of centrifugation. As mentioned in chapter 2.1, the solid fraction will contain the lipids and lutein while the soluble fraction contains the soluble proteins. The separation of lipids and lutein can be achieved by traditional solvent extraction and innovative supercritical fluid extraction (SFE). However, note that the simultaneous extraction of both lipids and lutein is still under development and that even at lab scale both processes are not yet optimised.

Within this study, the solvent extraction process for lipids and lutein as proposed by Prommuak et al. is simplified and made more environmental friendly using the knowledge of previous studies (Cerón et al. 2008; Li et al. 2002). The use of environmental harmful solvents is avoided and the number of treatment steps is kept to a minimum. The result is illustrated by Figure 4. The disrupted algal biomass is dried prior to extraction to avoid complications during the subsequent separation phase caused by the interaction between water, soap and glycerol (Prommuak et al. 2013). These products are formed after addition of KOH in methanol solution that enable direct transesterification and saponification of lipids and lutein respectively. After extraction of the free lipids and lutein by hexane, the esterified fatty esters and free lutein are separated by addition of an 85% ethanol flow. The biodiesel will remain dissolved in the hexane phase, while the lutein prefers the more polar ethanol in water phase. Lutein precipitates after addition of water and can be separated by filtration, while biodiesel is recovered by distillation of hexane. Also the diluted ethanol flow is evaporated and distilled to reach the initial 85% ethanol flow. The energy requirement of solvent extraction is thus related to mixing, filtration and heating for drying, distillation and for maintaining the solution at the required extraction temperature.

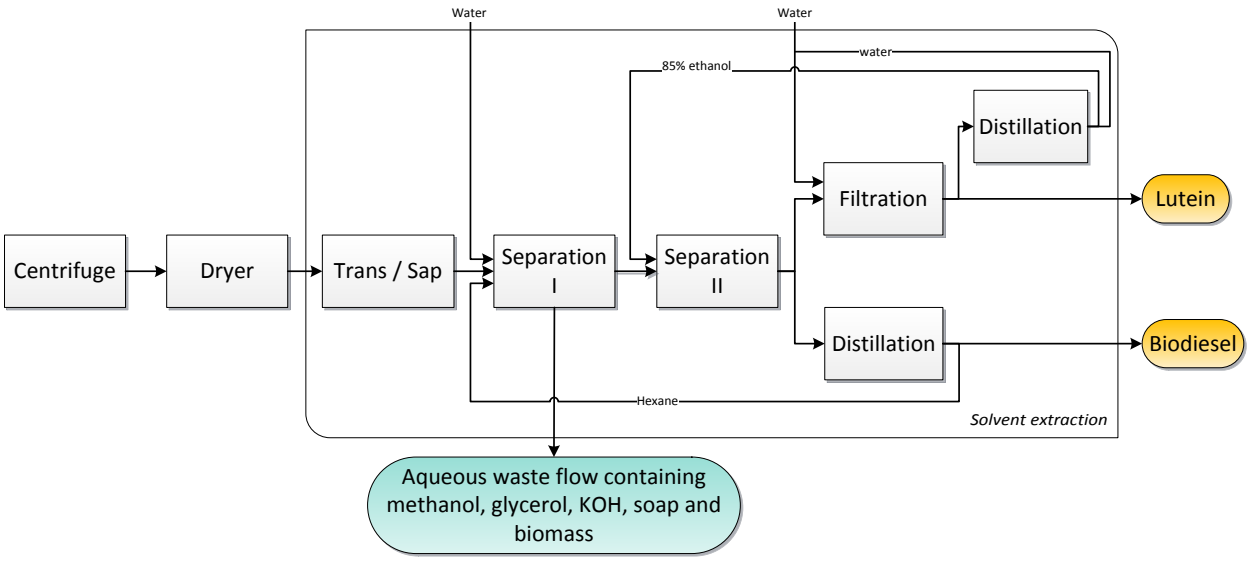

FIGURE 4, SCHEMATIC REPRESENTATION OF SOLVENT EXTRACTION FOR LIPID AND LUTEIN RECOVERY.

Supercritical fluid extraction (SFE) with CO_2 is also very suitable for lipid and carotenoid extraction (Mäki-Arvela et al. 2014; Macías-Sánchez et al. 2010). A supercritical fluid is a fluid when it is kept above the critical temperature and pressure (Pollet et al. 2014). In case of CO_2 this critical point is relatively easily obtained at 31 °C and 74 bar. CO_2 is also abundant, cheap, safe to use and recyclable and is therefore the most studied supercritical fluid. The model describing SFE within this biorefinery case study is based on the results obtained by Nobre et al. who investigated the simultaneous extraction of lipids and pigments (Nobre et al. 2013). Selective extraction of carotenoids is possible by the addition of a co-solvent, in this case ethanol. Using this result a proposed separation strategy was tested that first extracted a lipid fraction suitable for biodiesel and secondly a lipid fraction rich in carotenoids (Figure 5). This SFE method is performed on dried algae biomass at a temperature of 40 °C, 300 bar and a solvent flow rate of 30 $kg\ kg_{dw}^{-1}h^{-1}$. The lipid fraction for biodiesel is obtained using pure supercritical CO_2 and recovers 78% and 26% of the lipids and carotenoids present respectively. Within the second step the solvent flow is enriched with 20 wt% of ethanol. This results in recovery of 28% of the remaining lipids and 50% of the remaining carotenoids. These final fractions are however still dissolved in the ethanol fraction and therefore distillation of ethanol is required to obtain the end product. The total energy requirement of this SFE process thus depends on the energy demand for drying, pressurizing, heating and distillation.

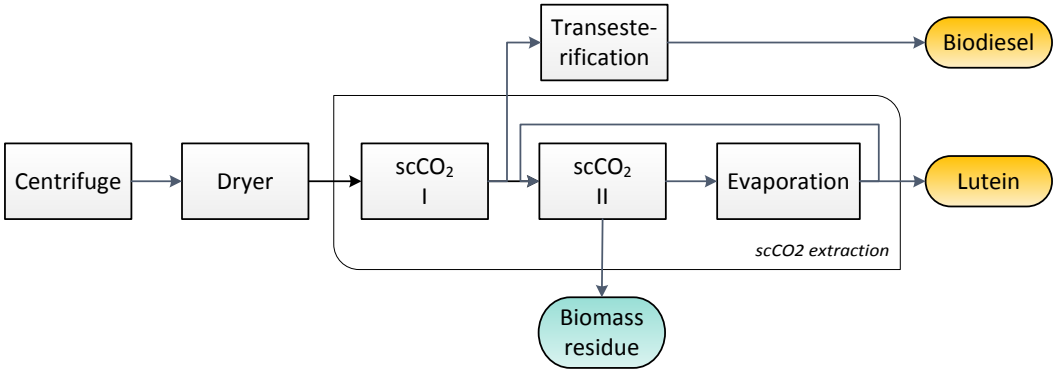

FIGURE 5, SCHEMATIC REPRESENTATION OF SFE FOR LIPID AND LUTEIN RECOVERY.

2.2.5. SEPARATION OF PROTEINS

The water soluble part contains useful products as well, such as functional proteins and carbohydrates. For the recovery of proteins also two different options are compared: shifting of pH and ultrafiltration (UF).

Precipitation of proteins by addition of an acid is a regularly used method to recover proteins (Figure 6). In case of protein separation after HPH treatment of *C. vulgaris* cells, acid treatment towards a pH of 4 resulted a protein extraction yield of about 55% w/w (Ursu et al. 2014). Recovery of the precipitated proteins is done by pressure filtration and afterwards the proteins are dried to obtain protein powder. The energy requirement of this process is thus dependent on mixing of the acid into the solution, pressurizing the membrane for filtration and drying.

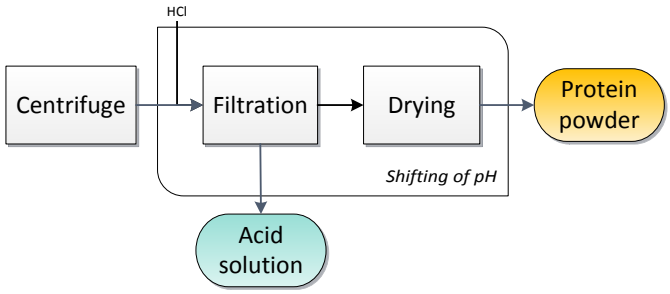

FIGURE 6, SCHEMATIC REPRESENTATION OF SHIFTING OF pH FOR PROTEIN RECOVERY.

Ultrafiltration is a known separation method which is implemented on industrial scale in for example water purification and food industries (Discart et al. 2014). For biorefinery purposes it is mainly used as a harvesting technique, though implementation for separation of microalgae components is under development (Gerardo et al. 2014). The membranes used for ultrafiltration have pore sizes varying from 1 to 100 nm. The model of ultrafiltration is based upon the two-step filtration method proposed by Safi et al. as illustrated by Figure 7 (Safi et al. 2014). The first separation is done with a membrane that retains compounds larger than 100 kDa. This involves a mixture of polysaccharides together with aggregated proteins. The permeate will pass through another membrane that retains compounds larger than 10 kDa. Due to the small pore size only sugars will pass through the membrane and the proteins stay in the retentate solution. The proteins which are now to a large extend separated from the carbohydrates have great emulsifying properties (Ursu et al. 2014). Based on recovery fractions observed by Safi et al., it is assumed that the first ultrafiltration step will retain 40% and 20% and the second step 100% and 10% of the proteins and carbohydrates present respectively. The energy input rate for both ultrafiltration steps is dependent on the pressure applied to the membrane and recirculation by pumping.

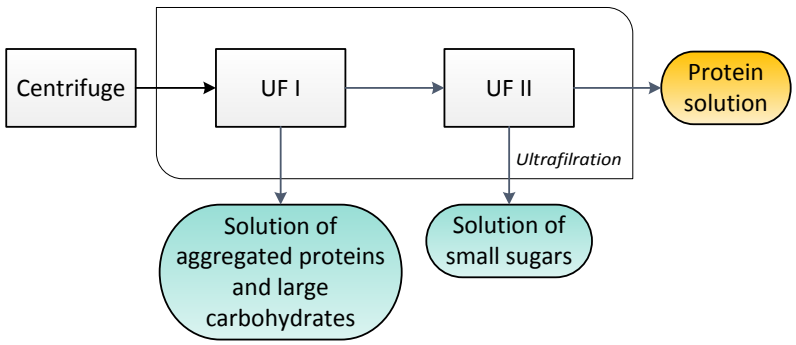

FIGURE 7, SCHEMATIC REPRESENTATION OF THE TWO-STEP UF METHOD FOR PROTEIN RECOVERY.

3. APPROACH

3.1. PROCESS MODELS

For each processing unit the characteristics of the outgoing flows and the energy consumption are calculated via mass and energy balancing. The overall mass balance is shown in equation (1).

$$F_{main,in} * C_{X,main,in} + F_{co,in} * C_{X,co,in} = F_{main,out} * C_{X,main,out} + F_{co,out} * C_{X,co,out} \quad (1)$$

Where F is the volumetric flow rate in m^3/h and C_x is the concentration of component X in kg/m^3 of the ingoing and outgoing main-stream and co-stream. The component X could represent either algae, lipid, lutein or protein.

Related to the mass balance are terms such as recovery efficiency R and concentration factor cf. R determines the mass fraction of a certain component within a flow and is implemented in all models to determine the efficiency of a processing unit. This value is often assumed, but can sometimes be calculated in case a relationship is found with other parameters. As the name implies, cf is needed to determine the concentration of an outgoing flow and is thus only concerned with harvesting and dewatering methods. The concentration factor is implemented as a decision variable and thus varied. The definitions of both parameters are used to solve the mass balances of each component and shown in equations (2) and (3).

$$R = \frac{F_{out} * C_{X,out}}{F_{in} * C_{X,in}} \quad (2)$$

$$cf = \frac{C_{A,out}}{C_{A,in}} \quad (3)$$

The overall energy balance is given by equation (4). The energy input rates of each processing unit concerns heating (H_h), regeneration of solvents (H_r), cooling (H_c), pressurising (H_{pr}), mixing (H_m), specific process requirements (H_s), pumping of the liquids to the next processing unit (H_p) and production of chemicals used (H_{ch}). Energy input rates are always calculated in J/s and the total energy consumption rate is given by H_{total}.

$$H_{total} = H_h + H_r + H_c + H_{pr} + H_m + H_s + H_p + H_{ch} \quad (4)$$

Though the operating conditions vary among different processing units, the equations to calculate these energy input rates are consistent. Within this process design heating is only required during the extraction of lipids and lutein. As shown in equation (5), H_h is dependent on the heat capacity Cp $(J/(kg * K))$, mass flow F_{mass} (kg/s) and difference in temperature ΔT (K) between the incoming flow temperature and the desirable extraction temperature.

$$H_h = Cp_X * F_{mass,X} * \Delta T \quad (5)$$

Within a drying step, the dried product is assumed to leave the dryer at a temperature of 323 K. Additionally, water is evaporated of which the energy requirement is described by equation (6). Here H_{evap} represents the heat of evaporation for water (J/kg), T_{air}, T_{in} and T_{out} represent the temperatures of the air, incoming and outgoing stream (K) and $(T_{air} - T_{out})/(T_{air} - T_{in})$ represents the energy efficiency in drying.

$$H_{evap} = H_{evap} * F_{mass,X} * \frac{T_{air} - T_{out}}{T_{air} - T_{in}} \quad (6)$$

In case of solvent extraction, extra energy input is needed for the regeneration of solvents via a distillation column (Wesselingh & Krijgsman 2013). The energy input for regeneration depends in such a case on the reflux ratio R_r, the volumetric flow rate F (m^3/s) and on the solvent's density ρ (kg/m^3), molecular weight MW (kg/mol) and heat of vaporization H_{vap} (J/mol).

$$H_r = R_r * F * \frac{\rho}{MW} * H_{vap} \quad (7)$$

While the extraction process requires heating, the preliminary disruption methods produce a lot of heat and therefore need cooling. The assumption is made that the energy input minus the theoretical energy demand for disruption, is lost in the form of heat (equation (8)). The reasoning behind this assumption is given in Appendix A3 on disruption methods. The energy input H differs per method, but the theoretical energy demand for cell disruption $E_{mass,A}$ (J/kg) is fixed to 763 J/kg (Lee et al. 2013). The subscript A stands for algae, so that C_A (kg/m^3) and F_A represent the concentration and flow of the algal biomass respectively.

$$H_c = H - (E_{mass,A} * C_A * F_A) \quad (8)$$

In case a processing unit requires an increase in pressure, the energy input rate can be easily calculated via equation (9), in which P represents the applied pressure in bar.

$$H_{pr} = F * P * 10^5 \quad (9)$$

Many processing units need a well-mixed solution in order to be effective. Mixing of the medium is done by means of a stirrer. The energy input rate of mixing H_m can be calculated according to equation (10), where N_p stands for power number, fixed to 0.4, ρ for the density of the flow (kg/m^3), v_s for the stirrer velocity $(1/s)$ and d for diameter of the stirrer (m). The size of the diameter is dependent on the volume that should be mixed, flow F (m^3/s) times time t (s), and can be derived using standard dimensions (equation (11)).

$$H_m = N_p * \rho * {v_s}^3 * d^5 \quad (10)$$

$$d = \left(\frac{2}{3} * \frac{F * t}{\pi}\right)^{\frac{1}{3}} \quad (11)$$

Some mechanical processing units have however a known standard or algebraically determined energy constant E, that could be used directly to calculate the energy input rate. E could be both defined as a volumetric energy requirement E_V (J/m^3) or a mass-related energy requirement E_{mass} (J/kg). The specific energy input rate of one operation unit is referred to as H_s (equation (12)).

$$H_s = E * F * cf \quad (12)$$

Apart from the energy demand of each processing unit, also energy is needed for the transportation of the flows towards the next processing step. The energy input rate of pumping H_p is influenced by many parameters, such as the density and flow rate of the solution, but also by the pumping distance and pipe diameter. Within this process design a fixed distance of 25 meters is assumed for pumping of the medium to the next processing unit. The relationship between these parameters and the rheological properties and energy demand of the transported medium, is in detail explained in the appendix.

3.2. OPTIMISATION APPROACH

The developed for the processing units are linked by taking the output of one processing unit as the input for the next. In this manner three routes were created for the harvesting and dewatering step and twelve routes for the disruption and separation step. As starting point a centrate flow of 150 m^3/h was taken for cultivation of *C. protothecoides* with a biomass concentration of 1.5 kg/m^3. After the harvesting and dewatering step a concentrated algae slurry is obtained with a fixed concentration of 150 kg/m^3. This is added as a constraint to the dewatering units and as a fixed input for the routes concerning disruption and separation. Based on the average results of the three different routes producing this algae slurry, also the inflow into the disruption unit was fixed to 1.5 m^3/h.

