Systematic analysis of anaerobic conversion of microalgal biomass into biomethane aiming for process efficiency optimization

Dissertation zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften (Dr. rer. nat.)

vorgelegt von

Viktor Klassen

Petropawlowka (Kasachstan)

Angefertigt an der Fakultät für Biologie der Universität Bielefeld
Lehrstuhl für Algenbiotechnologie und Bioenergie
unter der Betreuung von Herrn Prof. Dr. Olaf Kruse

April 2016

Erstgutachter: Prof. Dr. Olaf Kruse

Zweitgutachter: Prof. Dr.-Ing. Clemens Posten

Gewidmet in liebevoller Erinnerung,

meinem Großvater Johann Klassen

TABLE OF CONTENTS

Summary

Worldwide depletion of fossil fuel reserves advanced the search for environmental friendly and sustainable alternatives. The fact that microalgae perform very efficiently photosynthetic conversion of sunlight into chemical energy has moved them into the focus of regenerative fuel research, especially since algae cultivation, in contrast to land plants, is not restricted to arable land. Renewable fuel generation via anaerobic fermentation using microalgae biomass for biogas production, compared to biodiesel and bioethanol, is less intensive investigated.

This thesis provides a systematic analysis of parameters influencing the degradability of microalgae biomass in an anaerobic digestion process, with respect to algae species, biomass composition and culture conditions. The biodegradability of twenty different freshwater microalgae species possessing different cell wall characteristics, cultured under comparable conditions and harvested in the same growth phase, was observed to be relatively similar, corresponding to rather low conversion efficiencies of less than 53 % of the theoretical maximum. These findings suggested that the recalcitrance of the cell wall is not the only factor influencing anaerobic digestion, since not every algal species contains a rigid cell wall, further indicating that other parameters must influence the accessibility of algae cells towards decomposition by anaerobic microorganisms.

Naturally occurring nutrient starvation is a direct consequence of algae blooms in late summer, and therefore this natural phenomenon was simulated under controlled conditions and the impact on algae biomass degradability was investigated. Three scientifically and industrially relevant algae strains *Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Scenedesmus obliquus* were therefore cultured in low-nitrogen media (containing insufficient nitrogen source for extensive cell proliferation) and subjected at different growth stages to anaerobic fermentation in batch test. The results revealed a strong correlation of the cell starvation status and biodegradability to biogas, towards complete biomass disintegration at the maximum starvations level (indicated by max. C:N ratio). The feasibility of fermentation of “nitrogen starved” vs “nitrogen replete” microalgae biomass was furthermore investigated in a long term (160 days) continuous lab-scale simulation of an industrial biogas plant. The results of “nitrogen replete” biomass fermentation revealed low conversion efficiency and subsequent fermentations failure caused by high protein content in the biomass. The fermentation of “low nitrogen” biomass, on the contrary, was characterized by very stable process parameters and highly efficient biomass to methane conversion efficiency of 84 %. In comparison to “energy crops” (e.g. maize), usually used for biogas generation, the achieved

methane yield was 37 % higher on biomass basis and approximately 4.5 times higher based on areal productivity (conservative estimation).

In conclusion, this PhD work provides a simple and effective microalgae cultivation method for subsequent use of biomass as mono-substrate for anaerobic fermentation to methane. Highly efficient and stable fermentation process of this biomass was demonstrated in a continuous long-term experiment within this work and enables therefore an efficient industrial scale application.

Abbreviations

µg	microgram
µm	micrometer
AD	anaerobic digestion
ATP	adenosine-5´-triphosphate
BMP	biochemical methane potential
CHP	combined heat and power generator
C	carbon
C:N ratio	carbon to nitrogen ratio
C:N:P:S ratio	Carbon to nitrogen to phosphor to sulfur ratio
CMC	carboxymethyl cellulose
CO_2	carbon dioxide
COD	chemical oxygen demand
CSTR	continuously stirred tank reactor
d	day
DW	dry weight
EC	extracellular
FAN	free ammonia nitrogen
GMO	genetically modified organism
H_2O	water
ha	hectare
HRT	hydraulic retention time
kg	kilogram
L	liter
LCA	life-cycle analysis
LCFA	long chain fatty acid
LED	light-emitting diode
m	meter
mg	milligram
ml	milliliter
N	nitrogen
O_2	oxygen
ORL	organic loading rate
PBR	photobioreactor
PSI	photosystem I
PSII	photosystem II
RNA	ribonucleic acid
ROS	reactive oxygen species
Rubisco, Rbc	ribulose-1,5-bisphosphate carboxylase/oxygenase
SMY	specific methane yield
SRT	solids retention time
TAN	total ammonium nitrogen
TRIS	Tris(hydroxymethyl)aminomethane
VFA	volatile fatty acids
VS	volatile solids
WT	wild type
WWTP	waste water treatment plant

I. Introduction

1. Global energy demand – Photosynthetic energy conversion

Worldwide rising energy demand, limited fossil fuel sources and the threat of anthropogenic global warming have created enormous efforts in the development of renewable energy sources (IPCC, 2007; Martinot et al., 2007; REN21, 2011; REN21, 2015; Verbruggen and Al Marchohi, 2010). Renewable energy technologies using wind, water and solar energy currently cover only a portion of the global required energy demand and are still unprofitable due to high investment and material costs compared to the use of fossil fuels. For instance, nuclear fission represents an established technology for electricity generation and is often regarded as an alternative to fossil derived sources for energy provision with lower CO_2 emission. However, the application of this technology requires high-tech nuclear reactors and is accompanied by great risks and disposal difficulties of the high radioactive waste (BMUB, 2010). Renewable energy based on wind, water, sunlight or geothermal heat has distinct environment advantages, yet at the present time, they need to be financially subsidized for economic feasibility. Moreover, most of current alternative systems mainly produce electricity (e.g. photovoltaic, solar, thermal, nuclear and wind power), however the current global energy request accounts two-third as fuel (Rifkin, 2002).

Sunlight is the most abundant renewable energy source because the light energy reaching the surface of the earth exceeds the global primary energy demand several thousand times (Schenk et al., 2008). Photosynthetic organisms like vascular plants and algae harvest in a process called oxygenic photosynthesis, the light energy and use it to build up biomass. Oxygenic photosynthesis represents a process of sunlight capture and conversion into chemical energy by photoautotrophic organisms, involving the reduction of CO_2 to carbohydrates and the removal of electrons from H_2O, resulting in the release of O_2 and protons. Thereby, the photosynthetic reactions in plants and green algae occur mainly in the chloroplast (Nelson and Yocum, 2006) and are traditionally divided into the "light reactions", which consist of electron and proton transfer reactions and the "dark reactions", encompassing the biosynthesis of carbohydrates from CO_2 and utilizing reducing equivalents and ATP provided by the light reactions.

The use of plant biomass, obtained by the photosynthetic process, is generally regarded a resource alternative to fossil fuels (Brennan and Owende, 2010; Chisti and Yan, 2011; Costa and de Morais, 2011; Dragone et al., 2010; Singh et al., 2011; Stephens et al., 2010b), since equal amounts of CO_2 are assimilated during the photosynthetic growth and released during

biomass conversion, therefore photosynthetic energy sources have a favorable CO_2 balance compared with fossil fuels. First generation biofuels mostly derived from food and oil crops including corn, sugarcane, rapeseed oil, sugar beet, and maize as well as vegetable oils and animal fats, have now attained economic production levels (FAO, 2007; FAO, 2008). However, the use of alternate energy resources akin to terrestrial crops has led to highly controversy discussed food vs. fuel debate (Monbiot, 2004; Tomei and Helliwell, 2015), because this crops place an enormous pressure on world food markets, contribute to water shortages and precipitate the destruction of the world's forests (Amela, 2011; Chakravorty et al., 2009; Harrison, 2009; Hill et al., 2006). The advent of second generation biofuels is intended to produce fuels (e.g. bioethanol), instead of food crops, from the whole plant matter of ligno-cellulosic agricultural residues, forest harvesting residues or wood processing waste, which can be processed into feedstock by either gasification or by cellulolysis via cellulolytic bacteria as well as metabolic engineered yeast (Carere et al., 2008; Eisentraut, 2010; FAO, 2008; Naik et al., 2010; Rutz and Janssen, 2007). Even though very promising, the technology for conversion has not reached yet the scale for commercial exploitation (FAO, 2008; Timilsina and Shrestha, 2011). Third generation biofuels, specifically derived from microalgae, are also considered to be a viable alternative energy resource that is devoid of the major disadvantages associated with first and second generation biofuels (Borowitzka and Moheimani, 2013; Brennan and Owende, 2010; Chisti and Yan, 2011; Costa and de Morais, 2011; Dragone et al., 2010; Formighieri, 2015; Singh et al., 2011; Stephens et al., 2010b). Microalgae can grow on non-arable land using saline or waste water and produce lipids, proteins and carbohydrates in large amounts over short periods of time, which can be processed into biofuels (e. g. biodiesel, bio-ethanol, hydrogen, methane) and valuable co-products (Brennan and Owende, 2010; Gouveia, 2011; Gouveia and Oliveira, 2009; Kruse and Hankamer, 2010; Li et al., 2008; Mata et al., 2010; Mussgnug et al., 2010; Posten and Schaub, 2009; Rupprecht, 2009; Scott et al., 2010).

The conversion of photosynthetic biomass into usable energy forms can be achieved via different processes, leading to three main products: power/heat generation, transportation fuels and chemical feedstocks (McKendry, 2002). The most important established strategies are combustion to generate heat and electricity, conversion of carbohydrate-based compounds into bioethanol and lipids into biodiesel, as well as biomass conversion via anaerobic digestion (AD) into methane-rich biogas.

1.1. Microalga – sources for renewable substrates

Microalgae are often described as “lower” plants that never have true stems, roots, and leaves, and grow photoautotrophically by performing oxygenic photosynthesis (Hallmann, 2007), accounting for the net primary production of approximately 50% of the total organic carbon produced on earth each year (Field et al., 1998). They are mostly eukaryotic, although prokaryotic cyanobacteria are included in algae, and represent a highly heterogeneous group with up to 1,000,000 species, possessing different shapes and capabilities (e.g. flagella for motility, Figure 1), which inhabit all aquatic ecosystems but also some terrestrial habitats such as soils and bogs (Barsanti and Gualtieri, 2014).

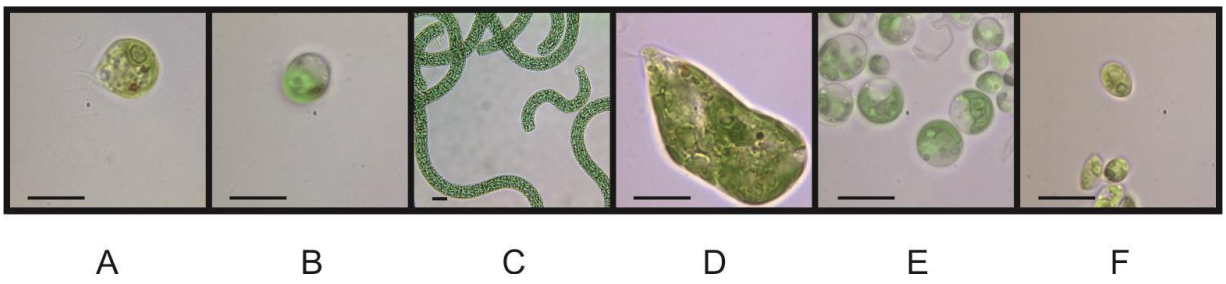

Figure 1: Light microscopic images of selected different microalgal species. (A) *Chlamydomonas reinhardtii*; (B) *Dunaliella salina*; (C) *Arthrospira platensis*; (D) *Euglena gracilis*; (E) *Parachlorella* (formerly *Chlorella*) *kessleri*; (F) *Scenedesmus obliquus*. Scale bars represent 10 µm. Figure modified from own work (Klassen, 2010; Mussgnug et al., 2010).

The most ancient group of photosynthetic eukaryotes is represented by the members of the green lineage (Viridiplantae) including the green algae and green plants, which acquired their plastids in an event of primary endosymbiosis (Keeling, 2010; Leliaert et al., 2012). In this event a heterotrophic eukaryotic host cell captured a photosynthetic prokaryote that became stably integrated and eventually turned into a plastid (Archibald, 2011; Keeling, 2010), leading to the rise of the Archeaplastida, which includes the green lineage (green algae (Chlorophyta) and green plants) as well as red algae (Rhodophyta) and glaucophytes (Glaucophyta) (Ball et al., 2011; Leliaert et al., 2012). Thenceforward, photosynthesis spread widely among diverse eukaryotic protists via secondary and tertiary endosymbiosis, involving captures of either green or red algae by non-photosynthetic protists (Keeling, 2010). Secondary endosymbioses have given rise to different eukaryotic groups of algae either by a single or multiple endosymbiotic events: the chlorarachniophytes (Chlorara-chniophyta), the photosynthetic euglenids (Euglenophyta) and the dinoflagellates (Dinoflagellata), the cryptophytes (Cryptophyta), haptophytes (Haptophyta), photosynthetic stramenopiles (Heterokonta e.g., diatoms, chrysophytes and brown seaweeds) (Archibald, 2011; Baurain et al., 2010; Bodył et al., 2009). All these highly diverse organisms are considered algae as they

are associated with each other through the presence of the plastid, thereby sharing a common link to the photosynthetic ancestor (McFadden, 2001).

This high level of diversity is also reflected in the protecting outer cell wall of different microalgae (Popper et al., 2011; Popper et al., 2014). To date, only little structural information is available for most of the species, but it was observed that microalgae mainly differ in terms of molecular components intra- and intermolecular linkages as well as the overall structure of their cell walls. Many species were described to possess a cell wall of high recalcitrant nature (Burczyk and Dworzanski, 1988; Takeda, 1991) and others are containing only a protecting membrane like the pellicle-complex in *Euglena gracilis* (Buetow and Schuit, 1968; Nakano et al., 1987). The cell wall analysis of different species revealed that they contain many different biopolymers such as proteins {Miller, 1972 #35;Goodenough, 1985 #387}, lipids (Gelin et al., 1999; Kodner et al., 2009), carotenoids (Burczyk et al., 1981) as well as carbohydrates (Kloareg and Quatrano, 1988) including cellulose (Bisalputra and Weier, 1963), chitin-/chitosan-like molecules (Kapaun and Reisser, 1995), hemicellulose (Domozych et al., 1980), pectin (Domozych et al., 2007), and lichenin (Ford and Percival, 1965). However the cell wall of the comparably well studied microalga *Chlamydomonas reinhardtii* (Fig. 1A) (Harris, 2001; Merchant et al., 2007) does not contain cellulose (Adair and Snell, 1990; Horne et al., 1971) and is solely composed of hydroxyproline-rich glycoproteins (Miller et al., 1972). On the other hand, species like *Parachlorella kessleri* and *Scenedesmus obliquus* (Fig. 1E, F), although phylogenetically not very distant from *C. reinhardtii* (Fig. 2), are considered to contain very rigid and recalcitrant polysaccharide-based cell walls.

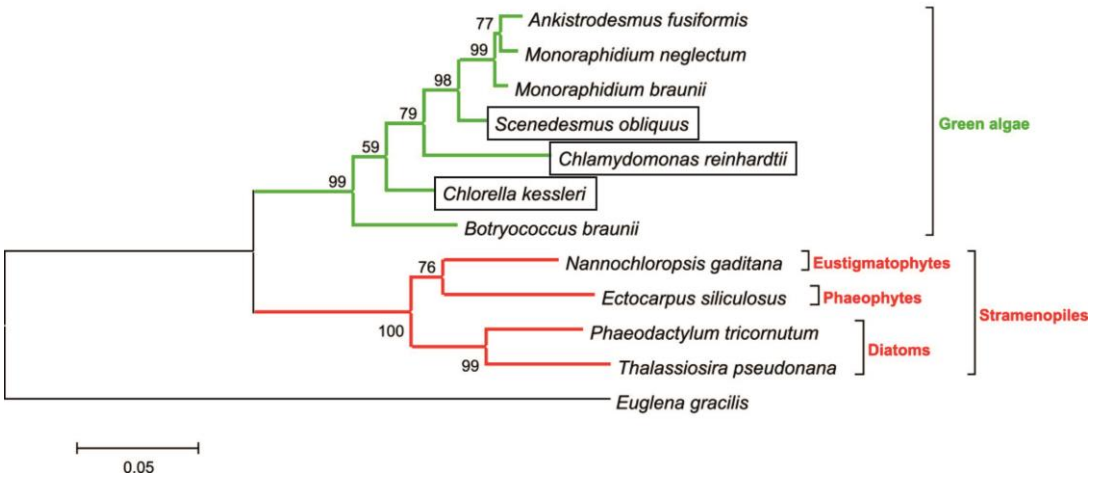

Figure 2: Phylogenetic analysis of selected microalgal strains based on the 18S rDNA sequences using the maximum likelihood method. Species of green algae highlighted with a box are of special interest within this work. Figure modified from (Bogen et al., 2013a).

The analysis of the cell wall constituents of *Scenedemus* sp. and *Chlorella* sp. revealed the presence of glucose, mannose, galactose, xylose as well as uronic acid (Burczyk and Dworzanski, 1988; Takeda, 1991; Takeda, 1996), however exclusively *Chlorella* was shown to contain glucosamine as common dominant cell wall breakdown product (Gerken et al., 2013; Huss et al., 1999; Juárez et al., 2011). This variety of sugar constituents indicates the presence of cellulose, hemicellulose, pectin, chitin and chitosan in microalgal cell walls (Gerken et al., 2013; Popper et al., 2011). Moreover, further analysis revealed that the high recalcitrance of microalgae is attributed to the formation of extraordinarily stable aliphatic polymers like sporopollenin (Burczyk and Dworzanski, 1988) and algaenan (Kodner et al., 2009; Scholz et al., 2014).

Several microalgae serve as model systems for scientific investigations or are of great economic and industrial importance (Fig. 2). For instance, the green unicellular biflagellate *Chlamydomonas reinhardtii* has long been used as a model system for studying photosynthesis, chloroplast biogenesis, flagella assembly and function, cell-cell recognition, circadian rhythm and cell cycle control because of its well-defined genetics, and the development of a comprehensive molecular toolkit (Breton and Kay, 2006; Grossman et al.; Harris, 2001; Harris et al., 2009; Mussgnug, 2015; Rochaix, 1995). Analysis of the complete nuclear genome sequence of *C. reinhardtii* significantly advanced the understanding of ancient eukaryotic features such as the function and biogenesis of chloroplasts, flagella and eyespots, and regulation of photosynthesis (Kreimer, 2009; Merchant et al., 2007; Peers et al., 2009). Several genome projects are ongoing and to date twenty complete microalgal genomes have been sequenced (Blanc-Mathieu et al., 2014; Blanc et al., 2012; Derelle et al., 2006; Fan et al., 2015; Ferris et al., 2010; Foflonker et al., 2015; Matsuzaki et al., 2004; Monier et al., 2012; Palenik et al., 2007; Pombert et al., 2014; Prochnik et al., 2010; Worden et al., 2009) among which many are of commercial/industrial interest like *Nannochloropsis gaditana* (Carpinelli et al., 2014; Radakovits et al., 2012), *Monoraphidium neglectum* (Bogen et al., 2013), *Auxenochlorella protothecoides* (Gao et al., 2014), *Chlorella variabilis* (Blanc et al., 2010), *Parachlorella kessleri* (Ota et al., 2016). The evaluation of the algal genome provides important informations for the understanding and future manipulation and design of molecular tools of the particular strain of biotechnological interest.

Among the eukaryotic, green microalgae of the class Chlorophyceae, the genera *Chlamydomonas*, *Chlorella*, *Scenedesmus*, *Haematococcus* and *Dunaliella* represent the most commonly utilized for current commercial applications (Rosenberg et al., 2008; Spolaore et al., 2006). Currently, microalgae are mainly cultivated as human/animal food source and used

in aquaculture and agriculture and/or as fertilizers. Furthermore, they are used for the production of high-value chemicals, pharmaceuticals, and cosmetics (e.g. polyunsaturated ω3-fatty acids). Moreover the algal biomass is very versatile and represents a rich source of proteins, biopolymers, and polysaccharides as agar, carrageenan, alginates, pigments, vitamins, and antioxidants (Barsanti and Gualtieri, 2014; Bux and Chisti, 2016; Hallmann, 2007; Spolaore et al., 2006).

Many microalgae studied so far are photosynthetic, whilst some are described to grow mixotrophically or heterotrophically by utilizing organic carbon sources like acetate or glucose (Dent et al., 2005; Harris et al., 2009; Lee, 2016). The general requirements for successful microalgal cultivation include light (photosynthetic and mixotrophic), carbon source, macronutrients such as nitrogen, phosphorus, magnesium and silicates as well as several micronutrients (Lee, 2016). A wide-ranging spectrum of phenotypes with specialized adaptation abilities exists within the microalgae, colonizing diverse ecological habitats, from freshwater to brackish, marine and hyper-saline, at different temperatures, pH, and nutrient availabilities (Barsanti and Gualtieri, 2014; Hallmann, 2007; Hu et al., 2008)

1.2. Microalgae mass cultivation methods

The use of microalgae for biofuel production has notable advantages, since they exhibit several attractive features (Formighieri, 2015; Formighieri and Bassi, 2013; Georgianna and Mayfield, 2012). First of all, being photoautotrophic organisms, they are able to produce biomass from solar energy, water and carbon dioxide, which are renewable and cheap components. According to experts, algae can grow faster than food crops, resulting in overall high area yields, containing more fuel than equivalent amounts of other biofuel sources such as soybean, canola or palm oil (Ullah et al., 2014). Microalgae can be grown almost anywhere like non-arable land using saline or waste water and since they require CO_2 for growth, they can be used for bio-fixation and bioremediation (Wigmosta et al., 2011).

