Optimization of the economic viability of the production and harvesting of microalgae by bioprocess engineering

by

Franziska Bleeke

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Approved Dissertation Committee

Prof. Dr. Mathias Winterhalter
Name and title of Chair

Prof. Dr. Roland Benz
Name and title of Committee Member

Prof. Dr. Gerd Klöck
Name and title of Committee Member

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Statutory Declaration

(on Authorship of a Dissertation)

I, Franziska Bleeke hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

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Abstract

Microalgae are microscopically small photoactive organisms with rising significance in the biotechnology sector. Even though microalgae show great potential for biomass production, industrial application has been limited in the past due to high production costs. Two major bottlenecks affecting cost effectiveness are the choice of a suitable production strain and the biomass harvest. Three manuscripts were published in the framework of the PhD Thesis, scientifically investigating new methods for strain selection and biomass harvest.

Microalgae exhibit an enormous biodiversity with between 200,000 and several million species. However, worldwide only 15 algae species are utilized in industrial production processes. The application of novel production strains with process-oriented properties, specifically fast growth and heat resistance, would improve production efficiency and reduce costs.

In the first publication "Isolation and Characterization of New Temperature Tolerant Microalgal Strains for Biomass Production", 130 environmental samples were taken in Germany, Spain, Italy and Portugal, purified and strains with a high growth rate and thermos tolerance were identified. The results showed that 21 of the isolated strains were able to grow at 40 °C with the highest growth rate of 1.16 per day (isolate T306A) and 13 of those were even growing at 45 °C with a maximum growth rate of 0.053 per day at 45 °C (isolate Sp13). All 21 strains were identified at molecular level by 18S rDNA sequencing. The sequences showed that the isolates were all chlorophytes belonging to four different families.

About one third of the algal biomass production costs are caused during the harvesting process. Usually centrifugation is the harvesting method of choice, requiring a high energy input for the separation of the small algal cells from a large volume of surrounding media. Increasing the efficiency at low energy demands is a major challenge which can be achieved by inserting a flocculation step. Flocculation can be achieved in several ways. In the second and third publications in this thesis two ways of flocculation, chemical- and electroflocculation were investigated and optimized.

In the second publication "Optimization of freshwater microalgal biomass harvest using polymeric flocculants", 15 polyelectrolytes were tested for their harvesting capability.

Cationic, anionic and non-ionic flocculants were analyzed at varying concentrations and incubation times. Three chlorophytes *Chlorella* sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* were tested to verify the influence of different sizes, morphologies

and motilities. In a recycling experiment a potential negative impact of flocculant residues was monitored over a period of eight weeks. The results showed that cationic flocculants were most effective with flocculant PK55H showing the highest efficiency at the lowest concentration. Anionic and non-ionic flocculants were ineffective.

In the third publication "Effect of voltage and electrode material on electroflocculation of *Scenedesmus acuminatus*", six electrode materials were tested for electroflocculation of *Scenedesmus acuminatus*. Besides the normally used aluminum and iron electrodes, magnesium, copper, zinc and brass electrodes were tested and compared. The influence of 10, 20, 30 and 40 V was examined and evaluated.

Electroflocculation was successful with all tested electrode materials. The maximum flocculation efficiency was reached with magnesium electrodes followed by Al, Zn, Cu, Fe and brass. Although a significant pH increase was monitored, the recycling experiment showed growth in all tested supernatants.

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Content

1 Introduction

1.1 Microalgae

Microalgae are photoactive eukaryotic organisms with unicellular or simple multicellular structure. Having a size of about 2-20µm they show a broad diversity of cell structures and shapes. In contrast to higher plants they are not differentiated into roots, stem or leaf systems. Due to their simple cell structure microalgae can adapt to various environmental conditions. Microalgae are mainly found in aquatic habitats where they build the basis of the marine food chain but can also be found in every ecosystem on earth, even in the extremes. From the arctic ice to the desert they populate nearly every habitat (Thomas & Dieckmann, 2002). In the course of time enormous biodiversity developed. It is estimated that there are between 200,000 and several million species (De Clerck, Guiry, Leliaert et al., 2013; Guiry, 2012; Spolaore, Joannis-Cassan, Duran et al., 2006). Although several algal strains show similar morphology, phylogenetically most strains are distantly related. Although apparently resembling each other, DNA analysis showed that due to primary and secondary endosymbiosis algae are represented in nearly every lineages of eukaryotes (Keeling, Burger, Durnford et al., 2005). Even if they show a similar morphology, biochemical and physiological properties differ. In contrast to eukaryotic microalgae, prokaryotic microalgae, also known as cyanobacteria, are lacking membrane bound cell organelles. Eukaryotic algal life presumably started with primary endosymbiosis. Primary endosymbiosis describes the process where a heterotrophic eukaryote engulfed and retained a cyanobacterium giving the host cell photosynthetic properties (Keeling, 2010). The prokaryotic cell became a plastid and due to an immense gene transfer between the host and the endosymbiont, the majority of the plastid genes were transferred into the nucleus (Keeling & Palmer, 2008). Three lineages of archaeplastida evolved out of this endosymbiosis comprising green algae (incl. land plants) red algae and glaucophytes. The majority of algal cells obtained their plastid during secondary endosymbiosis. In contrast to primary endosymbiosis, secondary endosymbiosis led to an immense genetic diversity on earth. During secondary endosymbiosis a eukaryotic cell (mostly a red algal cell) engulfs another eukaryote which has already undergone primary endosymbiosis. Three lineages derived from secondary endosymbiosis: euglenoids, chorarachniophytes and chromalveolates. The supergroup chromalveolated comprises the majority of algal lineages used in algal biotechnology.

Microalgae can either be autotrophic or heterotrophic. Autotrophic algae use inorganic CO_2 as carbon source whereas heterotrophic algae require organic carbon as energy source. Mixotrophic algae are capable of converting both inorganic and organic carbon into biomass. Autotrophic microalgae convert light energy into chemical energy by photosynthesis. Due to their photosynthetic activity marine microalgae made life on earth possible by producing oxygen. Today about 50% of the atmospheric oxygen is produced by algae (Posten & Walter, 2012a)

1.2 Microalgal cultivation

Large scale cultivation of microalgae was first conducted by Nihon Chlorella in Japan in the early 1960s (Spolaore et al., 2006). The cultivation was performed in raceway ponds, shallow water basins in the form of oval loops. The water depth is about 30cm and continuous mixing is provided by paddle wheels. These cultivation systems are the best investigated production systems although several other systems are available on market by now.

In the 1970s due to the oil crisis a lot of research was conducted in the framework of the aquatic species program (ASP) of the U.S. National Renewable Energy Laboratory (NREL). The aim of this project was to investigate microalgae as a potential renewable energy source. Besides the investigation of the lipid production a lot of attention was paid to improving reactor systems for large scale cultivation of microalgae. In contrast to photobioreactors, raceway ponds are less expensive to build and operate but more susceptible to weed algae or bacterial contamination.

High water losses due to evaporation, temperature shifts and mutual shading limit productivity. The cultivation in closed bioreactors allows a higher degree of process control but also results in higher acquisition costs. While progress on the large scale photobioreactor (PBR) design has been made in the recent years, the main production systems used are still open pond systems located in mainly in Asia (Milledge, 2011; Spolaore et al., 2006). The costs for both operation and acquisition for closed PBR systems are still too high, making the process uneconomical (Richardson, Johnson & Outlaw, 2012).

Morphologically unicellular algae, such as *Chlorella*, with cell wall and without any attachments or structures are most suited for large scale cultivation since this simplifies downstream processing and prevents biofouling. *Chlorella* is produced by more than 70 companies worldwide

with the largest company Taiwan Chlorella Manufacturing & Co. Ltd. with production sites located in Taiwan and China with an annual biomass production of 300 t (Rosello Sastre, 2012).

1.3 Potential and products

Photosynthetic biomass production requires light, CO_2, water, nutrients and generally temperatures between 20 and 30°C. Since light and nutrients can be taken up over the complete cell surface the biomass productivity per hectare is five times higher compared to terrestrial crops. In contrast to agricultural crops with 1.5% microalgae can convert about 5% light energy into chemical energy (Posten *et al.*, 2012a; Posten & Walter, 2012b). Compared to higher plants they have other advantages, such as the ability to be grown year round on non-arable land and the capacity to accumulate valuable products like lipids or pigments (Chisti, 2007; Mata, Martins & Caetano, 2010). They are easy to cultivate and show fast growth with low nutrient requirements. Wastewaters and flue gases from industry have been successfully applied as nutrient source (Chisti, 2007; Mallick, 2002; Mata et al., 2010; Wang, Li, Wu et al., 2008). Wastewaters are cheap byproducts often high in nitrogen (NH_4, NO_3) and phosphorous (PO_4). Algae use the contaminants as nutrients and thereby purify the wastewater in terms of bioremediation (Mata *et al.*, 2010). Microalgae are an interesting alternative for wastewater treatment because of their bioremediation potential which coupled with biomass production can be used for biofuel production.

No freshwater resources are required for growing microalgae. Depending on the species they can be cultivated in brackish or salty water. Due to their photosynthetic activity microalgae can fix up to 2.5kg CO_2 per kg dry biomass (Posten et al., 2012a). Industrial flue gases contain up to 15% CO_2, displaying a cheap source for micro algae cultivation. Simultaneously the sequestration of CO_2 from flue gas provides an alternative greenhouse gas mitigation strategy (Mata2010).

Although they show great potential, so far only a small number of microalgal strains have been described. Out of the estimated 200,000 to several million microalgal species on earth only about 44 000 have been described worldwide and less than 15 strains are used for large scale production (De Clerck *et al.*, 2013; Guiry, 2012; Spolaore *et al.*, 2006).

A vast array of microalgal products is available on the market. About 5000 tons / year of microalgal dry matter are produced resulting in a turnover of about $1.3x10^9$ US dollar (Pulz & Gross, 2004). The major part of the produced biomass is displayed with 3000 tons /year by the cyanobacterium *Spirulina* and the chlorophyte *Chlorella* with 2000 tons /year. Other strains like

Heamatococcus pluvialis, *Dunaliella salina*, *Isochrysis galbana*, *Phaeodactylum tricornutum* or *Porphorydium* sp. are also cultivated for industrial purposes but in limited amounts (Pulz et al., 2004).

About 75% of the produced biomass is utilized as food or feed additive in forms of powders, pills or capsules. As additive in pasta, bread or cookies, microalgae have an antioxidizing and prebiotic effect and are rich in proteins (about 60-70% for *Spirulina*), essential unsaturated fatty acids, minerals and vitamins.

The second largest application next to human nutrition is the market for animal feed. About 30% of the worldwide produced microalgal biomass is applied as feed additive. Adding up to 10% to domestic animal feed can positively affect health, skin, coat growth and fertility (Pulz et al., 2004; Spolaore et al., 2006). One of the most important applications of microalgae on the feed market is aquaculture. Since algae form the basis of the marine food chain several marine fish or shellfish feed directly or indirectly on microalgae. It is a direct food source for larvae, several crustaceans, mollusks and fish. Indirectly it serves as fish feed in form of zooplankton which was fed on microalgae or as feed additive in pellet form.

In the cosmetic industry microalgae can be found as an ingredient in vanishing cream, shampoo or facial masks. As natural fertilizer microalgal biomass can improve soil quality and fertility by adding nutrients and bioactive compounds. Such compounds might also positively affect the health of the plants by acting against diseases caused by bacteria or viruses (Pulz et al., 2004).

Several fine chemicals like pigments or polyunsaturated fatty acids extracted from algal biomass are available on the market. To improve light absorption several other pigments can be found in the antenna complex in the photosystems. Besides chlorophyll a and b phycobillins and carotenoids can be found.

Carotenoids are yellow, orange or red pigments naturally produced by some photosynthetic organisms, bacteria and fungi. In the microalgal biotechnology β-carotene and astaxanthin produced by *Dunaliella* and *Haematococcus* are mainly used as food colorants for humans or animals giving e.g. salmon the typically red flesh (Milledge, 2011; Spolaore et al., 2006). Due to their molecular structure, carotenoids have antioxidative potential which makes them highly interesting for the cosmetic industry.

Polyunsaturated fatty acids (PUFA) can only be synthesized by plants and are essential for humans. Since microalgae are at the basis of the food chain, they are the primary source of

PUFA. The biotechnologically most interesting PUFAs are eicosapentaenoic acid (EPA), linolenic-acid (GLA), arachidonic acid (AA) and docosahexaenoic acid (DHA). As nutritional supplement PUFAs directly deriving from microalgae have a promising biotechnological potential since in contrast to omega 3 or omega 6 fatty acids usually deriving from fish, they do not have the typical unpleasant fish odor and show a higher quality. Besides that a decrease of fish resources is predicted for the future which makes microalgae for PUFA production even more attractive.

The economically most important and best established microalgal products are polysaccharides in form of agar (E406), alginate (E401-E405) and carrageen (E407). Because of their gelling properties they are used in the food, cosmetic and pharmaceutical industry.

Because the resources for fossil fuels are limited recently a lot of attention has been paid to microalgae as a potential source for biofuel production (Chisti, 2007; Mata et al., 2010). The sustainable production of renewable energy is of increasing interest since the demand is rising constantly. The production of biofuels out of non-food raw materials such as microalgae offers great opportunities for the future (Mata et al., 2010). After extraction of the lipids, biomass can be used as feed supplement, organic fertilizer or as substrate for biogas production processes or as organic fertilizer (Wang et al., 2008).

1.4 Limitations

Although microalgal biotechnology shows great potential, several factors limit industrial application. The choice of the adequate bioreactor system is highly important. Although progress on the large scale photobioreactor (PBR) design has been made in the recent years, the main production systems used are still open pond systems located in mainly in Asia (Milledge, 2011; Spolaore *et al.*, 2006). On the technical side, the costs for both operation and acquisition for closed PBR systems are still too high, making the process uneconomical (Richardson *et al.*, 2012). On the biological side it has to be noted that even if an economical reactor system is found, the number of algal strains able to grow in this system is limited (Rawat, Kumar, Mutanda et al., 2013).

Besides the reactor system, there are two major bottlenecks affecting cost effectiveness: the choice of a suitable production strain and the biomass harvest (Uduman, Qi, Danquah *et al.*, 2010).

1.4.1 Strain selection

The increasing demand for microalgal products requires a process, product and environment oriented strain selection in order to make large scale production economical. Less than 15 strains of the above mentioned number of species are used worldwide for large scale production. The potential to improve strain selection is enormous (Spolaore *et al.*, 2006).

A process or environment orientated strain selection supports the use of either indigenous strains from the respective production site, or strains adapted to stress, which due to fast growth rates are able to outcompete predators or weed algae.

In warmer regions for example, strains with a high temperature and irradiance tolerance would improve biomass production efficiency. Heat produced by high or extreme temperatures in greenhouses, outdoor cultivation settings like hot deserts or photobioreactors illuminated by parabolic solar concentrators might negatively affect growth of many microalgal species (Ras, Steyer & Bernard, 2013; Salvucci & Crafts-Brandner, 2004). Constructing a temperature controlled environment would enhance algal growth but has been proven not to be sustainable due to high initial investment and operation costs. In coastal areas the use of marine microalgae allows the use of water in huge quantities without any water management problems. Freshwater strains should be cultivated in inland areas to avoid costs for salting and media preparation. The use of flue gas and waste water should always be considered to save costs. Establishment of cultivation sites in an industrial surrounding allows the use of flue gases as CO_2 source and waste waters as nutrient source but requires strains with a high CO_2 or nutrients acceptance. Additionally the screening for a contamination resistant strain might be interesting since contamination is present in every large scale outdoor facility.

In the product oriented strain selection the algal strain is selected according to the desired product instead of trying to manipulate an established strain in order to produce the desired product. There is a broad palette of microalgal metabolites and products available on the market. Pigments like e.g. astaxanthin are used as natural colorants in human food and cosmetics or as supplements in e.g. salmon feed in the aquaculture industry. Furthermore the aquaculture industry is searching for strains with high protein content and easily digestible cell walls in order to substitute fish meal in formulated feed. The food industry is looking for strains rich in polyunsaturated fatty acids since there is an increasing demand for products not deriving from fish.

The limiting factor in the production of microalgal biodiesel is the identification of a suitable strain accumulating high amounts of lipids under different culture conditions (Chen, Zhang, Song et al., 2009).

Since higher efficiency and product yield have been reported when cultivating the cells heterotrophically in the presence of organic carbon substrates, screening for heterotrophic strains could be performed to improve biomass production.

Biologically active compounds are being investigated e.g. antiviral substances or anti-cancer substances, several of them have already entered clinical trials (Niedermeyer & Brönstrup, 2012).

On the downstreaming side, the process oriented strain selection might prefer a strain with a certain morphology (e.g. filamentous) which simplifies cell harvest and therefore saves costs.

Strains with specific cell wall properties can simplify extraction procedures of algal products. Mechanically stable cell walls on the other hand might be advantageous in reactor systems with high shear stress or other mechanical influences. The right strain could be screened in order to improve efficiency for every location, reactor system or product.