Certain process parameters can have a large influence on both the recovery efficiency and the energy demand of a processing unit. As a result the characteristics of the outgoing flow change, which could have consequences for further processing steps. Such influential parameters are implemented within the model as decision variables for optimization and are shown in Table 1. The unit operation PEF and separation processes are not being optimized as these production processes are still under development.

The optimisation problem is defined as:

Find	decision variables X	
Such that	$E_{route} = H_{route}/M_{product}$	is minimised
Where	$H_{route} = H_{dew} + H_{disr} + H_{sep}$	
	$M_{product} = M_{biomass,initial} \cdot \eta_{dew} \cdot \sum(f_{comp} \cdot \prod \eta_{step,comp})$	∀ step, comp
Subject to	equations (1)-(12) in Chapter 3.1	
	$X_{LB} \leq X \leq X_{UB}$	

$X \in \{cf_f, cf_c, r_{air|solids}, P_{HPH}, N_{HPH}, u_{bm}\}$

comp ∈ $\{lipids, lutein, proteins\}$

step ∈ $\{disr, dryer, trans, sepI, sepII, hexane\ dist\}$ for lipids in case of solvent extraction

step ∈ $\{disr, dryer, scCO_2I\}$ for lipids in case of SFE

step ∈ $\{disr, dryer, trans/sap, sepI, sepII, hexane\ dist, filtration, ethanol\ dist\}$ for lipids and lutein in case of solvent extraction

step ∈ $\{disr, dryer, scCO_2I, scCO_2II, ethanol\ evap\}$ for lipids and lutein in case of SFE

step ∈ $\{disr, acid\ addition, drying\}$ for proteins in case of shifting of pH

step ∈ $\{disr, UFI, UFII\}$ for proteins in case of two-step UF method

Within the optimisation problem, the relative energy consumption per kilogram of recovered product is minimised for each route by varying the decision variables. Here the decision variables are constrained by an upper and lower boundary represented by X_{LB} and X_{UB} respectively and shown in Table 1. H_{route} represents the energy input in kWh and is calculated by adding the individual energy requirements for all processing units involved in a certain route. For the three routes concerning harvesting and dewatering, $M_{product}$ is defined as the amount of recovered biomass in kilogram. From this follows that E_{route} represents the relative energy requirements of the routes in $kWh/kg\ biomass$ recovered. For the twelve routes concerning disruption and separation, $M_{product}$is defined as the amount of recovered lipids, lutein and protein in kilogram. E_{route} represents in this case the relative energy requirements in $kWh/kg\ product$ obtained. For the scenario without lutein production, $M_{product}$only covers the weight of recovered lipids and proteins. The optimisation itself was performed in Matlab using the function *fmincon*, which is capable of finding a constrained minimum while dealing with multiple variables.

TABLE 1, OVERVIEW OF DECISION VARIABLES.

Unit operation	Decision variable	Symbol	Unit	Boundaries	
				X_{LB}	X_{UB}
Chitosan flocculation	Concentration factor	cf_f	-	1	20
Centrifuge	Concentration factor	cf_c	-	1	100
DAF	Air-to-solids ratio	$r_{air\|solids}$	$kg\ air/kg\ solids$	0.01	0.1
HPH	Pressure	P_{HPH}	bar	100	1500
	Number of passes	N_{HPH}	-	1	4
Bead mill	Agitator speed	u_{bm}	m/s	6	12

3.3. SUSTAINABILITY ANALYSIS

When developing biorefinery processing routes, the main focus is on obtaining the end products in an energy-efficient manner. However, the end products are a fraction of the total biomass and the remaining part is thus considered 'waste'. As stated within the introduction, this way of thinking should be replaced by a biorefinery approach in which all fractions of the biomass are regarded as valuable. Also during the processing steps should the waste produced and chemicals used be minimized. Within this study the potentials in this area are investigated.

Traditional separation methods based on the use of solvents or acids are compared with innovative separation methods working at high pressures or with membranes. All chemical waste flows are either recycled or further treated until a suitable application can be found. Recycling increases the energy demand of a processing step, but reduces significantly the amount of chemicals that need to be purchased. By treatment of waste flows valuable products are obtained which are otherwise lost to the wastewater treatment plant. Typically an LCA is used for quantitative analysis of each processing route. However, this is not possible since there is a shortage in knowledge on the fate of chemicals for the environmental impact assessment. Therefore, this study will give a descriptive analysis of the pathways that chemicals follow within the processing routes and treatment measures. In case solvents are used, also the additional energy consumption for recycling is included in the model. A processing route is considered 'green' when applications can be found for treated or untreated waste streams.

3.4. SENSITIVITY ANALYSIS

Wastewater is subjective to fluctuations in nutrient and chemical concentrations. Therefore, the biomass composition of *C. protothecoides* can vary as well. By means of a global sensitivity analysis, the impact of changes in lipid, lutein and protein fractions is evaluated for the relative energy consumption per kilogram of product. This is done for two routes: the most traditional and the most innovative separation options combined with disruption by HPH. The traditional route separates components by means of solvent extraction and shifting of pH, while the innovative route uses SFE combined with the two-step UF approach. The first order and total sensitivity indices are calculated for each fraction as described in literature (Saltelli 2002; Saltelli et al. 2010). The range in which the relative energy demand varies for changing fractions is determined by calculation of the standard deviation.

4. RESULTS

Within this chapter the results of the optimisation model, sustainability analysis and sensitivity analysis are presented. Routes linked to the same case are analysed together within the paragraphs. First the results of the routes concerning harvesting and dewatering are shown, followed by the routes for disruption and separation. Afterwards the influence of a high valuable compound is analysed by comparing the obtained results from the disruption and separation routes with the results in case lutein production is excluded. Also the influence of a varying biomass composition on the energy consumption is investigated by means of a sensitivity analysis.

4.1. HARVESTING AND DEWATERING

After cultivation, the dilute of *C. protothecoides* solution of 1.5 $kg\ biomass/m^3$ with a flow rate of 150 m^3/h, needs to be concentrated 100 times before it enters the disruption unit. Three different routes are compared: single-step centrifugation and two-step dewatering by centrifugation with either flocculation or DAF as preceding harvesting step. The modelling results in terms of energy consumption per recovered microalgae biomass are shown in Figure 8. Clearly, single-step centrifugation is the most energy consuming option. Flocculation prior to centrifugation seems to be the most energetically favourable route for dewatering. The reason that centrifugation requires more energy after DAF treatment is that DAF cannot concentrate the solution as far as flocculation, which results in a larger flow to be treated by the centrifuge. Another observation is that the energy for pumping is negligible, even though large volumes of water need to be transported. This is a consequence of the implementation of a maximum fluid velocity through a pipe that is standard for year round processing in order to minimize erosion (Wileman et al. 2012). Additional pipes are implemented in case a larger volume needs to be transported. This will simultaneously lower the total energy demand compared to transporting the same volume through one pipe at a higher velocity.

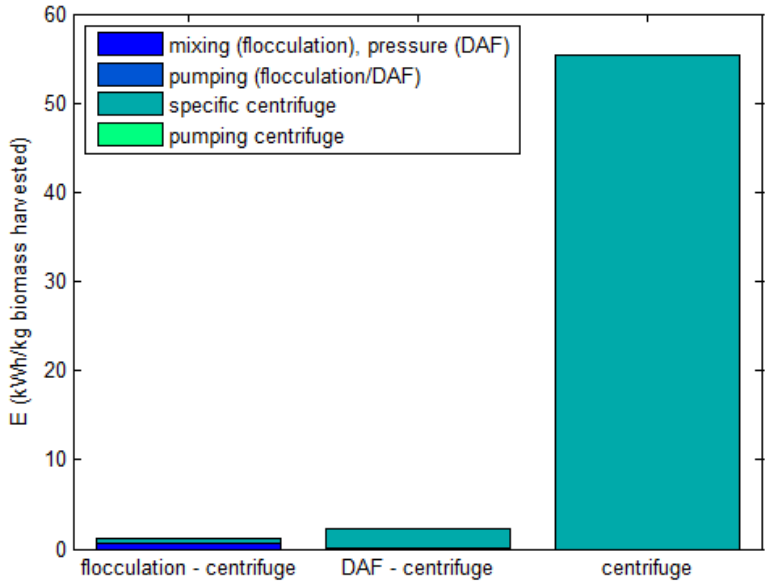

FIGURE 8, ENERGY CONSUMPTION OF THE THREE HARVESTING AND DEWATERING ROUTES.

Though energetically spoken the combination of flocculation and centrifugation is most favourable, there are other factors to be taken into account as well. For this reason both flocculation and DAF are compared by means of a SWOT analysis as shown in Table 2. Remarkable is that an increase in stirring speed during flocculation quickly

results in higher energy demands than the combination of DAF and centrifugation. DAF also requires mixing, but for a shorter period resulting in lower energy requirements. As for small changes in stirring speed the energy demand of DAF and flocculation become comparable, it is worth looking into other characteristics of both treatment options. The type of flocculant used has implications for the environment when looking at the production process. Chitosan was preferred over metal salts for flocculation, to minimize toxicity effects during further treatment of the biomass. However, the need of alkaline solutions for processing of chitin makes the production of chitosan is much more polluting than that of metal salts (Beach et al. 2012). Also other polymers are produced by energy intensive processes resulting in high greenhouse gas emissions (Udom et al. 2013). No literature can be found on the flocculant concentration remaining in the treated water after harvesting. In case the treated centrate needs to be recycled into the wastewater treatment plant due to insufficient nutrient depletion, the presence of flocculant could negatively affect the efficiency of the treatment process. It could change the final composition of the flocs, making them lighter and more feather-like, resulting in slower settling rates and an increased water content within the settled sludge (Bolto & Gregory 2007).

TABLE 2, SWOT ANALYSIS OF FLOCCULATION AND DAF AS HARVESTING MEASURES PRIOR TO CENTRIFUGATION.

	Strengths	Weaknesses	Opportunities	Threats
Flocculation	High concentration factor (16x) Chitosan is a very safe polymer	Energy consumption highly dependent on mixing speed Production process of chitosan has a high environmental impact	Frequently applied within wastewater treatment	Chance of chitosan effecting biorefinery processing steps
DAF	Low flocculant dosage Production process of aluminium sulfate has a low environmental impact	Low concentration factor (6x) Advanced equipment needed	Frequently applied within wastewater treatment	Chance of contamination of residual biomass

4.2. DISRUPTION AND SEPARATION OF LUTEIN, LIPIDS AND PROTEINS

The disruption steps work with a fixed average inflow of 1.5 m^3/h and an algae concentration of 150 kg/m^3, independent of the preceding harvesting and dewatering step. Figure 9 shows the energy consumption relative to the amount of products produced of the different disruption and separation routes. The amount of products obtained per kilogram of biomass entering the disruption unit is for each route given in Appendix B2. The route demanding the least energy involves HPH disruption followed by the traditional separation methods; solvent extraction for lipid and lutein recovery and shifting of pH to obtain the protein fraction. The energy gain by biodiesel formation only compensates for a minor fraction of the total energy consumption (Appendix B3). The most energy is consumed during lipid and lutein separation, while the energy required for protein separation is negligible. The choice of protein separation method has however a significant influence on the relative energy

consumption. In case of solvent extraction for lipid and lutein recovery, the combination with pH shifting results in the lowest relative energy consumption. SFE combines best with the two-step UF method, though the difference with pH shifting is minor.

When comparing the different disruption options, the bead mill is by far the most energy consuming option. Two different operation modes were compared for the bead mill: one aiming at 87.5% release of the soluble proteins and one aiming at complete disruption. The first mode consumes significantly less energy during bead milling, but fails to disrupt about 22% of the cells. This leads to a lower recovery of lipids and lutein and increases the energy consumption for separation (Appendix B4). For this reason, the complete disruption mode appears to be more beneficial in terms of energy consumption per total amount of recovered products. This mode was therefore used for comparison with the results obtained by the other routes (Figure 9). Also the routes relying on HPH for disruption demand a maximum energy input, damaging over 99% of the cells. PEF treatment cannot yet be optimized but is already compatible. The energy consumption for PEF is very low, but due to the fixed cell disruption efficiency of 90%, the final relative energy input rate per kilogram of recovered product is still slightly higher compared to HPH and bead milling.

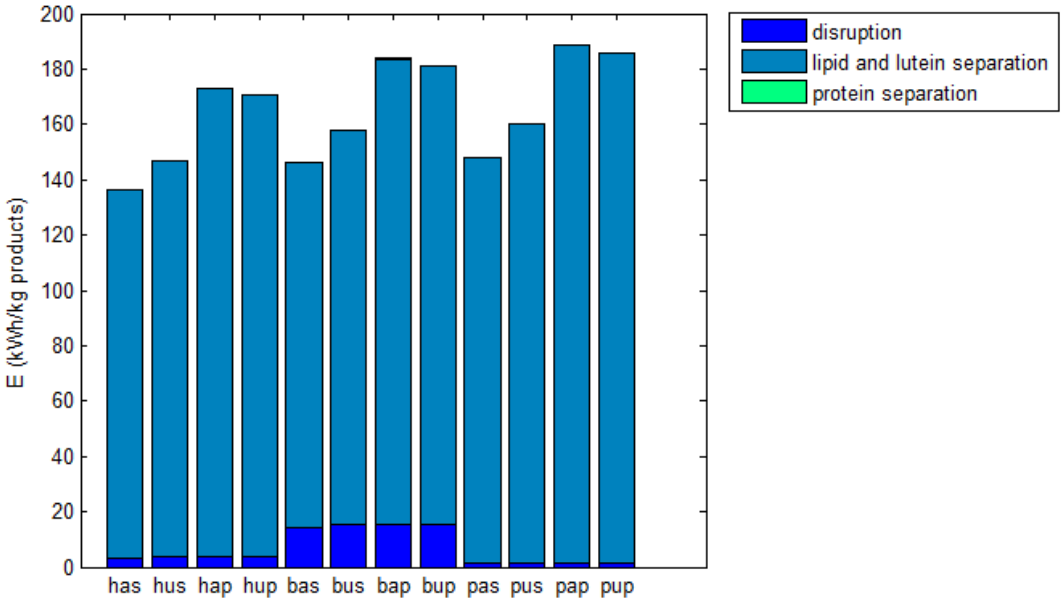

FIGURE 9, THE ENERGY CONSUMPTION PER KG PRODUCTS FOR DIFFERENT DISRUPTION AND SEPARATION ROUTES. THE SUBSCRIPT REFERS TO THE ROUTE TAKEN. THE FIRST LETTER SYMBOLIZES THE APPLIED DISRUPTION METHOD: 'H', 'B' AND 'P' STAND FOR HPH, BEAD MILL AND PEF TREATMENT RESPECTIVELY. THE SECOND LETTER SYMBOLIZES THE METHOD USED FOR PROTEIN SEPARATION: 'A' STANDS FOR PH SHIFTING TO ACIDIC ENVIRONMENT AND 'U' STAND FOR THE TWO-STEP ULTRAFILTRATION METHOD. THE THIRD LETTER REPRESENTS THE UNIT USED FOR SEPARATION OF LIPIDS AND LUTEIN: 'S' STANDS FOR SOLVENT EXTRACTION AND 'P' FOR SUPERCRITICAL FLUID EXTRACTION.

As the separation of lipids and lutein requires in general a lot of energy, it is interesting to see which steps during solvent extraction and supercritical fluid extraction are the most energy demanding (Figure 10). Within solvent extraction over 98% is used for distillation of the solvents, while SFE needs most energy (85%) for heating the solvents towards the extraction temperature of 40 °C under supercritical conditions.

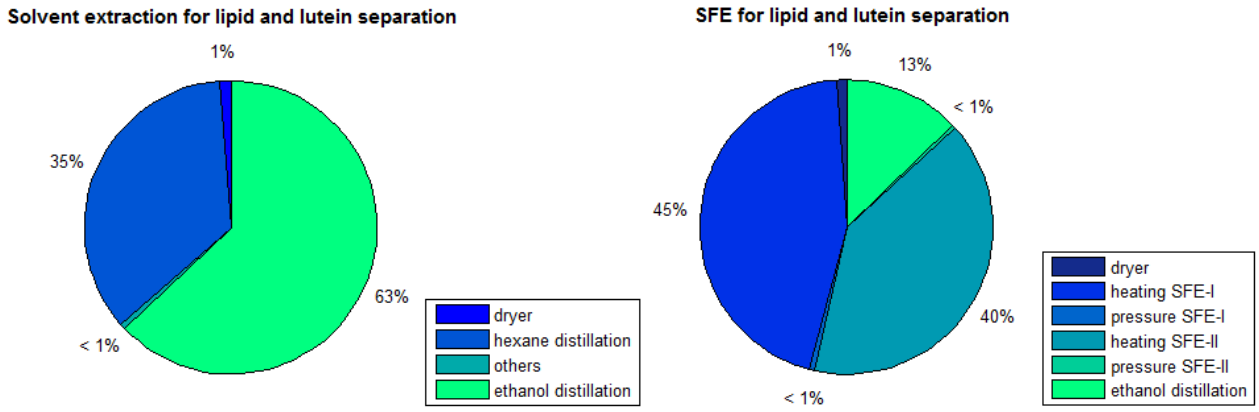

FIGURE 10, THE RELATIVE CONTRIBUTION OF ENERGY DEMANDING STEPS TO THE TOTAL ENERGY DEMAND OF LIPID AND LUTEIN SEPARATION DURING SOLVENT EXTRACTION (LEFT) AND SUPERCRITICAL FLUID EXTRACTION (RIGHT).