The perspective of large scale production of microalgae for biofuel applications is motivated by the high theoretically reachable productivity with an upper value of 263 tons ha^{-1} $year^{-1}$ (Chisti, 2007; Huntley and Redalje, 2007). Another work suggested the maximum theoretical biomass productivity of 0.077 kg m^{-2} d^{-1} (280 tons ha^{-1} $year^{-1}$), corresponding to a solar-to-biomass conversion efficiency of 8-10 % (Melis, 2009). In agricultural production, microalgae are cultivated in open ponds (Fig. 3 a, b), with sunlight driving photosynthetic growth (Chisti, 2016; Georgianna and Mayfield, 2012). In a well-operated raceway pond an average annual dry biomass productivity of around 0.025 kg m^{-2} d^{-1} (corresponding to 91.25

tons ha^{-1} $year^{-1}$) can be achieved (Chisti, 2012; Mendoza et al., 2013; Wolf et al., 2016), however during suitable weather conditions higher daily productivities with up to 0.05 kg m^{-2} d^{-1} (corresponding to 182.5 tons ha^{-1} $year^{-1}$) have been recorded (Grobbelaar, 2000; Moheimani and Borowitzka, 2007; Terry and Raymond, 1985; Weissman et al., 1989), thus representing a solar-to-biomass conversion efficiency of approximately 3 % (Melis, 2009) Nevertheless, 3% efficiency would still be greater in comparison to higher plants, where field trials reported 0.2% of solar to-biomass conversion efficiency and an average of 10 tons ha^{-1} $year^{-1}$ with sugarcane and switch grass (Macedo et al., 2008; Schmer et al., 2008).

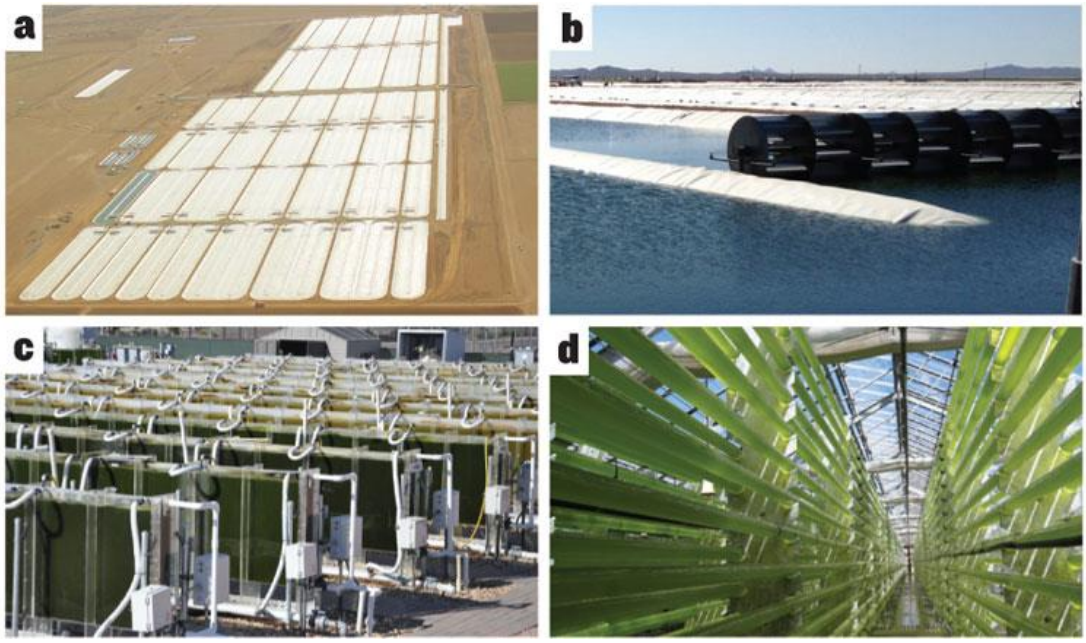

Figure 3: Algae cultivation methods. Figure modified from (Georgianna and Mayfield, 2012) **a**, Algal ponds of 0.5 ha and 1 ha are part of the first commercial-scale algal biofuel facility in the United States at Sapphire Energy's Integrated Algal BioRefinery. They cover an area 400 m wide by 1,600 m long at a location near Columbus, New Mexico. **b**, A single 1-million-litre paddle-wheel driven pond from the Columbus facility. **c**, A pilot-scale flat panel photobioreactor developed at the Laboratory for Algae Research and Biotechnology at Arizona State University in Mesa. **d**, A commercial-scale tubular photobioreactor designed and constructed by IGV and operated by Salata in Germany.

Naturally, not all algae are equally productive (Chisti, 2012) and photoautotrophic cultivation requires supplementation with carbon dioxide, which is insufficient in the ambient atmosphere for the achievement of high biomass productivities. One possible solution could be mixotrophic growth regime as is commonly encountered in high-rate algal ponds treating wastewater (Craggs et al., 2014; Craggs et al., 2012), where dissolved organic compounds contribute to growth leading to a generally higher productivity, reaching dry biomass productivities up to 0.0375 kg m^{-2} d^{-1} (corresponding to 136.88 tons ha^{-1} $year^{-1}$) (Fon Sing et al., 2014). Another impediment for open systems is that they are exposed to high risks of biological contamination by other microalgae species, bacteria, and/or predators owing to the

direct contact of the culture with the atmosphere (Pruvost et al., 2016). In order to overcome this and further obstacles associated with open systems, industrial production uses photobioreactors (Fig. 3c, d), which typically have a tubular or large, thin and flat panel design (Shen et al., 2009). Wolf and co-workers reported productivities of 0.0289 kg m^{-2} d^{-1} (corresponding to 105.49 tons ha^{-1} $year^{-1}$) for tubular reactor and up to 0.0408 kg m^{-2} d^{-1} (corresponding to 148.92 tons ha^{-1} $year^{-1}$) for flat panel reactor (Wolf et al., 2016).

With the use of photobioreactors (PBR) for the microalgal cultivation, many factors can be adjusted for productivity improvement and cost reduction, including culture media and nutrient sources, flow rates and sunlight exposure. Immobilized algal culture systems can be used for growth on a solid surface (Léonard et al., 2011; Shen et al., 2009) to prevent shading and improve photosynthesis, which is especially useful for algae that secrete fuel precursors (Georgianna and Mayfield, 2012). According to the calculations performed in a comparative life-cycle analysis (LCA) of the different growth systems, light-emitting diode (LED)-illuminated PBR produced significantly more biomass (244.67 kg ha^{-1}) than solar-illuminated PBR (8.26 kg ha^{-1}) and open ponds (4.96 kg ha^{-1}) (Georgianna and Mayfield, 2012). However, considering the production costs per kilogram of biomass, this order needs to be reversed: the estimated production costs in open ponds were reported to be about $3 for a kilogram, which was five times and eight times lower than the costs achieved with biomass from solar PBR and LED-PBR, respectively (Amer et al., 2011). From these results can be concluded, that despite higher biomass accumulation in PBR, open ponds are more cost effective because of their significantly lower construction costs, even though with lower total achievable biomass yield (Georgianna and Mayfield, 2012). However, growing algae efficiently and sustainably in fully exposed outdoor ponds remains difficult, and suitable cultivation systems and practices are still under development (Georgianna and Mayfield, 2012).

Nevertheless, for economic competitiveness of biofuels, the achievement of high biomass productivity through an improvement of photobioreactor systems and the photosynthetic efficiency (e.g. antenna engineering), are of great biotechnological relevance and interest in order to accomplish profitable generation of biofuels from microalgae (Beer et al., 2009; Carere et al., 2008; Kruse et al., 2005; Lehr and Posten, 2009; Melis, 2009; Mitra and Melis, 2008; Morweiser et al., 2010; Posten, 2009; Zhu et al., 2010).

1.3. Microalgae – promising feedstock for biofuels production

Microalgae are regarded as a promising feedstock for biofuels due to the high biomass productivity and high theoretical (10-12 %) and practical (~3%) photosynthetic efficiency (Melis, 2009). This group of microorganisms is very versatile and is able to produce large amounts (species dependent, up to 60%) of lipids, proteins and/or carbohydrates over short periods of time, by using only sunlight, carbon dioxide and water. The accumulated compounds can then further be processed into renewable biofuels (e. g. biodiesel, bio-ethanol, methane, hydrogen) and valuable co-products (Fig. 4) (Brennan and Owende, 2010; Bux and Chisti, 2016; Carballa et al., 2015; Gouveia, 2011; Gouveia and Oliveira, 2009; Kruse and Hankamer, 2010; Li et al., 2008; Mata et al., 2010; Posten and Schaub, 2009; Rosenberg et al., 2008; Rupprecht, 2009; Scott et al., 2010; Wijffels et al., 2010).

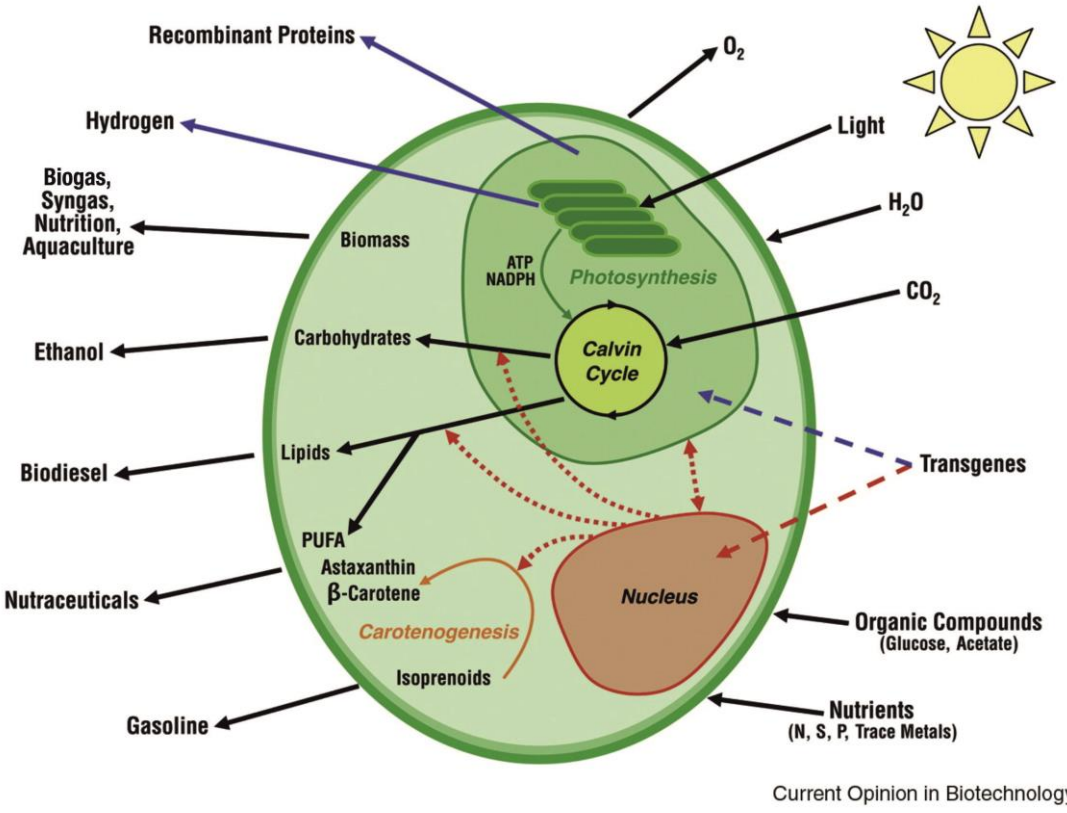

Figure 4: Schematic of commercially important metabolic pathways in microalgae, showing simplified cellular pathways involved in the biosynthesis of various products. Figure reprinted from (Rosenberg et al., 2008).

Confronted with stresses during culturing such as nutrient deprivation, microalgae store chemical energy in the form of oils such as neutral lipids or triglycerides (Hu et al., 2008). The algal oil can be extracted with organic solvents from the organisms and converted into biodiesel with complex processes like transesterification with short-chain alcohols (Amin, 2009; Chisti, 2007), or by hydrogenation of fatty acids into linear hydrocarbons

(Lestari et al., 2009). Furthermore, microalgae produce starch and other carbohydrates as storage compounds, which can be hydrolyzed by hydrolytic enzymes (e. g. amylases) or extracted via chemical or mechanical pretreatment to monomeric sugars and used in fermentation for the production of fuel alcohol like bio-ethanol and bio-butanol (Ellis and Miller, 2016). Diverse microorganisms including bacteria, yeast and filamentous fungi can ferment pentose and hexose sugars of microalgal carbohydrates to alcohols or other products such as acetone (Harun and Danquah, 2011; Harun et al., 2010; John et al., 2011).

Algae also synthesize directly other fuel products, such as hydrogen (Benemann, 2000; Kruse and Hankamer, 2010), ethanol (Deng and Coleman, 1999) and long-chain hydrocarbons, that resemble crude oil (Banerjee et al., 2002), or the algal biomass can be converted to biogas through anaerobic fermentation (Oswald and Golueke, 1960).

2. Biogas generation via anaerobic fermentation

Fermentative biogas generation via anaerobic digestion (AD) is a naturally occurring process that is readily observed when organic matter decomposes in anoxic milieu, e.g. in natural wetlands, rice fields as well as the intestinal tract of ruminants and termites (Deppenmeier, 2002; Walter et al., 2001). Human beings have been using anaerobic digestion processes for centuries, however the first documented digestion plant was constructed in Bombay, India in 1859 (Meynell, 1976). The first usage of biogas from a digester plant for street lightning was reported in 1895 in Exeter, England (McCabe and Eckenfelder, 1958). However, high fuel prices coupled with an increasing awareness of greenhouse gas emissions and global warming have promoted an interest in further anaerobic digestion research and industrial applications (Smith et al., 2001). Nowadays, the AD processes are regarded not only as techniques for treatment of sewage bio-solids, livestock manure, and concentrated wastes from food industry, but also as a potentially significant source of renewable fuel (Azman et al., 2015; Bohutskyi and Bouwer, 2013; Weiland, 2010).

The organic matter is usually composed of complex polymeric macromolecules (often in particulate or colloidal form), such as proteins, polysaccharides, lipids, and nucleic acids (Fig. 5). The ADP converts organic matter to the final products (methane and carbon dioxide), new biomass, and inorganic residue. Several groups of microorganisms (anaerobic bacteria and archaea) are involved in organic substrate transformation to methane, CO_2 and water, and the overall process comprises multiple stages with many intermediate products. Commonly, the process is simplified to four successive phases: (I) hydrolysis; (II) fermentation or

acidogenesis; (III) acetogenesis; and (IV) methanogenesis. The overall transformation, however, can be described rather by six distinct biological processes as shown in Figure 5 (Bohutskyi and Bouwer, 2013; Gujer and Zehnder, 1983).

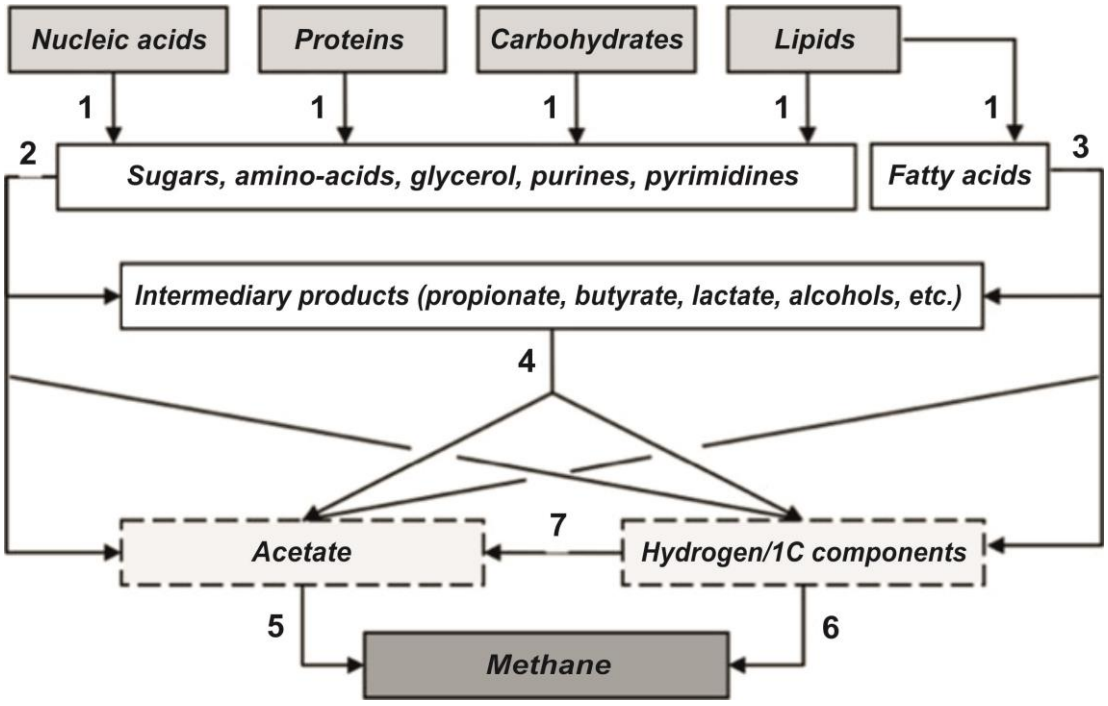

Figure 5: Flow diagram of complex organic matter anaerobic digestion. Figure modified from (Bohutskyi and Bouwer, 2013) (originally modified from (Gujer and Zehnder, 1983), where (1) hydrolysis; (2) fermentation; (3) ß-oxidation; (4) acetogenesis; (5) acetoclastic methanogens; (6) hydrogenophilic methanogens; (7) homoacetogenesis.

1. Hydrolysis of colloid and particulate biopolymers to monomers.

2. Fermentation or acidogenesis of amino-acids and sugars to intermediary products (propionate, butyrate, lactate, ethanol, etc.), acetate, hydrogen, and formate.

3. ß-oxidation of long-chain fatty acids and alcohol fermentation to volatile fatty acids (VFA) and hydrogen.

4. Anaerobic oxidation or acetogenesis of intermediary products, such as VFAs to acetate, carbon dioxide, and hydrogen. This reaction is performed by obligate and facultative hydrogen producing species.

5. Transformation of acetate into methane by acetoclastic methanogens.

6. Transformation of molecular hydrogen and carbon dioxide into methane by hydrogenophilic methanogens.

7. Conversion of variety of mono-carbon compounds (e.g. formate, methanol) to acetic acid, carried out by homoacetogenic bacteria (the same group of microorganisms that as primary fermenters perform the first three steps). These biological processes are sometimes referred to as acidogenesis or the acid-phase (Ghosh et al., 1975). The reduction of sulfur compounds to hydrogen sulfide by sulfur reducing bacteria represents another important biological process in AD.

During AD in biogas plants, organic biopolymers such as lipids, polysaccharides and proteins are converted by anaerobic hydrolytic bacteria into less complex compounds, which then can be further used by other microorganisms. According to microbiological examinations, hydrolytic species are found in a broad range of bacteria phyla and many of these bacteria have developed cell bound multi-enzyme complexes, known as the cellulosomes (Doi and Kosugi, 2004; Felix and Ljungdahl, 1993), for the decomposition of cellulose and hemicellulose containing substrates (Fontes and Gilbert, 2010; Lamed et al., 1983). Many bacterial species do not have cellulosomes (Blumer-Schuette et al., 2011), however they are able to secrete free hydrolases, containing multiple catalytic domains or they produce many other enzymes such as glucanases, hemicellulases, xylanases, amylases, lipases and proteases for efficient biomass hydrolysis (Azman et al., 2015; Weiland, 2010). The abundance of every hydrolytic bacterial species is dependent on the inoculum type of the digester, thus in biogas plants, the members of the phyla *Firmicutes* and *Bacteroidetes* are the most commonly found, while others belonging to *Fibrobacteres*, *Spirochaetes* or *Thermotogae* are less abundant (Azman et al., 2015). Thereby the members of the genus *Chlostridium* (*Firmicutes*) are described to usually dominate the bacterial community in the biogas plant (Burrell et al., 2004; Lucas et al., 2015; Nishiyama et al., 2009; Shiratori et al., 2009; Sundberg et al., 2013; Wirth et al., 2012; Zverlov et al., 2010).