Several studies have been conducted on screening for biodiesel production (Abomohra, Wagner, El-Sheekh *et al.*, 2013; Sydney, da Silva, Tokarski *et al.*, 2011; Talebi, Mohtashami, Tabatabaei *et al.*, 2013), essential fatty acids production (Yang, Lu, Chen *et al.*, 2010),

antioxidants (Natrah, Yusoff, Shariff et al., 2007), bioactive substances (Jiraskova, Poulickova, Novak et al., 2009; Ordog, Stirk, Lenobel et al., 2004; Scholz & Liebezeit, 2006; Volk, 2008) or the ability to remove nutrients from wastewaters (Sydney et al., 2011). Nevertheless a lot of potential gets lost since the above mentioned studies only focus on one, sometimes two characteristics. Furthermore most of the studies published screened already described species obtained from culture collections and not environmental samples leaving hundreds of thousands of species undiscovered (Abomohra et al., 2013; Ordog et al., 2004; Picardo, de Medeiros, Monteiro et al., 2013).

1.4.2 Bottlenecks of biomass harvest

The cell harvest contributes to 20-30% of the total production costs (Grima, Belarbi, Fernandez *et al.*, 2003; Mata *et al.*, 2010; Uduman *et al.*, 2010). Usually large volumes with a low cell concentrations of 0.5- 2.5 g l^{-1} have to be processed (Grima *et al.*, 2003).

There are several ways to harvest algal cells. The major technique applied is centrifugation or a combination of filtration, flotation or flocculation followed by a final centrifugation step. High energy inputs are needed to harvest the algae from the media, regularly beyond the energy content of the harvested biomass (Grima *et al.*, 2003; Uduman, Bourniquel, Danquah *et al.*, 2011; Wijffels & Barbosa, 2010).

Increasing the efficiency at low energy demands within the harvesting process is a major challenge in microalgal biotechnology.

Cell harvest by centrifugation is a well-established method in microalgal biotechnology (Golueke & Oswald, 1970). Within a few minutes high recovery rates of up to 90% can be reached but shear stress might damage the cell structure. Furthermore this method is expensive and time consuming since usually large volumes have to centrifuged (Grima *et al.*, 2003).

During flocculation, algal cells aggregate and larger particles are formed. These aggregates show a higher settling velocity due to the higher density of the flocks. At neutral pH the cell surface of microalgae is usually negatively charged due to functional groups on the algal surface (Golueke *et al.*, 1970). There are different flocculation techniques available for algal cells. Several studies have been published on the use of pH induced flocculation (Vandamme, Foubert, Fraeye *et al.*, 2012; Wu, Zhu, Huang *et al.*, 2012; Zheng, Gao, Yin *et al.*, 2012), bioflocculation using bacteria or filamentous fungi (Lee, Lewis & Ashman, 2009; Zhou, Min, Hu *et al.*, 2013). Even flocculation algae were tested to flock non-flocculation algae (Salim, Bosma, Vermue *et al.*, 2011). Chemical flocculation describes the process were a coagulant is added to the algal suspension for biomass harvest (Xu, Wang, Li *et al.*, 2010). There are organic an organic flocculants available resulting in different recovery rates (Gerde, Yao, Lio *et al.*, 2014; Granados, Acien, Gomez *et al.*, 2012; Papazi, Makridis & Divanach, 2010; Sirin, Trobajo, Ibanez *et al.*, 2012). Comparative studies were published revealing that higher biomass recovery rates were reached with polyelectrolytes than with metal salts (Gerde *et al.*, 2014; Granados *et al.*, 2012). Polyelectrolytes are typically applied in wastewater purification processes and favored for microalgae since lower quantities are required and non-toxic and biodegradable substances are vacant (Granados *et al.*, 2012). Numerous studies have been published on the use of electroflocculation for algal biomass harvest (Lee, Lewis & Ashman, 2013; Uduman *et al.*, 2011; Vandamme, Pontes, Goiris *et al.*, 2011).In electroflocculation the flocculant is produced directly in the algal culture by releasing metal ions from a sacrificial electrode (Vandamme *et al.*, 2011).

1.5 Aims and Outlines

The aim of this thesis was to improve cost effectiveness in microalgal biotechnology by scientifically investigating new methods for strain selection and biomass harvest. To reach this aim three hypotheses were constructed and scientifically investigated.

Hypothesis 1:
It is possible to isolate robust and efficient production strains for biomass production out of environmental samples.

Hypothesis 2:
It is feasible to harvest different microalgal strains by using commercially available polymeric flocculants and reuse the media after flocculation.

Hypothesis 3:
Apart from aluminum and iron other metals are suitable for electroflocculation independent of the voltage applied.

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2 Publications

2.1 Isolation and Characterization of New Temperature Tolerant Microalgal Strains for Biomass Production (Publication 1)

This manuscript was published in *Energies* on the 25th of November 2014:
Bleeke, F., Rwehumbiza, V. M., Winckelmann, D., Klöck, G. (2014). Isolation and Characterization of New Temperature Tolerant Microalgal Strains for Biomass Production. *Energies*, *7*, 7847-7856; doi:10.3390/en7127847

Summary

This article describes the isolation and characterization of new microalgal strains with a high temperature tolerance. Even though microalgae show an immense biodiversity of 500 000 to several million species, only a handful is used in biotechnological applications. The implementation of new process-oriented production strains which certain properties like fast growth and high temperature tolerance would improve biomass production efficiencies in warmer regions and thereby reduce costs. In the present study a screening was conducted investigating 130 environmental samples. Samples were taken in Germany, Spain, Italy and Portugal and incubated in liquid freshwater media. After the establishment of pure cultures growth experiments were done to determine the maximal tolerated growth temperature. The results showed that 21 strains were able to grow at 40 °C and 13 of those 21 strains even at 45 °C. The maximum growth rate at room temperature was 1.16 per day (isolate T306A) whereas increasing the temperature to 45°C resulted in a decrease of growth to 0.053 per day (isolate Sp13). 22 purified strains were investigated on molecular level via 18s rDNA sequencing and phylogenetically identified. The results showed that the tested strains belong to four different families (Scenedesmaceae, Chlorellaceae, Chlamydomonadaceae and Chlorococcaceae) and three different orders (Chlorococcales, Chlorellales and Volvocales).

Contribution of Ph. D. candidate

Franziska Bleeke contributed to experimental design, data acquisition, result analysis and manuscript preparation. Vincent M. Rwehumbiza contributed to result analysis and manuscript preparation. Dominik Winckelmann contributed to experimental design and data acquisition. Gerd Klöck contributed to experimental design, result analysis and manuscript preparation.

Isolation and Characterization of New Temperature Tolerant Microalgal Strains for Biomass Production

Franziska Bleeke [1,2], Vincent M. Rwehumbiza [2], Dominik Winckelmann [2] and Gerd Klöck [2,*]

[1] School of Engineering and Science, Jacobs University, 28759 Bremen, Germany; E-Mail: franziska.bleeke@hs-bremen.de

[2] Institute of Environmental Biology and Biotechnology, University of Applied Sciences Bremen, 28199 Bremen, Germany; E-Mails: v.rwehumbiza@gmail.com (V.M.R.); dominik.winckelmann@hs-bremen.de (D.W.)

* Author to whom correspondence should be addressed; E-Mail: Gerd.Kloeck@hs-bremen.de; Tel: +49-421-5905-4266, Fax: +49-421-5905-4245.

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Abstract: Microalgae exhibit great potential for biomass production. Although microalgae display an enormous biodiversity, surprisingly only 15 species are used for large scale production processes worldwide. The implementation of new production strains with good process-oriented properties, especially fast growth rate and heat resistance, could improve production efficiency and reduce costs. In this study 130 environmental samples collected in Germany, Spain, Italy and Portugal were investigated for fast growing thermotolerant photosynthetic species. Isolates were characterized and identified on a molecular level. In total 21 of the isolated freshwater strains were able to grow at 40 °C. Additionally, 13 of those 21 strains are able to grow at 45 °C. The highest growth rate at room temperature was 1.16 per day (isolate T306A), compared to 0.053 per day at 45 °C (isolate Sp13). In three thermotolerant strains pigment production was induced. Molecular identification by 18S rDNA sequencing revealed that the isolates were all chlorophytes belonging to four different families.

Keywords: screening; high temperature tolerance; biomass production; freshwater microalgae; pigments

1. Introduction

Microalgae are microscopically small photosynthetically active eukaryotic organisms with an increasing importance in the biotechnology sector. Compared to higher plants they have several advantages, such as faster growth, the ability to be grown on non-arable land and the capacity to accumulate valuable products such as lipids or pigments [1,2].

There are between 200,000 and several million microalgal species [3,4]. Even though microalgae display an enormous biotechnological potential due to their biodiversity and versatility, the industry is currently using only about 15 species for large scale production [5]. Due to the increasing demand for microalgal biomass, a process-oriented strain selection is essential in order to make large scale production economical. Process-oriented strain selection supports the use of either indigenous strains from the respective production site, or strains adapted to stress, which due to fast growth rates are able to outcompete predators or weed algae. Besides light exposure and nutrient availability, the temperature influences growth efficiency significantly. Due to high and favorable illumination the summer months usually lead to high biomass productivities [6]. The optimal growth temperature for common laboratory strains of microalgae varies among different species, but is usually stated to be between 20 and 30 °C [6–11].

Higher temperature conditions in greenhouses or outdoor cultivation settings during summer months may negatively affect growth of many microalgal species [6,8,12]. In greenhouses temperatures can reach up to 55 °C, resulting in maximum culture temperatures exceeding 35 °C [13]. For outdoor cultivation similar temperatures surpassing 35 °C and even 40 °C were reported [14–18]. Whereas temperatures below the optimum lead to a retained biomass production, temperatures above the optimum results in a steep decrease in productivity and possibly the total loss of the culture. The degradation and inactivation of enzymes involved in the photosynthetic process caused by heat stress results in the inhibition of growth or even programmed cell death [1,12,19]. Construction of temperature controlled environments for cultivation of microalgae would be ideal, but has been proven to not be sustainable due to high initial investment and operation costs. Therefore, acquiring microalgae strains with the ability to grow and propagate in

these severe heat conditions, especially during summer temperatures, is of utmost importance.

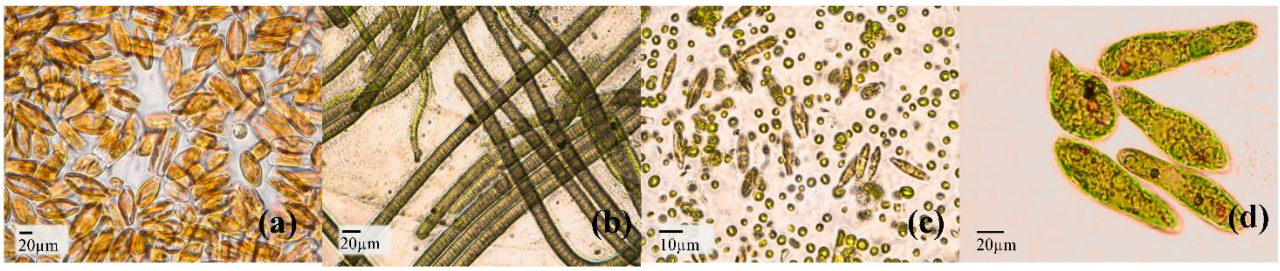
In the present study a simple screening protocol for environmental algal samples was developed. Within this screening the main focus was put on thermotolerant freshwater species with a high growth rate. 130 samples were collected in Germany, Italy, Spain, and Portugal and later analyzed. After an initial temperature tolerance test, pure cultures were obtained and characterized.

2. Results and Discussion

2.1. Sampling and Enrichment

One hundred and thirty samples from Germany, Italy, Spain and Portugal were collected and cultivated in test tubes containing Wuxal liquid medium (WM) [20]. The incubation resulted in algal growth in about 50% of the test tubes, leaving 66 samples for the screening procedure. Figure 1 shows a variety of cell types and shapes that could be observed in the test tubes using a Zeiss Axiostar Plus light microscope (Carl Zeiss, Oberkochen, Germany) investigated in Hellfeld mode at 40-fold magnification. The images were taken by a FinePix E550 digital camera (Fujifilm, Tokyo, Japan).

Figure 1. Morphological diversity of the mixed cultures in the test tubes. (**a**) mixed culture of pennate gold-brown single cells; (**b**) intense green filamentous microalgae; (**c**) mixed culture of coccoid and elongated single cells; and (**d**) flagellated protists with red eyespots visible.



2.2. Temperature Tolerance and Growth Kinetics

After incubation at 25 °C for 14 days, 7 (of 66) test tubes showed a decrease in coloration leaving

59 test tubes for further screening. In the following step the cultures were transferred to conical flasks (volume 100 mL) and exposed to 35 °C for seven days. This step left 40 samples for further investigations. After this initial enrichment of temperature tolerant samples, unialgal isolates were obtained by microbiological methods. These isolates were subjected to further temperature tolerance tests. When the growth temperature was further increased, 21 out of those 40 isolates were able to grow at 40 °C (Table 1), 13 of them were even growing at 45 °C. None of the isolates grew at 47 °C (Table 1, Figure 2).

Figure 2. Temperature tolerance test of purified isolates. Thirteen algae strains tolerated incubation at 45 °C. The highest growth could be observed for isolates Sp13, Sp14, I4, I5 and CS after a 48 h lag phase. A further temperature increase to 47 °C led to a decline in optical density after 48 h. Data points are the mean value of three replicates. Error bars represent standard deviation.

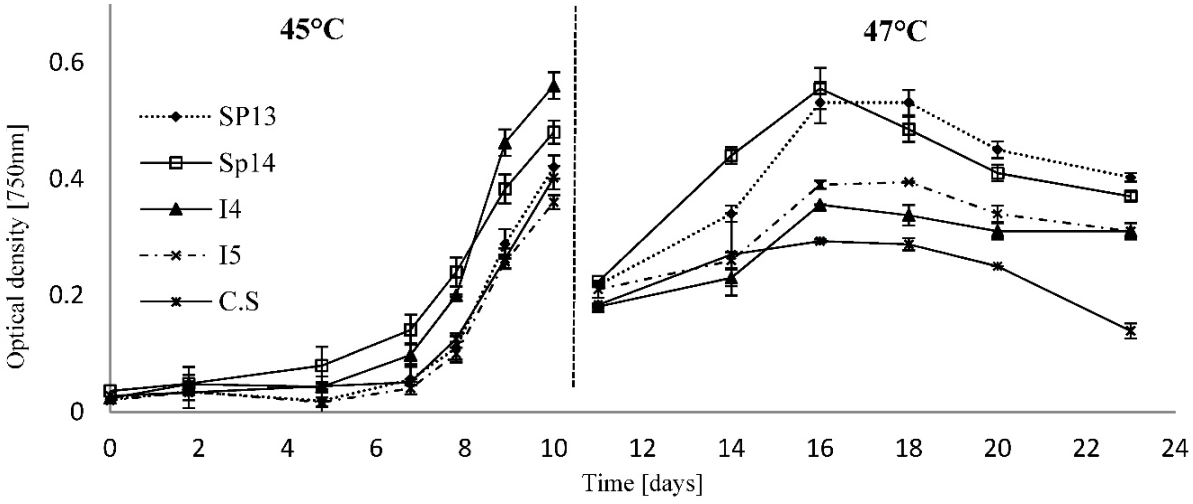


Table 1. Growth rates and the maximal temperature tolerated for biomass production of 22 unialgal isolates obtained after initial enrichment.

Isolate	μ day^{-1}	Maximal Temperature [°C]
T306A	1.163	45
Sp1	1.101	40
CS	0.943	45
T301	0.890	45
I5	0.832	45
Sp12	0.827	45
Sp13	0.766	45
Sp14	0.683	45
T308	0.667	40
Sp9	0.646	45
GDK	0.625	40
T302	0.585	45
T306B	0.564	45
T307	0.525	40
I4	0.469	45
Sp6	0.455	40
R6	0.423	45
R4	0.382	40
Ssp	0.371	40
R3	0.328	45
I1	0.144	35
I3	0.105	40

During incubation at 45 °C all isolates experienced a lag phase in the first 48 h. This lag phase was extended to 48 h for isolate I4, I5 and Sp13 and to 120 h for isolate CS. For most samples, the optical density measured at 750 nm wavelength started to rise after 48 h. The highest values were attained by I4 and Sp14 after 10 days of incubation. Determination of the cell number revealed the same trend (data not shown). Although isolate Sp13 showed the highest growth rate (Table 1), the final OD 750 value of 0.42 was lower compared to isolates I4 and Sp14 of 0.56 and 0.48, respectively. Five days of incubation showed an increase in cell number for all tested isolates. Comparing the growth rates of the isolates grown at room temperature and 45 °C, the effects of thermal induced stress were evident. The growth rates for CS, I4, I5, Sp13 and Sp14 at 45 °C were approximately 50% of the values determined at room temperature (Table 2). It was also noted that the samples cultivated at room temperature were light green while the ones grown at 45 °C were dark green.

Table 2. Comparison of growth rates at room temperature (22 ± 1 °C) and 45 °C.