SFE consumes significantly more energy than solvent extraction. However, SFE can be considered a more environmental friendly method due to the use of CO_2 as solvent which is abundant, non-toxic, recyclable and cheap. Hexane is also considered safe and recyclable, but it is obtained through fractional distillation of petroleum mixtures (Anon 2014), which makes it more expensive and non-renewable. Both methods also make use of ethanol which is necessary to separate lutein from the lipid fraction. High purity ethanol is also produced by the petrochemical industry. Fermentation can result in maximum 18% by volume ethanol which is suitable for alcoholic beverages or as fuel, but needs an energy-intensive upgrade process before it can be used as an solvent. The contribution of ethanol recovery from a dilute aqueous flow to the energy consumption within a processing route is clearly visible in Figure 10. The alternative solution is to further dilute this waste flow with clean water to obtain the maximum concentration allowed before entering a wastewater treatment plant. However, this is economically even less attractive since this would require a large increase in purchase of ethanol. It also implies that more ethanol should be produced which is disadvantageous in terms of sustainable processing.

What has not been included in the model, but will also significantly contribute to the total energy consumption, is the handling of the waste flow generated by transesterification. For a concentrated algae flow of 1.5 m^3/h as starting point, each solvent extraction route will produce about 3.7 m^3/h of aqueous methanol flow containing KOH, soap and glycerol. In practise, also the lipid fraction obtained by SFE has to undergo a transesterification step to meet the standards of biodiesel. The energy cost for cleaning this waste flow is not taken into account within the model since this would be equal for both routes. Due to the presence of methanol, this waste stream is considered as hazardous waste and further treatment is necessary. Direct separation of methanol could however reverse the transesterification reaction. Therefore the catalyst is first deactivated by addition of a strong acid that neutralizes the mixture. The acid also splits the soaps resulting in the formation of salts and free fatty acids. When phosphoric acid is used, the salts formed are potassium phosphate which after a washing step can be applied as fertilizer. The free fatty acids are insoluble in water and can be easily skimmed off or separated by centrifugation. Once recovered, they can find an application in biodiesel production or as animal feed (Van Gerpen 2007). What is left is a mixture of methanol and glycerol in water. Now the methanol can be separated by evaporation and fractional distillation and recycled back to the transesterification reaction. For successful transesterification it is important that the recycled alcohol is high in purity. Recycled methanol can obtain high purities and is therefore more practical in use compared to ethanol. Ethanol in water cannot reach higher purities than 95% via fractional distillation and will therefore need additional equipment (Gerpen 2005). After methanol recovery, the remaining glycerol in water solution has a purity of about 85%. This flow can be sold to companies that further refine the

glycerol towards 99.5%, but there are also possibilities on the wastewater treatment plant itself. One option would be to use the recovered glycerol as substrate together with the centrate and scale up the cultivation process. Multiple studies have showed that *C. protothecoides* is capable of accumulating lipids suitable for biodiesel production when growing on crude glycerol (Cerón-García et al. 2013; Feng et al. 2014; O'Grady & Morgan 2011). It can also be beneficial for the treatment of centrate since after cultivation still some nutrients are remaining. The use of glycerol as additional carbon source can result in complete consumption of these nutrients and thereby reduce the load of the wastewater treatment plant. Another option is to add the glycerol to the dewatered sewage sludge for anaerobic co-digestion. This was tested by Rivero et al. and resulted in enhanced volatile solids degradation and biogas production (Rivero et al. 2014). Presently research is ongoing in finding alternative applications due to the increased production of biodiesel-waste glycerol.

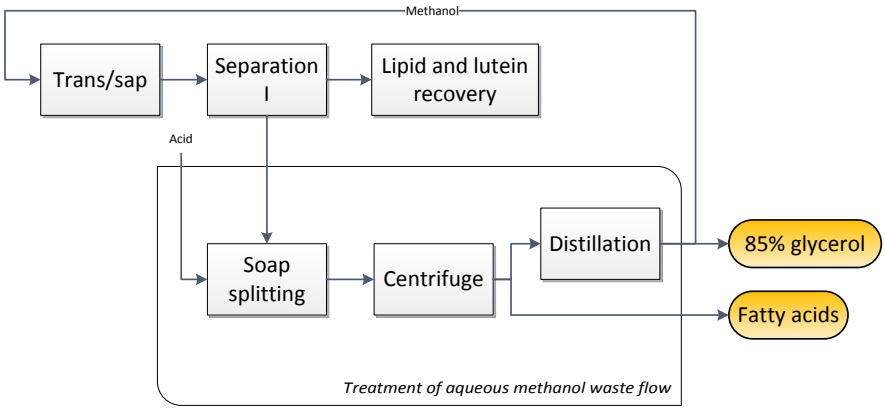

FIGURE 11, SCHEMATIC REPRESENTATION OF THE TREATMENT STEPS INVOLVED IN HANDLING OF THE AQUEOUS METHANOL WASTE FLOW PRODUCED DURING BIODIESEL FORMATION.

The microalgal biomass that is left after lipid extraction can be a useful substrate for fermentation processes (Podkuiko et al. 2014). This waste biomass consists mostly of disrupted cell wall material where carbohydrates and proteins are present. These carbohydrates are mainly large polysaccharides such as cellulose, xylose, arabinose and galactose, which need to be hydrolyzed prior to fermentation. Through hydrolysis the large polysaccharides are broken into separate sugar molecules that can serve as substrate during fermentation processes. The production of easily consumable sugars can be done through addition of acid, base or certain enzymes. Afterwards, yeast strains such as S. cerevisiae are capable of producing bioethanol. The resulting biomass waste is poor in carbohydrates and lipids, but relatively rich in protein. One option would be to anaerobically digest it for the production of biogas. Some algae can however produce bacteria repellent composites that can hinder the conversion towards methane. Another concern is the formation of ammonia due to the low carbon/nitrogen ratio of proteins. Also ammonia can inhibit the bacteria above a certain concentration. These complications can be prevented by adding additional carbon sources, such as the glycerol produced during transesterification. The digested biomass is rich in phosphor and nitrogen and therefore suitable as a fertilizer.

Another possibility is to directly ferment the lipid-poor biomass into hydrogen. Nobre et al. showed that the lipid-extracted biomass after SFE yielded slightly higher hydrogen production values than untreated biomass (Nobre et al. 2013). Results show that the optimum conditions applied during SFE for simultaneous lipid and lutein separation also lead to optimal production of hydrogen of 60.6 $L/kg\ biomass$. Lipid-extracted biomass even yielded slightly more hydrogen than untreated biomass. This can be related to the relatively higher sugar content due to the absence of lipids. Another interesting observation is that the fermentation efficiency increased with decreasing biomass concentration. The obtained mass fraction should thus be diluted before treatment. The

aqueous solutions obtained within both protein recovery routes are suitable for this purpose. They contain the soluble carbohydrate fraction which is the substrate for fermentation. In case the proteins are recovered by pH shifting the remaining acidic solution should first be neutralized (H. Wang et al. 2014), while after UF treatment no extra measures are needed. The addition of acid for protein precipitation has the advantage that it also partially hydrolyses the carbohydrates present. Of the two waste streams produced during UF, the sugar solution obtained after the second filtration step contains the most fermentable compounds. The other waste flow, containing the aggregated proteins and large carbohydrates, shows high emulsion stabilizing properties (Ursu et al. 2014). The use of protein-polysaccharide aggregates as emulsifier is researched by Schwenzfeier et al. It was showed that these charged complexes strengthen the emulsion stability by ensuring electrostatic repulsion between the emulsion droplets (Schwenzfeier et al. 2014).

4.3. INFLUENCE OF A HIGH VALUABLE COMPOUND

To observe the influence of lutein recovery on the energy consumption, a second scenario was modelled in which only the steps involved in lipid and protein separation were taken into account. By comparing the energy consumption including lutein separation (Figure 9) with the energy consumption excluding lutein separation (Figure 12), it can be concluded that more than half of the total energy is used for separating lutein from the lipid fraction. The contribution to the total energy consumption is especially large in case of solvent extraction, where 65% of the energy is required for lutein separation. After extraction of lutein by aqueous ethanol, the addition of water for lutein precipitation results in a large flow of diluted ethanol. Sustainable use of ethanol requires regeneration by distillation which is an energy intensive procedure (Figure 10). In the scenario without lutein production, the reduction in energy usage is the result of elimination of aqueous ethanol flow from the processing steps. No differences are observed between both scenarios in terms of type and amount of waste produced. The major waste flow is still the aqueous methanol stream obtained through the transesterification step. The residual biomass is considered to have the same composition.

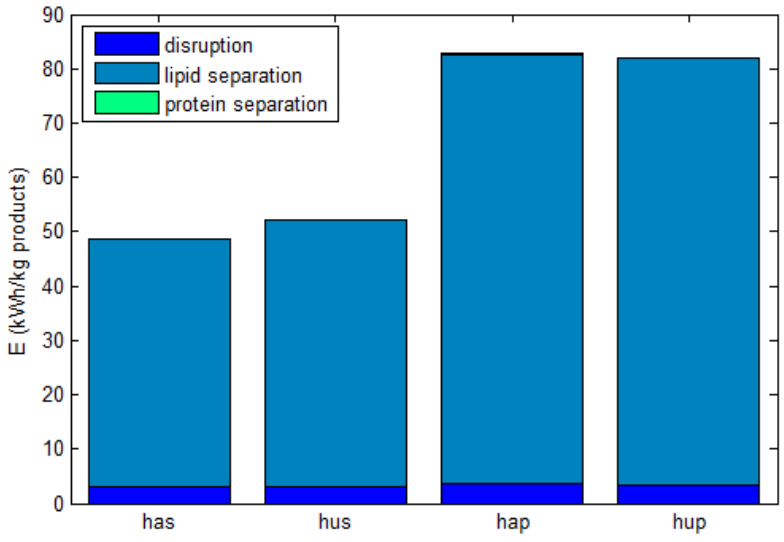

FIGURE 12, THE ENERGY CONSUMPTION PER KG PRODUCTS IN CASE OF ONLY LIPID AND PROTEIN RECOVERY. DISRUPTION IS ACHIEVED BY A HPH (FIRST LETTER 'H'). SEPARATION OF PROTEINS IS DONE VIA PH SHIFTING TOWARDS AN ACIDIC ENVIRONMENT (SECOND LETTER 'A') OR TWO-STEP UF (SECOND LETTER 'U'). SEPARATION OF LIPIDS IS ACHIEVED BY SOLVENT EXTRACTION (THIRD LETTER 'S') OR SFE (THIRD LETTER 'P').

4.4. SENSITIVITY TO CHANGING BIOMASS COMPOSITION

Wastewater typically has a fluctuating nutrient composition that could lead to different lipid, lutein and protein fractions inside the biomass. As a consequence, the relative energy consumption per kilogram of obtained products can vary. The uncertainty caused by a change in biomass composition is however dependent on the processing route. An uncertainty analysis was performed on the traditional route consisting of HPH, solvent extraction and shifting of pH (has), and on the innovative route consisting of HPH, SFE and the two-step UF method (hup). The fractions of components were varied between 0.25 and 0.45 for lipids, 0.001 and 0.01 for lutein and 0.1 and 0.3 for proteins. The combined fractions of lipids, proteins and 'others', which includes lutein, contribute to 70% of the microalgal biomass. The carbohydrate, ash and moist fractions are kept constant. The results in Figure 13 illustrate that the innovative route 'hup' brings more uncertainty and a higher relative energy consumption compared to the traditional route 'has'. A more detailed sensitivity analysis shows that the higher uncertainty of 'hup' is the result of a higher sensitivity of SFE towards changes in the lipid fraction (Appendix B5). The two-step UF method has however a lower sensitivity towards changing protein fractions compared to shifting of pH. Therefore, the least sensitive route to changes in biomass fractions consists of solvent extraction with UF for separation of components. The total sensitivity index also allows interactions between fractions. The contribution of lutein to the uncertainty is negligible as lutein is only a minor fraction within the obtained products. To reduce the uncertainty in energy consumption, the focus must be on lipid separation.

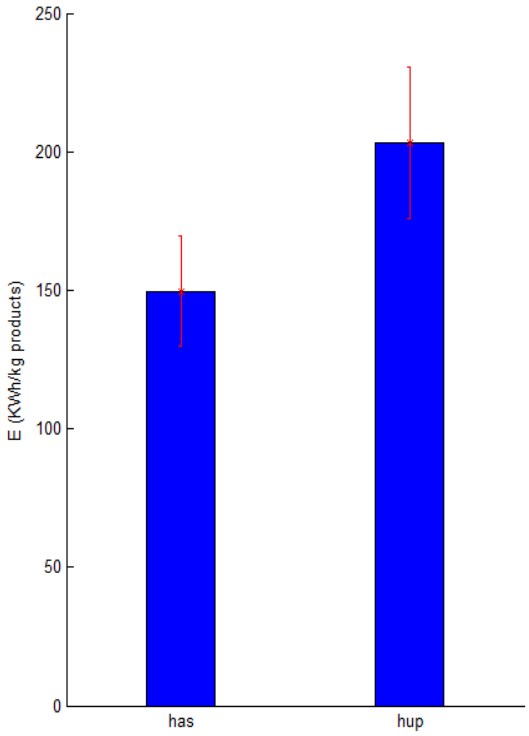

FIGURE 13, UNCERTAINTY ANALYSIS OF THE ROUTES HAS AND HUP. HAS STANDS FOR HPH, FOLLOWED BY ACID TREATMENT AND SOLVENT EXTRACTION. HUP STANDS FOR HPH, FOLLOWED BY UF STEPS AND SFE. THE RED LINES REPRESENT THE UNCERTAINTY CALCULATED BY THE STANDARD DEVIATION.

5. DISCUSSION

5.1. PERFORMANCE OF PROCESSING UNITS

It was expected that the dewatering route considering single-step centrifugation requires the most energy and that flocculation followed by centrifugation requires the least energy (Slegers et al. 2014). However, DAF treatment combined with centrifugation is also performing well in terms of energy usage. The model is currently run at optimum conditions, leading to a biomass recovery fraction of 0.9. Addition of more flocculant does not increase the recovery efficiency as the maximum biomass fraction of 10 wt% inside foam is already achieved. Increasing the recycle ratio above 20% to produce more air bubbles can in combination with additional flocculant usage harvest more microalgal biomass, but will also increase the energy demand of the air compressor. A way to simultaneously reduce the flocculant usage and energy consumption of DAF while increasing the concentration factor is to (partially) replace air by microspheres (Jarvis et al. 2009; Ometto et al. 2014). This innovation is specifically designed for harvesting of microalgae, but the concentrations of algae used in experiments are currently much lower than within this biorefinery case study. Both studies found however a major reduction in energy consumption and greenhouse gas emissions compared to traditional DAF treatment. The beads are recovered through rapid mixing or by use of a hydrocyclone. The collapse of foam to obtain a concentrated algae solution also demands energy, though this was not included in the model. The most effective and cheapest option would be to add chemical antifoam, under the condition that it is not affecting the quality of the final products. Examples of mechanical antifoaming methods are rotating paddles or beach drums (Edzwald 2010; Stevenson & Li 2014).

When comparing the disruption methods, HPH showed the best performance in terms of energy efficiency. Within the model the energy demand of the HPH is not dependent of the incoming biomass concentration, which is fixed to 150 kg/m^3. It is found that an increase in initial biomass concentration causes a decrease in degree of disruption. This is because the cells are moving at a lower velocity and thereby they experience less force during collision with the valve seat and the impact ring (Halim et al. 2013). Working with algae slurries can therefore significantly increase the energy consumption for obtaining sufficient disruption efficiencies. Almost complete disruption by HPH could still be achieved at 20-25% w/w when cells were weakened by incubation of 15 hours at 37°C (Olmstead et al. 2013). This study proves that HPH can also be implemented at highly concentrated slurries, but that this will increase the required processing time.