In the next steps, the acidogenesis and ß-oxidation, fermentative bacteria convert the breakdown products of hydrolysis to simple carbonic acids (e.g., propionate, butyrate, acetate, formate, succinate, and lactate), alcohols (e.g., ethanol, propanol and butanol), and other compounds (e.g., H_2, CO_2, VFAs and ketones). Some of these products (e.g., fatty acids longer than two carbon atoms, alcohols longer than one carbon atom, and aromatic fatty acids) are then used by acetogenic or syntrophic bacteria within the acetogenesis-step, for the conversion into acetate and C-1 compounds (Deppenmeier, 2002; Diekert and Wohlfarth, 1994; Drake et al., 1997). Hydrogen producing bacteria, like the homoacetogenic bacteria are *Acetobacterium woodii* and *Clostridium aceticum* are usually described to perform the acetogenesis, resulting in the generation of acetate, CO_2 and H_2 (Weiland, 2010). Even though many details of microbial metabolic networks in a methanogenic consortium are still unclear, there is evidence that hydrogen might be a limiting substrate for methanogens, since the addition of H_2-producing bacteria to the natural biogas-producing consortium increases the daily biogas production (Bagi et al., 2007; Weiland, 2010).

At the end of the degradation series, the metabolic activity of two groups of methanogenic bacteria, namely acetoclastic (utilizing acetate) or hydrogenotrophic (utilizing H_2, CO_2 or formate) methanogens produce methane. Only few species are acetoclastic methanogens, thus able to degrade acetate into CH_4 and CO_2, belonging to the order Methanosarcinales (e.g. *Methanosarcina barkeri* and *Methanotrix soehngenii*) and Methanococcales (e.g. *Methanonococcus mazei*), whereas all methanogenic bacteria are able to use hydrogen to form methane (Deppenmeier, 2002; Schink, 1997; Zinder, 1993). Methanogens of the orders Methanosarcinales, Methanomicrobiales and Methanobacteriales were usually the most abundant within the archaeal sub-community (Deppenmeier, 2002; Pope et al., 2013).

The biogas generation is a sequential process, where the overall digestion speed depends on the slowest, rate-limiting step. For simple degradable substrates like sugars, methanogenesis will often be rate limiting, whereas hydrolysis will often be rate limiting step in case of persistent lignocellulosic plant biomass (Azman et al., 2015; Speece, 1983; Weiland, 2010). In addition, for a balanced anaerobic digestion process, maintaining of valid environmental and operational process parameters for bacterial and archaeal communities is crucial for effective methane production.

2.1. Environmental and operational process parameter

An efficient AD process demands that both substrate degradation and methanogenesis are balanced. In case, the degradation step goes too fast, the acid concentration rises within the digester, and the pH drops below 7.0, which inhibits the methanogenic bacteria. If the methanogenesis runs too fast, methane production is limited by the hydrolytic stage. Thus, the rate-limiting step depends heavily on the particular substrate used for biogas production. However, usually within the anaerobic digester, Achaea receive only a limited energy amount from methanogenesis and possess the slowest growth rate among the anaerobic microorganisms (Bohutskyi and Bouwer, 2013). Altogether, the anaerobic digestion process needs to be controlled in terms of environmental and operational parameters as well as substrate characteristics (Bohutskyi and Bouwer, 2013; Weiland, 2010), since an unbalanced process would lead to an over-accumulation of certain intermediates or byproducts, such as VFAs, ammonia, and hydrogen sulfide, which can lead to inhibition of methane production (Chen et al., 2008; Yenigün and Demirel, 2013).

Important operational and environmental factors

Biogas plants are usually operated either at mesophilic (35–45°C) or thermophilic (45–60°C) conditions and at neutral pH levels. Fluctuating and changing temperatures or pH values within stable bioreactors may cause temporarily or constantly disturbance in methanogenic activity, leading to lower biogas production (Chae et al., 2008; Cioabla et al., 2012; Ferry, 1992).

Other essential parameters for an efficient-operating AD process are represented by organic loading rate (OLR), hydraulic (HRT) and solids retention time (SRT). For instance, the rapid increase of ORL, especially of easily digestible substrate, would cause fast acid formation, leading to alkalinity depletion and pH drop, since the methanogens cannot convert fast enough the produced acids to methane. The HRT determines the volume and capital cost for an AD system, whereas the SRT effects the volatile solids (VS) reduction and, thus the methane yield from biomass. Significant fluctuations in OLR, HRT, and SRT would lead to upset of the AD process and inhibition of the methane generation (Bohutskyi and Bouwer, 2013).

Substrate structure and accessibility (or substrate related factors)

All type of biomass, containing carbohydrates, proteins, lipids, hemicelluloses and cellulose as main components, would be suitable as substrate for biogas production. Only strong lignified organic substances, e.g., wood, are not suitable due to limited accessibility of hydrolases, and therefore, slow anaerobic decomposition (Vidal et al., 2011; Weiland, 2010). Today, most of the agricultural biogas plants digest manure from animals with the addition of co-substrates (e.g. harvest residues, organic wastes from agriculture-related industries, and food waste) in order to increase the content of organic material for the achievement of higher biogas yields (Weiland, 2010). Thereby, the composition of biogas and the methane yield depends on the feedstock type, the digestion system, and the retention time (Braun, 2007). Particle size of the substrate is one of the most important factors that influence the hydrolysis efficiency. Many studies showed increased hydrolysis rates with the particle size reduction (Pereira et al., 2012; Zhang et al., 2004) and consequently the overall digestion process improvement.

Furthermore, for stable maintenance of the microbial community, macro- and micronutrients are indispensable and the availability of carbon, nitrogen, phosphor, and sulfur should be in a relative ratio of 600:15:5:1 (Weiland, 2010). Trace elements like iron, nickel,

cobalt, selenium, copper, zinc and molybdenum, are also essential and have to be supplemented when they are not present in adequate amounts (Abdoun and Weiland, 2009; Kida et al., 2001; Noyola and Antonio, 2005). Additionally, the ratio of carbon and nitrogen (C:N ratio) is very important and should preferably be in the range of 15-30 (Braun, 1982; Weiland, 2010; Zubr, 1986), because ammonia accumulation as a consequence from protein rich (low C:N biomass substrate), is very detrimental and can lead to AD process failure (Bohutskyi and Bouwer, 2013). On the other hand, too high C:N-ratios (higher than 30, carbohydrate rich biomass) could lead to growth limitations of the microbial community within the digester and thereby also reduce the digestion efficiency.

2.2. Biogas generation from microalgae

Anaerobic digestion represents a promising application of algal biomass for methane production, since microalgae are considered an advantageous substrate due to high biomass productivity, low ash content and the reduced competition for arable land. Furthermore, AD has the potential for integration within an algae biorefinery for bioenergy provision as well as nutrients recovery (e.g. nitrogen, phosphorus) for reuse in microalgae cultivation (Bux and Chisti, 2016; Georgianna and Mayfield, 2012; Murphy et al., 2015).

The choice of optimal algal strains can conceivably lead to fast and efficient conversion of biomass to methane. For instance, the brown algae *Macrocystis pyrifera* has been long regarded for biomass-to-methane conversion because of its ease of harvesting and high growth rate as well as biogas yield (400,000 L per ton of VS) (Chynoweth, 2002). Several microalgae species have also been investigated for biogas production (Mussgnug et al., 2010), showing a strong species dependency for the biogas potential. Especially, the digestible nature of the microalgal cell, which in turn depends on the type of cell wall (chapter 1.1), is a key factor determining the biogas yield. Already, Golueke and colleagues demonstrated the problem of resilient algal cell walls by showing that a significant fraction of microalgal cells remained intact during the anaerobic fermentation (Golueke et al., 1957). Observations of *Chlorella vulgaris*, subjected to AD, revealed that based on the chlorophyll concentration increase, the cells continued growing for two weeks (Hernandez and Cordoba, 1993). Similar observations could also be made for *Scenedesmus obliquus*, where intact cells could be identified in an anaerobic digester after an experimental duration of up to six months (Klassen, 2010; Mussgnug et al., 2010). In the same study, six distinct microalgal species were compared regarding recalcitrance towards bacterial attack and it was shown that biogas yields correlated with the degree of cell decomposition. Species devoid of a rigid cell wall or

possessing a cell wall composed of glycoproteins and lacking cellulose or hemicellulose (e.g. *D. salina, C. reinhardtii, E. gracilis* and *A. platensis*) displayed a better digestibility than species with a rigid cell wall containing hemicellulose and sporopollenin (e.g. *C. kessleri* and *S. obliquus*) (Mussgnug et al., 2010). Regarding the biogas production potential, *C. reinhardtii* was shown to be the most efficient biogas substrate (587 mL g VS^{-1}), followed by *D. salina* (505 mL g VS^{-1}), representing an equivalent of 90 % and 77 % of the biogas yield from maize silage, respectively. Beside biogas yield, the relative amount of methane among biogas components determines the biogas quality, and all microalgae tested showed higher specific methane content (ranging from 61 to 67 %) compared to maize (54 %) (Mussgnug et al., 2010), suggesting the potential of algae for the production of quality biogas.

An easily degraded cell wall, or the total absence of a cell wall, does not necessarily imply that a microalga is a good substrate for AD. Other factors, such as productivity or sensitivity to contamination as well as the presence of inhibitory substances (e.g. toxic compounds produced by algae, high salts concentration, heavy metals) may also influence degradability and have to be considered (Hildebrand et al., 2012; Uggetti et al., 2016). However, when a specific microalgal strain of choise is considered for biogas production due to the high productivity and other valuable characteristics, but possesses a rigid cell wall, resistant to AD, a suitable pretreatment step might be necessary (e.g. thermal, biological or mechanical). Many studies dedicated to the anaerobic digestibility of microalgae after pretreatment have been performed and are summarized in numerous reviews to this topic (Bohutskyi and Bouwer, 2013; González-Fernández et al., 2012; Montingelli et al., 2015; Passos et al., 2014). However, for the decision-making whether a certain pretreatment is energetically worthwhile undertaking, improved estimates of the energy demands of the various pretreatments are required.

Additionally, the biogas production of microalgal biomass also depends on its compositions, for instance, high lipid content in the biomass can be advantageous because the theoretical biogas yield from lipids is generally higher (1390 L kg VS^{-1}) than proteins (800 L kg VS^{-1}) or carbohydrates (746 L kg VS^{-1}) (VDI-4630, 2006). However, excess lipid and/or protein content, which is usually observed in microalgae (Becker, 2007; Brown et al., 1997; Prochazkova et al., 2014), means also low C:N ratio in a range of 5-9 (Lardon et al., 2009; Yen and Brune, 2007) and may lead to accumulation of ammonia and long chain fatty acids (LCFAs) during AD, which are important inhibitors of anaerobic microorganisms (Angelidaki et al., 1999; Chen et al., 2008; Yenigün and Demirel, 2013). Co-digestion of microalgal biomass and carbon-rich substrates like corn stalks and waste paper, might be one possible

strategy to ensure more balanced C:N ratio (Park and Li, 2012; Shuchuan et al., 2012; Yen and Brune, 2007).

In conclusion, anaerobic digestion of microalgae is certainly promising, but requires pretreatment of the biomass and/or co-digestion with carbon-rich co-substrates. In the present state of knowledge, an AD of microalgae as mono-substrate is not feasible for industrial application.

II. Specific aims

Increase of the world human population and higher energy demand and simultaneously depletion of fossil fuel reserves have created an enormous demand of renewable energy source, especially CO_2 neutral sources represent an important issue in order to minimize the greenhouse effect (IPCC, 2015; REN21, 2015). Photosynthetic microalgae are increasingly considered as feedstock for renewable energy production such as biodiesel, bioethanol and biogas (Bux and Chisti, 2016; Daelman et al., 2016; Montingelli et al., 2015; Uggetti et al., 2016). However, due to comparably high biomass generation expenses and down-stream processing costs these systems are still not economically viable.

Biogas generation via anaerobic fermentation represents one of the most efficient biomass conversion processes, where most of the biomass compounds can be converted to methane and carbon dioxide with high efficiency (up to 88 % of energy can be transformed to methane (Raposo et al., 2011). However, due to natural recalcitrance of algae cells this efficiency can only be reached after energy or cost intensive pretreatment procedures. Moreover high protein content of the microalgae biomass complicates the fermentation process, by increasing the risk of ammonia inhibition and therefore the biomass must be co-fermented with other carbohydrate-rich substrates.

First aim of this work was to identify crucial parameters influencing algae biomass accessibility towards anaerobic decomposition, with respect to different algae species, since the identification of species with low cell wall recalcitrance and high methane yields can be useful in order to reduce process costs by avoiding all kinds of pretreatment. In order to elaborate a more universal applicable procedure, whereby different algae strains can be used, another strategy was persecuted. In this case natural occurring limitations in nutrient supply were investigated with respect to the influence of starvation impact on anaerobic degradability. Additionally, the possibility of altering of biomass composition by targeted deprivation of certain macronutrients was investigated. The primary aim was it, to reduce the protein content in the biomass without negative effect on biomass accumulation rate, in order to reduce anaerobic fermentation process imbalances caused by unbalanced C:N ratio in algae biomass.

Altogether, the optimization of methane productivity from algae biomass with the aim of cost reduction and process simplification was in the main focus of this work in order to allow later sustainable application at industrial scale.

III. Discussion

Microalgae are regarded as a potential biomass feedstock for the production of biofuels. This very diverse group of microorganisms performs photosynthetic conversion of sunlight into chemical energy and produces thereby large amounts of proteins, lipids and carbohydrates, which then can be converted into biofuels via anaerobic digestion. According to the present knowledge, the use of microalgae as substrate for anaerobic fermentation is not feasible for industrial application because of poor degradability of the algal biomass. Therefore for the efficient biogas production the biomass requires currently physical or biological pretreatments and co-digestion with carbon-rich substrates. The present work is dedicated to the systematic analysis and further understanding of anaerobic digestion of microalgae as mono-substrate into biomethane with the aim of the overall process efficiency optimization.

The five manuscripts presented in this thesis are:

[1] Blifernez-Klassen, O., **Klassen, V.**, Doebbe, A., Kersting, K., Grimm, P., Wobbe, L., Kruse, O. (2012) Cellulose degradation and assimilation by the unicellular phototrophic eukaryote *Chlamydomonas reinhardtii*. *Nature Communications* **3**: 1214

[2] Bogen, C., **Klassen, V.**, Wichmann, J., La Russa, M., Doebbe, A., Grundmann, M., Uronen, P., Kruse, O., Mussgnug, J.H. (2013) Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production. *Bioresource Technology* **133**: 622–626

[3] Klassen, V., Blifernez-Klassen, O., Hoekzema, Y., Mussgnug, J.H., Kruse, O. (2015) A novel one-stage cultivation/fermentation strategy for improved biogas production with microalgal biomass. *Journal of Biotechnology* 215, 2015: 44–51

[4] Klasen, V., Blifernez-Klassen, O., Wobbe, L., Schlütter, A., Kruse, O., Mussgnug, J.H. (2016) Efficiency and biotechnological aspects of biogas production from microalgal substrates. Submitted to *Journal of Biotechnology*

[5] Klassen, V., Blifernez-Klassen, O., Wibberg, D., Winkler, A., Chaudhari, S., Kruse, O. (2016) Highly efficient conversion of photoautotrophic grown algae biomass in to biomethane via anaerobic fermentation. (manuscript in preparation)

1. Microalgae potential for biofuel

Microalgae represent an extremely diverse taxonomic group (McFadden, 2001), including both cyanobacteria and eukaryotic organisms that occur in various natural habitats. Most of the microalgae investigated, are photoautotrophs, whereby some of them are also capable to grow mixotrophically or heterotrophically, consequently implying a wide genetic and metabolic diversity (Apt and Behrens, 1999). Microalgae accumulate a variety of metabolites and products within the cell (Hejazi and Wijffels, 2004; Pulz and Gross, 2004; Wijffels et al., 2013), but are also known to secrete enzymes into the aqueous environment. In addition to already well characterized extracellular enzymes such as carbonic anhydrases (Baba et al., 2011), the ability of the green algae *Chlamydomonas reinhardtii* to secrete hydrolytic enzymes was elucidated during this PhD thesis [**1**]. The investigation of the cellulose digestive system of *C. reinhardtii* revealed that this species secretes endo-1,4-ß-glucanases, which perform the degradation of cellulosic material to cellobiose and cellodextrins, and imports them into the cells, where they are converted to glucose by β-glucosidases [**1**]. In fact, it is well known that plants, some bacteria, fungi, protozoa, and sea squirts, which synthesize cellulose, should also be able to degrade or modify it during growth and development (Gilbert, 2010). However, the existence of cellulase encoding genes in the genome of *Chlamydomonas* cannot be explained by the requirement of cell wall rearrangement, because its cell wall is solely composed of hydroxyproline-rich glycoproteins (Adair and Snell, 1990; Horne et al., 1971; Miller et al., 1972). Accordingly the data provided within this work, suggest that *C. reinhardtii* is capable of cellulose degradation and the assimilation of the breakdown-products like cellobiose, mainly for mixotrophic growth especially under CO_2 limiting conditions [**1**].

This and other abilities of microalgae let these microorganisms appear very attractive for commercial use for biofuel and valuable product formation within a biorefinery complex (Bux and Chisti, 2016; Georgianna and Mayfield, 2012; Murphy et al., 2015). However, the formation capacity of the particular product is always dependent on algae strain and/or culture conditions, however the natural accumulation of some interesting metabolites like astaxanthin or α-tocopherol is relatively low (Lorenz and Cysewski, 2000; Tani and Tsumura, 1989), since the microalgae are in most cases evolutionary forced to cell division and biomass accumulation, rather than specific product formation. To overcome these drawbacks, genetic modification can be applied to certain algal species for the enhancement of distinct metabolic/enzymatic pathways or the establishment of new metabolic pathways in order to

increase product formation and enzyme secretion rates for customized solution (Gimpel et al., 2015; Lauersen et al., 2013; Rasala et al., 2012).

In general, photoautotrophic alga cultivation in large scale facilities is attractive for biofuel generation since it is, in contrast to bacteria or yeast not depended on heterotrophic energy source, and uses natural light energy, available in excess. Outdoor cultivation, however for optimal light-use efficiency requires more area compared to heterotrophic fermentation processes, which might be not easy and expensive for the cultivation of genetically modified organisms (GMO), since they have to be cultivated under controlled conditions in enclosed PBR (Henley et al., 2013). Given the fact, the large scale cultivation and use of microalga in the near future will be only possible with wild-type strains, accordingly with the associated drawbacks of biomass versus product formation.

Microalgae biomass is basically comprised of proteins, lipids and carbohydrates (Bux and Chisti, 2016), whereby the particular content of each can vary in dependence of strain and culture condition. Some of these compounds like proteins can be used for food industry, other like carbohydrates and lipids for fuel production, with the disadvantage that only this one part of the biomass is used, leaving the rest biomass as waste and for the extraction energy or solvent intensive methods have to be applied (Bux and Chisti, 2016; Chisti, 2007; Ellis and Miller, 2016; Spolaore et al., 2006). Another promising strategy could be fermentation of the biomass to biogas/methane via anaerobic digestion, since in this process most of the biomass can be converted in gaseous fuel (Uggetti et al., 2016).

1.1. Anaerobic fermentation of microalgal biomass

The total conversion efficiency of biomass to methane via anaerobic fermentation process can be quite high from 85 to 88 % (Raposo et al., 2011), whereby the rest is used for metabolic activity and cell growth of microbial community within anaerobic digester. However practically this is only true for biomass entirely accessible to anaerobic digestion process such as cellulose, starch or mung bean (Raposo et al., 2011). Microalgae, however, due to their natural habitats (aqueous or soil environments) where they have to resist to a variety of other microorganisms have evolved efficient protective mechanisms (Amaro et al., 2011; Senhorinho et al., 2015). The recalcitrance of microalga cells towards anaerobic microbes during the anaerobic fermentation was repeatedly observed by different authors (Golueke et al., 1957; Hernández and Córdoba, 1993; Klassen, 2010; Mussgnug et al., 2010). Thereby, the researchers described that microalga were not only able to resist the microbial disintegration but in some cases were able to proliferate under this mesophilic fermentation

conditions (Hernández and Córdoba, 1993). Within a comprehensive literature comparison regarding anaerobic digestion of microalgae biomass [**4**, chapter 4.2, Table 1], it became apparent that the fermentation of microalgae (without consideration of the individual species) is not efficient, since the achieved yields often do not exceed more than 50% of the theoretical methane potential (TMP) [**4**, chapter 4.2]. Nevertheless, some studies indicate that the choice of the species can be crucial for fermentation efficiency (Mussgnug et al., 2010), given the fact that cell wall composition of different species may vary considerably (Popper et al., 2011; Popper et al., 2014).

On the other hand, Frigon and colleagues reported significantly different methane yields for the same species *Scenedesmus sp*.-AMDD, which were achieved during fermentation, when the strain was cultured in different media (410, 340, 306 mL CH_4 g^{-1} VS for biomass grown in wastewater, municipal wastewater effluent and Bold´s 3N medium, respectively) (Frigon et al., 2013; McGinn et al., 2012). This strain was identified as the best performer (with respect to methane yield) from other 19 tested microalgae species in this study Frigon et al. 2013. Interesting is also the fact that other researcher testing *Scenedesmus* for biogas generation, reached much lower methane yields 38 % of TMP (mean from 10 other *Scenedesmus* stains 208±23 mL CH_4 g^{-1} VS) [**4**], thus qualifying this genus as one of the most recalcitrant tested so far [**4**]. This observation suggests that the biogas production potential of microalgae not only depends on the individual species, but might be rather more dependent on media and culture conditions applied for the biomass generation.