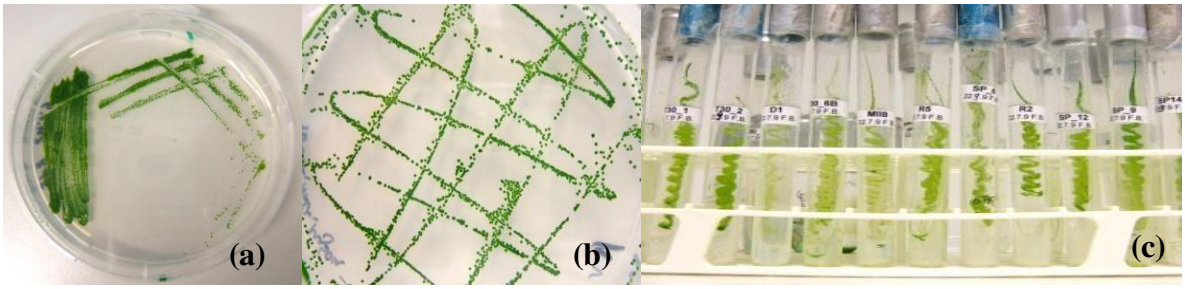
Isolate	μ day^{-1} (22 ± 1 °C)	μ day^{-1} (45 °C)
Sp13	0.766	0.53
CS	0.943	0.42
I4	0.469	0.4
I5	0.832	0.39
Sp14	0.683	0.37

Organisms inhabiting places in warmer regions are usually exposed to a wide variety of temperature conditions. Their capability to adapt to rapid changes in environmental stresses, such as temperature shifts, is an essential requirement for survival. In this study, the analyzed microalgae species responded to thermal stress exposure in various ways. The results of the temperature sensitivity tests revealed that 21 isolates out of 130 samples were able to grow at temperatures of 40 °C or even higher. The aim of this study was to obtain a greater biodiversity of thermotolerant strains and surprisingly 13 of the identified strains were genetically identified to belong to the family Chlorellaceae (Figure S1). It is well known that some *Chlorella* species tolerate a broad temperature range and have been used as model organisms for physiological studies on broad temperature tolerance of microorganisms [6,8,19,21]. This study confirms the reported ability of *Chlorella* strains to adapt and grow at high temperatures. Species belonging to genus *Chlorella* seems to be suitable for cultivation at high temperatures in outdoor settings or greenhouses.

2.3. Establishment of Unialgal Cultures

Twenty three monoalgal cultures were attained by using the thirteen-streak-method (Figure 3) [22]. The morphological features of the isolates were analyzed using a Zeiss Axiostar Plus light microscope (Figure 4). The samples were investigated in Hellfeld mode at 40-fold magnification. The images were taken using a Fujifilm FinePix E550 digital camera with macro function. Bacterial contamination occurred frequently during isolation making it necessary to repeat the procedure several times on agar media supplemented with antibiotics. No cyanobacteria were isolated as a result and fast growing colonies were selected and purified. This step makes it possible to adapt the screening procedure to other desired algae properties or products such as pigments.

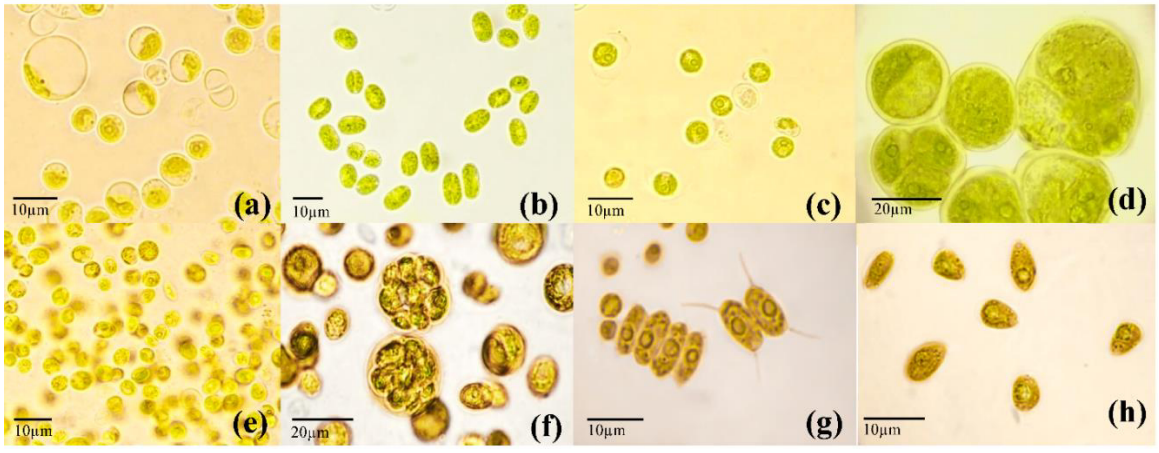
Figure 3. Establishment of pure cultures. (**a**) After purifying the environmental samples by using the thirteen-streak-method; (**b**) monocultures were obtained; (**c**) and kept in collection.



2.4. Molecular Identification

This work established the phylogenetic relationships of microalgae strains that could survive and/or grow between 35 and 45 °C relative to known microalgal genera and species. Twenty two isolates were genetically identified on molecular level via 18S rDNA sequencing and could be assigned to four different families (Scenedesmaceae, Chlorellaceae, Chlamydomonadaceae and Chlorococcaceae) and three different orders (Chlorococcales, Chlorellales and Volvocales) (Figure S1: Phylogenetic tree, Table S1: Accesion numbers).

Figure 4. Microscopic images of isolated species. (**a**) Sp14 (Chlorellaceae): Spherical cells with large vacuoles; (**b**) Sp1 (Chlorellaceae): elongated cylindrical cells; (**c**) T301 (Chlorellaceae): spherical single cells; (**d**) T309 (Chlorococcaceae): large coccoid cells containing zoospores; (**e**) R3 (Scenedesmaceae): spherical and ellipsoidal cells; (**f**) GDK (Scenedesmaceae): single cells and parental cells containing spores; (**g**) I3 (Scenedesmaceae): two and four cell cenobia with spines visible; (**h**) I1 (Chlamydomonadaceae): motile green ovoid cells with flagella.



The SSU rDNA (18S rDNA) is usually investigated for relationships between eukaryotic cells as the gene showns a slow evolutionary rate, bears well-conserved as well as rapidly evolving regions and is available in high copy numbers [23,24]. Using for example single gene analysis the small subunit ribosomal DNA (SSU rDNA) gene provides the basic structure for phylogenetic topologies but might be misleading in some aspects. Additional gene sequences could be investigated to improve the resolution of the tree, although the possibility of systematic errors increases with the number of genes and species involved [25].

3. Experimental Section

3.1. Sample Collection and Incubation

Samples were taken by using commercial cotton buds. After sampling, the cotton buds were wrapped into film to maintain moisture and then sent to the Laboratory of the University of Applied Sciences Bremen. The microalgae were inoculated by gently stirring the cotton buds into test tubes containing Wuxal liquid medium (WM) [20]. Test tubes were kept at 25 °C for two weeks in a light incubator to allow microalgal growth and any test tubes showing no algal growth after two weeks were removed.

3.2. Temperature Tolerance Test

An initial experiment increased the temperature to 35 °C for seven days. Test tubes showing a decrease in growth (macroscopically evaluated) were removed. In the following temperature increase experiment the mix cultures were transferred into 100 mL conical flasks and fresh WM was added. The flasks were incubated in a light incubator with a light/dark cycle of 12:12 h and exposed to higher temperatures for six hours per day. The cultures were illuminated with 105 µmol photons $m^{-2} \cdot s^{-1}$. Temperature pattern as well as light intensities during incubation were recorded using HOBO Data Logger (Onset, Linnich, Germany). For each sample a control was incubated at room temperature (22 ± 1 °C). Starting at 40 °C for seven days, the temperature was then increased daily by 1 °C increments up to 45 °C for ten days. Any cultures showing growth at 45 °C after ten days were exposed to 47 °C for another twelve days.

3.3. Establishment of Pure Cultures

Pure cultures were established by using the thirteen-streak-method [22]. Agar (15 g) was added to the liquid media before autoclaving to prepare solid WM. To inhibit bacterial growth an antibiotic cocktail was added to the agar by mixing penicillin (1.0 g), streptomycin (0.5 g) and chloramphenicol (0.1 g) with deionized water (16 mL), so that the final concentration of the antibiotic mix in the growth medium was 0.01% *v*/*v*. Single colonies were picked and recultured first on solid media and then in liquid media. The microalgae were cultivated at room temperature under sterile conditions in 50 mL WM liquid media in 100 mL conical flasks at 100 rpm. Fresh medium was added every 14 days. If no pure culture could be established after five purification cycles (thirteen-streak-method with subsequent plating), the sample was excluded from the screening.

3.4. Growth Kinetics

In order to compare the growth of the microalgae, the measured cell count data was standardized by calculating growth rates. Microalgae cultures were grown in batch cultures at an initial cell count of 1×10^6–1×10^7 cells mL^{-1}. These cultures were incubated at room temperature for approximately two weeks, until stationary phase was reached. Sampling was performed every two or three days by taking 1 mL of each culture in triplicate and measuring the optical density at 750 nm and to determine the cell count using the Thoma counting chamber (Paul Marienfeld GmbH + Co. KG, Lauda-Königshofen, Germany).

3.5. Molecular Identification

DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). The 18S rDNA amplification was performed in a T3 thermocycler (Biometra, Göttingen, Germany) with the primer pair EukA and EukB and additional internal primers (Table 3). The amplified products were purified using the MiniElute PCR Purification kit (Qiagen GmbH) then sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Residual nucleotides and PCR reagents were washed and removed by DyeEx Spin Kit (Qiagen) and sequencing was performed on an ABI 3100 Avant Sequencer (Applied Biosystems).

Signals generated by the capillary sequencer were investigated using the Sequencing Analysis Software v5.4 (Applied Biosystems). Single sequences were assembled to contigs using DNAStar (Madison, WI, USA) and consensus sequences were calculated. Using the BLAST (Basic Local Alignment Search Tool) algorithm (National Library of Medicine, Bethesda, MD, USA), related sequences were collected from the GenBank and a multiple sequence alignment was carried out using ClustalX (Conway Institute, UCD, Dublin, Ireland). The phylogenetic tree was constructed using Mega 4.1 (Center for Evolutionary Functional Genomics, Tempe, AZ, USA) with the neighbor joining clustering method based on the maximum likelihood model with 1000 bootstrap replicates.

Table 3. The oligonucleotide primers used for amplification and sequencing of 18S rDNA of selected microalgae strains.

Primer	Sequence	Reference
EukA	5'-AAC CTG GTT GAT CCT GCC AGT-3'	**[26], no polylinker**
EukB	5'-TGA TCC TTC TGC AGG TTC ACC TAC-3'	
1055f	5'-GGT GGT GCA TGG CCG TTC TT-3'	**[27]**
1055r	**5'-ACG GCC ATG CAC CAC CAC CCA T-3'**	

4. Conclusions

The screening for new thermotolerant microalgal strains for biomass production was successful.

Out of 130 environmental samples, 22 freshwater strains were isolated, cultivated and identified on a molecular level. It was found that the maximum temperature tolerated was 40 °C for eight strains and 45 °C for 13 strains, respectively. Although the results are promising, further experiments on biomass and secondary metabolite production must be conducted to give a more accurate prediction on the suitability of the isolates for industrial application. Process oriented strain selection is an important factor in making microalgal cultivation economical on an industrial scale. This study identifies new thermotolerant chlorophytes allowing cultivation under high or extreme temperature conditions in greenhouses or outdoor settings.

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Author Contributions

Franziska Bleeke contributed to experimental design, data acquisition, result analysis and manuscript preparation. Vincent M. Rwehumbiza contributed to result analysis and manuscript preparation. Dominik Winckelmann contributed to experimental design and data acquisition. Gerd Klöck contributed to experimental design, result analysis and manuscript preparation.

Conflict of Interest

The authors declare no conflict of interest.

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2.2 Optimization of freshwater microalgal biomass harvest using polymeric flocculants (Publication 2)

This manuscript was published in *International Aquatic Research* on the 10th of July 2015: Bleeke, F., Milas, M., Winckelmann, D., Klöck, G. (2014), Optimization of freshwater microalgal biomass harvest using polymeric flocculants. *International Aquatic Research*, DOI 10.1007/s40071-015-0108-8

Summary

This article describes the optimization of freshwater biomass harvest by testing different polymeric flocculants. Since the biomass harvest of microalgae contributes to up to 30% of the whole production costs, it is one of the major bottlenecks in microalgae biotechnology.

By chemical flocculation the cells coagulate and larger particles are produced with a higher settling velocity due to the higher density of the flocks. In this article 15 polyelectrolytes with cationic, anionic and non-ionic properties were examined and the flocculation efficiency of each flocculant determined. Three chlorophytes *Chlorella* sp, *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* were tested and the influence of different sizes, morphologies and motilities on the flocculation behavior verified. Since the reuse of the growth medium after cell separation significantly affects cost effectiveness, a recycling experiment was designed and the influence of possible flocculant residues on cell growth monitored over a period of eight weeks.

The results showed that the flocculation efficiency was considerably influenced by the net charge of the polymer. The highest flocculation efficiency was achieved using cationic flocculants. The best result was monitored using the cationic polyelectrolyte PK55H with a flocculation efficiency of 95% at concentrations of 1.5mg l^{-1}. The use of anionic and non-ionic flocculants resulted in no or insufficient flocculation. In the recycling experiment no limitation in culture growth and biomass production was detectable.

Contribution of Ph. D. candidate

Franziska Bleeke contributed to experimental design, data acquisition, result analysis and manuscript preparation. Malgorzata Milas contributed to data acquisition and result analysis. Dominik Winckelmann contributed to experimental design and manuscript preparation. Gerd Klöck contributed to experimental design, result analysis and manuscript preparation.

Optimization of freshwater microalgal biomass harvest using polymeric flocculants

Franziska Bleeke [1,2*], Malgorzata Milas[3], Dominik Winckelmann[2] and Gerd Klöck[2]

[1] Jacobs University, 28759 Bremen, Germany

[2] Institute of Environmental Biology and Biotechnology, University of Applied Sciences Bremen, 28199 Bremen, Germany

[3] Gdansk University of Technology, 80-233 Gdansk, Poland

* Author to whom correspondence should be addressed to: Franziska Bleeke: Franziskableeke@hotmail.de, Tel: +49 421 5905 2921, Fax: +49 421 5905 4245

Abstract:

Although microalgae show a great potential in the biotechnology sector, high production costs have limited industrial applications. Biomass harvest is one of the major bottlenecks in microalgae cultivation due to high energy inputs which are needed to separate the cells from the surrounding media. Chemical flocculation is considered to be a reliable resource to improve cost effectiveness in the down streaming processing. Flocculation efficiency is dependent on several factors such as the polymer type and charge as well as on the microalgae species. In the present study 15 polyelectrolytes were tested for their potential to harvest algal biomass. Cationic, anionic and non-ionic flocculants were tested in different amounts at varying incubation times to determine the adequate conditions needed. By testing the three chlorophytes *Chlorella sp*, *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii*, the influence of different sizes, morphologies and motilities of the flocculation efficiency was verified. Furthermore the biocompatibility of an efficient flocculant was tested in a recycling experiment over a period of eight weeks.

Keywords: microalgae, biomass harvest, chemical flocculation, polyelectrolytes, microalgal biotechnology, polymeric flocculants

1. Introduction

Microalgae are microscopically small photosynthetic protists with rising significance in the biotechnology sector. According to Posten & Walter 2012 microalgae have a 5 times higher biomass productivity per hectare compared to terrestrial crops (Posten *et al.*, 2012b). Furthermore they can be grown on non-arable land and produce biomass which can serve as food or feedstock or as potential substrate for biofuel production (Chisti, 2007; Mata *et al.*, 2010; Posten *et al.*, 2012b).

Although microalgae cultivation displays great potential, high production costs have limited industrial application. A major factor is the cell harvest, which contributes to 20-30% of the biomass production costs (Grima *et al.*, 2003; Mata *et al.*, 2010; Uduman *et al.*, 2010). Large volumes have to be processed, since the concentration of cells is usually low with 0.5- 2.5 g l^{-1} (Grima *et al.*, 2003). Commonly used separation processes are a combination of filtration, flotation or flocculation followed by a final centrifugation step. To separate the cells from the surrounding media high energy inputs are needed, often exceeding the energy content of the harvested biomass (Grima *et al.*, 2003; Uduman *et al.*, 2011; Wijffels *et al.*, 2010).

Increasing the efficiency at low energy demands within the harvesting process is a major challenge in microalgal biotechnology. In flocculation, the cells coagulate and larger particles are produced with a higher settling velocity due to the higher density of the flocks. Flocculation can be achieved in several ways. Numerous studies have been published on the use of electro coagulation (Lee *et al.*, 2013; Uduman *et al.*, 2011; Vandamme *et al.*, 2011), pH induced flocculation (Vandamme *et al.*, 2012; Wu *et al.*, 2012; Zheng *et al.*, 2012) or bioflocculation using bacteria (Lee *et al.*, 2009), filamentous fungi (Zhou *et al.*, 2013) or another flocculating algae to flock a non flocculating algae (Salim *et al.*, 2011) for biomass harvest.