The energy consumption of disruption with PEF is lower than with HPH and bead milling. In order to overall perform better, PEF needs to achieve a disruption efficiency of above 97%. However, in the model the disruption efficiency is fixed to 90% as no exact data is given. In literature, the increase in conductivity of the medium is given as measure for disruption. This information is relevant in case of protein separation as it proves release of soluble components. However, it gives no insight in the state of disruption. In some cases cells can recover from electroporation by closing the pores after PEF treatment (Luengo et al. 2014). The lipid and lutein fractions are thereby not accessible for recovery. Before PEF can achieve disruption efficiencies higher than 90%, more knowledge is needed on how to prevent cell survival.

Within separation processes, transesterification produces the largest aqueous waste flow containing methanol, KOH, soap and glycerol. As methanol needs to be recovered by distillation this contributes significantly to the energy consumption. The amount of alcohol needed during transesterification could be decreased by performing a two-step reaction (Gerpen 2005). Within this approach 80% of the alcohol and catalyst is added to the first CSTR. After removal of the formed glycerol the remaining 20% is added to the second CSTR. This two-step system increases the reaction efficiency greatly compared to the single-step systems and consumes thus less alcohol.

In case of lutein and protein recovery, different end products are obtained when using different separation methods. Solvent extraction results in lutein powder, while SFE recovers a lutein-rich oil fraction. Lutein powder obtained by solvent extraction showed a high purity in the range of 90-98% (Li et al. 2002). Within the oil enriched lutein fraction obtained by SFE also other pigments are present (Nobre et al. 2013). From these observations it can be concluded that SFE has a lower selectivity towards lutein compared to solvent extraction. In case of protein separation, the two-step UF approach obtains an aqueous protein solution, while shifting of pH causes precipitation of proteins. The precipitated proteins are easily dried which is beneficial for transportation. However, to solubilize these dried proteins addition of an alkaline solution is required (Ursu et al. 2014). The two-step UF approach is less suitable to obtain protein powder, but does not need the addition of chemicals and all flows can find a suitable application.

5.2. HEAT RECOVERY

The most energy demanding steps within the processing routes concern heating towards extraction temperature and distillation of either ethanol or hexane. The energy consumption can be significantly lowered by applying heat recovery. Figure 14 illustrates the opportunities of heat recovery within solvent extraction. It is assumed that a temperature difference of at least 10 °C is required as driving force for heat exchange in counter-current mode.

After distillation of ethanol, the ethanol vapour (H1) and water flow (H3) at 80°C need to be cooled down towards the operating extraction temperature of 40°C. The cooling liquid will then have a final temperature of 70°C. In case the cooling liquid is replaced by the diluted ethanol solution that needs to be heated towards 80°C prior to distillation (C1), this could save a lot of energy. Both the ethanol (H1) and water (H3) flow which are separated after distillation are needed to provide the heat for the diluted ethanol stream (C1). Only the final temperature difference of 70°C to 80°C needs to be taken care of by additional heating. The cooled ethanol and water flows will however still have a temperature of 50°C and need to be cooled further towards 40°C. These additional heat or cooling demands are in represented by the solid blue circles.

A similar scenario can be made for hexane distillation. Prior to distillation the hexane needs to be heated from 40°C to 70°C (C2). After evaporation the vapour hexane at 70°C needs to be cooled down again to 40°C (H2). In case these two streams exchange heat, the stream that needs to be cooled down will have a temperature of 50°C, while the stream that needs to be heated up will end at 60°C. The energy needed for the final heating step towards 70°C has to be provided additionally. The energy demand for cooling of the hexane is equal to what was needed for cooling of the purified ethanol. This gap can either be filled by an additional cooling source, or by the cooling liquid leaving the disruption unit (C3). During disruption the internal temperature does not exceed 30°C to prevent denaturation of proteins. As a result, the cooling liquid will heat up to 30°C and is afterwards sent to the wastewater treatment plant as these temperatures are very suitable for bacterial growth. Prior to treatment, this flow can be used to provide the required cooling.

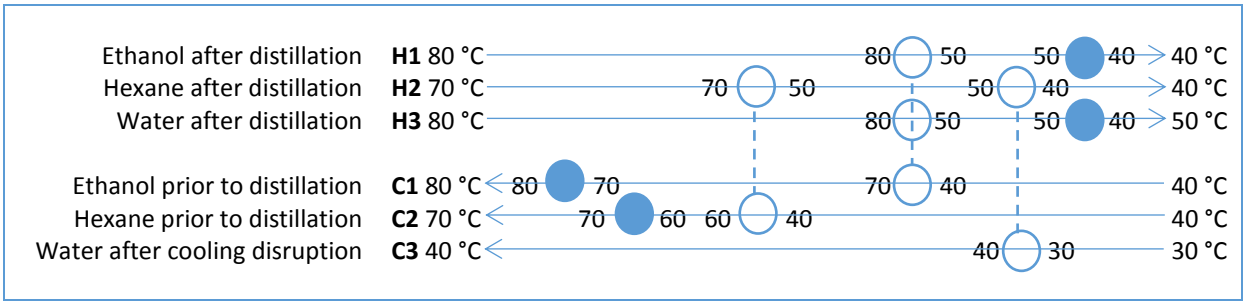

FIGURE 14, EXAMPLE OF A HEAT TRANSFER POSSIBILITY WITHIN SOLVENT EXTRACTION BASED ON A REQUIRED TEMPERATURE DIFFERENCE OF 10 °C. THE SOLID BLUE CIRCLES REPRESENT THE ADDITIONAL HEAT REQUIREMENT.

5.3. IMPACT OF FLOCCULANT USAGE

After harvesting it is expected that a small amount of flocculants remain in the treated centrate. This implies that the majority of flocculants stays attached to the algae biomass and could influence the succeeding disruption and separation steps. Therefore the fate and influence of flocculants on disruption and separation steps within this biorefinery case study is described.

For PEF treatment the effect of addition of a non-ionic flocculant has been tested on a yeast cell solution. At low cell concentrations the addition of a flocculant enhanced the state of permeability, while at higher cell densities the reverse effect was noticeable (El Zakhem et al. 2006). The hypothesis is that at a certain state of aggregation cells can hide from high electrical field sites and remain undamaged. Since PEF treatment is preferably performed on dense algae solutions, the use of flocculants during harvesting is not recommended. On the other two disruption methods, HPH and bead milling, the influence of flocculants is considered to be negligible. Cell aggregates are highly sensitive to shear and can break up due to extreme mixing or pumping conditions (Bolto & Gregory 2007). The harsh forces applied to the cells when using HPH or bead milling are sufficient to tear apart the cell walls and thus also to break apart the aggregates.

After disruption, the flocculants will partly separate from the biomass and could thereby influence both the protein, and the lipid and lutein separation after centrifugation. This centrifugation step can even break the flocs further when run at high shear conditions (Xu et al. 2013). Results of Utomo et al. show that chitosan flocculation of *Chlorella* sp. cells has a negative impact on lutein recovery (Utomo et al. 2013). It is hypothesized that the left and right hydroxyl groups of lutein bind to the amine group present inside chitosan. Another study investigated the effect of cationic and anionic flocculants on the lipid extraction and fatty acid composition (Borges et al. 2011). Though the same amount of lipids was extracted by a mixture of chloroform and methanol, a difference was observed in the fatty acid composition. The use of flocculants favours the extraction of saturated fatty acids which are suitable for biodiesel production. It is hypothesized that the complex lipids are entrapped by loops of flocculant. These are formed by partial attachment to the cell wall. In case of anionic flocculants, the flocculant binds only on a few places to the negatively charged cell wall and forms more loops that can collect complex lipids. Therefore, for biodiesel production the use of anionic flocculants is recommended.

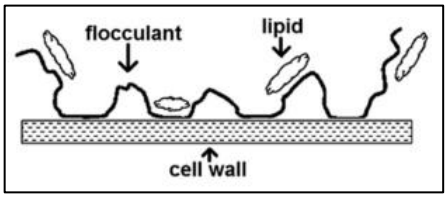

FIGURE 15, HYPOTHETICAL SCENARIO OF COMPLEX LIPID ENTRAPMENT BY FLOCCULANTS. (BORGES ET AL. 2011)

That flocculants have no influence on the lipid yield was also concluded by Anthony et al. (Anthony et al. 2013). Additionally, the effect of flocculants on further processing of the residual biomass and acidic aqueous phase was investigated. Results show that cationic starch is beneficial for bio-product formation, such as acetone, butanol and ethanol, while aluminium obtains on average lower yields. Inhibition of microbial activity in the presence of aluminium is also observed in other studies (Cabirol et al. 2002). Another disadvantage of using metal flocculants is that the resulting biomass cannot be utilized as feedstock material or as animal feed (Anthony et al. 2013).

Predicting the fate of organic flocculants is more difficult and type dependent. Chitosan is insoluble in water, alcohol and alkaline solutions but dissolves easily in mild acidic environments (Harith et al. 2009). Organic polymers are in general more active in acidic environments. Under alkaline conditions hydrolysis of the ester groups leads to a loss of cationic charge. The information above implies that during the transesterification step within the solvent extraction route the addition of a base would lead to degradation of the flocculants. The flocculant separates from the biomass and partly ends up in the aqueous waste flow together with methanol,

glycerol and soap particles. The first step in handling this waste flow is the addition of an acid to split the soaps and precipitate the formed free fatty acids. Acidic environments re-activate the flocculants which will then presumably end up within the precipitate. In case SFE is applied for lipid and lutein separation, it is expected that the flocculant remains inside the biomass fraction.

5.4. MULTIPRODUCT BIOREFINERIES

Biorefinery processes designed to obtain multiple end products have the potential of becoming economical competitive to fossil-based production processes. Currently several studies and reviews focus on such scenarios (da Silva et al. 2014; Hariskos & Posten 2014). However, none of the studies tried to combine biofuel production with the extraction of both a high valuable compound and proteins, while finding applications for the produced waste flows. In literature, the focus is mainly on biofuel production. Biodiesel is obtained through lipid extraction, and bioethanol and biohydrogen are obtained through fermentation and anaerobic digestion of the residual biomass respectively. Part of the studies focusses on energy efficient separation methods, such as wet solvent extraction for removal of the energy demanding drying step. Other studies focus on improving the sustainability of biofuel production by finding application for side streams such as the glycerol produced. The combination of biodiesel production with recovery of a high valuable compound, often pigments, is also receiving attention in literature. However, only few studies could be found that combined protein and lipid extraction.

In the current study, different separation routes are possible for this combination, depending on the choice of extraction units. For each processing route protein separation was performed at neutral pH, although at higher pH a larger fraction of proteins can be obtained. The reason for this is that alkaline conditions disturb lipid extraction due to saponification. To enable recovery of proteins at alkaline conditions, the defatted biomass can be mixed with the aqueous phase containing soluble proteins. After base addition, the proteins left inside the biomass will also become soluble. The next step is to separate the biomass again from the aqueous phase before protein recovery by means of pH shifting or UF steps. This scenario is suitable in case of SFE, but cannot be combined with solvent extraction because of the preceding transesterification step. During transesterification an alkaline waste flow is produced containing the remaining biomass, methanol, soap and glycerol. The biomass is separated before handling of this waste stream, but is deprived of proteins due to the presence of a base inside the media. One way to overcome this problem is to first extract lipids and lutein using a solvent and afterwards perform the transesterification step on the recovered lipid fraction. This will however yield a smaller fraction of free lutein and requires additional solvent usage to recover the FAME from the transesterification medium. It also yields less protein as part of the proteins is denatured by the organic solvent (Gerde et al. 2013). From this it can be concluded that alkaline protein extraction is only feasible in combination with SFE, but will also lead to higher energy requirements because an additional separation step is required to recover the biomass fraction.

Lutein extraction is also affected by the choice of unit operations. In this study transesterification was done prior to solvent extraction combined with saponification of lutein, so that the fraction of lutein available for extraction increased. A downside is that dry algal biomass is required to avoid saponification of lipids. Wet solvent extraction after separation does not require this energy intensive drying step. Research on organic solvent extraction on wet microalgal paste revealed that a water content of up to 20 vol% does not reduce the lipid extraction efficiency (Halim et al. 2014). However, the lutein yield obtained is decreased as saponification of lutein esters is not possible. An economical evaluation is necessary to decide which scenario is most beneficial. In case of SFE, research now focusses on realising *in situ* transesterification under supercritical conditions and the addition of a heterogeneous catalyst (Mac et al. 2014). This simplifies the recovery of methanol and purification products due to elimination of the neutralization, washing and drying steps. Although a high concentration of lutein in the oil fraction is expected due to the presence of an alcohol, separation of lipids and lutein is not achieved.

6. CONCLUSION

This research assessed the feasibility and sustainability of a biorefinery case study designed for lipid, lutein and protein production from microalgae grown on wastewater. This is done by comparing the performance of different routes in terms of energy consumption, waste production, chemical usage, sensitivity to a changing biomass composition and the complications which arise when designing routes aimed at multiple products.

Flocculation followed by centrifugation is energetically most favourable for the harvesting and dewatering step. However, the use of flocculants has a major impact on further processing of the biomass. Flocculation cannot be combined with PEF treatment for disruption, as cell aggregates can hide from electrical field sites and remain undamaged. The type of flocculant is also affecting the feasibility. Polymer flocculants have a low toxicity and require low dosage as high efficiencies are obtained. However, polymers are also capable of entrapping complex lipids or binding to pigments. This favours the extraction of lipids suitable for biodiesel production, but has a negative impact on lutein recovery. This phenomenon is not applicable in case flocculation is achieved by metal salts. The production of metal salts also demands significantly less energy compared to the production of polymer flocculants. A disadvantage is however that lower efficiencies are obtained and thus higher amounts are added. This has a toxic effect on processing of the residual biomass by fermentation and anaerobic digestion.

It is energetically beneficial if the disruption step causes almost complete disruption of the microalgae cells. Although this results in seemingly high energy consumption, separation of lipids and lutein is by far the most energy demanding step. SFE consumes a lot of energy for heating of supercritical fluids, while within solvent extraction almost all of the energy is put into distillation for recycling of the solvents. The energy requirement for protein separation is negligible, though the choice of protein extraction route has a significant influence on the relative energy consumption per kilogram of obtained product. The route demanding the least energy involved HPH disruption followed by the traditional separation methods; solvent extraction for lipid and lutein recovery and shifting of pH to obtain the protein fraction.

As the microalgae are cultivated using wastewater, varying fractions of lipid, lutein and protein are expected inside the biomass due to fluctuating nutrient compositions. The sensitivity in energy consumption is mostly determined by the lipid separation step. The least sensitive route to changes in biomass composition consists of solvent extraction with the two-step UF method for separation of components.

During design of different separation routes, it is important to consider the implementation of the transesterification step. Whether transesterification is performed prior to or after lipid extraction, has implications for protein and lutein recovery. Alkaline conditions result in undesired saponification of lipids. The combination of alkaline protein recovery and solvent extraction involving a preceding transesterification step is thus not possible. Performing the transesterification step after lipid recovery will however result in a lower lutein yield. In case of SFE, alkaline protein extraction can be performed by mixing the lipid-poor biomass with the aqueous phase containing soluble proteins.

In terms of environment friendly production, SFE is preferred over solvent extraction due to the use of CO_2 as solvent, which is abundant, safe and easily recyclable. Hexane is also a food-grade solvent, but requires an energy intensive recycling step and is produced by the petrol industry. The transesterification step during biodiesel production forms the biggest waste flow containing methanol, KOH, soap and glycerol in water. Handling of this waste stream contributes significantly to the total energy consumption, though an improvement can be made by performing a two-step transesterification reaction. The recovered glycerol can be sold for refining purposes, used as substrate to scale up the cultivation process or added to the dewatered sludge for anaerobic co-digestion. The

residual biomass fraction can be fermented for production of bioethanol, upon which the remaining biomass waste can also be added to the anaerobic digestion facility to produce biogas. The aqueous solutions obtained within both protein separation routes contain the soluble carbohydrate fraction and are therefore suitable for diluting the biomass prior to fermentation. In case of shifting of pH this solution first needs to be neutralized and thus requires additional chemicals. So, assessment of the environmental impact points out that biodiesel formation through transesterification is the most polluting step and that the residual biomass fraction can be made valuable through additional processing.

Apart from the two-step UF approach, all methods require additional energy or chemical input before the waste streams can be considered valuable. However, as suitable applications were found for the treated and untreated waste streams, in general microalgae biorefinery can be considered 'green'. The recovery of a high valuable compound, in this case lutein, contributes to about half of the energy requirements, but can be done without additional waste production. When designing the different routes for lipid, lutein and protein extraction, complications arose but could be overcome by simple rearrangements. Therefore, it can be concluded that the formation of a microalgae biorefinery process resulting in multiple end products is feasible and can be made sustainable by accepting increased energy consumption. The focus should however shift away from biodiesel production, towards the combined recovery of pigments, proteins and carbohydrates as this significantly reduces the energy needed for waste handling.