In order to test more systematically the influence of the applied microalgae species on methane productivity, 24 different strains were cultured under same conditions (with the exception for marine strains, the used media contained 500 mM NaCl compared to 17.1 mM for freshwater media), harvested in the mid logarithmic growth phase and subjected to AD batch test [**2**]. The resulting methane yields were in the range of 39 – 60 % of TMP and appeared to be very similar to the published values of other microalgal species [**4**, chapter 4.2]. At this point, it should be noted that all tested marine strains like *Navicula salinicola*, *Dunaliella tertiolecta*, *Dunaliella spec*. and *Spirulina platensis* showed the highest yields, representing 60 %, 57 %, 56 % and 56 % of TMP, respectively, whereas the fresh water species ranged between 39 and 53 % of TMP [**4**]. Slightly higher methane yields from marine microalgae can be attributed to the osmotic stress, to which the cells were exposed by inoculation in a mesophilic batch reactor. These results are also consistent with earlier findings where marine strains *Dunaliella salina* and *Arthrospira platensis* were shown to be very fast disintegrated after the addition to the anaerobe reactor (Mussgnug et al., 2010).

Despite the positive effect on disintegration and fermentation efficiency of marine algae in batch trails, no further investigations were performed within this work regarding marine species. The main reason was the possible severe inhibitory effect of NaCl (contained in culture media) on anaerobic microbial community in continuous fermentation trails (Chen et al., 2008). Theoretically, it is possible to eliminate the salt with sophisticated dewatering methods (Soomro et al., 2016), however, this work was primary aiming for the improvement of microalgae degradability without additional energy intensive steps (e.g. physical, chemical, enzymatically pretreatments), preferably associated with cost saving possibilities for later large scale applications of this technology.

Altogether, the achieved fermentation efficiency for the freshwater strains harvested in mid-logarithmic growth phase was observed to be in a similar range between 39 and 53 %, suggesting that all tested microalgal strains showed more or less comparably low degree of degradability [**2**]. Furthermore, the monitored methane values were comparable to the published data (< 50 % of TMP) for other microalgal species [**4,** chapter 4.2, Table 1]. Moreover, the studies with achieved high methane productivities (Bohutskyi et al., 2014; Frigon et al., 2013; Grimm et al., 2015; Mahdy et al., 2014b; Mussgnug et al., 2010) might be explained by the difference in the media composition, as indicated by Frigon et al. (Frigon et al., 2013), since indeed microalgae biomass composition can vary significantly depending on nutrition status and culture condition (Becker, 2007; Brown et al., 1997; Hu et al., 2008; Prochazkova et al., 2014). This has a direct impact on the theoretical methane potential (TMP) since different biomass compounds have a distinct energy density and accordingly different TMPs on the weight basis (Heaven et al., 2011). So, based on elemental composition of the compounds (protein, carbohydrates and lipids), the TMP potential can be calculated using the Buswell equation (Symons and Buswell, 1933). Thereby, TMP of carbohydrates, proteins and lipids is at the level of 415 mL, 446 mL and 1014 mL CH_4 g^{-1} VS, respectively. According to this, microalgae with high lipid content would possess higher TMP, and the biomass rich in carbohydrates and protein would possess a rather comparable TMP (Table 1).

Table 1: Theoretical methane potential (TMP) of microalgae biomass. Biomass composition is extrapolated on the basis of literature values [**4**], (Bux and Chisti, 2016; Markou et al., 2013).

Microalga biomass composition	Protein % of DW	Carbohydrates % of DW	Lipids % of DW	TMP CH_4 ml/g VS
typical composition	60	20	20	**553**
carbohydrate rich	20	60	20	**541**
lipid rich	20	20	60	**781**

However, the use of biomass with high lipid content for AD tests seems not be very frequent, since based on comprehensive literature examination [**4**], only biomass from *Nannochloropsis salina* (oleaginous marine microalgae), having high lipid content, was used for AD (Schwede et al., 2013a; Schwede et al., 2013b). Despite comparable high lipid content (36 % of DW), the attained methane yield was comparable low (200 mL CH_4 g^{-1} VS), corresponding to 31 % of TMP ([**4**], (Schwede et al., 2013b), indicating that other factors such as cell wall resistance might prevent the complete biomass disintegration. Authors were able to overcome natural cell wall resistance of the *Nanochoropsis* cells (Scholz et al., 2014) after applying thermal pretreatment, and achieved superior methane yields (570 mL CH_4 g^{-1} VS), in comparison to other research work [**4**, Table 2], (Schwede et al., 2013b). However the conversion efficiency of 89 % was only reached after energy intensive (thermal 120°C for 8 h) treatment, clearly showing that cell wall recalcitrance is the main drawback for an efficient AD [**4**], (Bohutskyi and Bouwer, 2013; González-Fernández et al., 2012; Montingelli et al., 2015; Passos et al., 2014; Uggetti et al., 2016), despite the high TMP.

In conclusion, the differences in the composition of the cultivation media and therewith associated differences in cell structure play a crucial role for anaerobic degradability, suggesting that algae cells under nutrient limitation stress, in contrast to replete conditions can be subjected to significant physiological changes (Hu et al., 2008; Prochazkova et al., 2014) and might therefore be more or less accessible for bacterial disintegration.

1.2. Anaerobic digestion of nitrogen limited microalgal biomass

It is generally accepted, that microalgal cells subjected to nitrogen or sulfur depleted conditions, recycle other, under these conditions less important proteins like RuBisCo or cell wall proteins for *de novo* synthesis of photosystem-related and protective proteins (González-Ballester et al., 2010; Grewe et al., 2014; Plumley and Schmidt, 1989; Sugimoto et al., 2007; Takahashi et al., 2001; Zhang et al., 2004), in order to further maintain photosynthetic activity and thereof accumulate storage compounds such as carbohydrates or lipids. However, this reshuffling of proteins may alter the cell wall function, usually serves as a barrier for other microorganisms. Indeed, some experiments, using different enzymatic pretreatments prior anaerobic digestion, elucidated the importance of protein compounds for cell wall recalcitrance [**4**, chapter 4.3.1]. Mahdy and co-workers treated three microalgae strains (*Chlamydomonas*, *Chlorella* and *Scenedesmus*) with carbohydrolases and proteases (Mahdy et al., 2014a; Mahdy et al., 2014b; Mahdy et al., 2015), whereby their results showed that protease application was more efficient than carbohydrolase-treatment [**4**]. Thus, despite the

general view that complex carbohydrates in the cell wall are responsible for the low digestibility of microalga, these studies suggest that the hydrolysis of cell wall proteins represents another equally important bottleneck for the AD process (Mahdy et al., 2014a; Mahdy et al., 2014b; Mahdy et al., 2015). Some further indications for the better biodegradability of nutrient starved phytoplankton biomass can be found in ecology related studies, where researchers often observe higher methanogenesis in natural or simulated environments (Tezuka, 1989; West et al., 2012; West et al., 2015), when nitrogen starved biomass is used. For instance, West and colleagues used *Scenedesmus* biomass, cultured under low-N and high-N condition for the biomass generation with high and low C:N ratios, respectively. (Here, it should be noted that microalgae cultivation in low-nitrogen media leads to the formation of biomass with high C:N ratio and *vice versa*). Subsequently, this biomass was then added to fresh water lake sediments and resulted in much higher methane production for high C:N ratio biomass compared to the low C:N ratio biomass (West et al., 2015). Since these results were acquired under psychrophilic conditions (temperature 4 °C), it was interesting to investigate, whether the same tendency can be observed under mesophilic fermentation conditions (temperature 38°C), which are more influential for industrial scale for biofuel generation.

For the investigation of the influence of the starvation degree of microalgal biomass on methane productivity (via AD) under mesophilic conditions, a novel experimental setup was designed and accomplished with three prominent (scientifically or industrial relevant) algae species (*Chlamydomonas reinhardtii* CC-1690, *Parachlorella kessleri* SAG 211-11h and *Scenedesmus obliquus* SAG 276-1) [**3**]. The technical innovatory degree in experimental setup was in minimizing AD test volume from 60 to 2 ml and thus minimizing by 30 times the required amount of algal biomass, because of the dispensation ability of microalga cells in aqueous environment no drawbacks in reproducibility could be observed (unpublished test experiments). Additionally, the setup envisaged direct application of algae for anaerobic test after harvesting and concentration, without need for storage (freezing, cryo-desiccation or drying), since this is a kind of pretreatment can lead to artificial results (Gruber-Brunhumer et al., 2015; Mussgnug et al., 2010), [**4**] regarding degradability and the subsequent methane yields. The main biological purpose of the experiments was to elucidate biochemical methane potential (BMP) of different microalgae species throughout all phases of nitrogen starvation, by periodically sampling the algae PBR, where the cells subsequently pass over in nitrogen starvation, and application of that biomass immediately to AD test (for more details see [**3**]).

The accomplished results showed a clear dependence of starvation state of the cells (indicated by C:N ratio) and methane formation potential [**3,** Fig. 2 C and Fig 4]. Interestingly, in the early cultivation stage, where nutrient limitation could not yet be observed, at least on biomass composition level, indicated by low (5-9) C:N ratio [**3,** Fig. 2 A, C], the achieved methane yields were quite low [**3**, Fig.4], thus indicating low conversion efficiency (<50 of TMP), and thereby confirming the values described in literature [**3** and **4,** Table 1]. Additionally, the so called "positive outliers", with inexplicably high methane yields (with BMP near the TMP) [**4**] (Bohutskyi et al., 2014; Frigon et al., 2013; Grimm et al., 2015; Mussgnug et al., 2010), could also be confirmed within the same experimental setup, since similarly high methane values could be achieved [**3**, Fig. 4] in the later cultivation phase, where nitrogen limitation was at maximum level (indicated by high C:N ration of 24-26) [**3,** Fig. 2 C]. These results enable a clarification of the previously described variety of fermentation data ([**4**], chapter 1.1): namely, it is the starvation state of cells, which impacts the anaerobic bio-degradability of the microalgal biomass and consequently leads to higher methane productivity. For the verification of these findings on other level than methane yields (since TMP can change significantly if lipids are accumulated, see Table 1), the degrading behavior of microalgae cells was observed and evaluated during the AD [**3**]. The observations confirmed the great effect of starvation status on algae cell disintegration efficiency [**3** Fig. 3]. Especially results achieved for *Chlamydomonas* strain were particularly clear, while nearly all cells from early growth phase could resist anaerobic disintegration [**3**, Fig. 3 time point 0], they were completely disintegrated after starvation [**3**, Fig. 3 time point 6 to 10]. Furthermore, the achieved maximal biomass to methane conversion efficiency rates (87 %, 82 % and 73 % of TMP for *Chlamydomonas*, *Parachlorell*a and *Scenedesmus*, respectively), were only comparable to the values reached after application of most efficient pretreatment strategies such as thermal and enzymatical [**4**, chapter 4.3.1, Table 2].

However, although the conversion efficiencies of *Chlorella* and *Scenedesmus* based on the evaluation of methane yields [**4**] were near the optimum (82 % and 73 % of TMP, respectively) [**3**], the assessment of the cell disintegrations level via cell count revealed that ~70% of *Chlorella* and ~50% of *Scenedesmus* cells or cell-shape-like structures could still be counted [**3**, Fig. 3]. At first glance, this finding was very contradictory, since the conversion efficiency and cell disintegration levels are normally expected to have the same degree. The main reason for this discrepancy was the manual cell count, where all particles (appearing as cell-shape-like structures) were precautionary counted as cells. In fact, many cells were partially or completely empty, [see **3**, Fig. 3, microscopic image of *Scenedesmus obliquus*

after fermentation, time point 10], where apparently only the cell wall was present and the cell content was absent. Furthermore, these results correlate well with the current literature knowledge (see Introduction chapter 1.1) regarding the differences in the cell wall composition of investigated algae strains *Chlamydomonas*, *Parachlorella* and *Scenedesmus*. The recalcitrant compounds like sporopollenin in *Scenedesmus* and chitosan in *Parachlorella* cell walls (Gerken et al., 2013; Juárez et al., 2011; Kapaun and Reisser, 1995) are well-known for their poor degradability under anaerobic conditions (Golueke et al., 1957; Mussgnug et al., 2010; Wieczorek et al., 2014), so that it is conceivable that the cell wall on itself might not be digestible. However, it might be that during the ongoing nitrogen starvation, the rearrangement/reshuffling of the cell wall compounds of *Parachlorella* and *Scenedesmus* enabled in some way the anaerobic microorganisms the access to compounds inside the cells, thereby consequently increasing the overall methane yields. On the other hand, the cell wall of *Chlamydomonas reinhardtii* is mainly composed of hydroxyproline-rich glycoproteins (Miller et al., 1972), which apparently are sufficient for the protection of the cells against microbial attack or disintegration under replete conditions but can be completely disintegrated after reshuffling under nitrogen limitation [see **3**, Fig. 3, microscopic image after ferm. *Chlamydomonas reinhardtii*, time points 0 and 10].

In conclusion, a simple and effective cultivation method was developed within this thesis, for the enhancement of microalgae biomass accessibility to anaerobic degradation, and thereby maximizing the methane production in batch fermentation to optimal levels [**3**, **4**]. The application of nitrogen limitation during photoautotrophic growth of the microalgae was not only increasing the accessibility of the biomass to higher methane conversions rates but also reduced the required nutrient input amount for biomass generation. However, despite the promising results achieved within the batch fermentation trails, the transferability of these findings from batch to continuous fermentation process is only partially possible, since batch procedures provide only information regarding the maximum possible methane potential and biological degradability of the particular biomass and do not allow any statements regarding the overall process stability or fermentability of mono-substrates for a continuously fed reactor. Therefore, a realistic assessment of the feasibility of above presented approach for industrial scale fermentation can only be performed after the implementation of continuous fermentation.

1.3. Anaerobic fermentation of microalgal biomass in semi-continuous mode

Biogas generation from plant material and different waste streams represents a well-established technology worldwide (Weiland, 2010), with the employment of different reactor types in dependence of substrate and process requirements. One of the most frequently applied reactor type is the continuously stirred tank reactor (CSRT), which allows very simple technical process implementation (VDI-4630, 2006). Despite the relatively simply technical implementation, the biological complexity of the process is comparably high, since different strict and facultative anaerobe microorganisms are involved in the substrate disintegration and methane formation process (Bohutskyi and Bouwer, 2013; Gujer and Zehnder, 1983) (see also Introduction chapter 2). For an efficient performance, all microbial participants have to cooperate with each other permanently (Azman et al., 2015; Deppenmeier, 2002; Goswami et al., 2016), and this evolutionary very old interplay among the microbial community seems to have evolved some self-regulating mechanisms. Nevertheless, utilization of various substrates can sometimes lead inevitably to process imbalances and thus to fermentations failure. Some substrate composition compounds (e.g. high protein content, antibiotics, toxic compound) are known to imbalance the fermentation process, others (e.g. unknown toxins, microelements, heavy metals) are not detailed tested yet, but are presumed to cause similar effects (Bohutskyi and Bouwer, 2013; Hildebrand et al., 2012; Uggetti et al., 2016). Additionally, factors like temperature, pH, organic loading rate (OLR), hydraulic retention time (HRT), nutrient availability, oxidation-reduction potential and substrate particle size can also be crucial for the continuous process (Speece, 1983). Therefore, new or modified substrates for biogas generation have first to be tested in a continuous fermentation setup in lab scale, before they can be transferred to industrial scale, since the results obtained in the lab have an enormous impact on the configuration and design of a large-scale plant (VDI-4630, 2006).

Continuous fermentation of microalgae as mono-substrate showed, according to literature [**4**, Table 3] very low biomass to methane efficiencies (Collet et al., 2011; Golueke et al., 1957; Passos et al., 2014; Ras et al., 2011; Schwede et al., 2013b; Wirth et al., 2015), most likely due to high recalcitrance of algae cells against anaerobic community. In order to overcome the resistance of microalgae cells, many researcher applied biomass pretreatments (successfully in increasing biomass degradability) prior the continuous fermentation (Mahdy et al., 2015; Markou et al., 2013; Mendez et al., 2015; Schwede et al., 2013b). Despite higher biomass accessibility as a consequence of the applied pretreatment, still the fermentation resulted in low efficiency and process instability [**4**, chapter 4.3.2]. The main reason for this poor productivity might be the high protein content of the substrate, which leads to

ammonium/ammonia accumulation within the reactor, thereby inhibiting primary the methanogenic community (Yenigün and Demirel, 2013). This is not surprising, since microalgae biomass is well-known to possess high protein content (50-60 % of DW) (Becker, 2007; Brown et al., 1997; Prochazkova et al., 2014), which is also indicated by low C:N ratio of microalgal biomass (Lardon et al., 2009; Yen and Brune, 2007) [**3**, **4**]. For an efficient fermentation, substrates with C:N ratios in the range from 15 to 30 are preferable (Weiland, 2010), and lower ratios were described to cause ammonia inhibition (Bohutskyi and Bouwer, 2013; Uggetti et al., 2016). Based on the results achieved in batch trails, where the importance of starvation degree and associated C:N ratio for efficient biomass degradability were elucidated, it was shown that biomass with higher C:N ratios un a range of 20-26 possess optimal accessibility to the microbial community in the digester ([**3**], chapter 1.2).

In order to investigate, whether nitrogen starved biomass is also fermentable in continuous manner, a long term setup with two reactors was designed, where beside the promising nitrogen starved biomass, also biomass cultured under replete nitrogen conditions was used [**5**]. The cultivation conditions for the microalgae biomass generation were based on previous results [**3**], with slight modifications: light intensity was lower 300 instead 400 µmol photons $m^{-2}s^{-1}$ and buffer substance TRIS was removed (in order to reduce associated costs) from media composition (remark: no negative growth effects could be observed in the test phase, unpublished result) [**3**, **5**]. According to batch trails biomass pretreatment via storage was avoided, for this directly after harvesting and concentration biomass was stored at 2 °C for maximal 2 weeks before feeding [**5**]. Microalgae growth performance was periodically monitored via gravimetrical measurements of organic dry biomass weight, and revealed no significant differences in biomass productivity until harvesting time point (6 d) [**5**, Fig. 1], suggesting that the usage of nitrogen limited medium had no disadvantageous effect on the biomass productivity. Furthermore, the analysis of algal biomass composition revealed significant differences, especially the protein content was considerably reduced in the biomass cultured with limited nitrogen amount (low-N biomass, 28 % DW), compared to biomass cultivated in nitrogen replete conditions (replete-N biomass, 61 % DW). Reduced protein content in low-N biomass led as expected to an increase of the C:N ratio (16.3) [**5**, Table 1], which is within the optimal range of 15-30 for an efficient fermentation (Weiland, 2010). On the other hand C:N ratio of replete-N biomass was with 6.9 significantly lower than desirable for continuous fermentation, however corresponding to typical range (5-9) described for microalgal biomass produced under nutrient replete conditions (Oh-Hama and Miyachi, 1988; Yen and Brune, 2007). Additionally, it is important to note that the lipid content did not vary

significantly in both types of biomass, leading to the estimation of similar TMP values of 554 and 552 $mL_N\ g^{-1}$ VS, for replete-N and low-N biomass, respectively [**5**, Table 1].

The operation of the continuous biomass fermentation was performed in consideration to the guideline (VDI-4630, 2006), where a sludge adaptation phase and stepwise organic loading rate (OLR) increase is recommended. The simultaneously process management of both types of algae biomass revealed significant differences regarding biogas productivity and all tested process parameters, despite the fact that the same microalgae strain (*Chlamydomonas reinhardtii* CC-1690) and same inoculum of anaerobic organisms (from waste water treatment plant (WWTP), Bielefeld, Heepen) were used [**5**].