In chemical flocculation a chemical coagulant is added to the algal suspension (Xu *et al.*, 2010). Organic and anorganic flocculants like polyelectrolytes and metal salts were investigated. Aluminum, ferric and zinc salts were tested (Papazi *et al.*, 2010) and compared with polyelectrolytes (Gerde *et al.*, 2014; Granados *et al.*, 2012; Papazi *et al.*, 2010; Sirin *et al.*, 2012). Comparative studies showed that higher flocculation efficiencies (FE) were achieved with polyelectrolytes than with metal salts (Gerde *et al.*, 2014; Granados *et al.*, 2012). Besides the lower FE, another disadvantage of the use of metal salts is the high concentration of metals in the algal biomass after harvest. Metal residues may impede the use of certain applications such as

animal feed (Grima *et al.*, 2003). Polymers are commonly used in wastewater purification processes and preferred for algal harvest because lower amounts are needed and non-toxic and biodegradable substances are available(Granados *et al.*, 2012). Polymeric flocculants are commercially available with cationic, anionic and non-ionic charges in different charge densities, whereas cationic flocculants are considered to be the most effective for algal harvest (Granados *et al.*, 2012; Tenney, Echelber.Wf, Schuessl.Rg *et al.*, 1969). The effect of different flocculants on several algae species has also been described. The results showed high variability in respect to the algae species and the nature of the flocculant (Gerde *et al.*, 2014; Granados *et al.*, 2012; Harith, Yusoff, Mohamed *et al.*, 2009; Papazi *et al.*, 2010; Rashid, Rehman & Han, 2013; Sirin *et al.*, 2012).

Chlorella sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* are commonly used laboratory strains and model organisms for algal research (Harris, 2001; Mandal & Mallick, 2009; Safi, Zebib, Merah *et al.*, 2014; Sanchez, Fernandez, Acien *et al.*, 2008). By testing these three chlorophytes the influence of different sizes, morphologies and motilities of the flocculation efficiency was analyzed.

In the present study 15 polymeric flocculants were compared to test the following hypotheses. Are cationic flocculants more effective compared to other polymeric flocculants (H1)? Do closely related species show similar flocculation behavior (H2)? Is a reuse of the media after cell separation by flocculation applicable (H3)?

2. Material and Methods

2.1. Microalgae and culture conditions

The experiments were carried out using the freshwater chlorophytes *Scenedesmus acuminatus*, *Chlorella sp.* and *Chlamydomonas reinhardtii* from the culture collection of the University of Applied Sciences Bremen. The cells were grown at 22°C ±1 in a culture volume of 2 liter in Wuxal liquid medium (WM)(Winckelmann, Bleeke, Bergmann *et al.*) without pH control. Bottles were equipped with aeration hoses providing continuous bubbling with compressed air at a flow rate of approximately 2.3 l min^{-1}. Every three days 600ml of the culture was used for the experiments and fresh medium was added to the remaining culture. Illumination was offered 24 hours per day by fluorescent lamps (OSRAM L 30W, warm white) placed in front and behind the algae cultures (light intensity, 50µmol photons m^{-2} s^{-1}).

2.2. Flocculants

Stock solutions of 15 polymeric flocculants with cationic, anionic and non-ionic characteristics were prepared according to the manufacturer's recommendation, dissolved in water and stored at 4°C for maximum 2 weeks.

Tab 1: The 15 tested flocculants. Commercial name, polymer type, net charge and supplier are listed. The prices stated are price indications subjected to daily fluctuations and dependent on the ordered quantities. NA= Price not available.

Commercial name	Polymer type	Net charge	Stock solution [g/l]	Supplier	Price [€/kg]
Emfloc KC 750	Starch	Cationic	10	Emsland-Stärke GmbH D- 49824 Emlichheim	1,25
Magnafloc LT 22S DWI	Polyacrylamide	Cationic	5	BASF Corporation Florhalm Park, USA	NA
POLY SEPAR® CFL 25	Tannine, quaternary ammonia compound	Cationic	10	Separ Chemie GmbH D-22926 Ahrensburg	2,20
POLY SEPAR® KW 100	Quaternary ammonia compound, free of Polyacrylamide	Cationic	10		2,40
POLY SEPAR® KW 45	Polyacrylamide	Cationic	10		2,40
POLY SEPAR® PK 55 H	Polyacrylamide	Cationic	2		3,20
POLY SEPAR® SK 72	Starch	Cationic	10		3,00
POLY SEPAR® KW 745 H	Polyacrylamide	Cationic	10		2,40
CFL 217	Poly DADMAC	Cationic	10		2,20
CFL 229	Poly DADMAC	Cationic	10		2,20
POLY SEPAR® AN 10 TW	Polyacrylamide	Anionic	10		3,50
Magnafloc LT 27	Polyacrylamide	Anionic	5	BASF Corporation Florhalm Park, USA	NA
Magnafloc LT 25	Polyacrylamide	Anionic	5		NA
Magnafloc LT 20	Polyacrylamide	Non-ionic	5		NA
POLY SEPAR®AN20	Polyacrylamide	Non-ionic	10	Separ Chemie GmbH D-22926 Ahrensburg	2,80

2.3. Flocculation experiments

Before each experiment, the pH of the sample from the algae culture was carefully adjusted to 7.0 by adding 1N HCl. The cell number was determined using the a Thoma counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and subsequently adjusted to 1x107 cells ml-1 by dilution with tap water. Flocculation experiments were evaluated using jar tests (Granados et al., 2012; Hudson & Wagner, 1981; Vandamme, Foubert, Meesschaert et al., 2010). 100ml Erlenmeyer flasks were filled with 100ml of algal suspension (Fig 2). The flasks were stirred at 150 rpm. One type and concentration was used in each flask. Once the flocculant was added, the culture was mixed for one minute at 250 rmp, to allow flocculant distribution. After one minute, the mixing speed was reduced to 50 rpm for two minutes, to support flock formation, followed by a settling phase without agitation. Samples were taken directly after the flocculant distribution and after 2, 10, 30 and 60 minutes from 3 cm under the culture surface. Optical density was measured at 750nm (Genesys 20, Thermo scientific, Walthman, USA) and the flocculation efficiency (FE) was determined as follows:

$$Flocculation\ efficiency = \left(\frac{ODt0 - ODt1}{ODt0}\right) \times 100$$

where ODT0 is the initial optical density before starting the flocculation and ODT1 is the optical density of the sample at a certain point of time during the process. Samples showing OD values above 0,5 were diluted with tap water for the measurements to assure linearity.

2.4. Media reuse after flocculation

A recycling experiment was conducted in order to investigate a potential negative impact of flocculant residues on the algal growth when the media is recycled. Flocculant PK55H was chosen for the media reuse experiment. The experiment was conducted in triplicate using the microalgae *Chlamydomonas reinhardtii* under the same cultivation setup as described in 2.1. Over a period of eight weeks the cultures were harvested weekly by flocculation (Fig 1,a) or centrifugation (Fig 1,b), whereas the centrifugation served as positive control displaying the growth without the impact of the flocculant. The volume of the recycled media was dependent on the optical density of the culture. After cell separation the culture supernatant was enriched with concentrated WM and led back into the culture vessel. The final optical density in the culture after media recycling was 1,5 at 750nm.

Cell growth was monitored by optical density measurements at 750nm, cell count determinations and dry biomass determination. For dry biomass determination 10ml of the algal culture where filtered through a previously balanced Whatman GF/C glass fiber filter (Whatman, Maidstone, UK). The filters were dried in an oven at 80°C for 12 hours and weighed afterwards. The difference in weight was calculated and multiplied by 100 resulting in the dry biomass expressed in g l-1.

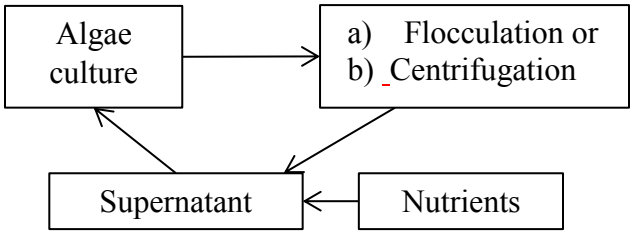


Fig 1: Media reuse experiment**.** Over a period of eight weeks the cultures were harvested weekly by a) flocculation or b) centrifugation, After cell separation the culture supernatant was enriched with concentrated WM and led back into the culture vessel.

3. Result and Discussion

The flocculation process consists of different stages as displayed in Fig 2. This shows the different phases during a flocculation process using *S.acuminatus* with the flocculant PK55H. After the addition of the polymer (a), the mixing speed is increased for 1 minute to allow flocculant distribution (b). The flocculant adsorbs to the cell surface and the suspension is destabilized (c) and flock formation can be observed (d,e). The flocks grow due to successive collisions and adsorption of microflocks (f-i). In a last phase the agitation is stopped and the flocks are allowed to settle (j). According to the manufacturer PK55H is a strong cationic polymer producing large dense flocks (Fig 5i; Fig 5c).

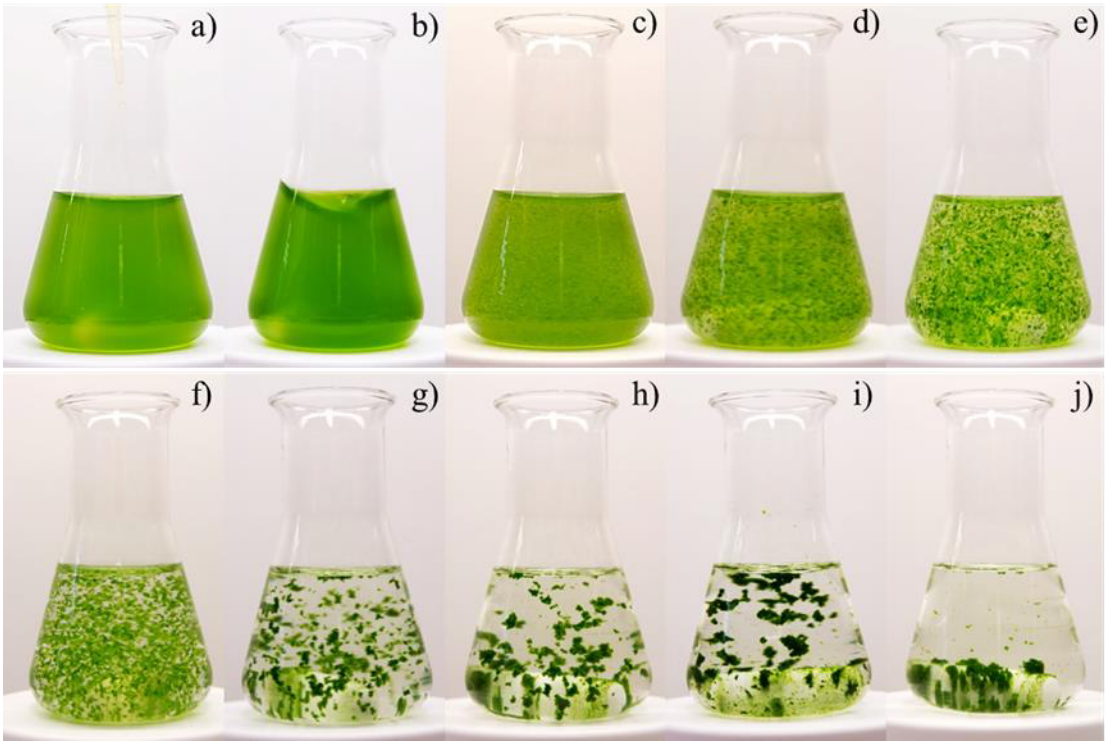


Fig 2: Flocculation of *Scenedesmus acuminatus* using flocculant PK55H. a) Addition of flocculant, b) flocculant distribution, c) destabilization of the suspension, d+e) flock formation, f-i) flock growth, j) settling of the flocks

The flocculant concentration in the suspension significantly affects the FE. If a destabilization of the algal culture is not visible after flocculant addition, the amount of flocculant can be increased.

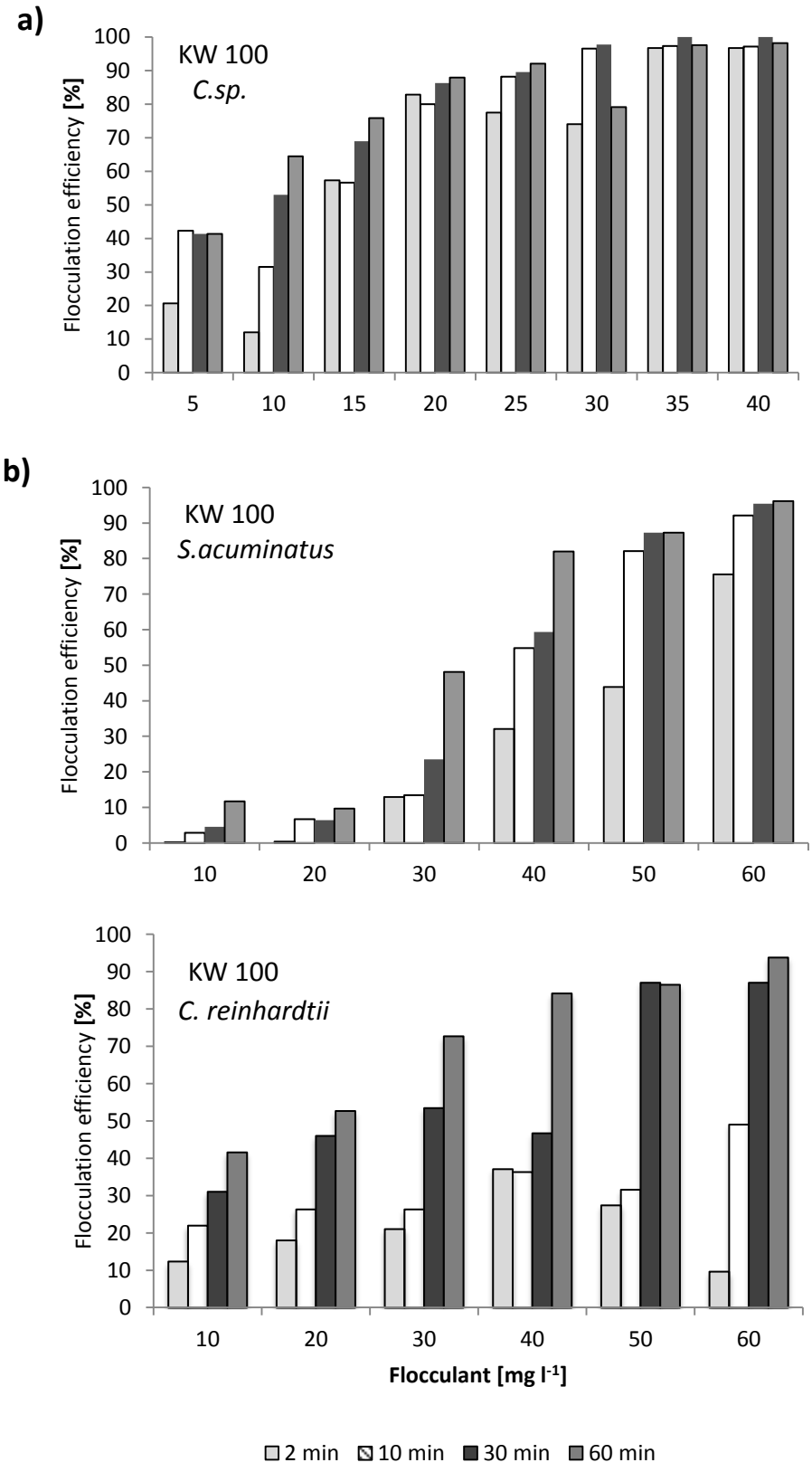


Fig 3: Example of a flocculation experiment using the cationic flocculant KW100. Flocculation efficiencies for harvesting *Chlorella sp* (a), *Scenedesmus acuminatus* (b) and *Chlamydomonas reinhardtii* (c) cultures are shown with different doses of flocculant (mg l^{-1}). Optical density measurements were conducted after 2, 10, 30 and 60 minutes.

Flocculation experiments were conducted determining the FE at different dosages for all 15 flocculants investigated (Tab 1). Furthermore the optimal incubation time was identified by monitoring the FE at different time point. In each experiment, the flocculant was added and the optical density was measured after 0, 2, 10, 30 and 60 minutes. The settling of the microalgae was monitored as a result of flocculation. Fig 3 shows the flocculation of *Chlorella* sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* with the cationic flocculant KW100. Increasing flocculant concentration causes the FE to rise. Fig 3a shows the results for *Chlorella* sp. at concentrations between 5- and 40mg l-1 of flocculant in the algae culture. Whereas 5mg l-1 did not even reach a FE of 50%, concentrations above 25mg l-1 resulted in a recovery rate of 90%. The incubation time however differed significantly. Increasing the amount of flocculant added reduces the incubation time needed to reach 90% FE. 60 minutes were needed at 25mg l-1; 10 minutes at 30mg l-1 and only 2 minutes at 35mg l-1 to reach the 90%. Higher amounts were needed for the flocculation of *S.acuminatus* and *C.reinhardtii* (Fig 3b and c). A FE of 90% was reached after 10- and 60 minutes respectively only after adding 60mg l-1. Lower doses of 15mg l-1 for *Chlorella* and 30mg l-1 for *Scenedesmus* and *Chlamydomonas* resulted in insufficient algae recovery. Different behavior in flocculation performance between the algae species was also observed in experiments displayed in Fig 4.