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NOMENCLATURE

$C_{A,foam}$	algae concentration in foam for DAF $(wt\%)$
E_V	energy requirement per volume (J/m^3)
E_{mass}	energy requirement per mass (J/kg)
E_{mol}	energy requirement per mole of air for DAF (J/mol_{air})
F_{mass}	mass flow rate (kg/s)
H_{evap}	heat of evaporation (J/kg)
H_{vap}	heat of vaporization (J/mol)
N_p	power number for stirring
R_r	reflux ratio for distillation
V_c	charging voltage of the transmission line for PEF (V/m)
c_{air}	molar concentration of air for DAF (mol/m^3)
f_F	Fanning friction factor
$r_{air\|solids}$	air-to-solids ratio for DAF (kg/kg)
v_s	stirrer velocity $(1/s)$
A	cross-sectional area (m^2)
C	concentration (kg/m^3)
Cp	heat capacity $(J/(kg*K))$
Cp'	capacity of the transmission line for PEF (F/m)
D	fraction of disrupted cells
F	volumetric flow rate (m^3/s)
H	energy input rate (J/s)
K	consistency index for pumping $(Poise/m)$
L	length (m)
MW	molecular weight (kg/mol)
N	number of passes for HPH, number of pulses for PEF
P	pressure (bar)
R	gas constant $(8.314\ J/(mol*K))$, recovery efficiency (kg/kg)
Re	Reynolds number
S	stirrer speed for flocculation (rpm)
T	temperature (K)
V	volume (m^3)
d	diameter (m)
f	frequency (Hz)
k	rate constant (s)
n	behaviour index for pumping
t	time (s)
u	speed of agitation for bead milling (m/s)
v	flow velocity (m^3/s)
y	fraction of intact cells for HPH
η	efficiency
ρ	density (kg/m^3)
τ	doubling time $(1/s)$

SUBSCRIPTS

A	microalgae biomass
H	hexane
bm	bead mill
ch	carbohydrates
dis	disintegration
$evap$	evaporation
li	lipids
lu	lutein
m	mixer
p	pump
pr	proteins
s	specific

APPENDIX A. DETAILED MODEL DESCRIPTIONS

A1. CULTIVATION

The biomass composition of microalgae is highly dependent on the culturing conditions. They can be grown heterotrophic, mixotrophic or autotrophic, in abundance of nutrients or under nutrient limiting conditions, and under different pH-levels and temperatures. Such choices determine the final characteristics of the algae flow that will be considered as the basis for this process design. Here the effects of different cultivation measures are evaluated for *Chlorella protothecoides* specifically.

The amount of centrate produced per wastewater treatment facility can differ greatly. In the cultivation experiments performed by Zhou et al. the centrate originated from St. Paul Municipal Wastewater Treatment Plant that generates 3800 m^3 of centrate per day (Zhou, Li, et al. 2012). However, average wastewater treatment plants produce around 450 m^3 of centrate per day (Metcalf & Eddy 2004). For the model the flow mentioned by Zhou et al. was taken as input. On centrate *C. protothecoides* is able to grow mixotrophically, leading to a growth rate of about one doubling per day which has been determined by many researchers (Wang et al. 2010; Ramos Tercero et al. 2014). Also similar growth rates have been found when performing cultivation on other substrates such as glucose, glycerol or acetate (Heredia-Arroyo et al. 2010; Sforza et al. 2012). Biomass concentration profiles of batch experiments show exponential growth during the first 4 days, followed by a stationary phase until the end of the experiment (Zhou, Min, et al. 2012). Different factors can cause a slow algal growth rate, such as nutrient or carbon source limitation, pH level, light intensity and temperature. Measurements indicate that after six days of cultivation still sufficient nitrogen and phosphorus is present in the wastewater (Zhou, Li, et al. 2012). It has also been proven that *C. protothecoides* can tolerate high pH levels, suggesting that a variation in pH is not one of the limiting factors (Li, Chen, et al. 2011; Zhou et al. 2011). Also temperature and light cannot have been limiting as the first one was kept constant and the second is never limiting during mixotrophic growth (J. Wang et al. 2014). Therefore, the only limiting factor in this case was the availability of organic carbon, which was indeed almost depleted after three days (Zhou, Li, et al. 2012). An organic carbon profile of autoclaved centrate indicates the presence of ethanol and volatile fatty acids, such as acetic acid, propionic acid and butyric acid (Zhou, Min, et al. 2012). Other suitable carbon sources for *C. protothecoides* are glucose and glycerol (Chen & Walker 2011), but these were not found in centrate.

Another possible limitation factor when using wastewater as a growth medium, is the presence of toxins and bacteria. However, no lag phase is observed when *C. protothecoides* is grown on centrate, proving the adaptability of this algae strain to the wastewater environment. It also provides evidence that *C. protothecoides* is a competitive microorganism. Within the centrate many bacterial species are present originating from the activated sludge process. Ma et al. investigated the effect of these bacteria on the growth and nutrient removal potential of algae (Ma et al. 2014). A positive effect was observed during the exponential growth phase as long as an initial concentration of at least 0.1 g/L of algae had been introduced. This can be attributed to the bacteria breaking down complex organic compounds that subsequently increase the amount of available organic carbon for algae. Next to an enhanced algal growth, the bacteria also cause an increase in the COD removal rate.

To overcome the shortage in carbon source observed under batch growth conditions, the microalgae should be grown continuous or semi-continuously. Zhou et al. developed a hetero-photoautotrophic two-stage cultivation process (Figure 16) that improved wastewater nutrient removal and enhanced algal lipid accumulation (Zhou, Min, et al. 2012). They also tested suitable hydraulic retention (HRT) times for semi-continuous growth mode (Zhou, Li, et al. 2012). Within the first stage, heterotrophic growth leads to reduction of chemical oxygen demand (COD) and

total organic carbon (TOC), until the usable organic carbon is depleted after three days and algal growth can no longer be maintained. The concentrations of nitrogen and phosphorus at that point have decreased extensively, though not sufficient for release of the wastewater into the environment. To meet the discharge standards, the centrate should be further treated. Therefore, after harvesting of the algae biomass by auto-flocculation, the wastewater will be reused for cultivation of *C. protothecoides* under autotrophic mode by addition of CO_2. This causes a rapid decrease in pH level within the second cultivation stage. In the first treatment phase, phosphorus was removed due to both algal metabolic uptake and precipitation at high pH. In the second stage, part of the precipitated phosphorus dissimilates and at the start a slight increase in phosphorus concentration is visible. However, due to autotrophic growth a final removal efficiency of 98% is achieved, much higher than what can be achieved in a single stage cultivation mode. The decrease in nitrogen concentration can be mostly attributed to the assimilation of ammonium that was completely removed after three days of autotrophic growth phase. The residual nitrogen could probably not be consumed by the algae, leading to a removal rate of 90% of nitrogen. During autotrophic growth the COD concentration is varying as photosynthesis results in organic carbon production and secretion. The final removal efficiency of COD of 75% is thus slightly lower than a single mixotrophic growth phase could achieve. However, the nutrient removal efficiencies of this hetero-photoautotrophic two-stage cultivation process are much higher than found in other literature (Wang et al. 2010; Li, Zhou, et al. 2011; Hu et al. 2012; Zhou, Li, et al. 2012). Implementation of this process under semi-continuous operation with a hydraulic retention time of 3 days is suggested as a promising cultivation mode (Zhou, Li, et al. 2012). In practice this means that 1/3 of the centrate will be replaced daily. Expected is that this combination will lead to an average biomass concentration in between of 1.5 g/L and 1.8 g/L. As a consequence, a safe estimation of the biomass production is 1.5 g/L/day.

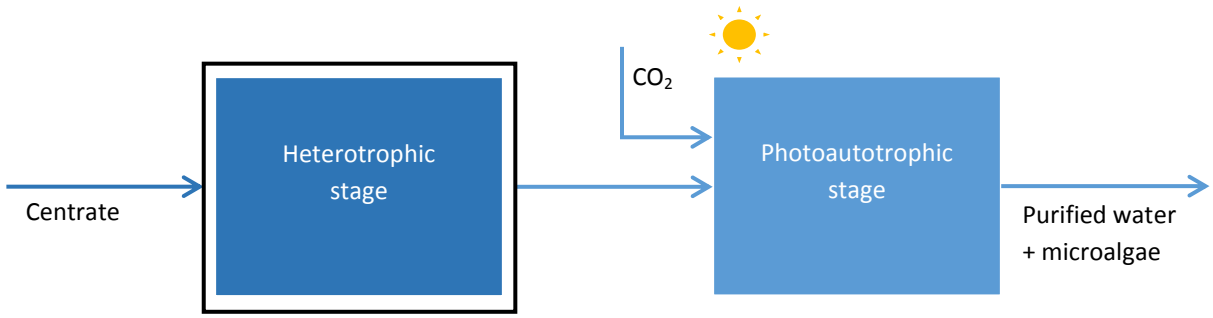


FIGURE 16, HETERO-PHOTOAUTOTROPHIC TWO-STAGE CULTIVATION MODE ON CENTRATE (ZHOU, MIN, ET AL. 2012)

The cell content of autotrophic and heterotrophic grown *C. protothecoides* was investigated by Miao et al. (Miao & Wu 2006). Clearly, lipids and carbohydrates tend to accumulate under heterotrophic growth, while proteins are formed under autotrophic growth. In particular the protein Rubisco is an important enzyme involved in fixation of CO_2. However, under nitrogen-limiting conditions autotrophic growth can also result in high lipid content, while the protein concentration decreases (Sforza et al. 2012; Guccione et al. 2014). This is because nitrogen shortage leads to consumption of intracellular nitrogen sources, such as chlorophyll or proteins (Campenni' et al. 2013). The lipid content of the biomass after the two-stage cultivation is about 35%, somewhat higher than what has been found in single stage mixotrophic cultivation (Li, Zhou, et al. 2011; Hu et al. 2012; Zhou, Li, et al. 2012). The final protein content is expected to be higher than during solely heterotrophic growth, but lower than with autotrophic growth. During the first stage of heterotrophic growth few proteins are present inside the cells, but this changes within the second stage due to autotrophic cultivation. The nitrogen-limiting conditions will however limit the production of proteins, resulting in an averaged expected protein content of 30% of the cell weight (Miao & Wu 2006; Guccione et al. 2014). The carbohydrate fraction is varying between 10 and 15% under autotrophic and heterotrophic cultivation respectively (Miao & Wu 2006). Under nitrogen limiting condition however, it was found

that *Chlorella* strains accumulate either lipids or carbohydrates, towards 50% of the cell content (Guccione et al. 2014). As *C. protothecoides* has shown to increase the lipid content but not to an extreme of 50%, a fraction of 15% of carbohydrates is expected. A reduction in starch granules after N-depreviation has indeed been observed in *C. protothecoides* (Li et al. 2013; Pasaribu et al. 2014). The ash and moisture content and fraction of other components are rather constant (Miao & Wu 2006). The lutein content under nitrogen limiting heterotrophic growth could contain as much as 5.35 mg/g biomass (Shi et al. 2002). As under varying cultivation conditions a lutein content of in between 4 and 5 mg/g is found (Shi et al. 2000; Shi et al. 2006), a safe estimation of 5 mg/g of lutein in the final biomass is considered acceptable. An overview of the collected design variables is given in Table 3. These values are implemented as initial conditions of the algae flow to be treated.

TABLE 3, OVERVIEW OF DESIGN VARIABLES FOR CULTIVATION OF *C. PROTOTHECOIDES*

Design variable	Symbol	Value	Unit	References
Growth rate	μ	1	$1/day$	(Wang et al. 2010; Ramos Tercero et al. 2014)
Biomass concentration	C_A	1.5	g/L	(Zhou, Li, et al. 2012)
Biomass production	P_A	1.5	$g/L/day$	(Zhou, Li, et al. 2012)
Centrate flow rate	$F_{centrate}$	7500	$m3/day$	
Density algae flow	ρ_A	1050	kg/m^3	(Bosma et al. 2003)
HRT*		3	$days$	(Zhou, Li, et al. 2012)
Nitrogen removal rate	r_N	90	%	(Zhou, Min, et al. 2012)
Phosphorus removal rate	r_P	98	%	(Zhou, Min, et al. 2012)
COD* removal rate	r_{COD}	75	%	(Zhou, Min, et al. 2012)
Lutein content**		5	mg/g	(Shi et al. 2002)
Lipid fraction		0.35	–	(Sforza et al. 2012; Zhou, Min, et al. 2012)
Protein fraction		0.30	–	(Miao & Wu 2006; Cerón-García et al. 2013)
Carbohydrate fraction		0.15	–	(Miao & Wu 2006; Cerón-García et al. 2013)
Ash fraction		0.06	–	(Miao & Wu 2006; Cerón-García et al. 2013)
Other components fraction		0.10	–	(Miao & Wu 2006)
Moisture fraction		0.04	–	(Miao & Wu 2006)

**HRT and COD stand for Hydraulic Retention Time and Chemical Oxygen Demand respectively.*
***The lutein content is included in the fraction 'Others'.*

A2. HARVESTING AND DEWATERING

Harvesting is the first step in concentrating the biomass by removal of water. It is often followed by a dewatering step to further concentrate the algae flow, as no single harvesting step can obtain the desired concentration factor. Harvesting and dewatering are together responsible for 20-30% of the total costs associated with processing of microalgae (Molina Grima et al. 2003). Since the suitability of a harvesting method is algal species specific (Milledge & Heaven 2012), many different techniques are being developed.

The mass balance of harvesting of microalgae consists of one flow in, the algae solution, and two flows out, the concentrated algae solution and the clarified water which is seen as the 'waste' flow.

$$0 = F_{A,in} * C_{A,in} - F_{A,out} * C_{A,out} - F_{A,waste} * C_{A,waste} \quad (13)$$

The recovery efficiency R, concentration factor cf and mass balance, described by equation (2), (3) and (13) respectively, together determine the outgoing flow characteristics.

$$C_{A,out} = C_{A,n} * cf \quad (14)$$

$$F_{A,out} = \frac{F_{A,in} * R}{cf} \quad (15)$$

$$C_{A,waste} = \frac{F_{A,in} C_{A,in} (1 - R)}{F_{A,in} - F_{A,out}} \quad (16)$$

Within this study the harvesting and dewatering performances of flocculation, Dissolved Air Flotation (DAF) and centrifugation and are evaluated. The first two methods have been selected as harvesting techniques for the favourable ratio of energy consumption to biomass recovery (Hagen 2014; Slegers et al. 2014). Flocculation has a very low energy demand, but also results in lower biomass recovery efficiencies and needs addition of chemicals. DAF has higher energy requirements, but also leads to a higher biomass recovery while using less chemicals than flocculation. Centrifugation has the highest energy demand but do not require the addition of chemicals. Three different harvesting and dewatering routes are compared: single-step centrifugation and two-step dewatering by centrifugation with either flocculation or DAF as preceding harvesting step.

A2.1. FLOCCULATION

Flocculation is a simple harvesting technique that involves the addition of a flocculant that attaches to the cells to form aggregates. As these aggregates start to increase in weight they tend to settle, forming a dense biomass layer at the bottom of a settling tank. The most influencing parameters determining the recovery efficiency are the chitosan concentration and speed of mixing (Riaño et al. 2012). Riaño et al. developed a second order polynomial equation that describes the relationship between these parameters (eq. (17)).

$$R = 84.3 + 17.5{C^*}_{chitosan} - 1.3S^* - 11.1{{C^*}_{chitosan}}^2 - 3.7S^{*2} - 2.6{C^*}_{chitosan}S^* \quad (17)$$

Where ${C^*}_{chitosan} = \frac{C_{chitosan} - 0.128}{0.086}$ and $S^* = \frac{S - 325}{194}$, where $C_{chitosan}$ is the chitosan concentration (kg/m^3) and S the stirring speed (rpm).

The only energy input required for this process is for mixing, which ensures that the flocculant is spread homogeneously among the algae solution. This is accomplished by a short period of rapid mixing followed by a longer period of slow mixing. Mixing energy requirements have been calculated using equation (10) and (11) for a

stirring speed of 60 rpm for 5 minutes, followed by 30 rpm for 25 minutes. These conditions have been chosen by comparing different literature studies (Granados et al. 2012; Riaño et al. 2012; Divakaran & Pillai 2002). Afterwards, the formed aggregates will have an hour to settle. The known algae flow rate $F_A$and total time of 90 minutes together determine the volume that should be treated within one settling tank.

As a sustainable production design is desired, the flocculants used for harvesting should be non-toxic, efficient, available and cheap, and should not affect the quality of the biomass and water. Granados et al. compared the recovery yield and concentration factor of different flocculants that satisfy these demands as they are commonly used in wastewater treatment.