Obvious differences could be observed during the continuous fermentation regarding biogas and methane productivities, although equal amounts of were fed and similar theoretical biogas values were estimated based on TMP [**5**], thus indicating lower conversion efficiency rate for the fermenter fed with replete-N biomass. Overall, higher biogas productivity was not only observed in the adaptation phase (OLR 1, fed with 1g $L^{-1}\ d^{-1}$), but also at OLR2 (fed with 2g $L^{-1}\ d^{-1}$) for low-N reactor reaching a biogas productivity of 761 $mL_N\ d^{-1}\ g^{-1}$ VS for low-N biomass and 634 $mL_N\ d^{-1}\ g^{-1}$ VS replete-N biomass [**5** Fig. 2, Table 2]. At higher loading rates (OLR 4, fed with 4g $L^{-1}\ d^{-1}$), productivity of the reactor fed with low-N biomass stayed on comparably high level (750 mL_N biogas $d^{-1}\ g^{-1}$ VS), while productivity in the control replete-N reactor gradually decreased [**5**, Fig. 2], resulting in a mean biogas productivity of only 203 $mL_N\ d^{-1}\ g^{-1}$ VS. However, for an accurate evaluation of the biomass conversion efficiency, methane productivities have to be taken into account, which were in the range of 464 and 462 $mL_N\ d^{-1}\ g^{-1}$ VS for low-N biomass and 416 and 131 $mL_N\ d^{-1}\ g^{-1}$ VS replete-N biomass in OLR2 and 4, respectively [**5** Fig. 2, Table 2]. Considering the theoretical methane potential (TMP) of the biomass, the achieved conversion efficiency of the process was at 84 % for low-N reactor (OLR2 and 4) and only at 75 % and 24 % for replete-N biomass reactor (OLR 2 and 4 respectively). Moreover, assuming the energy demands of the microbial community for maintenance, metabolic activity and *de novo* synthesis requires 12-15 % of chemical energy contained in the biomass (Raposo et al., 2011), the overall biomass-to-energy conversion of the low-N biomass was even more efficient (with 96-99 %) from the practical point of view [**5]**. Correspondingly to productivity, various process parameters (pH, TAN, FAN and VFA) were analysed and confirmed a well-balanced and stable fermentation in low-N reactor and subsequent ammonium (mainly indicated by TAN and FAN) increase in replete-N reactor [**5**]. Increasing ammonium concentrations within the fermenter contribute to raise of pH and formation of ammonia at higher concentration (> 50 mg L^{-1}), thereby inhibiting the

methanogens (Yenigün and Demirel, 2013). Since acetoclastic methanogenesis contributes up to 73 % of formed methane (Deppenmeier, 2002; Smith and Mah, 1966), inhibition of methanogens can led to volatile fatty acid (primary acetate) accumulation in the reactor supernatant, thus can accessory inhibit microbial community (Azman et al., 2015; Craggs et al., 2014; Uggetti et al., 2016; Weiland, 2010). This could also be observed within this work, in the replete-N reactor direct after the shift to ORL4, ammonia concentration increased to maximum value of 73 mg L^{-1} at day 112 and at the same day extraordinary VFA accumulation was initialized and continuously increased until the end of experiment (maximum 15 g L^{-1}) [**5** Fig. 3, replete-N BM]. However, similar ammonia concentration were also observed in the same reactor at OLR2, but for reasons currently not known, this raise did not lead to a significant increase in VFA concentration at this time point [**5**, Fig 3]. One crucial factor might be the lower TAN concentration (~1500 mg L^{-1}) at this OLR, which increased to 2249 mg L^{-1} at day 112 (OLR 4). Inhibitory TAN levels of 1700-1800 mg L^{-1} are considered as inhibitory (Yenigün and Demirel, 2013), to a not acclimated anaerobic community, which has been also confirmed by the results achieved during this PhD thesis.

The disturbing effect of the replete-N biomass on fermentation process was additionally observed in the analysis of the microbial community in both reactors via high-throughput 16S rDNA gene amplicon sequencing [**5**, Fig. 4]. The highest diversity of microorganisms (603 ± 52 OTUs) could be detected in the inoculum samples, which was not surprising, since WWTP are known to be confronted with very high substrate diversity, and therefore high microbial variety has to be involved for an efficient fermentation process. By application of a mono-substrate in continuous fermentation trails only proliferating species can survive in the reactor due to washout, since proliferation rate is determined especially by substrate availability (Blume et al., 2010; Carballa et al., 2015; Schlüter et al., 2008). Consequently, lower species diversity could be determined after 100 days in both reactors, low-N (269 OTUs) and replete-N (178 OUTs) biomass digester and after 160 days species diversity decreased even further to 111 and 177 OUT for replete-N and low-N reactors, respectively [**5**, suppl. Table S1]. However, qualitative differences in community composition are more important than quantitative changes. During the fermentation in reactors with optimal (no clear inhibition) performance (low-N OLR 2 and 4, replete-N OLR 2) the phyla *Bacteroidetes* dominated clearly the bacterial community, whereas the acetoclastic family of *Methanosaetaceae* was dominant among the Archaea. On the other hand, the inhibited fermentation process (replete-N OLR 4) was mainly predominated by the bacteria phyla *Firmicutes and Thermotogae* and archaeal populations changed from acetotrophic to hydrogenotrophic methanogenesis with the

species *Methanoculleus* sp. mainly replacing *Methanosaeta* sp. [**5**, Fig. 4, suppl. Fig. S5]. Similar observation could be made in other studies, suggesting that an unexpected shift of the predominant phyla within the bacterial community or the replacement of certain species, especially of the dominant *Methanosaeta* sp. by *Methanoculleus* sp., is to be interpreted as a potential warning indicator of acidosis (Blume et al., 2010; Carballa et al., 2015; Goux et al., 2015).

In summary, the data presented above clearly demonstrated the feasibility of using a simple method (nitrogen limitation), in order to change microalgae physiology from unfavorable towards optimal substrate for anaerobic fermentation. The fermentation of low-N algae biomass was shown to be a very stable and highly efficient process, in contrast to replete-N biomass. The method presented here, is in comparison to other attempts (pretreatment efforts) an all in one solution for algae biomass fermentation, since the hurdle of algae cell recalcitrance as well as the problem with the high protein content in the biomass is terminated at once (without energy investments in pretreatment). The feasibility of the continuous fermentations (of low protein biomass) changed towards stable and highly efficient, instead of inoperable in the case of the control fermentation (high protein biomass). Additionally, due to low protein content, significant amount of fertilizers can be saved, thereby significantly reducing cultivation costs.

2. Economic aspects of biogas generation from nitrogen limited microalgae biomass

According to the achieved results in this PhD thesis, it can be stated that microalgae biomass cultivated under nitrogen limited conditions [**3**, **5**], represents in contrast to other research accomplished under replete or undefined culturing conditions (Golueke et al., 1957; Mahdy et al., 2015; Markou et al., 2013; Melbinger et al., 1971; Mendez et al., 2014; Ras et al., 2011; Samson and Leduyt, 1986; Schwede et al., 2013b; Wirth et al., 2015) [**2**, **4**, **5**], a very productive substrate for anaerobic digestion.

In order to evaluate the economic perspectivity and feasibility for this technology, the knowledge obtained within this work will be combined with literature results, regarding microalgae outdoor cultivation and compared to economics of biogas generation from renewable plant material, as anestablished and economically applied/applicable technology. For this purpose, the methane yield of so called “energy crops” will be compared with methane productivity of microalgae biomass tested in this work. The methane productivity of plant material always depends on biological and physical parameters of the substrate, whereby lignin content (Givens and Deaville, 2001; Grabber et al., 2008; Grabber et al., 2009) and

substrate particle size (Cone et al., 2008) represent the most important factors, often lowering the degradability efficiency. This is also evident from the achieved methane yields (Table 2) of different energy crops, for instance plant biomass like maize, grass and sunflower, containing certain amounts of lignin (Demirbaş, 2002; Saxena and Stotzky, 2001; Vogel, 2008) for structural stability are less efficient convertible into methane compared to plants like sugar beet, fodder beet and wheat grain, which do not contain any significant amounts of lignin (Beaugrand et al., 2004; Foster et al., 2001).

Table 2: Methane yields of commonly used energy crops, calculated based on literature values from (Weiland, 2010).

Usually used energy crops	Methane yield ($mL_N\ g^{-1}$ VS)
Maize (*Zea mays*)	291 - 338
Maize cob	350 - 360
Grass (*Poaceae*)	286 - 324
Sunflower (*Helianthus*)	231 - 297
Red clover (*Trifolium pratense*)	297 - 347
Sugar beet (*Beta vulgaris*)	387 - 408
Fodder beet (root vegetable from *Beta vulgaris*)	398 - 424
Wheat (*Triticum*)	351 - 378
Wheat grain	371 - 398
Rye grain	297 - 413
Sorghum (*Sorghum bicolor*)	286 - 319
Triticale (*Triticum* x *Secale*)	319 - 335

For the yield maximization, drawbacks like high lignin content as well as substrate particle size can be overcome by application of various pretreatment strategies, however normally the energetic investment costs are higher than the return in additional methane productivity (Herrmann and Rath, 2012). On the contrary, microalgae biomass does not suffer from the drawbacks mentioned above, since the algal cells are typically unicellular and small in size and mostly do not contain lignin (Barsanti and Gualtieri, 2014; Domozych et al., 2007), therefore fermentation should yield in higher methane amounts. For instance, the comparison of the methane productivity of the most frequently used substrates like maize with 338 $mL_N\ CH_4\ d^{-1}\ g^{-1}$ VS and grass with 324 $mL_N\ CH_4\ d^{-1}\ g^{-1}$ VS (Table 2, (Weiland, 2010)) with the productivity achieved within this work for microalgae biomass (462 $mL_N\ CH_4\ d^{-1}\ g^{-1}$ VS) reveals a higher efficiency for microalgae biomass by 37 % and 43 % on the VS basis than maize or grass, respectively [**5**]. Furthermore, the microalgae biomass is also by 13 % and 22 % more productive compared to lignin free substrates like sugar beet and wheat with maximum reached productivities of 408 and 378 $mL_N\ d^{-1}\ g^{-1}$ VS, respectively (Table 2, (Weiland, 2010). However this comparison is based on VS-basis, whereby the areal

productivity (tons ha^{-1} $year^{-1}$) of biomass can have a more significant effect for commercial productivity and usability. For a more conservative comparison of the areal productivities of plant substrates suitable for biogas generation and microalgae biomass, only maximal productivities of plant material (Table 3) will be taken into account.

Table 3: Areal productivity of energy crops from different locations in Germany. (Data adopted from (Brauer-Siebrecht et al., 2016)).

Energy crops	Biomass productivity (tons ha^{-1} $year^{-1}$)
Maize (*Zea mays*)	20 - 27
Sugar beet (*Beta vulgaris*)	11 - 23
Wheat grain	8 - 9

Maize has the highest areal productivity with up to 27 tons ha^{-1} $year^{-1}$ (Table 3) and may therefore be the main crop grown for biogas production in Germany (Brauer-Siebrecht et al., 2016). Sugar beet is less productive with maximal 23 tons ha^{-1} $year^{-1}$, whereas wheat is performing worst of the tested plants and that is why it is not recommended by the authors for biogas application (Brauer-Siebrecht et al., 2016).

The biomass of microalgae, based on the theoretical calculations, can reach areal productivities of 263 tons ha^{-1} y^{-1} (Chisti, 2007; Huntley and Redalje, 2007) or even 280 tons ha^{-1} y^{-1} ((Melis, 2009) assuming solar-to-biomass conversion efficiency of 8-10 %. However, for a realistic economic evaluation only practically achieved productivities will be considered (Table 4).

Table 4: Microalge areal biomass productivity in different cultivation systems.

Microalgae cultivation system	Biomass productivity (tons ha^{-1} $year^{-1}$)	Literature source
Raceway pond	91	Chisti, 2012; Mendoza et al. 2013; Wolf et al. 2016
Raceway pond (optimal weather)	182	Moheimani and Borowitzka, 2007; Weissmann et al. 1989
High-rate pond (mixotrophic, wastewater)	137	Fon Sing et al. 2014
Photobioreactor	150	Carlsson et al. 2007
Photobioreactor (tubular)	105	Wolf et al. 2016
Photobioreactor (flat panel)	112-149	Wolf et al. 2016

Alga cultivation, in contrast to agriculture plants is not restricted to arable land, but this fact also means that culture vessels have to be installed (e.g. raceway ponds or photo-bioreactors). Thus, cultivation systems can have different productivities, for instance, closed photobioreactors are regarded to be more efficient due to more optimal light distribution and contamination prevention, which was also confirmed by practical experiments with biomass productivities up to 150 tons ha^{-1} $year^{-1}$ could be reached (Table 3, (Carlsson A.S. et al.,

2007; Fon Sing et al., 2014; Wolf et al., 2016). On the other hand, this cultivation method is also associated with higher acquisition costs compared to raceway pond cultivation systems (Georgianna and Mayfield, 2012). Generally, production of microalgae biomass in raceway ponds represents a less expensive and more established system, because it is used since 1966 for commercial production of algae biomass or high value products (Oren, 2005). The productivity of this system seems to be somewhat lower by 91 tons ha^{-1} $year^{-1}$ (Chisti, 2012; Mendoza et al., 2013; Wolf et al., 2016), however under optimal weather conditions (in the summer time) even more efficient values could be achieved (Moheimani and Borowitzka, 2007; Weissman et al., 1989) (Table 4). However, for the following calculations the more conservative and practically evaluated raceway pond productivity of 91 tons ha^{-1} $year^{-1}$ was considered.

Table 5: Comparison of areal methane productivities of energy crops and microalgae biomass. Microalgae methane yields are given on VS basis (experimental data from this PhD thesis [5]), other experimental data derived from (Brauer-Siebrecht et al., 2016; Weiland, 2010; Wolf et al., 2016).

Biomass	Methane productivity (m_N^3 ha^{-1} $year^{-1}$)
Maize (*Zea mays*)	9126
Sugar beet (*Beta vulgaris*)	9386
Wheat grain	3578
Microalgae	42042

In order to estimate the areal methane productivity by anaerobic fermentation of energy crops and microalgae biomass, experimentally proven biomass and methane yields were combined (microalgae methane yield on VS basis, experimental data from this PhD thesis [**5**], other experimental data from literature). The evaluation of the values shown in Table 5 clearly demonstrates that the methane productivity from microalgae biomass is approximately **4.5** times higher than values from the best energy crop. However, although areal methane productivity from microalgae is significantly higher, due to higher production costs associated with algae biomass generation (Introduction, chapter 1.2), this may represent an obstacle for economic use nowadays. Nevertheless microalgae are characterized by high diversity of by-products (Introduction, chapter 1.3), which can be generated concomitantly to biomass generation, and thereby improve significantly the total economics.

IV. Perspective

Wide-ranging application of microalgae for fuel generation, mainly driven by social and ecological motives (e.g. use of non-arable land and reduction of GHG emissions), depends in first place on positive economics (Georgianna and Mayfield, 2012; Jones and Mayfield, 2012; Stephens et al., 2010a). Highly efficient conversion of microalgae biomass to methane via anaerobic digestion might provide a universal basis for widespread application of algae biomass cultivation (Fig. 6).

Efficient microalgae growth is largely dependent on sufficient CO_2 availability as carbon source, which can be supplemented from biogas-producing plant directly, thereby upgrading the biogas (Ouyang et al., 2015) as well as from heat and power stations (exhaust gases), often used for biogas to electricity conversion or other CO_2 emitting facilities (Kao et al., 2012; Lindblom and Larsson, 2011). Since CO_2 emissions are restricted since 2005 in EU, sequestration is financially provided (Ellerman and Buchner, 2007) and can significantly improve the economical balance. Large part of nutrient requirements for biomass formation can be recycled from the fermentation sludge, which theoretically contains appropriate composition of macro- and micronutrients for generation of nitrogen-limited biomass (Larsen et al., 1991). Additionally, wastewaters from wastewater treatment plants can be used for the algae cultivation (Abou-Shanab et al., 2013; Bohutskyi et al., 2015; Cai et al., 2013; Chiu et al., 2015; Gokulan, 2014; Sharma et al.), which would not only save costs for fertilizers but also can be granted for water purification.

Natural ability of microalgae to secrete metabolites or proteins into the supernatant can substantially increase productivity of such systems. For instance, hydrogen production is regarded as promising CO_2 neutral renewable energy source, produced by microalgae under sulfur starvation (Doebbe et al., 2007; Kruse and Hankamer, 2010; Melis, 2009) as well as nitrogen deprivation (Philipps et al., 2012). Moreover, microalgae biomass naturally contains large amounts of high value products, that can be also purified as part of a biorefinery concept (Bux and Chisti, 2016; Georgianna and Mayfield, 2012; Murphy et al., 2015; Wijffels et al., 2010), whereby residual biomass can be fermented to biogas (Fig. 6). The use of genetic manipulation of microalgae might also be beneficial for product formation rates and appropriate disposal of residual GMO biomass is ensured by AD, since algae cells and DNA are completely disintegrated after fermentation [**3**, **5**].

Furthermore, secretion of endo-ß-1,4-glucanases for cellulose degradation [**1**] can be exploited for mixotrophic cultivation with cellulose containing waste streams, whereby the risk of

contamination (usually increased by mixotrophic cultivation) is minimized, since hydrolysis of the cellulose polymer is resulting in cellobiose, cellotriose, cellotetraose and cellopentaose, which is assimilated by algae cell and no glucose is present in the supernatant [**1**]. Genetic modification can also lead to fundamental alleviation of the overall biorefinery process efficiency, e.g. by using natural secretion mechanisms for proteins secretion into supernatant [**1**], for highly variable customized protein production (Gimpel et al., 2015; Lauersen et al., 2013; Rasala et al., 2012), whereby costs for extraction and purification from supernatant are expected to be significantly reduced. Due to large dimensions of the cultivation facility different classes of protein products can be targeted, e.g. catalytic enzymes chemical industry (bulk product) can be produced in raceway ponds and proteins for medical treatment in closed photobioreactor alongside, whereby cell biomass is always recycled via anaerobic digestion and methane is generated.

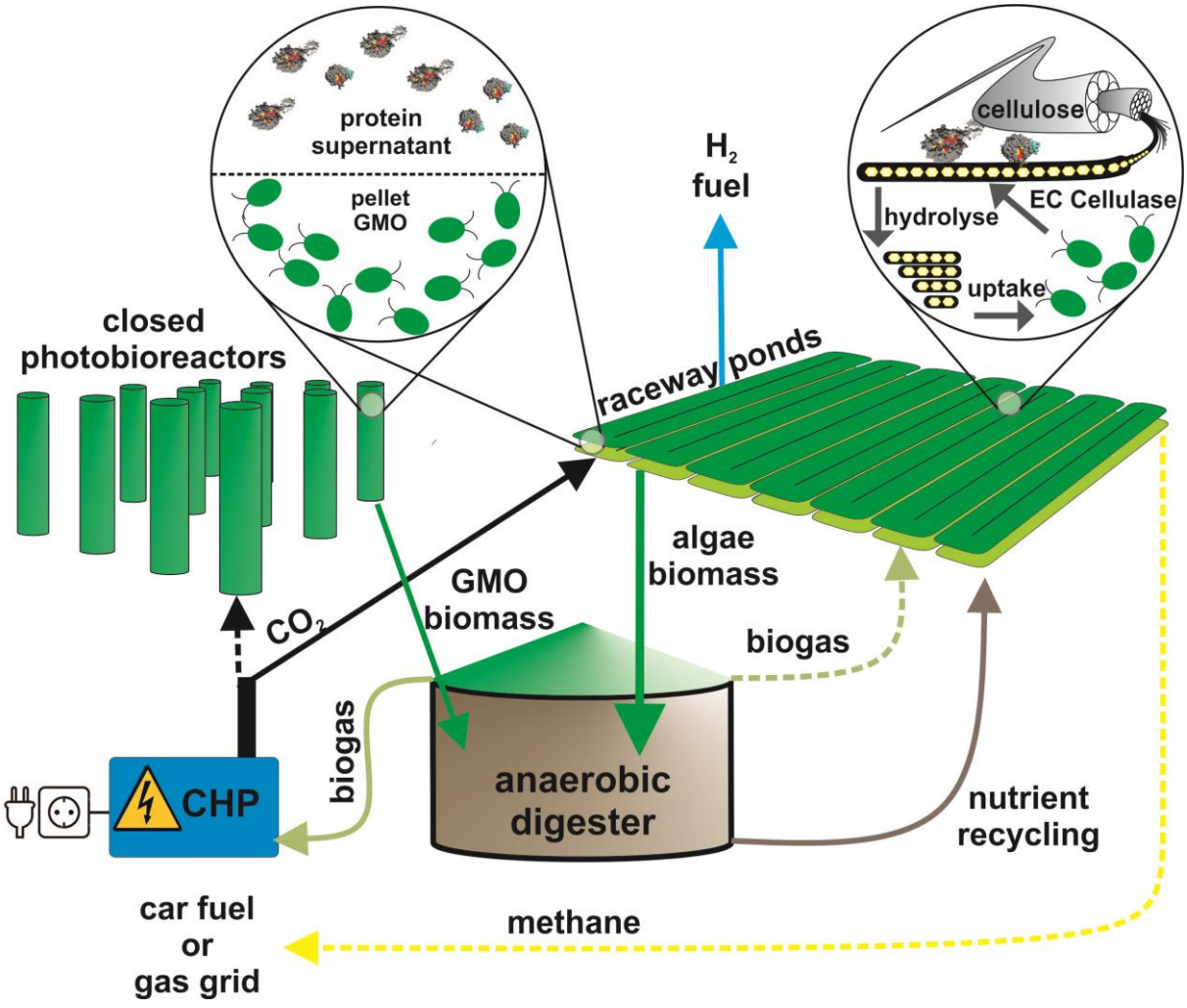

Figure 6: Perspective for future microalgae cultivation and fermentation concept. (CHP = combined heat and power generator. GMO = genetic modified organisms. EC = extracellular)

V. References

Abdoun, E. and Weiland, P. (2009). Optimization of monofermentation from renewable raw materials by the addition of trace elements. *Bornimer Agrartechnische Berichte* **68**, 69-78.

Abou-Shanab, R. A., Ji, M.-K., Kim, H.-C., Paeng, K.-J. and Jeon, B.-H. (2013). Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production. *Journal of environmental management* **115**, 257-264.

Adair, W. S. and Snell, W. J. (1990). The *Chlamydomonas reinhardtii* cell wall: structure, biochemistry and molecular biology. In *Organization and Assembly of Plant and Animal Extracellular Matrix*, (ed. R. P. M. a. W. S. Adair), pp. 15-84. Orlando: Academic Press.

Amaro, H. M., Guedes, A. C. and Malcata, F. X. (2011). Antimicrobial activities of microalgae: an invited review. *Science against microbial pathogens: communicating current research and technological advances* **3**, 1272-1284.