Cationic, anionic and non-ionic flocculants were compared as regards the flocculation efficiency of the three algal species. First the minimum concentration needed to achieve the highest FE was determined.

Fig 4 shows the highest FE achieved within the experiments together with the corresponding amount of flocculant applied. The concentration is given in mg l-1 and is indicated on top of each bar. Whereas Fig 4a and b show the results of cationic flocculants, Fig 4 c shows the FE for anionic and non-ionic flocculants. PK55H reached the best FE at the lowest flocculant concentration. According to the manufacturers' information PK55H is a strongly cationic polymer of high molecular weight. Even at low PK55H concentrations of 1.5 mg l-1 for *Chlorella* and 2- and 4 mg l-1 for *Scenedesmus* and *Chlamydomonas*, high FE of <95% were attained (Fig 4b). This flocculant is suitable for all of the three cell types, resulting in large and dense flocks (Fig 5).

The cationic charge of a flocculant is dependent on the amount of cationic charged monomers bound to the polymer chain and can vary between 0 and 100% (Gerde et al., 2014). According to

the manufacturers' information KW 100 and KW 45 are both polymers with similar polymer structure, merely differing in the amount of cationic groups attached to the polymer chain.

a)

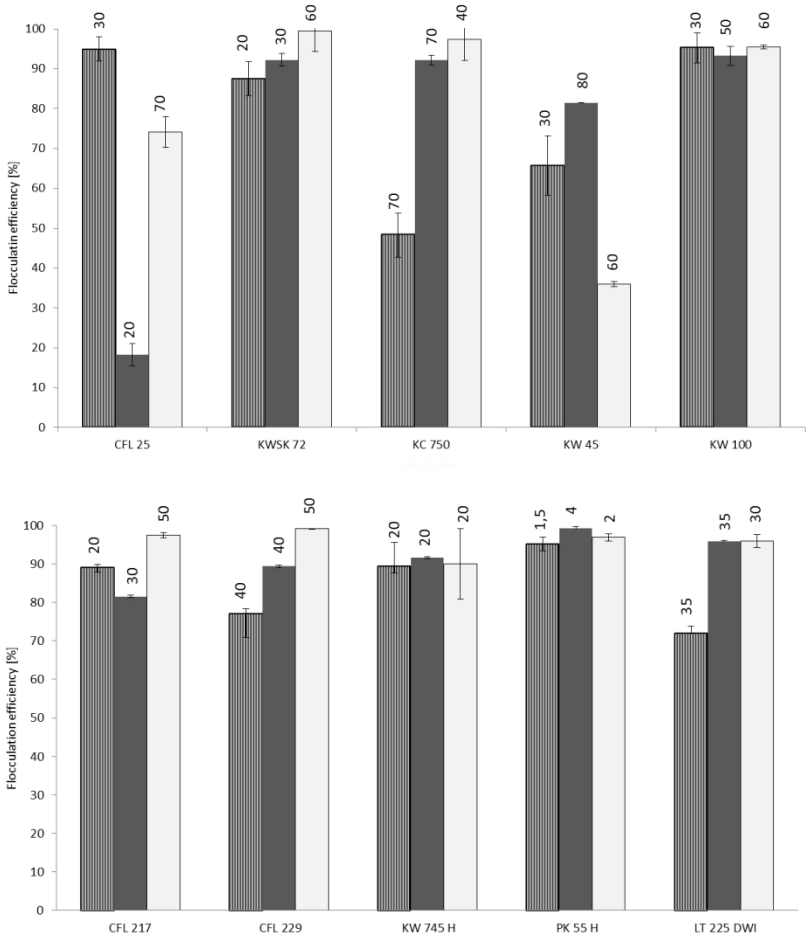
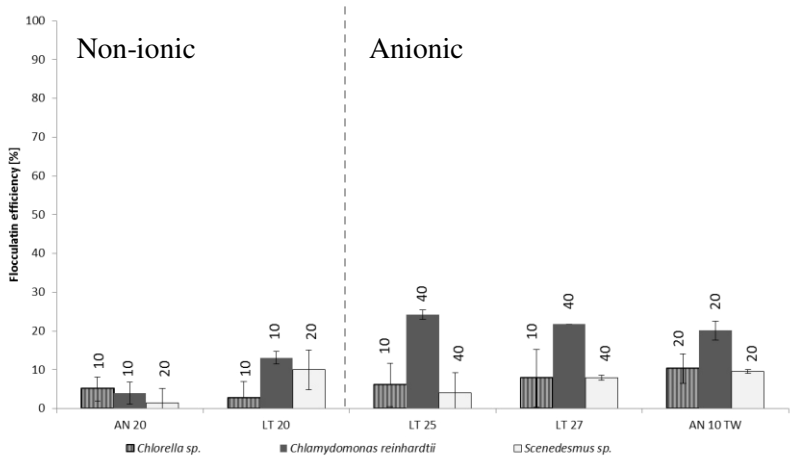


Fig 4: Flocculation efficiencies of 15 polyelectrolytes with *Chlorella sp, Chlamydomonas reinhardtii* and *Scenedesmus acuminatus*. Cationic (a and b), anionic and nonionic (c) flocculants were tested and the most efficient concentration (mg l^{-1}) determined and displayed on top of each bar. The incubation time was 30 minutes in all experiments

b)



The polymer chain of KW100 is completely substituted with cationic groups resulting in FE <90%. In the case of KW45 only 35% of the available sites are covered with cationic groups showing lower FE between 40 and 80%. This result confirms the assumption that the degree of cationic ionization affects harvesting efficiencies. A higher cationic charge of a polymer results in better FE.

CFL217, CFL 229 and CFL 25 belong to the group of polyDADMACS (Diallyldimethyl ammonium chloride). According to the manufacturer's information the molecular weight of these polymers is low with a high cationic charge. The results showed FE of 80 – 100% with the best results for Scenedesmus. In the case of CFL25 the results for the three tested microalgae differ (Fig 4). *Chlorella* cells were coagulated with a FE of < 90% resulting in very fine and small flocks (Fig 5). *Chlamydomonas* cells however only achieved FE of 20%. The specific surface area of the cells is another important factor to consider. The smaller size of *Chlorella* cells (2 – 10µm) give them a smaller surface area compared to *Scenedesmus* and *Chlamydomonas* cells(10 – 30µm). Polymers of low molecular weight can entirely absorb onto the cell surface forming regions with cationic nature. These regions can bind negatively charged regions of other algal cells. Larger cells might not be bound effectively resulting in lower FE.

Several microalgae have been subjected to flocculation experiments like *Chlorella*, *Chlamydomonas*, *Scenedesmus*, *Schizochytrium*, *Muriellopsis*, and *Phaeodactylum* resulting in different FE per mg of flocculant (Gerde et al., 2014; Granados et al., 2012; Harith et al., 2009; Papazi et al., 2010; Rashid et al., 2013; Sirin et al., 2012). *Chlorella* sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* are commonly used laboratory strains and model organisms for algal research (Harris, 2001; Mandal et al., 2009; Safi et al., 2014; Sanchez et al., 2008). By testing these three chlorophytes the influence of different sizes, morphologies and motilities of the flocculation efficiency should be verified. Considering the varying flocculant concentrations needed to flock the different algal species the hypothesis that related species (Chlorophyceae) show similar floccculation behavior cannot be positively proved.

Fig 4c shows the results gained with nonionic (AN 20, LT20) and anionic flocculants (LT25, LT27 and AN10TW). No flocculation was observed for these flocculants, whatever dose used.

At neutral pH the cells surface of microalgae is usually negatively charged due to functional groups on the algal surface (Golueke et al., 1970).Therefore generally positively charged flocculants are recommended to neutralize the negatively charged cell surface of the microalgae

(Granados et al., 2012; Tenney et al., 1969). Flocculation with polymers of the same charge can also be achieved by other mechanisms than charge neutralization, such as inter-particle bridging with long polymers. Tenney and co-workers reported the attachment of anionic polymers to the algal surface, but no flock formation in the solution was observed. Strongly anionic polymers can be effective in basic media. Harith et al described an effective use of anionic flocculants only after algal surface charge neutralization by pH adjustments. The application of flocculant LT25 and LT 27 at a pH of 10.2 with the microalgae Chaetoceros calcitrans resulted in flocculation efficiencies above 90% (Harith et al., 2009). The increase of pH before flocculation might improve FE but negatively affects media recycling and thereby cost effectiveness.

Based on the results, the use of anionic and nonionic flocculants cannot be recommended for harvesting of *Chlorella*, *Scenedesmus* and *Chlamydomonas* cells. The results gained positively prove the hypothesis that cationic flocculants are more effective than anionic or non-ionic polymers in respects of FE.

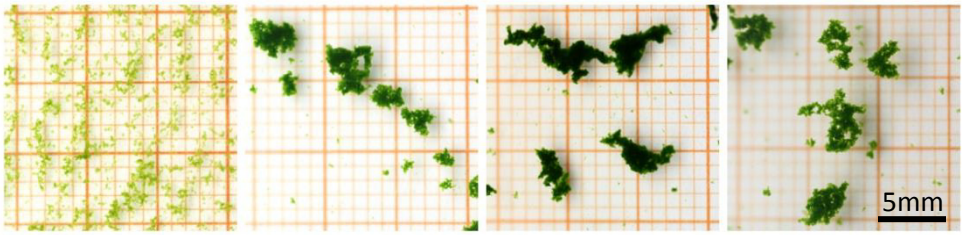


Fig 5: Flocks produced by the flocculants CFL25, KW100, PK55H and KC750 (from left to right). The picture shows the result of a flocculation using *Scenedesmus acuminatus*.

For industrial application the recycling of the growth media after cell harvest is an important factor in cost effectiveness. Residues and color changes after flocculation experiments were reported indicating that medium recycling after flocculation was not feasible (Sirin et al., 2012). It was necessary to verify if remaining flocculant particles do not negatively affect cell growth if the media is led back into the culture vessel after cell separation. A recycling experiment was conducted over a period of eight weeks using *Chlamydomonas* cultures. The biomass was harvested weekly using the flocculant PK55H or centrifugation and the supernatant was returned into the bioreactor. Fig 6 shows the results of the growth of the microalgae by optical density (OD) measurements. After each harvesting the optical density (750nm) was adjusted to 1.5 to

improve the light conditions for the algae (2.4). No differences between the flocculated cultures and the cultures harvested by centrifugation were visible by comparing OD values.

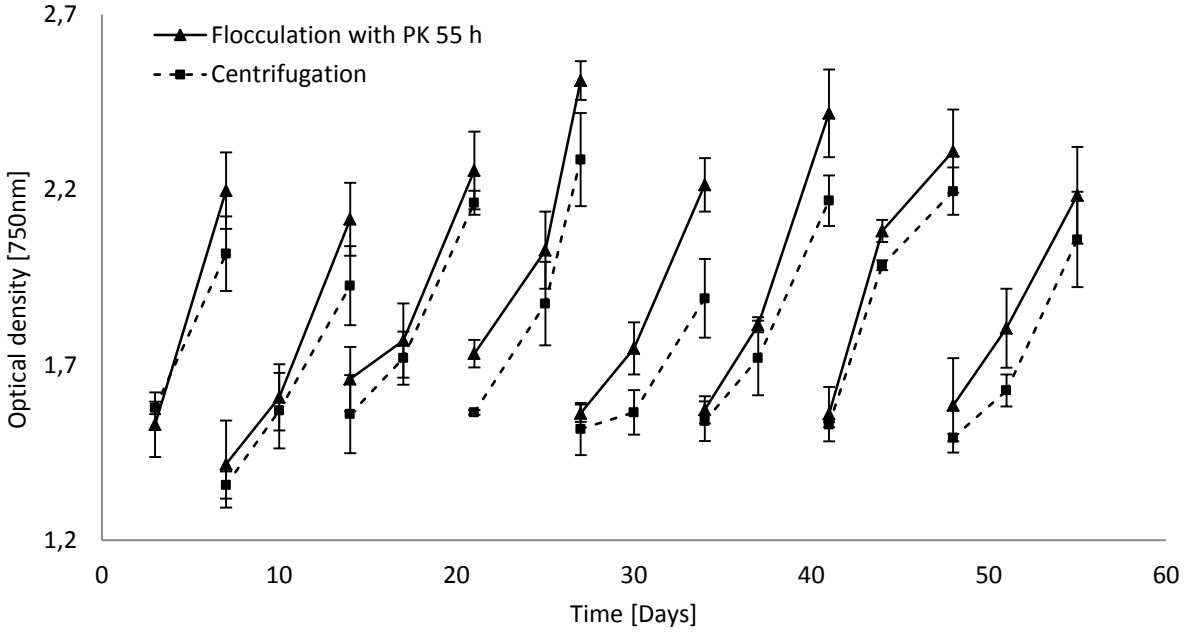


Fig 6: Media reuse after flocculation. Over a period of eight weeks the culture was harvested weekly by flocculation or centrifugation and the supernatant enriched with nutrients was led back into the bioreactor. The graph displays the optical density measurements registered three times per week.

Although the OD values between flocculated and centrifuged cultures remained similar, the biomass concentration changed After 10 days the formation of aggregates was visible under the microscope and the ratio between OD and BTM changed (Tab.2, Fig.6). Although die OD values remained the same, due to the aggregates, more cells were present in the cultures, resulting in higher dry biomass values.

Tab 2 lists the dry biomass values measured every week before harvesting. The data from the control and the recycling experiment differed significantly. After eight weeks of cultivation the dry biomass of the recycling experiment was 1gl-1 higher than that of the control cultures, although the OD values remained similar. The growth rates however indicate that neither the added flocculant, nor a possible accumulation of algal products negatively affected cell growth.

Tab 2: Dry biomass concentration in g l^{-1}. During the media reuse growth experiment the dry biomass was determined every week before harvesting.

Day	Flocculation			Centrifugation		
	MV	±	SD	MV	±	SD
0	1,52	±	0	1,52	±	0
3	1,49	±	0,16	1,47	±	0,04
10	2,18	±	0,22	1,73	±	0,01
17	2,14	±	0,03	1,60	±	0,18
25	2,36	±	0,17	1,50	±	0,11
30	2,09	±	0,04	1,34	±	0,10
37	2,42	±	0,12	1,59	±	0,04
44	2,53	±	0,08	1,68	±	0,22
51	2,75	±	0,18	1,72	±	0,14

Over a period of eight weeks the culture media was successfully recycled after flocculation. The hypothesis that the reuse of the media is applicable was positively proved.

4. Conclusion

The flocculation efficiency of microalgae for cell harvest was significantly influenced by the net charge of the tested polymer. Cationic flocculants achieved the highest flocculation efficiencies, whereas anionic and non-ionic flocculants resulted in no or insufficient flocculation. The amount of flocculant needed varied among the chemicals from 1.5- to 70mg l-1 but also between the algae species (e.g. CFL25 with 30mg l-1 for *Chlorella* and 70mg l-1 for *Scenedesmus*). Because of high flocculation efficiencies above 95% at low concentrations of 1.5mg l-1, the cationic polyelectrolyte PK55H was selected for a recycling experiments. No limitation in culture growth and biomass production was detected although an aggregation of the cells was visible. The results showed that chemical flocculation is a simple efficient and inexpensive method for harvesting different types of microalgae.

5. Acknowledgements

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2.3 Effect of voltage and electrode material on electroflocculation of *Scenedesmus acuminatus* (Publication 3)

This manuscript was published in *Bioresources and Bioprocessing* on the 12th of August 2015:

Bleeke, F., Quante, G., Winckelmann, D., Klöck, G. (2015), Effect of voltage and electrode material on electroflocculation of *Scenedesmus acuminatus*. *Bioresources and Bioprocessing 2015*, 2:36 ; DOI:10.1186/s40643-015-0064-6

Summary

In this study the effect of electro flocculation using different electrode materials was tested on the harvesting efficiency of the chlorophyte *Scenedesmus acuminatus*. One of the major challenges in microalgal biotechnology is to improve cost-effectiveness by decreasing harvesting costs. Commonly used separation techniques like centrifugation require high energy demands often exceeding the energy content of the desired algal product. Several studies have been published on the use of electroflocculation for algal biomass harvest using iron and or aluminum electrodes. In this article the use of aluminum, iron, magnesium, copper, zinc and brass electrodes was tested and compared. The flocculation efficiency was determined at four different voltages (10, 20, 30 and 40 V). The results showed that the highest harvesting efficiency was monitored for magnesium electrodes followed by Al, Zn, Cu, Fe and brass. The higher the voltage applied, the faster the cells were harvested. In a recycling experiment the reuse of the supernatant was tested to verify whether remaining flocculant might negatively affect cell growth. Reuse of the supernatant showed no adverse effect on algal growth. This study showed that besides the commonly used electrode materials like magnesium, copper, zinc and brass can be successfully used for microalgal biomass harvest. For some applications like food additive the use of magnesium shows clear advantages because of its lower toxicity even at high concentrations.