Metal salts obtain lower recovery efficiencies (Granados et al. 2012; Beach et al. 2012). Therefore they are often used in high quantities to maintain acceptable flocculation efficiency. This may result in contamination of the biomass and could affect further processing (Pragya et al. 2013). In case of anaerobic digestion of sludge from wastewater, the presence of aluminium and sulphate has been found to inhibit methanogenic activity (Cabirol et al. 2002). Aluminium salts also caused cell lysis of about 25% and 10% of the total number of cells for $AlCl_3$ and $Al_2(SO_4)_3$ respectively. With ferric chloride as flocculant the culture changed colour from green to brown-yellow (Papazi et al. 2009). An effect on pigments is however not desirable, since the pigment lutein is one of the final products. Therefore, metal salts are not used within this processing step.

Polyelectrolytes are polymeric flocculants that include ionic and non-ionic species, which can be of natural or synthetic origin. The high charge density and large surface area of polyelectrolytes increase the effectivity of algae harvesting (Granados et al. 2012). As a result less flocculant is needed, which consequently lowers the probability of harmful contamination. There can be made a division between cationic, anionic and non-ionic polymers. All are used in water treatment and have a low toxicity, though cationic polymers are considered more toxic to aquatic species (Bolto & Gregory 2007). The effect of cationic starch, an example of an organic cationic polymer, on the formation of acetone, butanol, ethanol by fermentation and lipid extraction of harvested microalgae was studied by Anthony et al. (Anthony et al. 2013). No toxicity was observed during fermentation and also the lipid recovery had not been affected. Since even enhanced product formation was observed, they could conclude that the use of cationic starch is beneficial within downstream processing of microalgae. Cationic polymers also show higher recovery rates compared to the anionic and non-ionic polymers for *Chlorella* species (Granados et al. 2012). This is because microalgae cells have a negatively charged surface that need a positive charge for interaction (Uduman et al. 2010). Within this process design it has been decided to evaluate the performance of the biodegradable cationic organic polymers chitosan.

Chitosan is a polymer of acetylglucosamine produced from crustacean shell waste of fishing industries, which makes it a renewable source (Beach et al. 2012). It is a very safe polymer with many applications, also in the pharmaceutical industry (Ravi Kumar 2000). The use of a natural occurring polymer has environmental benefits over the use of synthetic or inorganic polymers. However, the production process of chitosan from chitin is still quite energy demanding. Highly alkaline solutions are needed for deacetylation of chitin. Sodium hydroxide is produced via an energy-intensive process called chloralkali electrolysis (Beach et al. 2012). From a life-cycle assessment performed by Beach et al follows that in total the production of chitosan consumes 8.33 MJ/kg of chitosan. Another downside of chitosan is the high pH dependency of the flocculant capacity. Therefore research is done on modification of natural polymers, such as chitosan. These grafted flocculants are less sensitive to pH changes and have higher aggregating power. Modification of chitosan involves energy-intensive procedures such as high energy radiation, UV-radiation or microwave based methods (Lee et al. 2014). Within this research the use of grafted flocculants is not considered since flocculation is selected as harvesting method for its low energy consumption.

Chitosan is effective as a flocculant at alkaline pH levels near the neutralization point at 7.9 (Gualtieri et al. 1988). In acidic environment the amine groups of chitosan are protonated and repel each other, causing chitosan to form an extended chain which results in smaller and looser flocks. Near the neutralization point of chitosan are the amine groups no longer protonated and chitosan tends to coil and precipitate. At similar pH levels algal cells have the highest negative charge, resulting in the formation of large and dense flocks (Harith et al. 2009; Chen et al. 2014). Therefore pH control is needed in order to obtain high recovery efficiencies. Other research states that a pH of below 7 chitosan activity and flocculation efficiency increase (Xu et al. 2013). These observation differences might be the result of different culturing conditions and specific strain properties. The chemical composition of the wastewater also influences the performance of the flocculant (Uduman et al. 2010). For that reason it is important to first test the optimum conditions for flocculation by chitosan on *C. protothecoides* before implementation.

A2.2. Dissolved Air Flotation

Within dissolved air flotation (DAF) an aqueous flow saturated with air is kept under high pressure before release into the algae solution. The sudden pressure release causes air bubble formation with an average diameter of 10-100 µm. The size also depends on the equipment set-up, flocculant concentration and gas flow rates. The bubbles rise to the surface while attaching particles via hydrophobic interactions. Part of the clarified water phase is saturated with air and recycled back into the flotation tank (Uduman et al. 2010). The addition of a flocculant is essential for high harvesting efficiencies. In absence of coagulation the ratio of cells to bubbles is too large and a small fraction of the cells will be harvested. A reduction in the number of particles and an increase in flock size both increase the chance of capture by bubbles (Zhang et al. 2014).

DAF is a cheap harvesting method that can be applied at large scale and is capable of handling dilute streams (Rawat et al. 2013). It is already researched extensively for the removal of pollution from wastewater, such as phosphates and metals (Peleka & Matis 2008; Edzwald 2010). It has also been implemented for harvesting of microalgae that serve as biological treatment for nutrient removal from wastewater (Ometto et al. 2013). Unfortunately, in these cases the biomass concentrations are far more dilute than within algae production systems. A study that focussed on harvesting of 1.5 g/l of a *Chlorella* species by dissolved air flotation is considered as a suitable reference point (Zhang et al. 2014). Zhang et al. investigated the efficiency of harvesting for varying flocculant doses, culture conditions, biomass concentrations and recycle ratios. As expected, an increase in flocculant concentration is highly beneficial for the harvesting efficiency. Higher initial cell concentrations and lower recycle ratios lead to lower efficiencies. This reveals that the number of air bubbles could be a limiting factor.

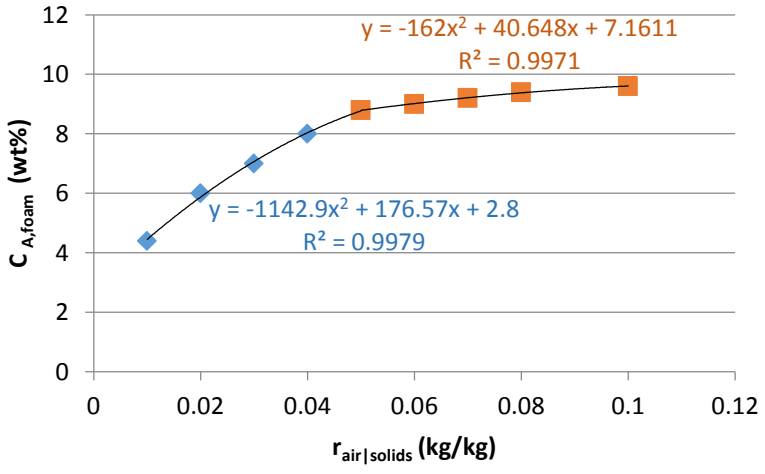


FIGURE 17, AIR-TO-SOLIDS RATIO VERSUS FLOAT CONCENTRATION (RAWAT ET AL. 2013).

The flows into the DAF unit implemented within this model are the main flow of algae solution after cultivation and two co-streams of flocculant, in this case aluminium sulfate, and recycled water saturated with air. Outgoing are a concentrated algae flow recovered from the top foam layer and a clarified water flow of which part is recycled back into the DAF system. A recycle ratio of 8 to 12% is

commonly applied in wastewater treatment systems. Harvesting of cultivated microalgae needs however more air bubbles and for this reason a recycle ratio of 20% of the outgoing flow is needed (Metcalf & Eddy 2004). The microalgae recovery R of DAF reaches 90% when combined with 50 mg/g aluminium sulfate as flocculant (Zhang et al. 2014). Addition of more flocculant does not further increase the recovery. For that reason the necessary amount of flocculant is determined by this ratio and the initial biomass concentration.

After DAF treatment, the fraction of cells present within the foam can comprise up to 10 wt% (Rawat et al. 2013). This depends on the amount of air entering the system, also defined by the air-to-solids ratio $r_{air|solids}$ in kg/kg. Figure 17 shows the relation between $r_{air|solids}$ and the float concentration of cells within the foam s $C_{A,foam}$. The final concentration of algal cells within the outflow is calculated by equation (18).

$$C_{A,out} = C_{A,foam} * \rho_{foam} * R \quad (18)$$

This implies that the concentration factor cf in this case also is determined by $r_{air|solids}$. As this parameter influences the characteristics of the concentrated algae flow and the energy input rate at the same time, it has been chosen as a decision variable (table decision variables).

The energy balance involves energy consumption for pressurizing (H_{pr}), mixing (H_m) and for pumping of the main ($H_{p,main}$), waste ($H_{p,waste}$) and recycle stream ($H_{p,recycle}$).

$$H_{DAF} = H_{pr} + H_m + H_p + H_{p,waste} + H_{p,recycle} \quad (19)$$

Prior to DAF treatment, the flocculant is mixed through the algae solution to enhance the formation of flocs. This mixing step is shorter compared to flocculation and thus requires significantly less energy. During DAF treatment, the work done by an air compressor is calculated according to equation (20) used by Coward et al. (Coward et al. 2013).

$$E_{mol} = \frac{RT}{\gamma - 1}\left[\left(\frac{P_2}{P_1}\right)^{\frac{\gamma-1}{\gamma}} - 1\right] \quad (20)$$

Here E_{mol} is the energy demand in J/mol_{air}; R represents the gas constant of 8.314 $J\ K^{-1}mol^{-1}$; T stands for the temperature in Kelvin (293 K); γ is the ratio specific heat of air which is assumed to be 1.4; and P_1 and P_2 represent the pressure in bar outside (1 bar) and inside (5.5 bar) of the compressor respectively. In order to obtain the related energy consumption (H_{pr}), the efficiency of the air compressor (η) and the molar concentration (c_{air}) and flow (F_{air}) of air should be known. The molar concentration of air at 5.5 bar and 293 K can be calculated using the ideal gas law, resulting in a value of 225.8 mol/m^3. The flow of air is quantified by the selected air-to-solids ratio and initial biomass concentration. The efficiency of the compressor is assumed to be 80%, a safe estimation for packed saturators (Edzwald 2010). The final calculation of the energy requirement for pressurizing is shown in equation (21).

$$H_{pr} = E_{mol} * c_{air} * F_{air} * \frac{1}{\eta} \quad (21)$$

A2.3. CENTRIFUGE

The centrifuge separates particles based on density difference by generating a centrifugal force. This can be accomplished very quickly and a centrifuge has therefore a much lower footprint compared to settling processes relying on gravity such as flocculation. The energy consumption of a centrifuge depends on the incoming algae concentration and flow rate and on the desired concentration factor, as described by equation (12) and (24) (Boxtel 2015).

$$E_V = 0.2032 * 10^{0.0218 * C_{A,in}} \quad (22)$$

A3. DISRUPTION

For disruption the choice fell on three mechanical options: high pressure homogenizer (HPH), bead mill and pulse electric field (PEF). These three disruption method have in common that they do not produce a waste stream. The flow entering the disruption apparatus is equal to the outgoing flow with the only change that a large part of the cells present has been damaged. Hereby the intracellular components become available for extraction. The mass balance for each component (subscript X) is shown in equation (23).

$$0 = F_A * C_{A,in} * f_X - F_A * C_{A,out} * f_X - F_A * C_{X,out} \quad (23)$$

Here F_A stands for the algae flow, $C_{A,in}$ and $C_{A,out}$ represent the incoming and outgoing algae concentration and f_X refers to the fraction of component X present inside the cells. These fractions have been determined within the chapter on cultivation. The outgoing concentration of component X ($C_{X,out}$) and $C_{A,out}$ are determined by the disruption efficiency D according to equation (24) and (25).

$$C_{X,out} = C_{A,in} * D * f_X \quad (24)$$

$$C_{A,out} = C_{A,in} * (1 - D) \quad (25)$$

The evaluated disruption methods make no use of chemicals and therefore the products will not suffer from contamination. They also have the potential to be implemented on a large scale. The differences between these processing units lay within the energy demand and time of operation. The consequence of not using chemicals or enzymes is that cell disruption will require a much higher energy input. The theoretical energy requirement to disrupt an individual cell has been studied by Lee et al. They discovered that disruption of dry microalgal biomass would theoretically need 763 J/kg (Lee et al. 2013). Most mechanical treatment processes use at least 5 orders of magnitude more energy, meaning that over 99% of the energy input is converted to heat (Doucha & Lívanský 2008; Lee et al. 2012). The two main reasons for this inefficient use of energy are: (a) the low efficiency in creating a cavitation of sufficient intensity that could cause cell disruption and (b) the low probability of such a force meeting an intact cell (Lee et al. 2012). In order to increase the efficiency of energy intensive disruption methods, the second cause could be countered by increasing the cell density. This will as well create a more concentrated product solution that would decrease the costs of recovery. It should however be taken into account that additional equipment and energy input is required to achieve these higher densities. Together with the increasing energy demand for pumping, there will be an optimum in terms of minimal energy usage leading towards the desirable algae concentration.

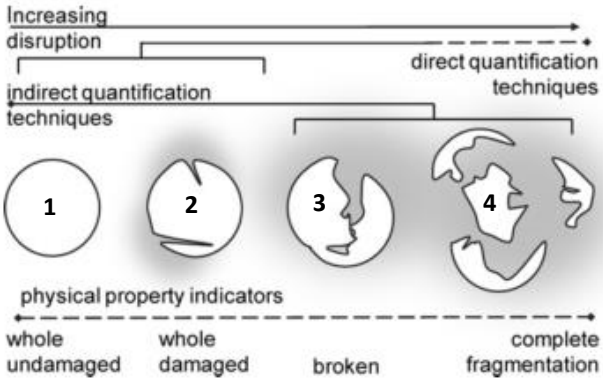


FIGURE 18, SIMPLIFIED DEPICTION OF CELLULAR DISRUPTION FROM WHOLE CELLS TO COMPLETELY FRAGMENTED CELLS (ERIN M. SPIDEN ET AL. 2013)**.**

In order to compare disruption efficiencies of several methods, Spiden et al. discussed the use of different indicators for cell disruption of *S. cerevisiae*. Cell counting was considered the most accurate measure, with the disadvantage of having a significant analysis time (Erin M. Spiden et al. 2013). As in other literatures studies cell count was often used as indicator of cell disruption, these results were also implemented in the models to determine disruption efficiencies. The consequence of using a direct quantification technique is that no

distinction can be made between intact cells (stage 1) and damaged cells that remain whole but release cellular components (stage 2). Therefore it can be stated that using cell count as an indicator will lead to a conservative estimate of disruption. The different stages of disruption are shown in Figure 18. All disruption methods used in this study are at least capable of breaking the cells (stage 3). This result in release of the intracellular components such as water soluble proteins and the lipids and lutein present inside the cell wall become available for extraction.

A3.1. High Pressure Homogenizer

As the name implies, a high pressure homogenizer (HPH) disrupts algal biomass by placing it under high pressure, usually up to 1500 bar. The flow of algae collides against a valve seat and impact ring and when leaving the HPH it encounters a sudden release of pressure. Although the exact cause of disruption is not known, the mechanism induces a range of theories involving turbulence; viscous and high pressure shear; the sudden decrease in pressure and subsequent release of gas bubbles which burst inside the cells; and collision of cells on the hard surfaces of the valve seat and impact ring (Lee et al. 2012).

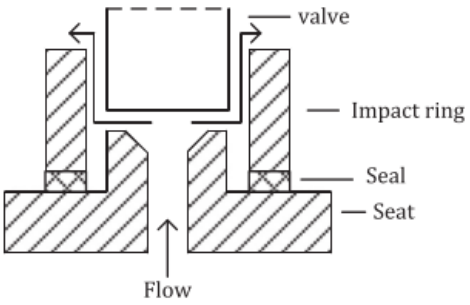
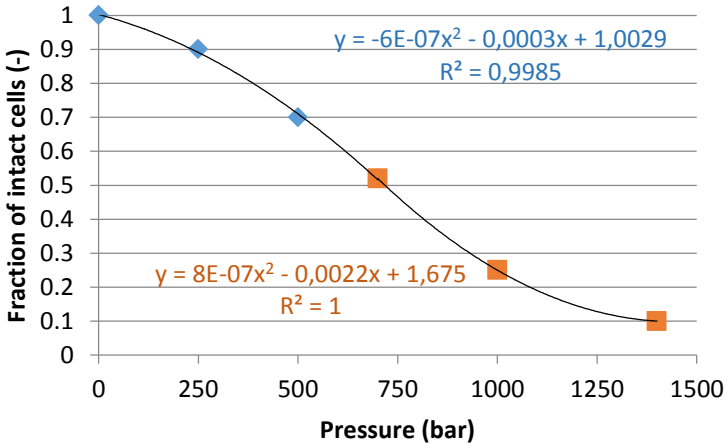


FIGURE 19, A TYPICAL HPH VALVE SEAT (LEE ET AL. 2012).

Multiple parameters play a role in the disruption efficiency, such as process temperature, medium flow rate, valve and orifice design, number of passes and applied pressure. The last mentioned has the highest influence and determines the release of cell contents for each pass.