Amela, A. (2011). Biofuels versus food production: Does biofuels production increase food prices? *Energy* **36**, 2070-2076.

Amer, L., Adhikari, B. and Pellegrino, J. (2011). Technoeconomic analysis of five microalgae-to-biofuels processes of varying complexity. *Bioresource Technology* **102**, 9350-9359.

Amin, S. (2009). Review on biofuel oil and gas production processes from microalgae. *Energy Conversion and Management* **50**, 1834-1840.

Angelidaki, I., Ellegaard, L. and Ahring, B. K. (1999). A comprehensive model of anaerobic bioconversion of complex substrates to biogas. *Biotechnology and Bioengineering* **63**, 363-372.

Apt, K. E. and Behrens, P. W. (1999). Commercial developments in microalgal biotechnology. *Journal of Phycology* **35**, 215-226.

Archibald, J. M. (2011). Origin of eukaryotic cells: 40 years on. *Symbiosis* **54**, 69-86.

Azman, S., Khadem, A. F., Van Lier, J. B., Zeeman, G. and Plugge, C. M. (2015). Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. *Critical Reviews in Environmental Science and Technology* **45**, 2523-2564.

Baba, M., Suzuki, I. and Shiraiwa, Y. (2011). Proteomic analysis of high-CO2-inducible extracellular proteins in the unicellular green alga, Chlamydomonas reinhardtii. *Plant and Cell Physiology* **52**, 1302-1314.

Bagi, Z., Ács, N., Bálint, B., Horváth, L., Dobó, K., Perei, K. R., Rákhely, G. and Kovács, K. L. (2007). Biotechnological intensification of biogas production. *Applied Microbiology and Biotechnology* **76**, 473-482.

Ball, S., Colleoni, C., Cenci, U., Raj, J. N. and Tirtiaux, C. (2011). The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis. *Journal of Experimental Botany* **62**, 1775-1801.

Banerjee, A., Sharma, R., Chisti, Y. and Banerjee, U. (2002). Botryococcus braunii: a renewable source of hydrocarbons and other chemicals. *Critical reviews in biotechnology* **22**, 245-279.

Barsanti, L. and Gualtieri, P. (2014). Algae: anatomy, biochemistry, and biotechnology: CRC press.

Baurain, D., Brinkmann, H., Petersen, J., Rodríguez-Ezpeleta, N., Stechmann, A., Demoulin, V., Roger, A. J., Burger, G., Lang, B. F. and Philippe, H. (2010). Phylogenomic evidence for separate acquisition of plastids in cryptophytes, haptophytes, and stramenopiles. *Molecular Biology and Evolution* **27**, 1698-1709.

Beaugrand, J., Crônier, D., Thiebeau, P., Schreiber, L., Debeire, P. and Chabbert, B. (2004). Structure, chemical composition, and xylanase degradation of external layers isolated from developing wheat grain. *Journal of agricultural and food chemistry* **52**, 7108-7117.

Becker, E. (2007). Micro-algae as a source of protein. *Biotechnology Advances* **25**, 207-210.

Beer, L. L., Boyd, E. S., Peters, J. W. and Posewitz, M. C. (2009). Engineering algae for biohydrogen and biofuel production. *Current Opinion in Biotechnology* **20**, 264-271.

Benemann, J. R. (2000). Hydrogen production by microalgae. *Journal of Applied Phycology* **12**, 291-300.

Bisalputra, T. and Weier, T. (1963). The cell wall of *Scenedesmus quadricauda*. *American Journal of Botany*, 1011-1019.

Blanc-Mathieu, R., Verhelst, B., Derelle, E., Rombauts, S., Bouget, F.-Y., Carré, I., Château, A., Eyre-Walker, A., Grimsley, N., Moreau, H. et al. (2014). An improved genome of the model marine alga Ostreococcus tauri unfolds by assessing Illumina de novo assemblies. *BMC Genomics* **15**, 1-12.

Blanc, G., Agarkova, I., Grimwood, J., Kuo, A., Brueggeman, A., Dunigan, D. D., Gurnon, J., Ladunga, I., Lindquist, E., Lucas, S. et al. (2012). The genome of the polar eukaryotic microalga Coccomyxa subellipsoidea reveals traits of cold adaptation. *Genome Biology* **13**, R39-R39.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J., Polle, J. et al. (2010). The Chlorella variabilis NC64A Genome Reveals Adaptation to Photosymbiosis, Coevolution with Viruses, and Cryptic Sex. *The Plant Cell* **22**, 2943-2955.

Blume, F., Bergmann, I., Nettmann, E., Schelle, H., Rehde, G., Mundt, K. and Klocke, M. (2010). Methanogenic population dynamics during semi-continuous biogas fermentation and acidification by overloading. *Journal of applied microbiology* **109**, 441-450.

Blumer-Schuette, S. E., Ozdemir, I., Mistry, D., Lucas, S., Lapidus, A., Cheng, J.-F., Goodwin, L. A., Pitluck, S., Land, M. L. and Hauser, L. J. (2011). Complete genome sequences for the anaerobic, extremely thermophilic plant biomass-degrading bacteria Caldicellulosiruptor hydrothermalis, Caldicellulosiruptor kristjanssonii, Caldicellulosiruptor kronotskyensis, Caldicellulosiruptor owensensis, and Caldicellulosiruptor lactoaceticus. *Journal of bacteriology* **193**, 1483-1484.

BMUB. (2010). Sicherheitsanforderungen an die Endlagerung wärmeentwickelnder radioaktiver Abfälle., (ed.: German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB).

Bodył, A., Stiller, J. W. and Mackiewicz, P. (2009). Chromalveolate plastids: direct descent or multiple endosymbioses. *Trends Ecol. Evol* **24**.

Bogen, C., Al-Dilaimi, A., Albersmeier, A., Wichmann, J., Grundmann, M., Rupp, O., Lauersen, K. J., Blifernez-Klassen, O., Kalinowski, J., Goesmann, A. et al. (2013a). Reconstruction of the lipid metabolism for the microalga Monoraphidium neglectum from its genome sequence reveals characteristics suitable for biofuel production. *BMC Genomics* **14**, 1-18.

Bogen, C., Klassen, V., Wichmann, J., La Russa, M., Doebbe, A., Grundmann, M., Uronen, P., Kruse, O. and Mussgnug, J. H. (2013b). Identification of Monoraphidium contortum as a promising species for liquid biofuel production. *Bioresource Technology* **133**, 622-626.

Bohutskyi, P., Betenbaugh, M. J. and Bouwer, E. J. (2014). The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains. *Bioresource Technology* **155**, 366-372.

Bohutskyi, P. and Bouwer, E. (2013). Biogas production from algae and cyanobacteria through anaerobic digestion: A review, analysis, and research needs. In *Advanced biofuels and bioproducts*, (ed. J. W. Lee), pp. 873-975. New York ; London: Springer.

Bohutskyi, P., Liu, K., Nasr, L. K., Byers, N., Rosenberg, J. N., Oyler, G. A., Betenbaugh, M. J. and Bouwer, E. J. (2015). Bioprospecting of microalgae for integrated biomass production and phytoremediation of unsterilized wastewater and anaerobic digestion centrate. *Applied Microbiology and Biotechnology* **99**, 6139-6154.

Borowitzka, M. A. and Moheimani, N. R. (2013). Algae for Biofuels and Energy. Dordrecht: Springer Netherlands.

Brauer-Siebrecht, W., Jacobs, A., Christen, O., Götze, P., Koch, H.-J., Rücknagel, J. and Märländer, B. (2016). Silage Maize and Sugar Beet for Biogas Production in Rotations and Continuous Cultivation: Dry Matter and Estimated Methane Yield. *Agronomy* **6**, 2.

Braun, R. (1982). Grundlagen und Anwendungasbeispiele, Innovative Energietechnik, (ed.: Springer Verlag Wien-New York.

Braun, R. (2007). Anaerobic digestion: a multi-faceted process for energy, environmental management and rural development. In *Improvement of crop plants for industrial end uses*, pp. 335-416: Springer.

Brennan, L. and Owende, P. (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14**, 557-577.

Breton, G. and Kay, S. A. (2006). Circadian rhythms lit up in Chlamydomonas. *Genome Biology* **7**, 215.

Brown, M., Jeffrey, S., Volkman, J. and Dunstan, G. (1997). Nutritional properties of microalgae for mariculture. *Aquaculture* **151**, 315-331.

Buetow, D. and Schuit, K. (1968). Phosphorus and the growth of Euglena gracilis. *The Journal of protozoology* **15**, 770-773.

Burczyk, J. and Dworzanski, J. (1988). Comparison of sporopollenin-like algal resistant polymer from cell wall of *Botryococcus*, *Scenedesmus* and *Lycopodium clavatum* by GC-pyrolysis. *Phytochemistry* **27**, 2151-2153.

Burczyk, J., Szkawran, H., Zontek, I. and Czygan, F.-C. (1981). Carotenoids in the outer cell-wall layer of Scenedesmus (Chlorophyceae). *Planta* **151**, 247-250.

Burrell, P., O'sullivan, C., Song, H., Clarke, W. P. and Blackall, L. (2004). Identification, detection, and spatial resolution of Clostridium populations responsible for cellulose degradation in a methanogenic landfill leachate bioreactor. *Applied and environmental microbiology* **70**, 2414-2419.

Bux, F. and Chisti, Y. (2016). Algae Biotechnology: Products and Processes: Springer.

Cai, T., Park, S. Y., Racharaks, R. and Li, Y. (2013). Cultivation of Nannochloropsis salina using anaerobic digestion effluent as a nutrient source for biofuel production. *Applied Energy* **108**, 486-492.

Carballa, M., Regueiro, L. and Lema, J. M. (2015). Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus. *Current Opinion in Biotechnology* **33**, 103-111.

Carere, C. R., Sparling, R., Cicek, N. and Levin, D. B. (2008). Third generation biofuels via direct cellulose fermentation. *Int J Mol Sci* **9**, 1342-60.

Carlsson A.S., Van Bilein J.B., Möller R., Clayton D. and D., B. (2007). Outputs from EPOBIO Project: micro- and macroalgae utility for industrial application., (ed. York,UK.

Carpinelli, E. C., Telatin, A., Vitulo, N., Forcato, C., D'Angelo, M., Schiavon, R., Vezzi, A., Giacometti, G. M., Morosinotto, T. and Valle, G. (2014). Chromosome scale genome assembly and transcriptome profiling of Nannochloropsis gaditana in nitrogen depletion. *Molecular Plant* **7**, 323-335.

Chae, K., Jang, A., Yim, S. and Kim, I. S. (2008). The effects of digestion temperature and temperature shock on the biogas yields from the mesophilic anaerobic digestion of swine manure. *Bioresource Technology* **99**, 1-6.

Chakravorty, U., Hubert, M. H. and Nøstbakken, L. (2009). Fuel Versus Food. *Annual Review of Resource Economics* **1**, 645-663.

Chen, Y., Cheng, J. J. and Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresource Technology* **99**, 4044-4064.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances* **25**, 294-306.

Chisti, Y. (2012). Raceways-based production of algal crude oil. In *Microalgal biotechnology: Potential and production*, (ed. C. Posten and C. Walter), pp. pp. 113-146. Berlin: de Gruyter.

Chisti, Y. (2016). Large-Scale Production of Algal Biomass: Raceway Ponds. In *Algae Biotechnology*, pp. 21-40: Springer.

Chisti, Y. and Yan, J. Y. (2011). Energy from algae: Current status and future trends Algal biofuels - A status report. *Applied Energy* **88**, 3277-3279.

Chiu, S.-Y., Kao, C.-Y., Chen, T.-Y., Chang, Y.-B., Kuo, C.-M. and Lin, C.-S. (2015). Cultivation of microalgal Chlorella for biomass and lipid production using wastewater as nutrient resource. *Bioresource Technology* **184**, 179-189.

Chynoweth, D. P. (2002). Review of biomethane from marine biomass. *University of Florida*, 1-207.

Cioabla, A. E., Ionel, I., Dumitrel, G.-A. and Popescu, F. (2012). Comparative study on factors affecting anaerobic digestion of agricultural vegetal residues. *Biotechnology for Biofuels* **5**, 1.

Collet, P., Helias, A., Lardon, L., Ras, M., Goy, R. A. and Steyer, J. P. (2011). Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresource Technology* **102**, 207-214.

Cone, J., Van Gelder, A., Van Schooten, H. and Groten, J. (2008). Effects of chop length and ensiling period of forage maize on in vitro rumen fermentation characteristics. *NJAS-Wageningen Journal of Life Sciences* **55**, 155-166.

Costa, J. A. V. and de Morais, M. G. (2011). The role of biochemical engineering in the production of biofuels from microalgae. *Bioresource Technology* **102**, 2-9.

Craggs, R., Park, J., Heubeck, S. and Sutherland, D. (2014). High rate algal pond systems for low-energy wastewater treatment, nutrient recovery and energy production. *New Zealand Journal of Botany* **52**, 60-73.

Craggs, R., Sutherland, D. and Campbell, H. (2012). Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. *Journal of Applied Phycology* **24**, 329-337.

Daelman, M. R., Sorokin, D., Kruse, O., van Loosdrecht, M. C. and Strous, M. (2016). Haloalkaline Bioconversions for Methane Production from Microalgae Grown on Sunlight. *Trends in biotechnology*.

Demirbaş, A. (2002). Fuel characteristics of olive husk and walnut, hazelnut, sunflower, and almond shells. *Energy Sources* **24**, 215-221.

Deng, M.-D. and Coleman, J. R. (1999). Ethanol synthesis by genetic engineering in cyanobacteria. *Applied and environmental microbiology* **65**, 523-528.

Dent, R. M., Haglund, C. M., Chin, B. L., Kobayashi, M. C. and Niyogi, K. K. (2005). Functional genomics of eukaryotic photosynthesis using insertional mutagenesis of Chlamydomonas reinhardtii. *Plant Physiology* **137**, 545-556.

Deppenmeier, U. (2002). The unique biochemistry of methanogenesis. *Progress in nucleic acid research and molecular biology* **71**, 223-283.

Derelle, E., Ferraz, C., Rombauts, S., Rouzé, P., Worden, A. Z., Robbens, S., Partensky, F., Degroeve, S., Echeynié, S. and Cooke, R. (2006). Genome analysis of the smallest free-living eukaryote Ostreococcus tauri unveils many unique features. *Proceedings of the National Academy of Sciences* **103**, 11647-11652.

Diekert, G. and Wohlfarth, G. (1994). Energetics of acetogenesis from C1 units. In *Acetogenesis*, pp. 157-179: Springer.

Doebbe, A., Rupprecht, J., Beckmann, J., Mussgnug, J. H., Hallmann, A., Hankamer, B. and Kruse, O. (2007). Functional integration of the HUP1 hexose symporter gene into the genome of C. reinhardtii: impacts on biological H 2 production. *Journal of Biotechnology* **131**, 27-33.

Doi, R. H. and Kosugi, A. (2004). Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nature Reviews Microbiology* **2**, 541-551.

Domozych, D. S., Ciancia, M., Fangel, J. U., Mikkelsen, M. D., Ulvskov, P. and Willats, W. G. (2007). The cell walls of green algae: a journey through evolution and diversity. *Current challenges in plant cell walls*, 73.

Domozych, D. S., Stewart, K. D. and Mattox, K. R. (1980). The comparative aspects of cell wall chemistry in the green algae (Chlorophyta). *Journal of molecular evolution* **15**, 1-12.

Dragone, G., Fernandes, B., Vicente, A. A. and A., T. J. (2010). Third generation biofuels from microalgae. *Applied Microbiology* **2**, 1355-1366.

Drake, H. L., Daniel, S. L., Küsel, K., Matthies, C., Kuhner, C. and Braus-Stromeyer, S. (1997). Acetogenic bacteria: what are the in situ consequences of their diverse metabolic versatilities? *Biofactors* **6**, 13-24.

Eisentraut, A. (2010). Sustainable Production of Second-Generation Biofuels: Potential and Perspectives in Major Economies and Developing Countries, (ed. IEA Energy papers 2010/1: OECD Publishing.

Ellerman, A. D. and Buchner, B. K. (2007). The European Union emissions trading scheme: origins, allocation, and early results. *Review of environmental economics and policy* **1**, 66-87.

Ellis, J. T. and Miller, C. D. (2016). Fuel Alcohols from Microalgae. In *Algae Biotechnology*, pp. 143-154: Springer.

Fan, J., Ning, K., Zeng, X., Luo, Y., Wang, D., Hu, J., Li, J., Xu, H., Huang, J., Wan, M. et al. (2015). Genomic Foundation of Starch-to-Lipid Switch in Oleaginous Chlorella spp. *Plant Physiology* **169**, 2444-2461.

FAO. (2007). Sustainable Bioenergy: A Framework for Decision Makers. In *FAO. The State of Food and Agriculture.*, vol. http://www.fao.org/docrep/010/a1094e/a1094e00.htm (ed. Rome: Food and Agriculture Organization of the United Nations.)

FAO. (2008). Biofuels: Prospects, Risks and Opportunities. In *FAO. The State of Food and Agriculture.* , vol. <http://www.fao.org/sof/sofa/index_en.html>. (ed. Rome.)

Felix, C. R. and Ljungdahl, L. G. (1993). The cellulosome: the exocellular organelle of Clostridium. *Annual Reviews in Microbiology* **47**, 791-819.

Ferris, P., Olson, B. J. S. C., De Hoff, P. L., Douglass, S., Casero, D., Prochnik, S., Geng, S., Rai, R., Grimwood, J., Schmutz, J. et al. (2010). Evolution of an Expanded Sex-Determining Locus in Volvox. *Science* **328**, 351-354.

Ferry, J. G. (1992). Methane from acetate. *Journal of bacteriology* **174**, 5489-5495.

Field, C. B., Behrenfeld, M. J., Randerson, J. T. and Falkowski, P. (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. *Science* **281**, 237-40.

Foflonker, F., Price, D. C., Qiu, H., Palenik, B., Wang, S. and Bhattacharya, D. (2015). Genome of the halotolerant green alga Picochlorum sp. reveals strategies for thriving under fluctuating environmental conditions. *Environmental Microbiology* **17**, 412-426.

Fon Sing, S., Isdepsky, A., Borowitzka, M. and Lewis, D. (2014). Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant Tetraselmis sp. in raceway ponds under increasing salinity: a novel protocol for commercial microalgal biomass production. *Bioresource Technology* **161**, 47-54.

Fontes, C. M. and Gilbert, H. J. (2010). Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. *Annual review of biochemistry* **79**, 655-681.

Ford, C. and Percival, E. (1965). 1298. The carbohydrates of phaeodactylum tricornutum. Part I. Preliminary examination of the organism, and characterisation of low molecular weight material and of a glucan. *Journal of the Chemical Society (Resumed)*, 7035-7041.

Formighieri, C. (2015). Solar-to-fuel conversion in algae and cyanobacteria. Cham: Springer International Publishing.

Formighieri, C. and Bassi, R. (2013). Algae, a New Biomass Resource. In *Renewable Energy Systems*, pp. 1-26: Springer.

Foster, B. L., Dale, B. E. and Doran-Peterson, J. B. (2001). Enzymatic hydrolysis of ammonia-treated sugar beet pulp. *Applied biochemistry and biotechnology* **91**, 269-282.

Frigon, J. C., Matteau-Lebrun, F., Abdou, R. H., McGinn, P. J., O'Leary, S. J. B. and Guiot, S. R. (2013). Screening microalgae strains for their productivity in methane following anaerobic digestion. *Applied Energy* **108**, 100-107.

Gao, C., Wang, Y., Shen, Y., Yan, D., He, X., Dai, J. and Wu, Q. (2014). Oil accumulation mechanisms of the oleaginous microalga Chlorella protothecoides revealed through its genome, transcriptomes, and proteomes. *BMC Genomics* **15**, 1-14.

Gelin, F., Volkman, J., Largeau, C., Derenne, S., Damsté, J. S. and De Leeuw, J. (1999). Distribution of aliphatic, nonhydrolyzable biopolymers in marine microalgae. *Organic Geochemistry* **30**, 147-159.

Georgianna, D. R. and Mayfield, S. P. (2012). Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature* **488**, 329-335.

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

Ghosh, S., Conrad, J. R. and Klass, D. L. (1975). Anaerobic acidogenesis of wastewater sludge. *Journal (Water Pollution Control Federation)*, 30-45.

Gilbert, H. J. (2010). The biochemistry and structural biology of plant cell wall deconstruction. *Plant Physiol* **153**, 444-55.

Gimpel, J. A., Hyun, J. S., Schoepp, N. G. and Mayfield, S. P. (2015). Production of recombinant proteins in microalgae at pilot greenhouse scale. *Biotechnology and Bioengineering* **112**, 339-345.

Givens, D. and Deaville, E. (2001). Comparison of major carbohydrate fractions and cell wall digestibility in silages made from older and newer maize genotypes grown in the UK. *Animal feed science and technology* **89**, 69-82.

Gokulan, R. (2014). Best dilution ratio and GC-MS analysis for the removal of nutrient from municipal wastewater by microalgae. *International Journal of ChemTech Research* **6**, 663-672.

Golueke, C. G., Oswald, W. J. and Gotaas, H. B. (1957). Anaerobic digestion of Algae. *Appl Microbiol* **5**, 47-55.