Contribution of Ph. D. candidate

Franziska Bleeke contributed to experimental design, data acquisition, result analysis and manuscript preparation. Gunnar Quante contributed to result analysis and manuscript preparation. Dominik Winckelmann contributed to experimental design and data acquisition. Gerd Klöck contributed to experimental design, result analysis and manuscript preparation.

Effect of voltage and electrode material on electroflocculation of *Scenedesmus acuminatus*

Franziska Bleeke [1,2*] , Gunnar Quante[2], Dominik Winckelmann[2], Gerd Klöck[2]

1) Jacobs University Bremen, Bremen, Germany
2) Institute of Environmental Biology and Biotechnology, University of Applied Sciences Bremen, Bremen, Germany

*****Corresponding author

E mail addresses: Franziska Bleeke: franziskableeke@hotmail.de
Gunnar Quante: gunnar.quante@googlemail.com
Dominik Winckelmann: d.winckelmann@gmx.de
Gerd Klöck: gerd.kloeck@hs-bremen.de

Abstract

Background:

Microalgae are a promising new source for biomass production. One of the major challenges in regards to cost effectiveness is the biomass harvest. High energy input is required for the separation of the small algal cells from a large volume of surrounding media. Electroflocculation is reported as a promising harvesting technique to improve cost effectiveness within the downstream process.

In the present study, six electrode materials were tested for electroflocculation of *Scenedesmus acuminatus*. Besides the commonly used aluminum and iron electrodes, magnesium, copper, zinc and brass electrodes were tested for biomass harvest and compared. The influence of four different voltages (10, 20, 30 and 40 V) was investigated and evaluated.

Results:

Electroflocculation was applicable with all tested electrode materials. The highest flocculation efficiency was achieved using magnesium electrodes followed by Al, Zn, Cu, Fe and brass. Using magnesium, 90 % of the suspension was clarified at 40 V, 30 V, 20 V, and 10 V after 9.2, 12.5, 18.5, and 43 minutes respectively. All electrode materials showed the fastest flocculation at 40 V

and the lowest at 10 V. The pH increased from 7.5 to values between 9.3 and 11.9 during the flocculation processes. Reuse of the supernatant showed no adverse effect on algal growth. The highest cell counts after 12 days of incubation were achieved with iron at $1.86x10^7$ cells ml^{-1} and the lowest with copper at $1.23x10^7$ cells ml^{-1}.

Conclusion:
Besides the commonly used iron and / or aluminum electrodes, other materials like magnesium, copper, zinc and brass can be successfully used for microalgal biomass harvest. For special biomass applications like food or feed additives, metals like magnesium have other advantages besides their high flocculation efficiency such as their low toxicity at high concentrations. Higher voltages increased the maximum flocculation efficiency but also increased the required energy input.

Keywords: Electroflocculation, Microalgae, Biomass harvest, *Scenedesmus acuminatus*, Flocculation

Background

Microalgae are eukaryotic, photosynthetic microorganisms that convert sunlight into chemical energy. The produced biomass can be used as food, feedstock or as potential substrate for biofuel production (Chisti, 2007; Mata *et al.*, 2010). They show a broad application in biotechnology since they grow fast at low nutritional and environmental requirements (Chisti, 2007; Mallick, 2002; Mata *et al.*, 2010; Wang *et al.*, 2008). Although they are easy to cultivate, the bottleneck which often makes microalgal cultivation uneconomical is the downstream processing which contributes to 20-30 % of the biomass production costs (Grima *et al.*, 2003; Mata *et al.*, 2010; Uduman *et al.*, 2010). Separation of the cells (2 -10 µm) from the surrounding growth media requires high energy inputs (Grima *et al.*, 2003). Large volumes must be processed, since the concentration of cells is very low at around 0.5 to 2.5 g l^{-1}. Common separation processes combine filtration or flotation with a final centrifugation step (Grima *et al.*, 2003; Uduman *et al.*, 2011), often exceeding the energy content of the harvested biomass (Wijffels *et al.*, 2010). Increasing the efficiency at low energy demands within the harvesting process is a major

challenge. By flocculation, the cells coagulate and larger particles are produced with a higher settling velocity.

Several studies are available on the use of chemical flocculation using metal salts or polyelectrolytes (Gerde *et al.*, 2014; Granados *et al.*, 2012; Papazi *et al.*, 2010; Tenney *et al.*, 1969), pH induced flocculation (Vandamme *et al.*, 2012; Wu *et al.*, 2012; Zheng *et al.*, 2012), and bioflocculation using bacteria or filamentous fungi for biomass harvest (Zhou *et al.*, 2013). In electroflocculation, the flocculant is produced by releasing metal ions from a sacrificial electrode (Vandamme *et al.*, 2011). Numerous studies have been published on the use of electroflocculation for algal biomass harvest (Lee *et al.*, 2013; Uduman *et al.*, 2011; Vandamme *et al.*, 2011); however, these experiments used primarily aluminium electrodes (Kim, Ryu, Kim *et al.*, 2012; Lee *et al.*, 2013; Vandamme *et al.*, 2011; Xu *et al.*, 2010) and/or iron electrodes (Uduman *et al.*, 2011; Vandamme *et al.*, 2011). Very little information can be found on the use of other electrode materials like Mg, Zn, Cu or brass for electroflocculation of microalgae.

The microalgae *Scenedesmus* is one of the most common genera found in freshwater ecosystems. These polymorphic chlorophytes show a broad application in wastewater treatment (Chinnasamy, Bhatnagar, Hunt *et al.*, 2010; Hodaifa, Martinez & Sanchez, 2008; Martinez, Sanchez, Jimenez *et al.*, 2000), biodiesel production (Mandal *et al.*, 2009; Tang, Han, Li *et al.*, 2011) and in the production of high value pigments like lutein (Sanchez *et al.*, 2008). Flocculation of *Scenedesmus* by metal salts, biofloculants, and cationic polymers has been tested in prior experiments, but little research has been done on electroflocculation of *Scenedesmus* (Mallick, 2002; Uduman *et al.*, 2010; Vandamme *et al.*, 2012; Vandamme *et al.*, 2011; Wang *et al.*, 2008).

In the present study, six electrode materials were tested at four different voltages. It was verified whether metals other than aluminum and iron are suitable for the electroflocculation process (H1) and if the flocculation efficiency increases at higher voltages applied (H2).

Material and Methods

Culture Media and Microalgae Cultivation

All experiments were carried out using the freshwater chlorophyte *Scenedesmus acuminatu*s from the culture collection of the University of Applied Sciences Bremen. The cells were grown in 2 litres of liquid Wuxal medium (WM) (Winckelmann *et al.*). Aeration was provided by compressed air, which was introduced into the culture by aeration hoses. Light was emitted by

fluorescent lamps (OSRAM L 30W, warm white) placed in front and behind the algae cultures for 24 hours per day.

Electroflocculation

The flocculation experiments were carried out at room temperature in 100 ml beakers filled with 90 ml of algae suspension. The initial cell concentration of the algae was $1x10^7$ cells ml^{-1}. The electrode plates were cut from commercial grade metal sheets. Before use, they were mechanically polished using abrasive paper and placed parallel and vertically into the algae suspension. The distance between the cathode and anode was 2.5 cm and the depth of immersion was 4 cm resulting in a submerged area of 51.2 cm^2 (40 mm x 32 mm x 4 electrode sides) for both electrodes. All experiments were carried out with the same area immersed in water and the same electrode distance. During the flocculation process, the culture was gently stirred at 100 rpm using a magnetic stirrer.

The electrodes were connected to a DC power supply (Hewlett Packard, Model 6205b, USA) and the voltage was adjusted to 10 V, 20 V, 30 V or 40 V.

To determine the flocculation efficiency (FE), 1 ml samples were taken before the flocculation and every 2.5 minutes during the process. Samples were taken from 3cm below the surface. Optical densities for each sample were measured at a wavelength of 750 nm in a Genesys 20 (Thermo scientific, Walthman, USA) photometer and pH values were recorded.

FE was calculated as follows:

$$Flocculation\ efficiency = \left(\frac{ODt0 - ODt1}{ODt0}\right) \times 100$$

OD_{t0} is the initial optical density before starting the flocculation and OD_{t1} is the optical density of the sample at a certain point of time during the process.

After the flocculation, the biomass and supernatant were separated and kept at -20 °C for recycling experiments. Before and after each flocculation, the electrodes were dried at 60 °C for two hours.

Iron, magnesium, aluminium, zinc, copper and brass electrodes were tested and compared. Each material served as both the anode and cathode at the same time. For comparison, a continuous function of the flocculation efficiency depending on the flocculation time, an interpolation, was used based on the following assumption:

The flocculation process can be described by a sigmoid function with an upper limit of 100 %, leading to the following function:

$$Efficiency(t) = \left(\frac{100}{1 + a^{b-t}}\right)$$

The coefficients a and b were determined for each voltage and electrode material, respectively, using a numerical curve fitting tool. By determining the coefficients a and b, it was possible to solve the efficiency function for the time when the curve reached values of 90 %. Those values were used in order to compare the various flocculation experiments.

The influence of each electrode material was tested and the best material regarding FE at 90 % was determined.

The mass loss as a function of time was assumed to be linear. This assumption allowed calculating the amount of mass lost during the time until a flocculation efficiency of 90% was reached. This mass was multiplied by the exchange prices of the corresponding electrode materials in order to estimate the costs of each flocculation process (exchange).

Media Reuse After Flocculation

A batch-recycling experiment was conducted over 12 days in 100 ml Erlenmeyer flasks, filled with 50 ml of algae cells incubated in flocculation supernatant. Cell growth was monitored every other day by optical density and cell count measurements. Cell number was determined using the a Thoma counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and adjusted to $2x10^6$ cells ml^{-1} by the addition of concentrated cells to the cell-free supernatant. For positive control the cells were diluted in fresh Wuxal Media. All experiments were carried out in triplicate.

Results and Discussion

Iron, magnesium, aluminium, zinc, copper and brass electrodes were tested and their corresponding FEs compared. Figure 1 shows an example setup of the flocculation experiment. The electrodes were connected to a DC power supply (Hewlett Packard, Model 6205b, USA) and the voltage was adjusted to 10 V, 20 V, 30 V or 40 V depending on the recent experiment (a, b). During the flocculation process, the suspension became milky white and gas visibly formed at the

electrodes resulting in foam formation on the top of the liquid surface (c, d). The flocculation was successful when the liquid phase was clear and algal cells were located in the foam on top (d).

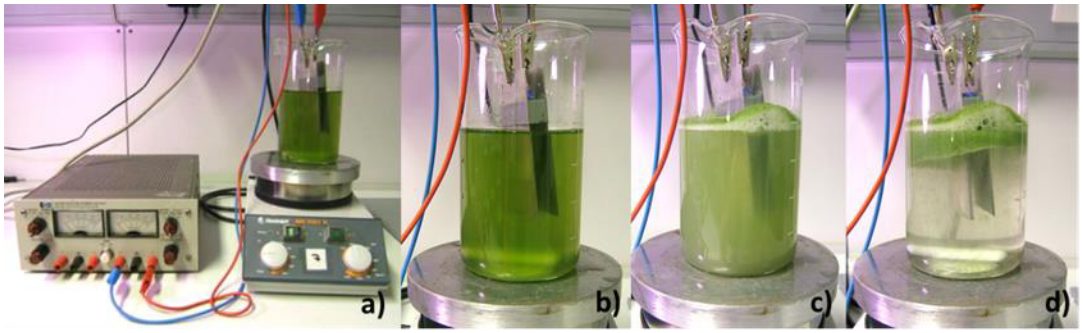

Figure 1: Electroflocculation process. a) Experimental setup: The flocculation experiments were carried out in 100-ml beakers filled with 90 ml of algae suspension. The electrodes were connected to a DC power supply (Hewlett Packard, Model 6205b, USA), b) Algae culture before flocculation, c) during the flocculation process and d) after the flocculation.

In Figure 2, the performance (FE) of the different electrode materials at different voltages over time are shown. All graphs show the fastest flocculation at 40 V and the slowest at 10 V; the higher the applied voltage, the faster the maximal flocculation efficiency. Similar results were published by other authors (Alfafara, Nakano, Nomura *et al.*, 2002; Poelman, DePauw & Jeurissen, 1997; Vandamme *et al.*, 2011; Xu *et al.*, 2010; Zhang, Yu, Li *et al.*, 2015).

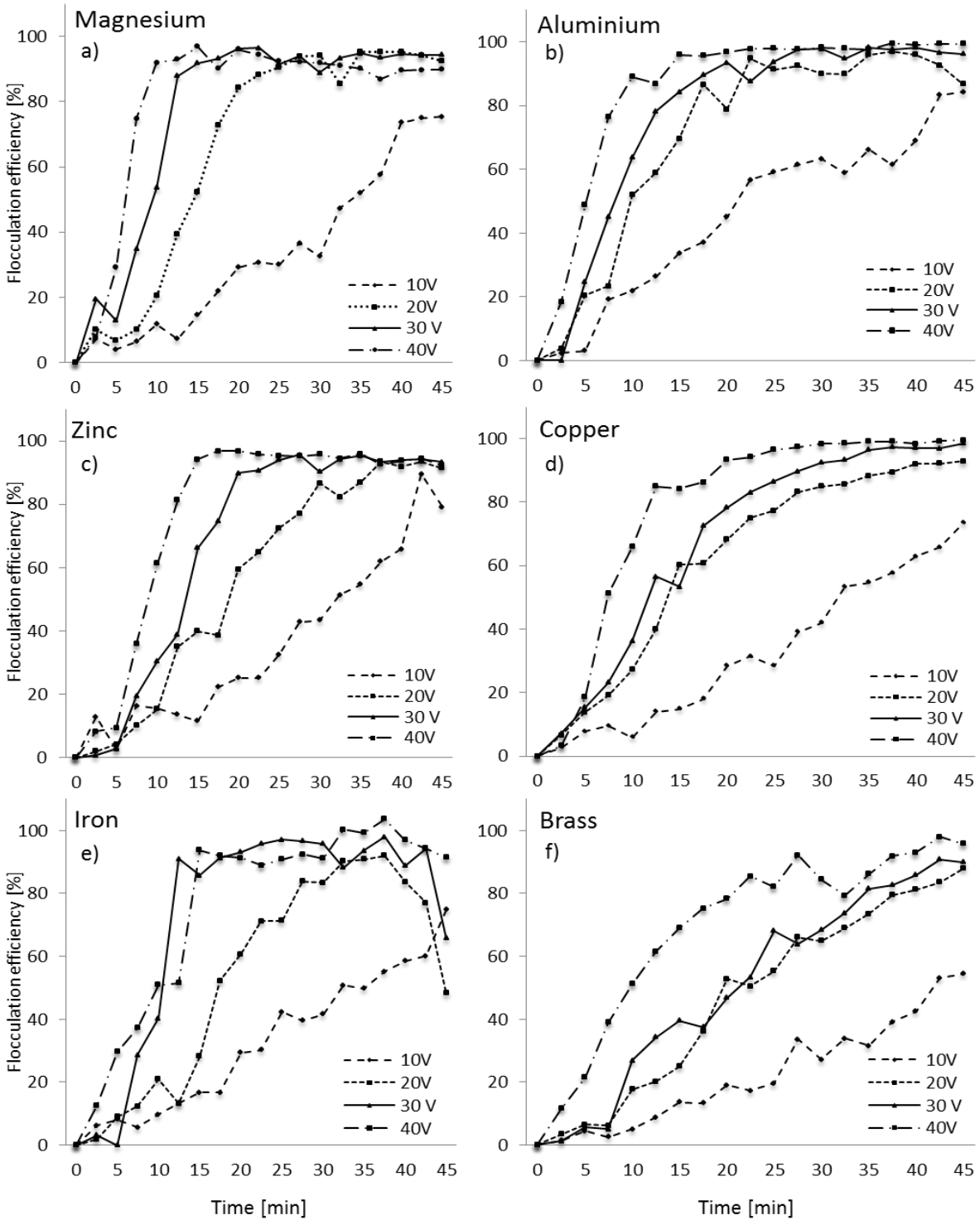

Figure 2: Effect of electrode material and applied voltage on the recovery rate during the electroflocculation process. Flocculation experiments were conducted using different electrode materials: magnesium (a), aluminum (b), zinc (c), copper (d), iron (e) and brass (f). The graphs were arranged according to the calculated electrode efficiency from high (a) to low (f). The voltage was adjusted to 10 V, 20 V, 30 V or 40 V and the flocculation efficiency was measured every 90 seconds.

The highest FE was observed when using Mg electrodes (Figure 2 a). Ninety percent of the suspension was clarified at 40 V after 9.2 minutes (Table 1). When the voltage was decreased to 30V, the graph levelled slightly, and the recovery time increased. At 30V, a FE of 90 % is reached after 12.5 min, at 20 V after 18.5 min and at 10 V only after 43 min (Table 1).