Yap et al. tested HPH treatment on *Chlorella* sp. biomass for the purpose of lipid recovery by solvent extraction (Yap et al. 2014). They measured the percentage of intact cells for different pressures applied, resulting in two polynomial functions that together describe these measurements perfectly (Figure 20).

FIGURE 20, RELATION BETWEEN THE DEGREE OF DISRUPTION AND PRESSURE APPLIED DURING HPH TREATMENT (YAP ET AL. 2014).



These results show a higher disruption efficiency for each pressure applied than observed in a previous experiment with *Chlorella* sp. biomass (Erin M Spiden et al. 2013). However the measurements are still comparable as the pressure needed to obtain certain disruption efficiency is within the same range of magnitude. In order to account for the number of passes, a simple formula is used to calculate the final level of disruption.

$$D_{HPH} = 1 - y^N \quad (26)$$

In which D_{HPH} represents the fraction of disruption, N represents the number of passes and y is fraction of intact cells and thus the outcome of the polynomial functions shown in Figure 20.

The energy input rate for pressurization H_{pr} for a HPH is determined by the pressure and amount of passes applied to a certain algae flow.

$$H_{pr} = F_A * P * 10^5 * N \quad (27)$$

Within this equation F_A, P and N represent the algae flow in (m^3/s), the pressure applied (bar) and N the number of passes, respectively.

A3.2. BEAD MILLING

A bead mill consists of a vessel that is filled with beads that collide with microalgae while the vessel is being shaken or rotated. Though this grinding causes the cells to disrupt, bead milling is considered a mild method as all products maintain functional. As long as the vessel is well equipped with cooling jackets since a lot of heat is produced during operation that could cause denaturation of proteins (Doucha & Lívanský 2008).

The efficiency of disruption is dependent on the size, density and amounts of the beads, the speed and design of the agitator and on the residence time and characteristics of the feed, such as temperature, viscosity and biomass concentration (Lee et al. 2012). Doucha et al. tested various types of bead mills and discovered that the most influential parameters are the biomass concentration and agitator speed. They found opposing effects of increased cell concentrations in different bead mills. For the Dyno-Mill KD-Pilot, the disruption efficiency decreased significantly with increasing biomass concentration starting from about 100 kg/m^3. They also concluded that the specific energy consumption (J/kg) decreased with increasing feed rate and increased with increasing degree of cell disintegration (Doucha & Lívanský 2008).

The change in specific energy consumption for the Dyno-Mill has been further investigated by Postma et al. (Postma et al. 2014). Here they focussed on what had been described by Doucha et al. as the most influential parameters: the biomass concentration and the agitator speed. The model used for simulation of the bead mill is based upon the obtained results. To cover the findings in second order polynomial regression models, they coded the process parameters according to equation (28) and (29). Here the coded biomass concentration (C_x) is X_1 and the coded agitator speed (u) resembles X_2.

$$X_1 = \frac{C_x - 85}{60} \quad (28)$$

$$X_2 = \frac{u - 9}{3} \quad (29)$$

Using these coded parameters two different rate constants are determined: one for the fraction of disintegration of the cells (k_{dis}) and one for water soluble protein release (k_{pr}). From this last rate constant the running time of the bead mill (τ_{bm}) is calculated, that would lead to release of 87.5% of the total water soluble proteins content (equivalent to 3 times the doubling time). The time of operation in turn determines the degree of disruption (D). The energy consumption in J/kg required for 87.5% release of water soluble proteins is shown in equation (34) by the second order polynomial. To convert it to the energy demand in J/m^3 (E_V) this value is multiplied with the amount of Joules in a kWh and the biomass concentration (C_x) in kg/m^3. Multiplication with the flow rate (F) in m^3/s leads to the specific energy input rate in J/s (H_s) .

$$k_{dis} = 10\text{^}(-1.8014 + 0.1385X_1 + 0.0602X_2 - 0.1126X_2^2) \quad (30)$$

$$k_{pr} = 10\text{^}(-1.6994 + 0.176X_1 + 0.0491X_2 - 0.0945X_2^2) \quad (31)$$

$$\tau_{bm} = 3 * k_{pr}^{-1} * \ln(2) \quad (32)$$

$$D_{bm} = 1 - e\text{^}(-k_{dis} * \tau_{bm}) \quad (33)$$

$$E_V = (1.319 - 2.0553X_1 + 0.689X_2 + 0.8435X_1^2 - 0.2779X_1X_2) * 10^6 * C_x \quad (34)$$

$$H_s = E_V * F \quad (35)$$

Results show that the biomass concentration has a relatively large influence on the disruption efficiency compared to the agitation speed (Figure 21). The disruption efficiency is however only slightly fluctuating and it is assumed that the degree of disintegration is still sufficient for lipid and lutein extraction. The specific energy demand decreases substantially with increasing biomass concentration, until a minimum is reached. Within the experiment performed by Postma et al. no increase in specific energy demand had been observed as the highest biomass concentration they worked with was 145 kg/m^3. Higher biomass concentrations were more difficult to handle with respect to biomass losses and mixing properties (Postma et al. 2014). Due to problems with operation, it is expected that further increase in biomass concentration will require more energy. The minimum in specific energy demand observed at about 150 kg/m^3 is therefore acceptable, though no scientific prove could be found (Figure 21).

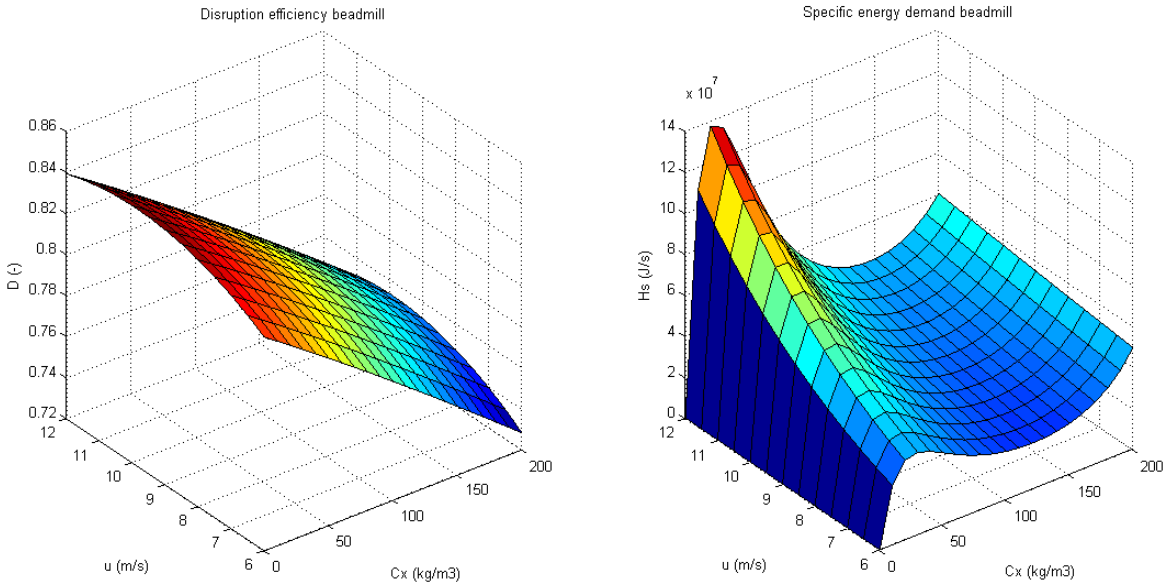


FIGURE 21, ANALYSIS OF BEAD MILL PERFORMANCES FOR VARYING AGITATION SPEEDS AND BIOMASS CONCENTRATIONS. THE LEFT GRAPH SHOWS THE DISRUPTION EFFICIENCY AND THE RIGHT GRAPH THE SPECIFIC ENERGY DEMAND.

A3.3. Pulse Electric Field

Pulse electric field (PEF) is a disruption method that uses high intensity electric field pulses to disintegrate the cell membranes. Due to selective concentration of the electric field on membranes, the phospholipids start to change their position which causes a membrane to become permeable (El Zakhem et al. 2006). This phenomena is called 'electroporation' and the damage could be either temporary or permanent (Luengo et al. 2014). As electroporation of membranes is difficult to analyse by means of electroscopic observations, the degree of electroporation is measured by increase in conductivity of the medium.

The efficiency of PEF treatment is mainly dependent on the specific energy input E_{mass} (J/kg) and on the elapse time between recovery of components and PEF treatment. The specific energy input is in turn dependent on the

biomass concentration, field strength and on the shape, duration and amount of pulses applied (Goettel et al. 2013). The relation between these parameters is shown in equation (36).

$$E_{mass} = \frac{0.5 * N * Cp' * V_C^2}{C_x} \quad (36)$$

In which N represents the number of pulses applied, Cp' the capacity of the transmission line cable (F/m), V_c the charging voltage of the transmission line (V/m) and C_x is the biomass concentration (kg/m^3) (Eing et al. 2009).

Experiments with *C. protothecoides* showed release of soluble compounds above a specific energy input of 50 kJ/kg_{sus} and the concentration of released cellular components started to saturate above 150 kJ/kg_{sus} (Goettel et al. 2013; Eing et al. 2013). PEF treatment did not cause excretion of lipids. In a succeeding experiment the yield of lipids had been measured by means of solvent extraction with ethanol (70 $wt\%$). This was done using biomass treated by PEF at different energies resulting in a required minimum specific energy input of 75 kJ/kg_{sus}. However, to obtain reasonable lipid yields of above 0.75 the biomass should be treated with at least 150 kJ/kg_{sus}. The higher energy requirement for lipid recovery is hypothesized to be necessary for obtaining irreversible membrane permeabilization (Eing et al. 2013). As these experiments were performed using an algae solution of 100 kg/m^3, the specific energy input rate H_s is calculated according to equation (12) using an energy requirement E_{mass} of 1.5 MJ/kg_{dw} within this model.

A way to improve the energy efficiency of PEF treatment would be to lower the total amount of suspension that needs to be treated. This can be done by extensive dewatering and will result in higher biomass densities. Zakhem et al. investigated the influence of the biomass concentration of *S. cerevisiae* on the perforation efficiency by PEF and observed a linear relationship. They measured a certain threshold concentration of 16 $wt\%$ after which the saturated electrical conductivity abruptly decreased. From this observation they concluded that above the threshold concentration a fraction of cells can be hidden at low electrical field sites and remain undamaged (El Zakhem et al. 2006). Working at higher biomass concentrations would thus lead to lower PEF efficiencies. Goettel et al. performed a similar experiment on *C. protothecoides,* though with a field strength of almost ten times higher. *S. cerevisiae* is a microorganism with similar dimensions to oleaginous algae, such as *C. protothecoides* (Erin M. Spiden et al. 2013). Within the experiment of Goettel et al., they too observed a linear relationship between the final conductivity and biomass concentration. The threshold value found for *S. cerevisiae* was not exceeded, but at 160 kg/m^3 no decrease in PEF efficiency had been observed. However, as a more intensive field strength was used, a higher threshold concentration can be expected. Goettel et al. also tested the effect of the biomass concentration on the amount of released intracellular substances. Higher biomass concentrations could theoretically lead to the release of more intracellular substances. This phenomena was evaluated according to a release-factor R, which is defined as the ratio between released substances and the biomass concentration. A slight increase in R was measurable, meaning that higher biomass concentrations seem to improve the extraction efficiency (Goettel et al. 2013). There is however an optimum when taking into account the disadvantages of high density slurries like difficulties with pumping and the existence of a threshold concentration. Therefore a rough estimation is made that PEF operated under the conditions of Goettel et al. will perform well at biomass concentrations up to 200 kg/m^3.

Next to the PEF treatment chamber, also a mixer is involved in order to keep the suspension well mixed after treatment. The energy input rate of mixing H_m is calculated according to equation (10). As mentioned before, the elapse time between treatment and product recovery has a significant influence on the obtained yield. During resting intracellular substances continue to leak out of the microalgal cells, until the conductivity saturates after about two hours. This resting time is especially important to increase the availability of lutein. Lutein is situated

inside the chloroplast and the chloroplast membrane takes some time to become permeable (Luengo et al. 2014). Mixing is of importance to prevent the formation of equilibrium conditions near the cell membrane. A steep gradient results in faster leakage of soluble compounds into the solution. During treatment it is assumed that pumping of the algae solution into the PEF treatment chamber will cause the necessary turbulence to counter the formation of a boundary layer around the membranes. During resting time the solution will be stirrer with a speed of 100 rpm for two hours. As the incoming flow rate is known, the required dimensions of the stirrer equipment can be calculated according to equation (11).

A4. SEPARATION OF LIPIDS AND LUTEIN

A4.1. SOLVENT EXTRACTION

The method used within this production process design is based upon the results of three studies (Cerón et al. 2008; Li et al. 2002; Prommuak et al. 2013). Li et al. developed a method in which lutein was extraction from dried algal biomass by dichloromethane after saponification and further purified using a solution of ethanol and water (Li et al. 2002). Cerón et al. was the first to describe a large-scale process that would lead to the recovery of lutein from microalgae (Cerón et al. 2008). Alkaline treatment on dry biomass for saponification of lutein was followed by extraction with hexane in an efficient counter-current manner. Prommuak et al. took it a step further and proposed a process design that extracted both lipids and lutein from *Chlorella vulgaris* (Prommuak et al. 2013). This resulted in a rather complicated process consisting of simultaneously performed reactions and multiple extraction and product separation steps. Within this study, the process proposed by Prommuak et al. is simplified and made more environmental friendly using the knowledge of previous studies.

The first step consists of drying of the biomass, of which the energy demand is calculated according to equations (5) and (6). To the dried biomass 4% w/v KOH in methanol is added in a 16:1 v/w biomass ratio. This will support the transesterification and saponification reactions towards biodiesel and free lutein respectively (Cerón et al. 2008; Prommuak et al. 2013). During this combined transesterification and saponification step, only energy is needed for 10 minutes mixing at 200 rpm as described by equation (10). The biodiesel and free lutein are extracted from this mixture by addition of hexane in the first separation step. Hexane is added in a 3:1 v/v ratio for a six step counter-current extraction procedure (Cerón et al. 2008). After phase separation the hexane will contain biodiesel and free lutein, while the aqueous phase holds the methanol, KOH and reaction side products such as soap and glycerol. Within this step again energy is needed for mixing and pumping, but also for heating of the fraction of hexane lost during recovery towards the extraction temperature of $313K$ (equation (5)). The separation of biodiesel and free lutein is accomplished by addition of a 85% ethanol solution to the hexane flow in a 2:1 v/v ratio (Prommuak et al. 2013). Now only the fraction of 85% aqueous ethanol flow that is lost during recovery (1%) needs to be heated to $313K$, next to the usual energy demands for mixing and pumping. After the second phase separation, the biodiesel can be obtained by evaporation of hexane. To do so, the hexane is first heated towards the vaporization temperature of 70 $°C$ according to equation (5), and then distilled as described by equation (7). The free lutein is obtained from the ethanol solution by further addition of water, until the solution is diluted to 8.5% ethanol and lutein precipitates (Li et al. 2002). By means of pressure filtration the lutein powder is obtained. The energy for pressure filtration is calculated via equation (12) with a volumetric specific energy constant of 2 MJ/m^3. The 8.5% ethanol stream is recycled through distillation (equation (7)). A full recovery of ethanol is assumed and a 85% aqueous ethanol stream is obtained by further addition of water.

A4.2. SUPERCRITICAL FLUID EXTRACTION

The model that describes supercritical fluid extraction (SFE) of lutein and lipids is based upon the results of Nobre et al. (Nobre et al. 2013). SFE was performed on *Nannochloropsis* sp. microalgae with the use of CO_2 as supercritical solvent. By testing the effect of ethanol addition on lipid and lutein extraction yields, it was possible to design a process that extracts lipid fractions with a low and a high lutein content. As a result, a lipid fraction suitable for biodiesel and an oil enriched pigment fraction is produced. Analysis of the composition of pigments showed an increase in the presence of lutein compared to acetone extraction and SFE extraction without the addition of ethanol. The proportion of chlorophyll a was significantly smaller and only increased a little in the presence of ethanol. As *C. protothecoides* is known to accumulate lutein in high quantities, an even larger fraction of lutein is expected to be present in the obtained lipid fraction than what was observed by Nobre et al.

Before the biomass can enter the SFE processing unit, a drying step is required of which the energy demand is calculated via equation (5) and (6). The process is operated at a temperature of 40 $°C$, a pressure of 300 bar and a solvent flow rate of 0.62 g/min. The lipid fraction for biodiesel is obtained using pure supercritical CO_2 and recovers 78% and 26% of the lipids and carotenoids present respectively. Within the second step the solvent flow is enriched with 20 wt% of ethanol. This results in recovery of 28% of the remaining lipids and 50% of the remaining carotenoids. For both separation steps, the total energy demand is determined by the energy needed for pressurizing the solvent towards 300 bar (equation (9)) and for heating of the fraction of solvent that is lost during recovery (1%) towards the extraction temperature (equation(5)). The ethanol flow used in the second extraction step for lutein recovery needs to be evaporated to obtain the oil enriched lutein fraction. Evaporation of ethanol is again calculated through equations (5) and (7).