González-Ballester, D., Casero, D., Cokus, S., Pellegrini, M., Merchant, S. S. and Grossman, A. R. (2010). RNA-seq analysis of sulfur-deprived Chlamydomonas cells reveals aspects of acclimation critical for cell survival. *The Plant Cell* **22**, 2058-2084.

González-Fernández, C., Sialve, B., Bernet, N. and Steyer, J. (2012). Thermal pretreatment to improve methane production of *Scenedesmus* biomass. *biomass and bioenergy* **40**, 105-111.

González-Fernández, C., Sialve, B., Bernet, N. and Steyer, J. P. (2012). Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. *Biofuels, Bioproducts and Biorefining* **6**, 205-218.

Goodenough, U. W. and Heuser, J. E. (1985). The Chlamydomonas cell wall and its constituent glycoproteins analyzed by the quick-freeze, deep-etch technique. *The Journal of cell biology* **101**, 1550-1568.

Goswami, R., Chattopadhyay, P., Shome, A., Banerjee, S. N., Chakraborty, A. K., Mathew, A. K. and Chaudhury, S. (2016). An overview of physico-chemical mechanisms of biogas production by microbial communities: a step towards sustainable waste management. *3 Biotech* **6**, 1-12.

Gouveia, L. (2011). Microalgae as a Feedstock for Biofuels, pp. 1-69: Springer Berlin Heidelberg.

Gouveia, L. and Oliveira, A. (2009). Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology & Biotechnology* **36**, 269-274.

Goux, X., Calusinska, M., Lemaigre, S., Marynowska, M., Klocke, M., Udelhoven, T., Benizri, E. and Delfosse, P. (2015). Microbial community dynamics in replicate anaerobic digesters exposed sequentially to increasing organic loading rate, acidosis, and process recovery. *Biotechnology for Biofuels* **8**, 1.

Grabber, J. H., Hatfield, R. D., Lu, F. and Ralph, J. (2008). Coniferyl ferulate incorporation into lignin enhances the alkaline delignification and enzymatic degradation of cell walls. *Biomacromolecules* **9**, 2510-2516.

Grabber, J. H., Mertens, D. R., Kim, H., Funk, C., Lu, F. and Ralph, J. (2009). Cell wall fermentation kinetics are impacted more by lignin content and ferulate cross-linking than by lignin composition. *Journal of the Science of Food and Agriculture* **89**, 122-129.

Grewe, S., Ballottari, M., Alcocer, M., D'Andrea, C., Blifernez-Klassen, O., Hankamer, B., Mussgnug, J. H., Bassi, R. and Kruse, O. (2014). Light-harvesting complex protein LHCBM9 is critical for photosystem II activity and hydrogen production in Chlamydomonas reinhardtii. *The Plant Cell* **26**, 1598-1611.

Grimm, P., Risse, J. M., Cholewa, D., Müller, J. M., Beshay, U., Friehs, K. and Flaschel, E. (2015). Applicability of Euglena gracilis for biorefineries demonstrated by the production of α-tocopherol and paramylon followed by anaerobic digestion. *Journal of Biotechnology* **215**, 72-79.

Grobbelaar, J. U. (2000). Physiological and technological considerations for optimising mass algal cultures. *Journal of Applied Phycology* **12**, 201-206.

Grossman, A., Harris, E., Hauser, C., Lefebvre, P., Martinez, D., Rokhsar, D., Shrager, J., Silflow, C. and Stern, D. Vallon 0, et al (2003) Chlamydomonas reinhardtii at the crossroads of genomics. *Eukaryot Cell2*, 1137-1150.

Gruber-Brunhumer, M., Jerney, J., Zohar, E., Nussbaumer, M., Hieger, C., Bochmann, G., Schagerl, M., Obbard, J., Fuchs, W. and Drosg, B. (2015). Acutodesmus obliquus as a benchmark strain for evaluating methane production from microalgae: Influence of different storage and pretreatment methods on biogas yield. *Algal Research* **12**, 230-238.

Gujer, W. and Zehnder, A. J. B. (1983). Conversion Processes in Anaerobic Digestion. *Water Science and Technology* **15**, 127-167.

Hallmann, A. (2007). Algal transgenics and biotechnology. *Transgenic Plant J* **1**, 81-98.

Harris, E. H. (2001). *Chlamydomonas* as a model organism. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 363-406.

Harris, E. H., Stern, D. B. and Witman, G. (2009). The chlamydomonas sourcebook: Cambridge Univ Press.

Harrison, R. W. (2009). The Food versus Fuel Debate: Implications for Consumers. *Journal of Agricultural and Applied Economics* **41**, 493-500.

Harun, R. and Danquah, M. K. (2011). Influence of acid pre-treatment on microalgal biomass for bioethanol production. *Process Biochemistry* **46**, 304-309.

Harun, R., Danquah, M. K. and Forde, G. M. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. *Journal of Chemical Technology and Biotechnology* **85**, 199-203.

Heaven, S., Milledge, J. and Zhang, Y. (2011). Comments on 'Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable'. *Biotechnology Advances* **29**, 164-167.

Hejazi, M. A. and Wijffels, R. H. (2004). Milking of microalgae. *Trends in biotechnology* **22**, 189-194.

Henley, W. J., Litaker, R. W., Novoveská, L., Duke, C. S., Quemada, H. D. and Sayre, R. T. (2013). Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. *Algal Research* **2**, 66-77.

Hernandez, E. P. S. and Cordoba, L. T. (1993). Anaerobic digestion of Chlorella vulgaris for energy production. *Resources Conservation and Recycling* **9**, 127-132.

Hernández, E. S. and Córdoba, L. T. (1993). Anaerobic digestion of Chlorella vulgaris for energy production. *Resources, conservation and recycling* **9**, 127-132.

Herrmann, A. and Rath, J. (2012). Biogas production from maize: current state, challenges, and prospects. 1. Methane yield potential. *BioEnergy Research* **5**, 1027-1042.

Hildebrand, M., Davis, A. K., Smith, S. R., Traller, J. C. and Abbriano, R. (2012). The place of diatoms in the biofuels industry. *Biofuels* **3**, 221-240.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Sciences* **103**, 11206-11210.

Horne, R. W., Davies, D. R., Norton, K. and Gurneysm.M. (1971). Electron microscope and optical diffraction studies on isolated cells from *Chlamydomonas*. *Nature* **232**, 493-&.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant Journal* **54**, 621-639.

Huntley, M. E. and Redalje, D. G. (2007). CO2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. *Mitigation and adaptation strategies for global change* **12**, 573-608.

Huss, V. A. R., Frank, C., Hartmann, E. C., Hirmer, M., Kloboucek, A., Seidel, B. M., Wenzeler, P. and Kessler, E. (1999). Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). *Journal of Phycology* **35**, 587-598.

IPCC. (2007). Climate change 2007. In *Fourth Assessment Report*, (ed. Intergovernmental Panel on Climate Change: IPCC.

IPCC. (2015). Climate Change 2014: Mitigation of Climate Change: Cambridge University Press.

John, R. P., Anisha, G., Nampoothiri, K. M. and Pandey, A. (2011). Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresource Technology* **102**, 186-193.

Jones, C. S. and Mayfield, S. P. (2012). Algae biofuels: versatility for the future of bioenergy. *Current Opinion in Biotechnology* **23**, 346-351.

Juárez, Á. B., Vélez, C. G., Iñiguez, A. R., Martínez, D. E., Rodríguez, M. C., Vigna, M. S. and de Molina, M. d. C. R. (2011). A Parachlorella kessleri (Trebouxiophyceae, Chlorophyta) strain from an extremely acidic geothermal pond in Argentina. *Phycologia* **50**, 413-421.

Kao, C.-Y., Chiu, S.-Y., Huang, T.-T., Dai, L., Hsu, L.-K. and Lin, C.-S. (2012). Ability of a mutant strain of the microalga Chlorella sp. to capture carbon dioxide for biogas upgrading. *Applied Energy* **93**, 176-183.

Kapaun, E. and Reisser, W. (1995). A chitin-like glycan in the cell wall of a Chlorella sp.(Chlorococcales, Chlorophyceae). *Planta* **197**, 577-582.

Keeling, P. J. (2010). The endosymbiotic origin, diversification and fate of plastids. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **365**, 729-748.

Kida, K., Shigematsu, T., Kijima, J., Numaguchi, M., Mochinaga, Y., Abe, N. and Morimura, S. (2001). Influence of Ni 2+ and Co 2+ on methanogenic activity and the amounts of coenzymes involved in methanogenesis. *Journal of Bioscience and Bioengineering* **91**, 590-595.

Klassen, V. (2010). Systematische Characterisierung der anaeroben, bakteriellen Fermentation von Mikroalgen. In *Faculty of Biology, Algaebiotechnology and Bioenergy*, vol. Master Thesis (ed.) Bielefeld: Bielefeld University.

Kloareg, B. and Quatrano, R. (1988). Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *OCEANOGRAPHY AND MARINE BIOLOGY: AN ANNUAL REVIEW.* **26**, 259-315.

Kodner, R. B., Summons, R. E. and Knoll, A. H. (2009). Phylogenetic investigation of the aliphatic, non-hydrolyzable biopolymer algaenan, with a focus on green algae. *Organic Geochemistry* **40**, 854-862.

Kreimer, G. (2009). The green algal eyespot apparatus: a primordial visual system and more? *Current genetics* **55**, 19-43.

Kruse, O. and Hankamer, B. (2010). Microalgal hydrogen production. *Current Opinion in Biotechnology* **21**, 238-243.

Kruse, O., Rupprecht, J., Mussgnug, J. H., Dismukes, G. C. and Hankamer, B. (2005). Photosynthesis: a blueprint for solar energy capture and biohydrogen production technologies. *Photochem Photobiol Sci* **4**, 957-70.

Lamed, R., Setter, E. and Bayer, E. A. (1983). Characterization of a cellulose-binding, cellulase-containing complex in Clostridium thermocellum. *Journal of bacteriology* **156**, 828-836.

Lardon, L., Helias, A., Sialve, B., Steyer, J. P. and Bernard, O. (2009). Life-cycle assessment of biodiesel production from microalgae. *Environmental Science & Technology* **43**, 6475-6481.

Larsen, A., Funch, F. and Hamilton, H. (1991). The use of fermentation sludge as a fertilizer in agriculture. *Water Science and Technology* **24**, 33-42.

Lauersen, K. J., Berger, H., Mussgnug, J. H. and Kruse, O. (2013). Efficient recombinant protein production and secretion from nuclear transgenes in Chlamydomonas reinhardtii. *Journal of Biotechnology* **167**, 101-110.

Lee, Y. K. (2016). Microalgae Cultivation Fundamentals. In *Algae Biotechnology*, pp. 1-19: Springer.

Lehr, F. and Posten, C. (2009). Closed photo-bioreactors as tools for biofuel production. *Curr Opin Biotechnol* **20**, 280-5.

Leliaert, F., Smith, D. R., Moreau, H., Herron, M. D., Verbruggen, H., Delwiche, C. F. and De Clerck, O. (2012). Phylogeny and Molecular Evolution of the Green Algae. *Critical Reviews in Plant Sciences* **31**, 1-46.

Léonard, A., Dandoy, P., Danloy, E., Leroux, G., Meunier, C. F., Rooke, J. C. and Su, B.-L. (2011). Whole-cell based hybrid materials for green energy production, environmental remediation and smart cell-therapy. *Chemical Society Reviews* **40**, 860-885.

Lestari, S., Mäki-Arvela, P., Beltramini, J., Lu, G. and Murzin, D. Y. (2009). Transforming triglycerides and fatty acids into biofuels. *ChemSusChem* **2**, 1109-1119.

Li, Y., Horsman, M., Wu, N., Lan, C. Q. and Dubois-Calero, N. (2008). Biofuels from Microalgae. *Biotechnology Progress* **24**, 815-820.

Lindblom, J. and Larsson, M. (2011). Algal Flue Gas Sequestration and Wastewater Treatment: An Industrial Experiment.

Lorenz, R. T. and Cysewski, G. R. (2000). Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends in biotechnology* **18**, 160-167.

Lucas, R., Kuchenbuch, A., Fetzer, I., Harms, H. and Kleinsteuber, S. (2015). Long-term monitoring reveals stable and remarkably similar microbial communities in parallel full-scale biogas reactors digesting energy crops. *FEMS Microbiol Ecol* **91**.

Macedo, I. C., Seabra, J. E. and Silva, J. E. (2008). Green house gases emissions in the production and use of ethanol from sugarcane in Brazil: The 2005/2006 averages and a prediction for 2020. *biomass and bioenergy* **32**, 582-595.

Mahdy, A., Mendez, L., Ballesteros, M. and Gonzalez-Fernandez, C. (2014a). Autohydrolysis and alkaline pretreatment effect on *Chlorella vulgaris* and *Scenedesmus* sp. methane production. *Energy* **78**, 48-52.

Mahdy, A., Mendez, L., Ballesteros, M. and Gonzalez-Fernandez, C. (2014b). Enhanced methane production of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* by hydrolytic enzymes addition. *Energy Conversion and Management* **85**, 551-557.

Mahdy, A., Mendez, L., Ballesteros, M. and González-Fernández, C. (2015). Protease pretreated Chlorella vulgaris biomass bioconversion to methane via semi-continuous anaerobic digestion. *Fuel* **158**, 35-41.

Markou, G., Angelidaki, I. and Georgakakis, D. (2013). Carbohydrate-enriched cyanobacterial biomass as feedstock for bio-methane production through anaerobic digestion. *Fuel* **111**, 872-879.

Martinot, E., Dienst, C., Weiliang, L. and Qimin, C. (2007). Renewable energy futures: Targets, scenarios, and pathways. *Annu. Rev. Environ. Resour.* **32**, 205-239.

Mata, T. M., Martins, A. A. and Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable & Sustainable Energy Reviews* **14**, 217-232.

Matsuzaki, M., Misumi, O., Shin-i, T., Maruyama, S., Takahara, M., Miyagishima, S.-y., Mori, T., Nishida, K., Yagisawa, F., Nishida, K. et al. (2004). Genome sequence of the ultrasmall unicellular red alga Cyanidioschyzon merolae 10D. *Nature* **428**, 653-657.

McCabe, J. and Eckenfelder, W. W. (1958). Biological treatment of sewage and industrial wastes: Reinhold.

McFadden, G. I. (2001). Primary and secondary endosymbiosis and the origin of plastids. *Journal of Phycology* **37**, 951-959.

McGinn, P. J., Dickinson, K. E., Park, K. C., Whitney, C. G., MacQuarrie, S. P., Black, F. J., Frigon, J.-C., Guiot, S. R. and O'Leary, S. J. (2012). Assessment of the bioenergy and bioremediation potentials of the microalga Scenedesmus sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode. *Algal Research* **1**, 155-165.

McKendry, P. (2002). Energy production from biomass (part 2): conversion technologies. *Bioresource Technology* **83**, 47-54.

Melbinger, N., Donnellon, J. and Zablatzky, H. R. (1971). Toxic Effects of Ammonia Nitrogen in High-Rate Digestion [with Discussion]. *Journal (Water Pollution Control Federation)*, 1658-1670.

Melis, A. (2009). Solar energy conversion efficiencies in photosynthesis: Minimizing the chlorophyll antennae to maximize efficiency. *Plant Science* **177**, 272-280.

Mendez, L., Mahdy, A., Ballesteros, M. and González-Fernández, C. (2015). Biomethane production using fresh and thermally pretreated Chlorella vulgaris biomass: A comparison of batch and semi-continuous feeding mode. *Ecological Engineering* **84**, 273-277.

Mendez, L., Mahdy, A., Demuez, M., Ballesteros, M. and Gonzalez-Fernandez, C. (2014). Effect of high pressure thermal pretreatment on *Chlorella vulgaris* biomass: Organic matter solubilisation and biochemical methane potential. *Fuel* **117**, 674-679.

Mendoza, J., Granados, M., De Godos, I., Acién, F., Molina, E., Banks, C. and Heaven, S. (2013). Fluid-dynamic characterization of real-scale raceway reactors for microalgae production. *biomass and bioenergy* **54**, 267-275.

Merchant, S. S. Prochnik, S. E. Vallon, O. Harris, E. H. Karpowicz, S. J. Witman, G. B. Terry, A. Salamov, A. Fritz-Laylin, L. K. Marechal-Drouard, L. et al. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **318**, 245-251.

Meynell, P.-J. (1976). Methane: planning a digester. In *C.T.T. series*, (ed. Dorchester: Prism Press.

Miller, D. H., Miller, M. and Lamport, D. T. A. (1972). Hydroxyproline heterooligosaccharides in *Chlamydomonas*. *Science* **176**, 918-&.

Mitra, M. and Melis, A. (2008). Optical properties of microalgae for enhanced biofuels production. *Opt. Express* **16**, 21807-21820.

Moheimani, N. R. and Borowitzka, M. A. (2007). Limits to productivity of the alga Pleurochrysis carterae (Haptophyta) grown in outdoor raceway ponds. *Biotechnology and Bioengineering* **96**, 27-36.

Monbiot, G. (2004). Feeding Cars, Not People. In *The Guardian*, http://www.monbiot.com/2004/11/23/feeding-cars-not-people/.

Monier, A., Welsh, R. M., Gentemann, C., Weinstock, G., Sodergren, E., Armbrust, E. V., Eisen, J. A. and Worden, A. Z. (2012). Phosphate transporters in marine phytoplankton and their viruses: cross-domain commonalities in viral-host gene exchanges. *Environmental Microbiology* **14**, 162-176.

Montingelli, M., Tedesco, S. and Olabi, A. (2015). Biogas production from algal biomass: a review. *Renewable and Sustainable Energy Reviews* **43**, 961-972.

Morweiser, M., Kruse, O., Hankamer, B. and Posten, C. (2010). Developments and perspectives of photobioreactors for biofuel production. *Appl Microbiol Biotechnol* **87**, 1291-301.

Murphy, J. D., Drosg, B., AllEn, E., JErnEy, J., XiA, A. and Herrmann, C. (2015). A perspective on algal biogas. *IEA Bioenergy*, 1-38.

Mussgnug, J. H. (2015). Genetic tools and techniques for Chlamydomonas reinhardtii. *Applied Microbiology and Biotechnology* **99**, 5407-5418.

Mussgnug, J. H., Klassen, V., Schluter, A. and Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J Biotechnol* **150**, 51-6.

Naik, S. N., Goud, V. V., Rout, P. K. and Dalai, A. K. (2010). Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews* **14**, 578-597.

Nakano, Y., Urade, Y., Urade, R. and Kitoka, S. (1987). Isolation, Purification, and Characterization of the Pellicle of Euglena gracilisz. *Journal of biochemistry* **102**, 1053-1063.

Nelson, N. and Yocum, C. F. (2006). Structure and function of photosystems I and II. *Annu. Rev. Plant Biol.* **57**, 521-565.

Nishiyama, T., Ueki, A., Kaku, N. and Ueki, K. (2009). Clostridium sufflavum sp. nov., isolated from a methanogenic reactor treating cattle waste. *International journal of systematic and evolutionary microbiology* **59**, 981-986.

Noyola, A. and Antonio, T. A. (2005). Anaerobic thermophilic digestion of sludge from enhanced primary treatment of municipal wastewater. *Proceedings of the Water Environment Federation* **2005**, 8031-8042.

Oh-Hama, T. and Miyachi, S. (1988). *Chlorella*. Cambridge: Cambridge University Press.

Oren, A. (2005). A hundred years of Dunaliella research: 1905–2005. *Saline systems* **1**, 1.

Oswald, W. J. and Golueke, C. G. (1960). Biological transformation of solar energy. *Adv. Appl. Microbiol* **2**, 223-262.

Ota, S., Oshima, K., Yamazaki, T., Kim, S., Yu, Z., Yoshihara, M., Takeda, K., Takeshita, T., Hirata, A., Bišová, K. et al. (2016). Highly efficient lipid production in the green alga Parachlorella kessleri: draft genome and transcriptome endorsed by whole-cell 3D ultrastructure. *Biotechnology for Biofuels* **9**, 1-10.

Ouyang, Y., Zhao, Y., Sun, S., Hu, C. and Ping, L. (2015). Effect of light intensity on the capability of different microalgae species for simultaneous biogas upgrading and biogas slurry nutrient reduction. *International Biodeterioration & Biodegradation* **104**, 157-163.

Palenik, B., Grimwood, J., Aerts, A., Rouzé, P., Salamov, A., Putnam, N., Dupont, C., Jorgensen, R., Derelle, E., Rombauts, S. et al. (2007). The tiny eukaryote Ostreococcus provides genomic insights into the paradox of plankton speciation. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 7705-7710.

Park, S. and Li, Y. (2012). Evaluation of methane production and macronutrient degradation in the anaerobic co-digestion of algae biomass residue and lipid waste. *Bioresource technology* **111**, 42-48.

Passos, F., Uggetti, E., Carrere, H. and Ferrer, I. (2014). Pretreatment of microalgae to improve biogas production: A review. *Bioresource Technology* **172**, 403-412.

Peers, G., Truong, T. B., Ostendorf, E., Busch, A., Elrad, D., Grossman, A. R., Hippler, M. and Niyogi, K. K. (2009). An ancient light-harvesting protein is critical for the regulation of algal photosynthesis. *Nature* **462**, 518-521.