During the EF process, metal ions are continuously released from the anode by electrolytic oxidation. In H_2O these ions immediately react and form metal hydroxides. These metal hydroxides represent the flocculant which reacts with the algae cells. For the magnesium electrodes the following reaction occurs:

$Mg \longrightarrow Mg^{2+} + 2e^-$ $\quad 2H_2O \longrightarrow 4H^+ + O_2 + 4e^-$ (Anode)

$M(aq)\ n^+ + ne^- \longrightarrow M(s)$ $\quad 2H_2O + 2e^- \longrightarrow H_2 + 2OH^-$ (Cathode)

At higher applied voltages, more ions are produced, and consequently more flocculant is available in the algal solution (Mollah, Morkovsky, Gomes *et al.*, 2004; Mollah, Schennach, Parga *et al.*, 2001). The positively charged metal hydroxides react with the negatively charged cell surface of the microalgae. The potential of the algal cell is increased, and the surface charge is neutralized (Henderson, Parsons & Jefferson, 2008). The suspension becomes destabilized, and flocks are formed. At 10 V a short lag phase could be detected in all experiments, which is assumed to be due to insufficient flocculant availability at the start the flocculation process. After 5 – 10 minutes, the values start to rise. The shortened lag phase present at higher voltages is due to the faster ion release from the electrodes. Magnesium is bivalent and therefore forms extra stable hydrogen bonds, resulting in an effective flock formation.

The contamination of the harvested biomass and the remaining media with metal particulates might interfere with further processing steps or the use of the biomass as food or feed additive. Here the use of magnesium shows another advantage. Accepted magnesium limits are higher when compared to aluminium, iron, copper, or brass. In the German Drinking Water Ordinance, for example the limits for magnesium and also for zinc were removed since a negative impact for human health was considered as very low, whereas concentrations of 0,2 mg l^{-1} for aluminium and iron and 2 mg l^{-1} for copper are not to be exceeded (Ordinance, 2001).

Extensive research has been conducted regarding the use of aluminium electrodes (Chen *et al.*, 2009; Kim *et al.*, 2012; Lee *et al.*, 2013; Vandamme *et al.*, 2011; Xu *et al.*, 2010) and iron electrodes (Uduman *et al.*, 2011; Vandamme *et al.*, 2011). Aluminium was found to have the second highest FE (Figure 2b). Ninety percent FE was reached after 9, 11.4, 20.6 and 42 minutes

at 40, 30, 20 and 10 V (Table 3). In literature the use of aluminium electrodes is compared to the use of iron electrodes in coagulation processes with (Vandamme *et al.*, 2011) or without algae (Zongo, Maiga, Wethe *et al.*, 2009) involved. The results of this present study agree with the data reported that the use of aluminium is more efficient than the use of iron electrodes (Figure 2e). The lower efficiency of the iron electrode compared to aluminium might be explained by the lower current efficiency of the iron electrode (Zongo *et al.*, 2009).

The lowest recovery efficiency was found using brass electrodes (Figure 2f). After 15 minutes at 40 V only approximately 70 % of the biomass was recovered. Ninety percent was reached after 30 minutes which is believed to have been caused by the high pH value during the process. Figure 3a shows that after 11.5 minutes, a pH of 11.8 was observed. It is suspected that algal recovery efficiency decreases at pH values of 12 because of algal lysis (Contreras, Pieber, Delrio *et al.*, 1981; Xu *et al.*, 2010).

Table 3: Calculated incubation time until a 90 % flocculation efficiency is reached. The incubation time is given for each electrode material at 10, 20, 30 and 40 Volts.

	Magnesium	Aluminium	Zinc	Copper	Iron	Brass
10 V	43.2	42.1	53.6	61.0	53.7	71.1
20 V	18.5	20.6	30.6	31.3	27.9	43.9
30 V	12.5	11.4	21.7	20.3	16.0	40.7
40 V	7.3	9.0	14.2	14.6	46.9	30.9

Electroflocculation technologies are currently in use at wastewater treatment facilities (Mollah *et al.*, 2001). In industrial wastewater cleaning, the initial pH is one of the most important factors influencing the EF process (Mouedhen, Feki, Wery *et al.*, 2008). In Figure 3, the pH evolution during the EF with different electrode materials at 40V is shown. Figure 3a shows the data for Al, Mg and Zn while Figure 3b shows the values monitored with Fe, Cu and brass. In all of the experiments, the pH increased during the flocculation process.

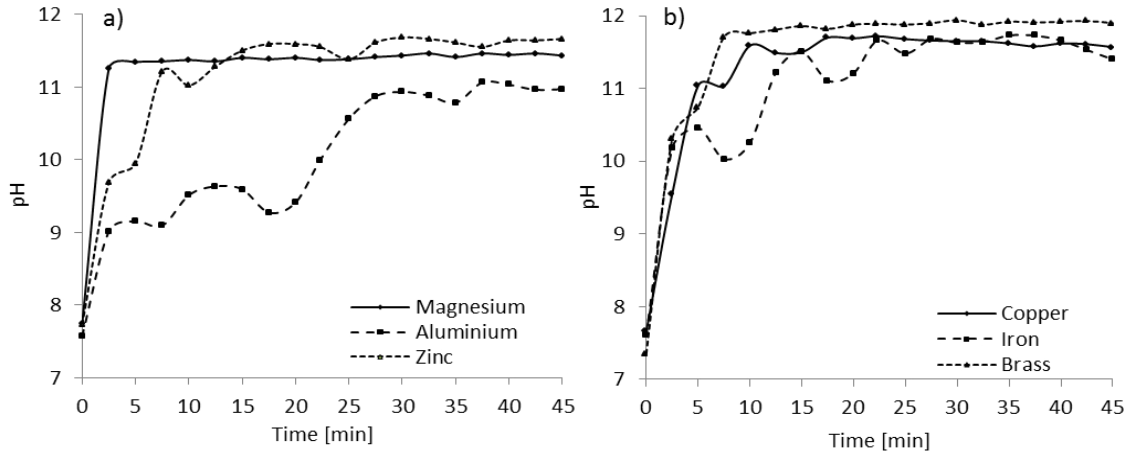

Figure 3: Effect of electroflocculation on the pH value using aluminum, iron and brass (a) and zinc, copper and magnesium (b) for electrode material. Measurements were recorded during a flocculation at 40 V.

A fast pH increase up to 10 – 12 was monitored in all samples except for Al. The highest pH value was reached during the flocculation using brass electrodes. The lowest pH was recorded in the samples using Al electrodes. Since the pH is a measure for the hydrogen and hydroxide concentration, it is expected that the produced metal hydroxides were immediately bound and thereby not contributing to pH increase. During the flocculation process, the algae recovery rate increased in the beginning but started to decrease after 20 minutes of flocculation (Figure 2e). Similar effects were observed by other authors. Xu 2010 described a decrease in recovery time and efficiency from pH 7 to 11 and an increase of recovery time at pH 12 which is expected to be because of algal lysis (Contreras *et al.*, 1981; Xu *et al.*, 2010). The fluctuations in the Al and Fe graphs might be due to insufficient mixing during sample taking.

Table 4: pH values recorded after 30 minutes of electroflocculation. The table lists the pH values gained with aluminum-, brass-, magnesium-, zinc-, copper-, and iron electrodes at 10, 20, 30 and 40 V starting with the lowest values (left) to the highest (right).

	Aluminium	Brass	Magnesium	Zinc	Copper	Iron
10 V	9.3	10.7	11.4	11.5	11.5	11.6
20 V	10.3	11.5	11.5	11.3	11.6	11.7
30 V	9.7	11.9	11.4	11.6	11.7	11.6
40 V	10.4	11.9	11.5	11.7	11.6	11.6

Figure 4 shows the mass loss and the calculated material costs until a flocculation efficiency of 90% is reached. All tested electrodes lost weight between 1.1 g for magnesium and 101 g for

iron. The highest mass loss was recorded for Fe with 101g at 40 V and for brass at 20 and 30 V. Although the mass loss of the iron electrodes is high, the costs for the flocculation with iron are comparably low. Since brass is the most expensive electrode material showing the lowest FE, it cannot be recommended for algal harvest using electro flocculation. The most economical efficient flocculation was achieved with Mg, Al and Fe.

For industrial applications, reuse of the growth media after cell separation is an important factor regarding cost effectiveness. A recycling experiment was conducted to investigate if the high pH value and/or remaining metal residues negatively affect cell growth if the media is led back into the culture vessel after cell harvest. The growth of the *Scenedesmus* cells was monitored in a batch experiment over twelve days of cultivation.

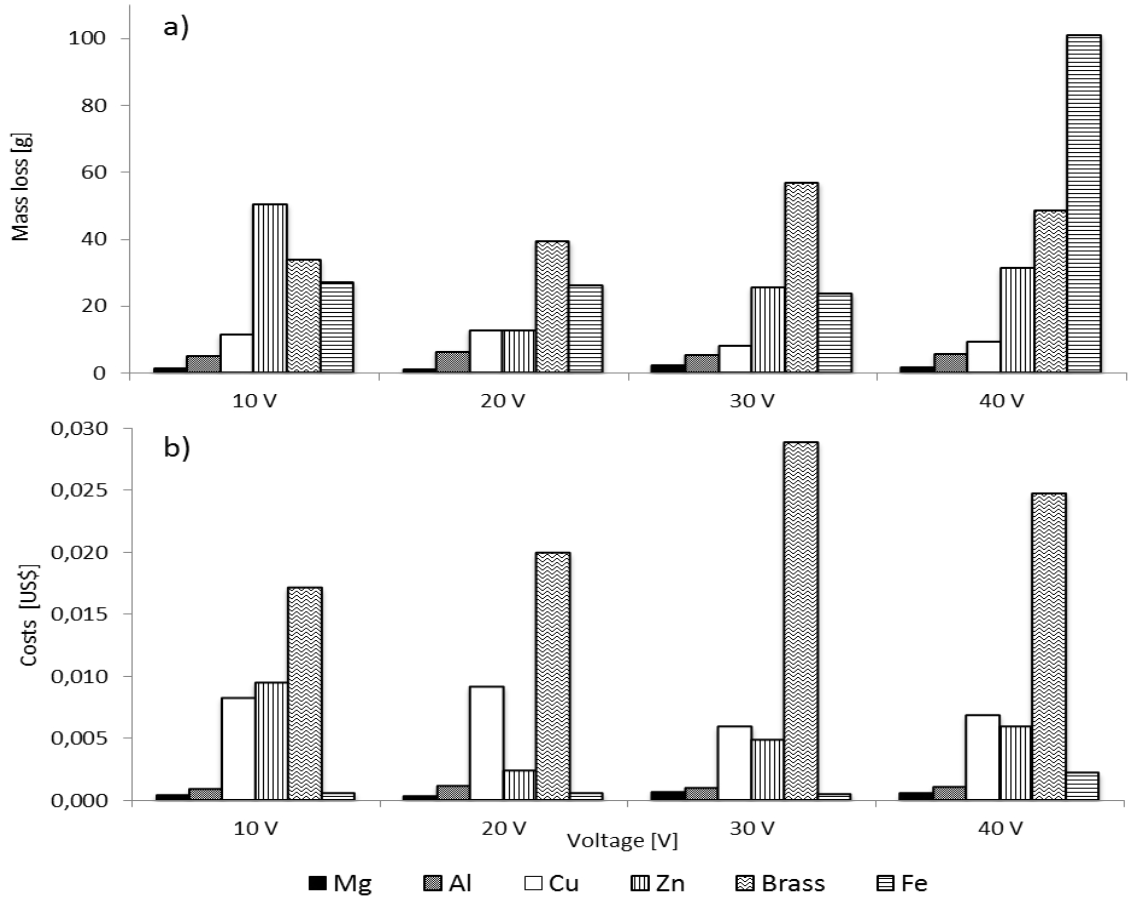

Figure 4: Mass loss and electrode costs. Mass loss of the electrodes during a flocculation experiment (a) and the corresponding metal costs (b). The metal prices were stated by the London metal exchange and are subjected to daily fluctuations.

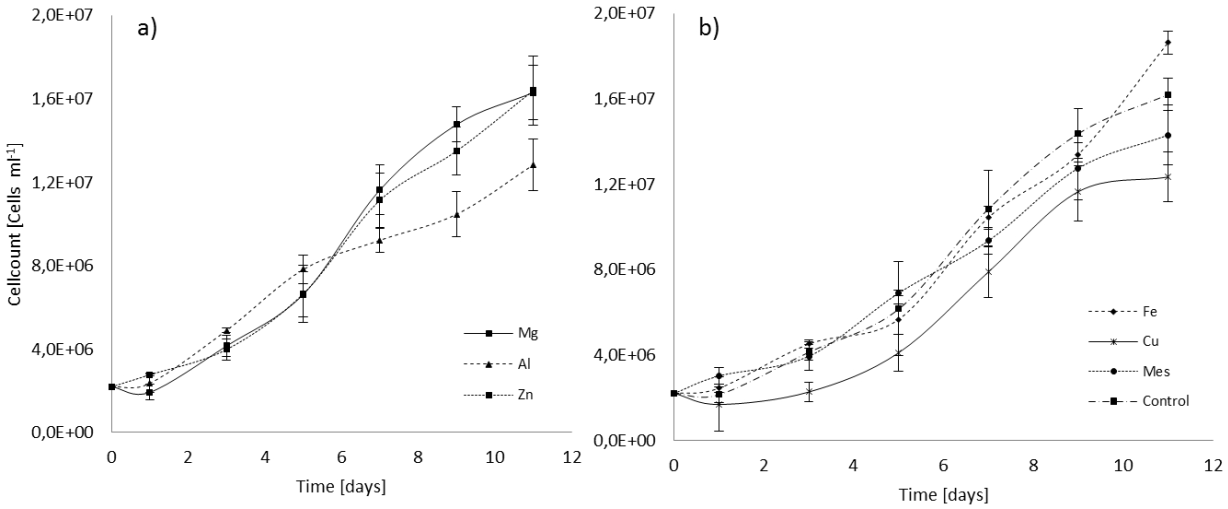

Figure 5: Media reuse after flocculation. For 12 days *Scenedesmus* cells were incubated in flocculation supernatant enriched with nutrients. The graphs show the cell growth displayed the cell growth measured in cells ml^{-1} monitored every other day.

Cell growth was monitored in all of the experiments (Figure 5). The best result was achieved with Iron supernatant and a final cell concentration of $1.86x10^7$ cells ml^{-1}. The control experiments with fresh media resulted in final cell concentration of $1.62x10^7$ cells ml^{-1}.The lowest cell concentration was reached with the copper supernatant with $1.23x10^7$ cells ml^{-1}. Although copper is an essential micronutrient for plants, higher concentrations are known to inhibit photosynthetic reactions (Kupper, Gotz, Mijovilovich *et al.*, 2009). The reuse of all supernatants showed similar growth behavior within the 12 days of cultivation.

Several experiments have been published on the use of electroflocculation for algal biomass harvest (Lee *et al.*, 2013; Uduman *et al.*, 2011; Vandamme *et al.*, 2011) mainly using aluminium electrodes (Kim *et al.*, 2012; Lee *et al.*, 2013; Vandamme *et al.*, 2011; Xu *et al.*, 2010) and/or iron electrodes (Uduman *et al.*, 2011; Vandamme *et al.*, 2011). Magnesium, copper, zinc or brass electrodes have so far not been used for this purpose. This study revealed that besides iron and aluminium, magnesium shows high potential for algal harvest by electroflocculation. Besides a high FE and cost effectiveness, magnesium is non-toxic and can be utilized in a broad range of application.

Conclusion

Besides the commonly used iron and or aluminum electrodes other materials like magnesium, copper, zinc and brass can be successfully used for microalgal biomass harvest by electroflocculation. The most cost effective flocculation was achieved with Mg, Al and Fe as electrode material. For special biomass applications like food or feed additives metals like Magnesium have other advantages besides their high flocculation efficiency such as their relative harmlessness even at higher concentration. A higher voltage increased the maximum flocculation efficiency but also increased the energy input needed. Recycling of the supernatant was shown to be possible but should be repeated in a long term experiment comprising several harvesting steps.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FB contributed to experimental design, data acquisition, result analysis and manuscript preparation. GQ contributed to result analysis and manuscript preparation. DW contributed to experimental design and data acquisition. GK contributed to experimental design, result analysis and manuscript preparation.

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3 Discussion

There is an increasing scientific interest in microalgal research not least because of the shortcomings of fossil fuels and the associated rising demand in renewable energy sources where microalgae are considered to be a highly interesting raw material. A lot of attention is paid to the development and economical improvement of up- and downstream processes of the algal products.

3.1 Hypothesis 1: It is possible to isolate robust and efficient production strains for biomass production out of environmental samples

Beginning at the upstream stage during the production process, industry is trying to apply well understood laboratory strains for outside large scale cultivations. It is often neglected to investigate whether these strains are able to withstand outdoor culture conditions. Identifying more new and productive strains is expensive in both time and costs in a rapid evolving industry. A lot of money is spent on methods and equipment to adapt the environment to the conditions needed by algae. Huge amounts of water and energy are wasted by cooling outside facilities during summer or heating in winter. Money is spent either to shade the bioreactors in order to avoid temperature increase and photo inhibition of dilute cultures or to artificially illuminate cultures to avoid mutual shading in dense cultures or to elongate the natural light phases. Several attempts are made to try to adapt the environment to the need of the chosen production strains. Choosing the strain according to the environmental requirements would bypass several of the above mentioned difficulties and would help to make the process more economically efficient.