A5. SEPARATION OF PROTEINS

A5.1. SHIFTING OF PH

The most common method to recover proteins from a solution is precipitation by addition of acid. Protein separation after HPH treatment of *C. vulgaris* cells through acid treatment until a pH of 4 yielded 57% w/w of extracted proteins (Ursu et al. 2014). The model used within this research is based on this result. An acidic solution is added to the aqueous solution containing proteins under mixed conditions (equation (10)). After precipitation occurs, the proteins are separated through pressure filtration (equation (12)). For pressure filtration a volumetric specific energy constant of 2 MJ/m^3 is used. The remaining water content in the recovered protein fraction is removed by drying (equations (5) and (6)).

A5.2. ULTRAFILTRATION

Ultrafiltration is a known separation method which is implemented on industrial scale in for example water purification and food industries (Discart et al. 2014). For biorefinery purposes it is mainly used as a harvesting technique, though implementation for separation of microalgae components is under development (Gerardo et al. 2014). The membranes used for ultrafiltration have pore sizes varying from 1 to 100 nm.

Safi et al. proposed a two-stage ultrafiltration process for separation of microalgae components after cell disruption (Safi et al. 2014). The experiments were conducted on *Tetraselmis suecica* but it was mentioned how adaptation of the membrane pore sizes could make it suitable for other species, such as *Chlorella.* The cells were pre-treated by HPH for release of soluble compounds and subsequently centrifuged to separate the biomass from the suspension. The remaining liquid was further filtered in two steps. For the first separation a membrane was used that retained compounds larger than 100 kDa. This resulted in a retentate consisting of a mixture of polysaccharides, mainly starch, together with aggregated proteins. Also some pigments are present that are attached to small parts of cell debris or dissolved in oil droplets. The permeate was a mixture of small sugars and proteins that could be further separated by a second ultrafiltration step. Here the membrane retained compounds of larger than 10 kDa, which resembled all the proteins and a small part of the sugars. The permeate therefore consisted of a pure sugar solution. A similar set-up was tested by Marcati et al. for the separation of high molecular weight carbohydrates, proteins and sugars derived from *Porphyridium cruentum* (Marcati et al. 2014).

The recovered fractions are dependent on the size of the molecules present inside the microalgae. Ursu et al. analyzed the molecular weight of proteins from *C. vulgaris* which is expected to have a comparable protein composition as *C. protothecoides*. After cell disruption by HPH they measured the presence of complex soluble aggregates of over 670 kDa, consisting of proteins and chlorophyll (Ursu et al. 2014). Protein measurements performed at neutral pH conditions further led to the appearance of a clear band at 25 and 60 kDa. It is therefore expected that the proteins which are not aggregated will pass through the first membrane of 100 kDa, but will be retained by the second membrane of 10 kDa; similar to the observations by Safi et al. As a large part of the proteins after cell disruption are expected to be aggregated, it is assumed that 60% of the soluble proteins will pass through the first membrane. As *C. protothecoides* is grown under N-deprived conditions a low starch content is expected (Pasaribu et al. 2014), meaning that most of the carbohydrates present will be small sugars. Therefore the assumption is made that the 100 kDa and 10 kDa membranes will retain 20% and 10% of the incoming carbohydrates respectively. The small amount of starch present will also decrease the chances of fouling as large-sized polysaccharides often create a polarized layer causes resistance (Morineau-Thomas et al. 2002).

The model of ultrafiltration is based upon these assumptions and the results obtained by Safi et al. The energy input rate for both ultrafiltration steps is described by equation (9) as it is dependent on the pressure. In both cases a pressure equal to 2 bar is assumed to be applied.

APPENDIX B. ADDITIONAL RESULTS

B1. DECISION VARIABLES

Route	Decision variable	Value	Unit
flocculation-centrifuge	cf_f	16.7	-
	cf_c	6	-
DAF-centrifuge	$r_{air\|solids}$	0.1	$kg\ air/kg\ solids$
	cf_c	17	-
Centrifuge	cf_c	100	-
has/hus	P_{HPH}	1240	bar
	N_{HPH}	4	-
hap/hup	P_{HPH}	1256	bar
	N_{HPH}	4	-
bas/bus/bap/bup	u_{bm}	6	m/s

B2. PRODUCT RECOVERY

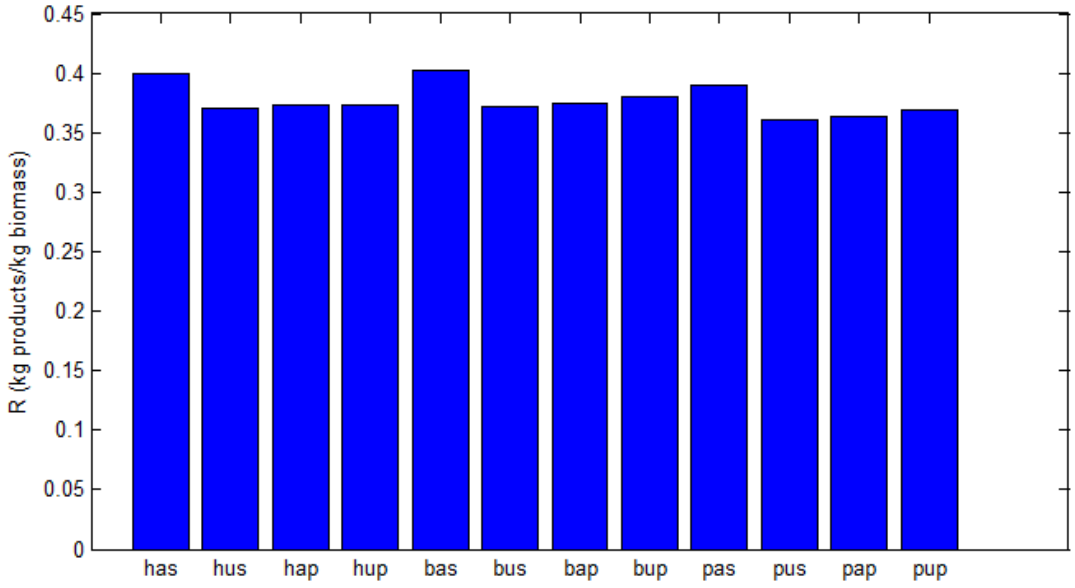


FIGURE 22, KILOGRAM OF RECOVERED END PRODUCTS PER KILOGRAM OF BIOMASS ENTERING THE DISRUPTION UNIT. USEFUL FOR COMPARISON OF ENERGY INPUT RESULTS OF THE HARVESTING AND DEWATERING STEP AND THE DISRUPTION AND SEPARATION STEPS.

B3. NET ENERGY RATIO (NER)

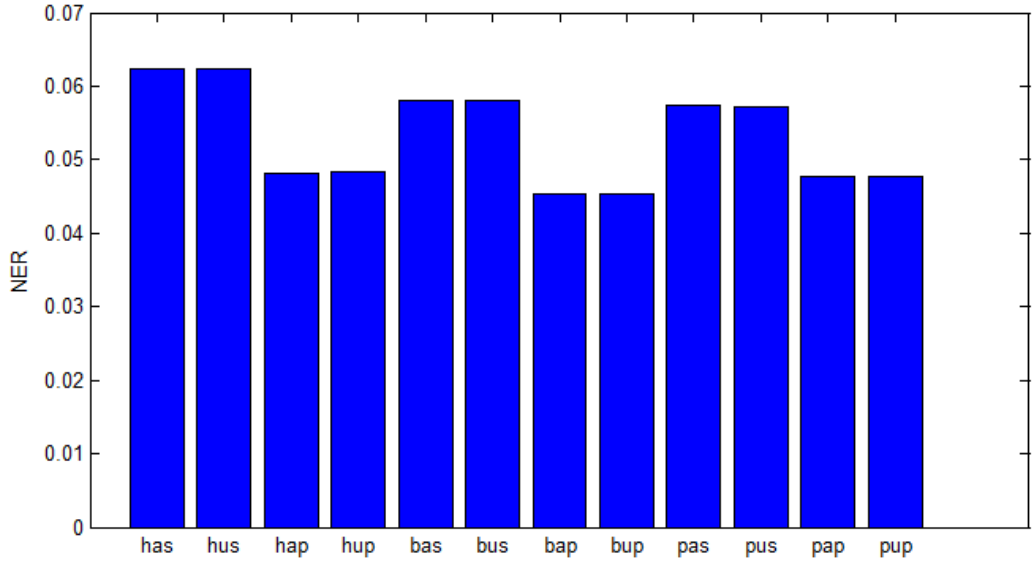


FIGURE 23, NET ENERGY RATIO OF EACH ROUTE. THE NER VALUES WERE CALCULATED BY DIVIDING THE ENERGY GAIN THROUGH BIODIESEL PRODUCTION BY THE RELATIVE ENERGY DEMAND.

B4. MILD BEAD MILLING

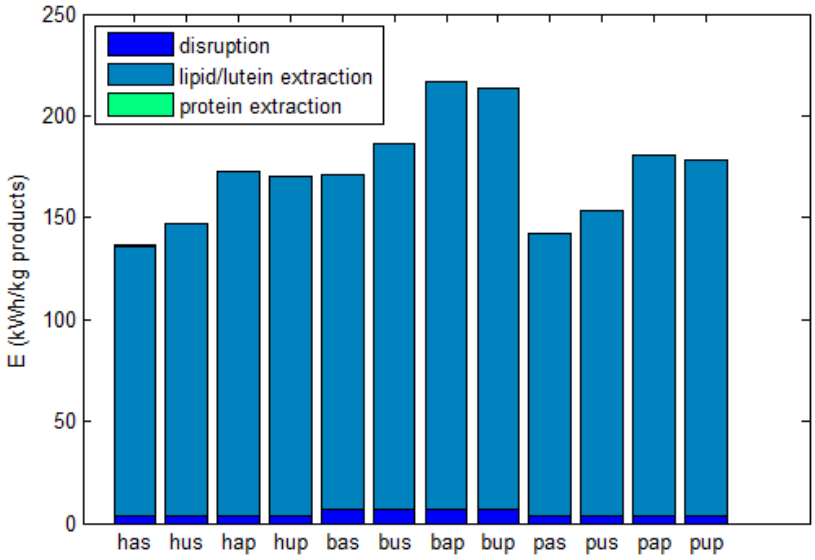


FIGURE 24, THE ENERGY CONSUMPTION PER KG PRODUCTS FOR DIFFERENT DISRUPTION AND SEPARATION ROUTES. IN THIS SCENARIO THE BEAD MILL AIMS AT 87.5% RELEASE OF THE SOLUBLE PROTEIN CONTENT. THE SUBSCRIPT REFERS TO THE ROUTE TAKEN. THE FIRST LETTER SYMBOLIZES THE APPLIED DISRUPTION METHOD: 'H', 'B' AND 'P' STAND FOR HPH, BEAD MILL AND PEF TREATMENT RESPECTIVELY. THE SECOND LETTER SYMBOLIZES THE METHOD USED FOR PROTEIN SEPARATION: 'A' STANDS FOR PH SHIFTING TO ACIDIC ENVIRONMENT AND 'U' STAND FOR THE TWO-STEP ULTRAFILTRATION METHOD. THE THIRD LETTER REPRESENTS THE UNIT USED FOR SEPARATION OF LIPIDS AND LUTEIN: 'S' STANDS FOR SOLVENT EXTRACTION AND 'P' FOR SUPERCRITICAL FLUID EXTRACTION.

B5. SENSITIVITY ANALYSIS

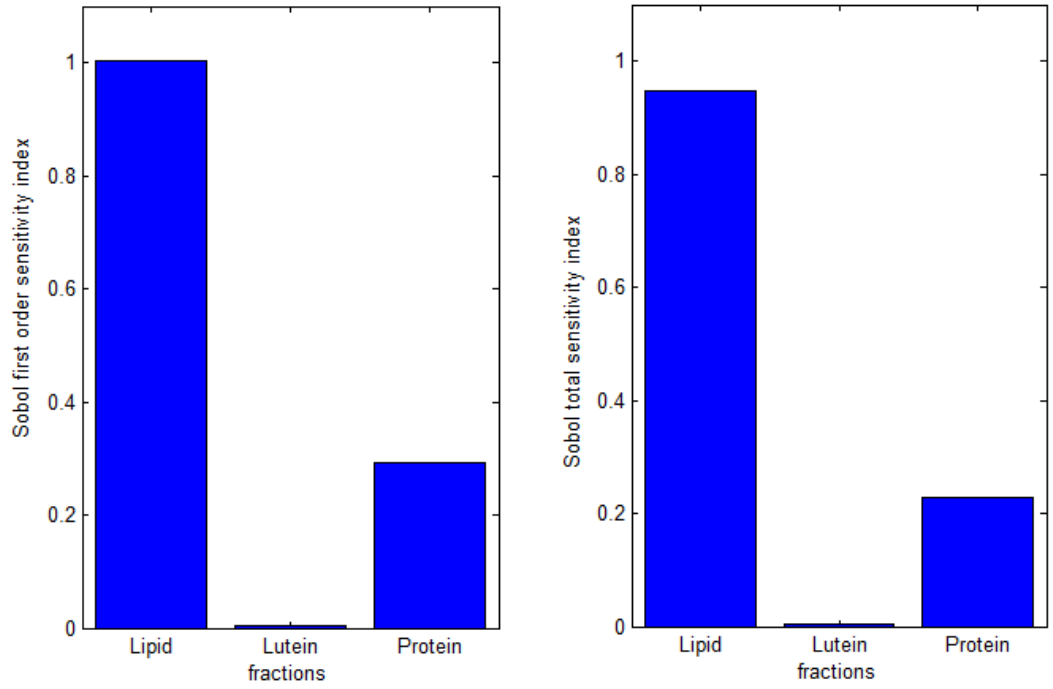


FIGURE 25, SENSITIVITY ANALYSIS OF THE LIPID, LUTEIN AND PROTEIN FRACTIONS ON THE ENERGY CONSUMPTION OF THE ' HAS' ROUTE.

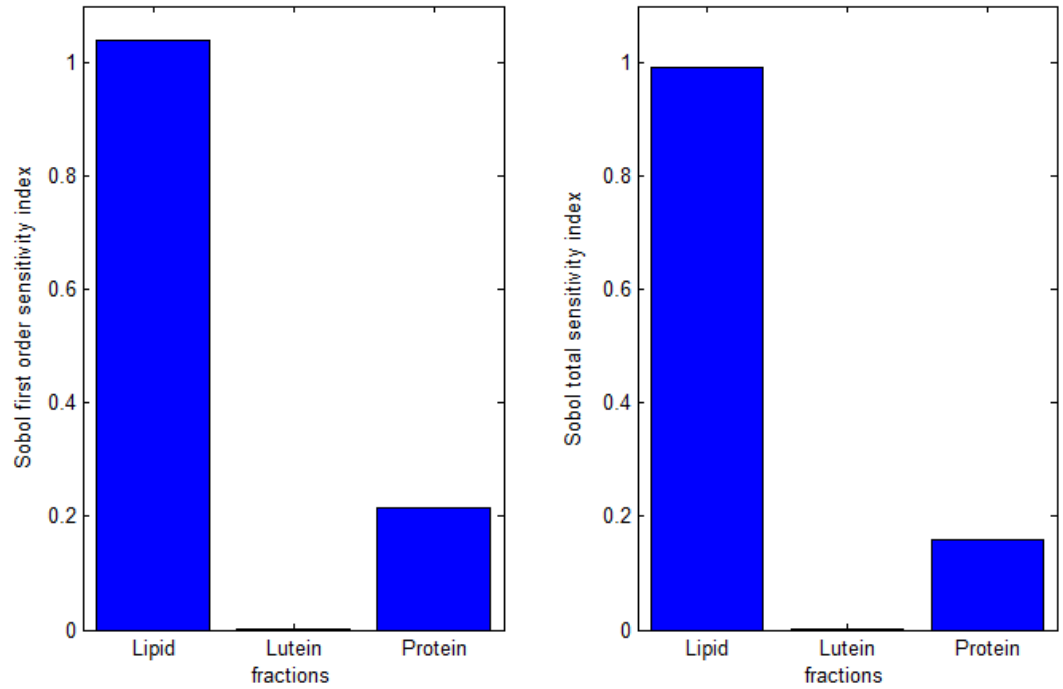


FIGURE 26, SENSITIVITY ANALYSIS OF THE LIPID, LUTEIN AND PROTEIN FRACTIONS ON THE ENERGY CONSUMPTION OF THE ' HUP' ROUTE.