Pereira, C. P., Castanares, G. and Van Lier, J. (2012). An OxiTop® protocol for screening plant material for its biochemical methane potential (BMP). *Water Science and Technology* **66**, 1416-1423.

Philipps, G., Happe, T. and Hemschemeier, A. (2012). Nitrogen deprivation results in photosynthetic hydrogen production in Chlamydomonas reinhardtii. *Planta* **235**, 729-745.

Plumley, F. G. and Schmidt, G. W. (1989). Nitrogen-dependent regulation of photosynthetic gene expression. *Proceedings of the National Academy of Sciences* **86**, 2678-2682.

Pombert, J.-F., Blouin, N. A., Lane, C., Boucias, D. and Keeling, P. J. (2014). A Lack of Parasitic Reduction in the Obligate Parasitic Green Alga Helicosporidium. *PLoS Genetics* **10**, e1004355.

Pope, P. B., Vivekanand, V., Eijsink, V. G. and Horn, S. J. (2013). Microbial community structure in a biogas digester utilizing the marine energy crop Saccharina latissima. *3 Biotech* **3**, 407-414.

Popper, Z. A., Michel, G., Herve, C., Domozych, D. S., Willats, W. G. T., Tuohy, M. G., Kloareg, B. and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: From algae to flowering plants. *Annual Review of Plant Biology, Vol 62* **62**, 567-588.

Popper, Z. A., Ralet, M.-C. and Domozych, D. S. (2014). Plant and algal cell walls: diversity and functionality. *Annals of botany* **114**, 1043-1048.

Posten, C. (2009). Design principles of photo-bioreactors for cultivation of microalgae. *Engineering in Life Sciences* **9**, 165-177.

Posten, C. and Schaub, G. (2009). Microalgae and terrestrial biomass as source for fuels—A process view. *Journal of Biotechnology* **142**, 64-69.

Prochazkova, G., Branyikova, I., Zachleder, V. and Branyik, T. (2014). Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. *Journal of Applied Phycology* **26**, 1359-1377.

Prochnik, S. E., Umen, J., Nedelcu, A. M., Hallmann, A., Miller, S. M., Nishii, I., Ferris, P., Kuo, A., Mitros, T., Fritz-Laylin, L. K. et al. (2010). Genomic Analysis of Organismal Complexity in the Multicellular Green Alga Volvox carteri. *Science (New York, N.Y.)* **329**, 223-226.

Pruvost, J., Cornet, J.-F. and Pilon, L. (2016). Large-Scale Production of Algal Biomass: Photobioreactors. In *Algae Biotechnology*, pp. 41-66: Springer.

Pulz, O. and Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology* **65**, 635-648.

Radakovits, R., Jinkerson, R. E., Fuerstenberg, S. I., Tae, H., Settlage, R. E., Boore, J. L. and Posewitz, M. C. (2012). Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropsis gaditana. *Nature communications* **3**, 686.

Raposo, F., Fernández-Cegrí, V., De la Rubia, M. A., Borja, R., Béline, F., Cavinato, C., Demirer, G., Fernández, B., Fernández-Polanco, M., Frigon, J. C. et al. (2011). Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study. *Journal of Chemical Technology & Biotechnology* **86**, 1088-1098.

Ras, M., Lardon, L., Bruno, S., Bernet, N. and Steyer, J.-P. (2011). Experimental study on a coupled process of production and anaerobic digestion of Chlorella vulgaris. *Bioresource Technology* **102**, 200-206.

Rasala, B. A., Lee, P. A., Shen, Z., Briggs, S. P., Mendez, M. and Mayfield, S. P. (2012). Robust expression and secretion of Xylanase1 in Chlamydomonas reinhardtii by fusion to a selection gene and processing with the FMDV 2A peptide. *PLoS One* **7**, e43349.

REN21. (2011). Renewables In *Global Status Report*, (ed. Paris.)

REN21. (2015). REN21. Renewables Global Status Report., (ed.)

Rifkin, J. (2002). The hydrogen economy : the creation of the world-wide energy web and the redistribution of power on earth, (ed., pp. 294. New York: J.P. Tarcher/Putnam.

Rochaix, J.-D. (1995). Chlamydomonas reinhardtii as the photosynthetic yeast. *Annual review of genetics* **29**, 209-230.

Rosenberg, J. N., Oyler, G. A., Wilkinson, L. and Betenbaugh, M. J. (2008). A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. *Current Opinion in Biotechnology* **19**, 430-436.

Rupprecht, J. (2009). From systems biology to fuel—Chlamydomonas reinhardtii as a model for a systems biology approach to improve biohydrogen production. *Journal of Biotechnology* **142**, 10-20.

Rutz, D. and Janssen, R. (2007). Biofuel technology handbook. Munich, Germany: WIP Renewable Energies.

Samson, R. and Leduyt, A. (1986). Detailed study of anaerobic digestion of Spirulina maxima algal biomass. *Biotechnology and Bioengineering* **28**, 1014-1023.

Saxena, D. and Stotzky, G. (2001). Bt corn has a higher lignin content than non-Bt corn. *American Journal of Botany* **88**, 1704-1706.

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O. and Hankamer, B. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *BioEnergy Research* **1**, 20-43.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* **61**, 262-280.

Schlüter, A., Bekel, T., Diaz, N. N., Dondrup, M., Eichenlaub, R., Gartemann, K.-H., Krahn, I., Krause, L., Krömeke, H. and Kruse, O. (2008). The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *Journal of Biotechnology* **136**, 77-90.

Schmer, M. R., Vogel, K. P., Mitchell, R. B. and Perrin, R. K. (2008). Net energy of cellulosic ethanol from switchgrass. *Proceedings of the National Academy of Sciences* **105**, 464-469.

Scholz, M. J., Weiss, T. L., Jinkerson, R. E., Jing, J., Roth, R., Goodenough, U., Posewitz, M. C. and Gerken, H. G. (2014). Ultrastructure and composition of the Nannochloropsis gaditana cell wall. *Eukaryotic Cell* **13**, 1450-1464.

Schwede, S., Kowalczyk, A., Gerber, M. and Span, R. (2013a). Anaerobic co-digestion of the marine microalga Nannochloropsis salina with energy crops. *Bioresource Technology* **148**, 428-435.

Schwede, S., Rehman, Z. U., Gerber, M., Theiss, C. and Span, R. (2013b). Effects of thermal pretreatment on anaerobic digestion of *Nannochloropsis salina* biomass. *Bioresource Technology* **143**, 505-511.

Scott, S. A., Davey, M. P., Dennis, J. S., Horst, I., Howe, C. J., Lea-Smith, D. J. and Smith, A. G. (2010). Biodiesel from algae: challenges and prospects. *Curr Opin Biotechnol* **21**, 277-86.

Senhorinho, G. N., Ross, G. M. and Scott, J. A. (2015). Cyanobacteria and eukaryotic microalgae as potential sources of antibiotics. *Phycologia* **54**, 271-282.

Sharma, R., Tiwari, A. K., Kumar, G. S. and Lamba, B. Y. Cost effective and economic method for cultivation of Chlorella pyrenoidosa for the simultaneous treatment of biogas digester wastewater and biogas production.

Shen, Y., Yuan, W., Pei, Z., Wu, Q. and Mao, E. (2009). Microalgae mass production methods. *Transactions of the ASABE* **52**, 1275-1287.

Shiratori, H., Sasaya, K., Ohiwa, H., Ikeno, H., Ayame, S., Kataoka, N., Miya, A., Beppu, T. and Ueda, K. (2009). Clostridium clariflavum sp. nov. and Clostridium caenicola sp. nov., moderately thermophilic, cellulose-/cellobiose-digesting bacteria isolated from methanogenic sludge. *International journal of systematic and evolutionary microbiology* **59**, 1764-1770.

Shuchuan, P., Chenghu, H., Jin, W., TianHu, C., XiaoMeng, L. and ZhengBo, Y. (2012). Performance of anaerobic co-digestion of corn straw and algae biomass from lake Chaohu. *Transactions of the Chinese Society of Agricultural Engineering* **28**, 173-178.

Singh, A., Nigam, P. S. and Murphy, J. D. (2011). Renewable fuels from algae: An answer to debatable land based fuels. *Bioresource Technology* **102**, 10-16.

Smith, A., Brown, K., Ogilvie, S., Rushton, K. and Bates, J. (2001). Waste management options and climate change. Final report to the European Commission. *Final Report ED21158R4* **1**, 205.

Smith, P. H. and Mah, R. A. (1966). Kinetics of acetate metabolism during sludge digestion. *Applied microbiology* **14**, 368-371.

Soomro, R. R., Ndikubwimana, T., Zeng, X., Lu, Y., Lin, L. and Danquah, M. K. (2016). Development of a Two-Stage Microalgae Dewatering Process – A Life Cycle Assessment Approach. *Frontiers in Plant Science* **7**, 113.

Speece, R. E. (1983). Anaerobic biotechnology for industrial wastewater treatment. *Environmental Science & Technology* **17**, 416A-427A.

Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* **101**, 87-96.

Stephens, E., Ross, I. L., King, Z., Mussgnug, J. H., Kruse, O., Posten, C., Borowitzka, M. A. and Hankamer, B. (2010a). An economic and technical evaluation of microalgal biofuels. *Nature Biotechnology* **28**, 126-128.

Stephens, E., Ross, I. L., Mussgnug, J. H., Wagner, L. D., Borowitzka, M. A., Posten, C., Kruse, O. and Hankamer, B. (2010b). Future prospects of microalgal biofuel production systems. *Trends Plant Sci* **15**, 554-64.

Sugimoto, K., Sato, N. and Tsuzuki, M. (2007). Utilization of a chloroplast membrane sulfolipid as a major internal sulfur source for protein synthesis in the early phase of sulfur starvation in Chlamydomonas reinhardtii. *FEBS letters* **581**, 4519-4522.

Sundberg, C., Al-Soud, W. A., Larsson, M., Alm, E., Yekta, S. S., Svensson, B. H., Sørensen, S. J. and Karlsson, A. (2013). 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS microbiology ecology* **85**, 612-626.

Symons, G. and Buswell, A. (1933). The methane fermentation of carbohydrates1, 2. *Journal of the American Chemical Society* **55**, 2028-2036.

Takahashi, H., Braby, C. E. and Grossman, A. R. (2001). Sulfur economy and cell wall biosynthesis during sulfur limitation of Chlamydomonas reinhardtii. *Plant Physiology* **127**, 665-673.

Takeda, H. (1991). Sugar Composition of the Cell-Wall and the Taxonomy of *Chlorella* (Chlorophyceae). *Journal of Phycology* **27**, 224-232.

Takeda, H. (1996). Cell wall sugars of some *Scenedesmus* species. *Phytochemistry* **42**, 673-675.

Tani, Y. and Tsumura, H. (1989). Screening for tocopherol-producing microorganisms and α-tocopherol production by Euglena gracilis Z. *Agricultural and biological chemistry* **53**, 305-312.

Terry, K. L. and Raymond, L. P. (1985). System design for the autotrophic production of microalgae. *Enzyme and Microbial Technology* **7**, 474-487.

Tezuka, Y. (1989). The C: N: P ratio of phytoplankton determines the relative amounts of dissolved inorganic nitrogen and phosphorus released during aerobic decomposition. *Hydrobiologia* **173**, 55-62.

Timilsina, G. R. and Shrestha, A. (2011). How much hope should we have for biofuels? *Energy* **36**, 2055-2069.

Tomei, J. and Helliwell, R. (2015). Food versus fuel? Going beyond biofuels. *Land Use Policy*.

Uggetti, E., Passos, F., Solé, M., García, J. and Ferrer, I. (2016). Biogas from Algae via Anaerobic Digestion. In *Algae Biotechnology*, pp. 195-216: Springer.

Ullah, K., Ahmad, M., Sofia, Sharma, V. K., Lu, P., Harvey, A., Zafar, M., Sultana, S. and Anyanwu, C. N. (2014). Algal biomass as a global source of transport fuels: Overview and development perspectives. *Progress in Natural Science: Materials International* **24**, 329-339.

VDI-4630, V. D. I. (2006). 4630: Fermentation of organic materials, characterisation of the substrate, sampling, collection of material data, fermentation tests. *Verein Deutscher Ingenieure (VDI), editor. VDI Handbuch Energietechnik. Berlin: Beuth Verlag GmbH*.

Verbruggen, A. and Al Marchohi, M. (2010). Views on peak oil and its relation to climate change policy. *Energy Policy* **38**, 5572-5581.

Vidal, J. B. C., Dien, B. S., Ting, K. and Singh, V. (2011). Influence of feedstock particle size on lignocellulose conversion—a review. *Applied biochemistry and biotechnology* **164**, 1405-1421.

Vogel, J. (2008). Unique aspects of the grass cell wall. *Current Opinion in Plant Biology* **11**, 301-307.

Walter, B. P., Heimann, M. and Matthews, E. (2001). Modeling modern methane emissions from natural wetlands 1. Model description and results. *Journal of Geophysical Research* **106**, 34189-34206.

Weiland, P. (2010). Biogas production: current state and perspectives. *Applied Microbiology and Biotechnology* **85**, 849-860.

Weissman, J. C., Tillet, D. M. and Goebel, R. P. (1989). Design and operation of an outdoor microalgae test facility., vol. Report SERI/STR-232-3569. (ed. Golden, CO.: Solar Energy Research Institute.

West, W. E., Coloso, J. J. and Jones, S. E. (2012). Effects of algal and terrestrial carbon on methane production rates and methanogen community structure in a temperate lake sediment. *Freshwater Biology* **57**, 949-955.

West, W. E., McCarthy, S. M. and Jones, S. E. (2015). Phytoplankton lipid content influences freshwater lake methanogenesis. *Freshwater Biology* **60**, 2261-2269.

Wieczorek, A., Hetz, S. and Kolb, S. (2014). Microbial responses to chitin and chitosan in oxic and anoxic agricultural soil slurries. *Biogeosciences* **11**, 3339-3352.

Wigmosta, M. S., Coleman, A. M., Skaggs, R. J., Huesemann, M. H. and Lane, L. J. (2011). National microalgae biofuel production potential and resource demand. *Water Resources Research* **47**.

Wijffels, R. H., Barbosa, M. J. and Eppink, M. H. (2010). Microalgae for the production of bulk chemicals and biofuels. *Biofuels, Bioproducts and Biorefining* **4**, 287-295.

Wijffels, R. H., Kruse, O. and Hellingwerf, K. J. (2013). Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. *Current Opinion in Biotechnology* **24**, 405-413.

Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rákhely, G. and Kovács, K. L. (2012). Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. *Biotechnology for Biofuels* **5**, 1.

Wirth, R., Lakatos, G., Böjti, T., Maróti, G., Bagi, Z., Kis, M., Kovács, A., Ács, N., Rákhely, G. and Kovács, K. L. (2015). Metagenome changes in the mesophilic biogas-producing community during fermentation of the green alga Scenedesmus obliquus. *Journal of Biotechnology* **215**, 52-61.

Wolf, J., Stephens, E., Steinbusch, S., Yarnold, J., Ross, I. L., Steinweg, C., Doebbe, A., Krolovitsch, C., Müller, S., Jakob, G. et al. (2016). Multifactorial comparison of photobioreactor geometries in parallel microalgae cultivations. *Algal Research* **15**, 187-201.

Worden, A. Z., Lee, J.-H., Mock, T., Rouzé, P., Simmons, M. P., Aerts, A. L., Allen, A. E., Cuvelier, M. L., Derelle, E., Everett, M. V. et al. (2009). Green Evolution and Dynamic Adaptations Revealed by Genomes of the Marine Picoeukaryotes Micromonas. *Science* **324**, 268-272.

Yen, H. W. and Brune, D. E. (2007). Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresour Technol* **98**, 130-4.

Yenigün, O. and Demirel, B. (2013). Ammonia inhibition in anaerobic digestion: a review. *Process Biochemistry* **48**, 901-911.

Zhang, Z., Shrager, J., Jain, M., Chang, C.-W., Vallon, O. and Grossman, A. R. (2004). Insights into the survival of Chlamydomonas reinhardtii during sulfur starvation based on microarray analysis of gene expression. *Eukaryotic Cell* **3**, 1331-1348.

Zhu, X. G., Long, S. P. and Ort, D. R. (2010). Improving Photosynthetic Efficiency for Greater Yield. *Annual Review of Plant Biology* **61**, 235-261.

Zinder, S. H. (1993). Physiological ecology of methanogens. In *Methanogenesis*, pp. 128-206: Springer.

Zubr, J. (1986). Methanogenic fermentation of fresh and ensiled plant materials. *Biomass* **11**, 159-171.

Zverlov, V. V., Hiegl, W., Köck, D. E., Kellermann, J., Köllmeier, T. and Schwarz, W. H. (2010). Hydrolytic bacteria in mesophilic and thermophilic degradation of plant biomass. *Engineering in Life Sciences* **10**, 528-536.

VI. Publications

The five manuscripts presented in this thesis are:

[1] Cellulose degradation and assimilation by the unicellular phototrophic eukaryote *Chlamydomonas reinhardtii*

Olga Blifernez-Klassen, Viktor Klassen, Anja Doebbe, Klaudia Kersting, Philipp Grimm, Lutz Wobbe and Olaf Kruse

Nature Communications 3, 2012: 1214

[2] Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production

Christian Bogen, Viktor Klassen, Julian Wichmann, Marco La Russa, Anja Doebbe, Michael Grundmann, Pauliina Uronen, Olaf Kruse, Jan H. Mussgnug

Bioresource Technology 133, 2013: 622–626

[3] A novel one-stage cultivation/fermentation strategy for improved biogas production with microalgal biomass

Viktor Klassen, Olga Blifernez-Klassen, Yoep Hoekzema, Jan H. Mussgnug, Olaf Kruse

Journal of Biotechnology 215, 2015: 44–51

[4] Efficiency and biotechnological aspects of biogas production from microalgal substrates

Viktor Klassen, Olga Blifernez-Klassen, Lutz Wobbe, Andreas Schlüter, Olaf Kruse, Jan H. Mussgnug

Journal of Biotechnology 234, 2016: 7-26

[5] Highly efficient methane generation from untreated microalgae biomass

Viktor Klassen, Viktor Klassen, Olga Blifernez-Klassen, Daniel Wibberg, Anika Winkler, Jörn Kalinowski, Clemens Posten, Olaf Kruse

Biotechnology for Biofuels 2017 10(1)

Acknowledgements

Eigene Publikationen

2017 | Journal Article | PUB-ID: 2913038
Highly efficient methane generation from untreated microalgae biomass
Viktor Klassen, Olga Blifernez-Klassen, Daniel Wibberg, Anika Winkler, Jörn Kalinowski, Clemens Posten, Olaf Kruse
DOI: 10.1186/s13068-017-0871-4
Biotechnology for Biofuels 10(1)

2016 | Journal Article | PUB-ID: *2904809*
Efficiency and biotechnological aspects of biogas production from microalgal substrates
Klassen V, Blifernez-Klassen O, Wobbe L, Schlüter A, Kruse O, Mussgnug JH (2016)
DOI: 10.1016/j.jbiotec.2016.07.015
Journal of Biotechnology 234: 7-26

2015 | Journal Article | PUB-ID: 2753171
A novel one-stage cultivation/fermentation strategy for improved biogas production with microalgal biomass
Klassen V, Blifernez-Klassen O, Hoekzema Y, Mussgnug JH, Kruse O (2015)
DOI: 10.1016/j.jbiotec.2015.05.008
Journal of Biotechnology 215: 44-51.

2013 | Journal Article | PUB-ID: 2579037
Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production
Bogen C, Klassen V, Wichmann J, La Russa M, Doebbe A, Grundmann M, Uronen P, Kruse O, Mussgnug JH (2013)
DOI: 10.1016/j.biortech.2013.01.164
Bioresource Technology 133: 622-626.

2012 | Journal Article | PUB-ID: 2541470
Cellulose degradation and assimilation by the unicellular phototrophic eukaryote *Chlamydomonas reinhardtii*
Blifernez-Klassen O, Klassen V, Doebbe A, Kersting K, Grimm P, Wobbe L, Kruse O (2012)
DOI: 10.1038/ncomms2210
Nature communications, 3.

2010 | Journal Article | PUB-ID: 1897327
Microalgae as substrates for fermentative biogas production in a combined biorefinery concept
Mussgnug JH, Klassen V, Schlüter A, Kruse O (2010)
DOI: 10.1016/j.jbiotec.2010.07.030
Journal of Biotechnology 150(1): 51-56.

2007 | Journal Article | PUB-ID: 1631729
Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion
Mussgnug JH, Thomas-Hall S, Rupprecht J, Foo A, Klassen V, McDowall A, Schenk PM, Kruse O, Hankamer B (2007)
DOI: 10.1111/j.1467-7652.2007.00285.x
PLANT BIOTECHNOLOGY JOURNAL 5(6): 802-814.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst angefertigt habe und nur die angegebenen Quellen und Hilfsmittel verwendet habe. Alle aus der Literatur ganz oder annähernd entnommenen Stellen habe ich als solche kenntlich gemacht.

Weiterhin erkläre ich, dass die vorliegende Dissertation weder vollständig noch teilweise einer anderen Fakultät mit dem Ziel vorgelegt worden ist, einen akademischen Titel zu erwerben. Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Universität Bielefeld.

Bielefeld, den 20. April 2016