In the framework of the first publication “Isolation and Characterization of New Temperature Tolerant Microalgal Strains for Biomass Production” the question whether it is feasible to isolate new production strains out of the environment was scientifically investigated and analyzed. 130 environmental samples were collected and investigated, focusing on high temperature tolerance.
During the isolation procedure antibiotics had to be applied to get rid of bacterial contamination. In most cases the addition of antibiotics delayed bacterial growth and sometimes inhibited microalgal growth. At times, bacterial growth seemed to support microalgal growth. Metabolites produced by the bacteria might have served as nutritional or growth supplement enhancing algal

propagation. Bitrophic interactions between microalgae and Bacteria have been reported (Croft et al., 2005; Keshtacherliebson et al., 1995). Croft et al 2005 surveyed 326 algae species from 8 different algae groups for their ability to synthesize vitamin B12. Half of them required exogenous vitamin B12 for growth gained throughout symbiotic interactions with bacteria. The algae supplied fixed carbon in return for vitamin B12. This kind of symbiosis is one of the reasons why many algal cultures are maintained non axenically in culture collection. As a result of the addition of antibiotics during the isolation process, no cyanobacteria were isolated in this study.

21 strains were able to grow at 40°C. 13 of these 21 strains were even able to grow at 45°C. Monitoring the growth rates showed that by increasing the temperature, the growth rate decreased. Molecular identification showed that 13 of the identified strains could be assigned to the chlorellaceae. *Chlorella* sp. is known to tolerate a broad temperature range and is widely used as a model organism for physiological studies on broad temperature tolerance of microorganisms. For instance, a low temperature strain of *C. sacchorophila* is very resistant to temperature variations as cell growth has been found to occur between 5 and 30 °C (Hanagata, Takeuchi, Fukuju et al., 1992; Kessler, 1985; Zuppini, Andreoli & Baldan, 2007). *Chlorella* species seem to be appropriate for cultivation at high temperatures in outdoor bioreactors or greenhouses. The reported results can be confirmed by this study.

But although fast growing algal strains could be isolated the strains were not tested in a large scale outdoor facility. Even if higher temperatures or seasonality does not affect the algal growth, there are several other parameters which might negatively impact cell growth. Beside a high temperature acceptance other characteristics like metabolite accumulation or the initialization of synthesis of metabolites should be further investigated. A product oriented strain selection would support the use of strains with the ability to accumulate high value products. The aquaculture industry is searching for strains with high protein content and easily digestible cell walls in order to substitute fish meal in formulated feed. Besides the ability to grow phototrophic, some microalga can also divide and metabolize without light. In this mode of culture, organic carbon sources like sugars or organic acids are used as sole carbon and energy source. Searching for algae with the ability to grow heterotrophically might result in a cost effective alternative with numerous advantages (Chen, 1996).

Several details were published about higher efficiencies and product yield when cultivating the cells heterotrophically in the presence of an organic carbon substrate (Gladue & Maxey, 1994; Schmidt, Wiebe & Eriksen, 2005; Tan & Johns, 1996; Wen & Chen, 2003). A high value product would help to make the process economical feasible. In the recent years a lot of attention has been paid to microalgae as a potential source for biofuel production (Chisti, 2007; Mata *et al.*, 2010). The limiting factor in the process is the identification of a suitable production strain accumulating high amounts of lipids under different culture conditions (Chen *et al.*, 2009). For example Nile red staining would provide a fast, sensitive and versatile screening method for lipid production in microalgae in the framework of a screening procedure (Chen et al., 2009; Huang, Chen & Chen, 2009). Compared to solvent extraction and gravimetric determination (Bligh & Dyer, 1959) which takes about three days, the Nile red staining is simple, less time consuming and smaller amounts of biomass are required (Huang et al., 2009).

On the down-streaming side the process oriented strain selection might prefer a strain with a certain morphology (e.g. filamentous) which simplifies cell harvest and therefore saves costs. For example the isolation of strains with cell walls which can be lysed in order to extract specific products. Mechanical stable cell walls on the other hand might be advantageous in reactor systems with high shear stress or other mechanical influences.

For every location, reactor system or product the right strain could be screened in order to improve efficiency. Several screening studies have been conducted. These studies mostly searched for one characteristic, sometimes for two. Furthermore most of the studies published screened already described species obtained from culture collections (Abomohra *et al.*, 2013; Ordog *et al.*, 2004; Picardo *et al.*, 2013) and not environmental samples.

This thesis showed that it is possible to isolate potential production strains out of environmental samples and that the identification of new thermotolerant chlorophytes was successful.

3.2 Hypothesis 2: It is feasible to harvest different microalgal strains by using commercially available polymeric flocculants and reuse the media after flocculation

Besides the algal strain selection, the biomass production process shows several possibilities for improvement in order to create a more cost effective process. One of these possibilities is

displayed within the down-stream processing by the harvesting technique. About 30% of the total production costs are caused in the harvesting step (Grima, Belarbi, Fernandez et al., 2003; Mata et al., 2010; Uduman, Qi, Danquah et al., 2010). Although a lot of research has been conducted to develop better, more efficient methods, the major part of the biomass nowadays is still harvested using separation processes like centrifugation. The advantages of centrifugation or separation are a fast harvesting result with a high efficiency. The high energy costs of this technique however are often exceed the energy amount of the produced biomass (Grima *et al.*, 2003; Uduman *et al.*, 2011; Wijffels *et al.*, 2010). The combination of centrifugation with a prior flocculation step would help to decrease energy input.

This research showed that the use of polymeric flocculants for microalgal biomass harvest is applicable on a laboratory scale. Fifteen flocculants with different chemical properties were tested and the optimal concentration and incubation time for each flocculant verified. The net charge of the tested polymer mainly influenced the success of a flocculation. Ten of the 15 tested polymers carried a cationic charge and the flocculation using these chemicals resulted in good or sufficient flocculation performances. The application of anionic flocculants resulted in no or insufficient FEs. The surface charge of algal cells at a pH of 7 is usually negative due to the attachment of functional groups on the algal cell surface (Golueke et al., 1970). Therefore generally positively charged flocculants are recommended to neutralize the negatively charged cell surface of the microalgae (Granados et al., 2012; Tenney et al., 1969).

The measuring and monitoring of the zeta potential before, during and after flocculation would be ideal for further investigation. Attempts were made to measure the Zeta-potential. Unfortunately the flocs were too large to be measured with a Nano-ZS ZEN3600 zetasizer (Malvern Instruments, UK). In aqueous media, a transitional layer is formed around the cells due to potential differences between the algae and the surrounding media. Another more diffuse layer is separated from the first layer by a shear plane. The difference in electric potential between this shear plane and the surrounding media is called zeta potential. The dimension of the zeta potential shows the extent of electrostatic repulsion among the algal cells. By the addition of cationic flocculants, the increase of cationic concentration in the media reduces the zeta potential of the algal cells by compressing the diffuse layer (Ives, 1959). By neutralizing the zeta potential, the cells can collide with each other and the solution is destabilized.

The best flocculation performance at the lowest concentration was achieved by the strongly cationic polymer PK55H at concentrations of 1.5 mg l^{-1} for *Chlorella*, 2mg l^{-1} for *Scenedesmus* and 4 mg l^{-1} for *Chlamydomonas.* The addition of 1.5mg l^{-1} of the flocculant resulted in FEs above 95%. Although these are good results it has to be considered that PK55H is a polyacrylamide which might hinder the use of the biomass as food additive or animal feed (Friedman, 2003). Inhibitory effects of acrylamide during biodiesel production have not been reported.

Numerous algal strains were exposed to flocculation experiments like *Scenedesmus, Chlamydomonas*, *Chlorella*, *Phaeodactylum, Schizochytrium* and *Muriellopsis* resulting in different FE per mg of flocculant (Gerde *et al.*, 2014; Granados *et al.*, 2012; Harith *et al.*, 2009; Papazi *et al.*, 2010; Rashid *et al.*, 2013; Sirin *et al.*, 2012). In this study three different chlorophytes, *Chlorella sp*, *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* were tested. *Chlorella* cells show a size of (2 – 10µm) resulting in a smaller surface area compared to *Scenedesmus* and *Chlamydomonas* cells (10 – 30µm). In contrast to *Chlorella* and *Scenedesmus*, *Chlamydomonas* cells are flagellated and motile. All three of them are model organisms for algal research and frequently used as laboratory strains (Harris, 2001; Mandal *et al.*, 2009; Safi *et al.*, 2014; Sanchez *et al.*, 2008). This study showed that the morphology, motilities and cell sizes have an impact on the FE. The FEs for the polyDADMACS (Diallyldimethyl ammonium chloride) CFL25 differed among the different algae species. Whereas the flocculation of *Chlorella* cells resulted in a FE of > 90%, the flocculation of *Chlamydomonas* cells only achieved a FE of 20%. According to the manufacturer's information CFL25 has a low molecular weight with a high cationic charge. Compared to PK55H it is a small polymer which can be entirely absorbed on the algal surface but cannot effectively form bridges between algal cells by binding to more than one cell surface. If the polymer is entirely absorbed to the surface, cationic regions are formed which can bind to the negative cell surface of other cells. For smaller sized cells like *chlorella* this seems to be effective but not for larger cells like *Chlamydomonas*.

Since optimal flocculants were identified in this study, for further improvement the mixing speed and time might be investigated for each flocculant. Usually there is a three step procedure composed of a first phase at high agitation to allow flocculant distribution, the second phase where the agitation is reduced to allow flock formation and a third phase, the settling phase, without agitation. In literature different values for the initial two phases can be found. Granados

et al. 2012 used a flocculant distribution at 150 rpm for two minutes and a flock formation at 20 rpm for 5 minutes. Harith *et al*.2009 applied 200 rpm for flocculant distribution for one minute and 50 rpm for two minutes. Vandamme *et al*. 2010 even used 1000 rpm for distribution for five minutes and 250 rpm for 30 minutes for flock formation. The nature of the tested flocculant should determine the choice of the relevant conditions. Whereas polymer solutions with a low molecular weight show a high viscosity (in the presented results for example displayed by the polyDADMACs) and are easily distributed, the distribution of polymer solutions with a low viscosity (for example starches or PK55H) requires more agitation. In this manuscript it was chosen to use 250 rpm for flocculant distribution and 50rpm for flock formation. Since longer polymers are more susceptible to shear stress the mixing time was reduced to one minute within the flocculant distribution to avoid polymer breaking.

A recycling experiment was conducted over a period of eight weeks to verify whether the tested flocculant has a negative effect on the algal growth if the media is reused. The reuse of the growth media is an important factor which has to be considered regarding the cost effectiveness of the production process. The experiment was conducted using *Chlamydomonas reinhardtii* in combination with the cationic flocculant PK55H. No adverse effect was monitored during the experiment. For metal salts secondary pollution by residues may limit follow up applications like food or feed additive due to high concentrations of metals in both biomass and culture media (Sirin *et al.*, 2012). Interference with biodiesel production was also reported (Rwehumbiza, Harrison & Thomsen, 2012). No adverse effects are reported for natural organic flocculants like modified starches or tannin based polymers. In contrast higher biodiesel and biogas production rates from the harvested biomass were reported (Anthony, Ellis, Sathish et al., 2013; Gutierrez, Ferrer, Garcia et al., 2015) and some are approved for food contact and for use in treatment of drinking water (Krentz, Lohmann, Schwarz et al., 2006; Pal, Mal & Singh, 2005). Even though a lot of research on microalgal biotechnology has been done within the recent years, only little information can be found on the effect of the applied flocculant or harvesting method in general on the quality of the desired algal product (Anthony et al., 2013). In the recycling experiment it was however found that after ten days of incubation, aggregate formation became visible under the microscope. While the OD values stayed similar, due to the formation of aggregates more *Chlamydomonas* cells were present in the cultures, leading to higher dry biomass values.

The results of this study demonstrated that flocculation using cationic polymeric flocculants is an inexpensive and feasible method to improve algal biomass harvest. The hypothesis that it is feasible to harvest different microalgal strains by using commercially available polymeric flocculants and reuse the media after flocculation was herewith scientifically proven.

3.3 Hypothesis 3: Apart from aluminum and iron other metals are suitable for electroflocculation independent of the voltage applied

In this study six electrode materials were investigated to test the hypothesis that other metals besides aluminum and iron are suitable for electroflocculation. The literature contains several studies examining the application of electroflocculation for algal biomass harvest (Lee *et al.*, 2013; Uduman *et al.*, 2011; Vandamme *et al.*, 2011). In these flocculation tests mainly aluminum electrodes and iron electrodes were analyzed, sometimes a combination of both (Kim *et al.*, 2012; Lee *et al.*, 2013; Uduman *et al.*, 2011; Vandamme *et al.*, 2011; Xu *et al.*, 2010). Although the use of other electrode materials shows high potential in several application fields, no or very little data has been published on the application of further electrode materials like Mg, Zn, Cu or brass. The best biomass recovery was achieved when using Mg electrodes. Magnesium is bivalent and consequently builds extra stable hydrogen bonds, causing an effective floc formation. After 9 minutes, 90% of the suspension was clarified at 40V. By decreasing the voltage to 30V, the flocculation time needed to reach 90% increased to 12,5 minutes. The higher the applied voltage, the faster the flocculation efficiency of 90% was reached in all experiments. The electrodes were connected to a DC power supply and submerged in the algal suspension. During the process the flocculant was directly formed in the suspension by the release of metal ions from the sacrificial anode by electrolytic oxidation. In aqueous media the ions immediately react and form metal hydroxides which can react with the negative cell surfaces and destabilize the suspension. By increasing the applied voltage, more ions are released from the metals and consequently more flocculant is produced (Mollah *et al.*, 2004; Mollah *et al.*, 2001). A short lag phase was monitored at lower voltage, which is to be expected because of insufficient flocculant availability at the beginning of the flocculation experiment. The lag phase was shortened at higher voltages.
Remaining metal residues in the harvested biomass might hinder the use of the biomass as food or feed additive or interfere with downstream processing steps like extraction methods. The

accepted limits are generally higher for magnesium than for iron or aluminium making magnesium as electrode material even more interesting for industrial applications. The impact on human health is considered low. The German Drinking Water Ordinance, for instance, does not limit the content of magnesium in the drinking water. However the accepted concentrations are not allowed to exceed 0,2 mg l^{-1} for aluminium and iron and 2 mg l^{-1} for copper (Ordinance, 2001).

In all samples a pH increase from 7 to 11 was monitored within the initial ten minutes of flocculation at 40V. The fast increase of the pH might negatively affect both the biomass use and the media recycling. The high pH can cause cell lysis resulting in lower biomass gains and / or destruction of desired metabolites (Contreras *et al.*, 1981; Xu *et al.*, 2010). The reuse of the culture media after cell separation is also an important factor to consider. A batch experiment tested whether the high pH affects cell growth. The cell free supernatant was enriched with nutrients and *Scenedesmus* cells cultivated for twelve days. In all culture vessels cell growth was detectable with the best result achieved with the supernatant of the iron flocculation having a final cell concentration of $1.86x10^7$ cells ml^{-1}. This value even exceeded the cell counts of the control culture, indicating that the iron residues might have a fertilizing effect on the cell growth. With $1.23x10^7$ cells ml^{-1} copper showed the lowest growth performance. At low dosage copper is a vital micronutrient for plants. However at higher concentration the photosynthetic reaction is inhibited (Kupper *et al.*, 2009). To confirm these test results a recycling experiment comprising several harvesting steps should be done then a possible accumulation of metals, metabolites or the influence of a constantly high pH can be monitored.

Since the applied voltage significantly influences the FE, energy costs should be considered. In further experiments the electrical current flowing during the experiments should be measured and an estimation about the energy costs conducted.

The hypothesis, that other electrode materials than iron and aluminium can be applied for electroflocculation, was positively tested. Magnesium, copper, zinc and brass can be effectively used for microalgal biomass harvest.

4 Outlook and Conclusion

The overall aim of this thesis was successfully achieved in terms of isolating and identifying new temperature tolerant biomass production strains and improving the biomass harvest by chemical- and electroflocculation.

Within the screening for a biomass production strain, 130 environmental samples were collected and analyzed. Stains with a fast growth and high temperature acceptance were isolated and identified allowing the cultivation in outdoor facilities or green houses. This study demonstrated that a process oriented strain selection is possible. More research should be conducted on the production of high value products like lipids or pigments which would help to improve cost effectiveness in industrial applications.

To reduce the energy costs during the harvesting process two different ways of flocculation were effectually investigated. During chemical flocculation polymeric cationic flocculants achieved higher flocculation efficiencies compared to anionic or non-ionic polymers. Three chlorophytes were tested: *Chlamydomons reinhardtii*, *Chlorella* sp. and *Scenedesmus acuminatus.* The amount of flocculant needed varied between the algae species (e.g. CFL25 with 30mg l-1 for *Chlorella* and 70mg l-1 for *Scenedesmus*) and the flocculants (1.5- to 70mg l^{-1}). The lowest concentration at the highest flocculation efficiency (above 95%) was monitored cationic polyelectrolyte PK55H with 1.5mg l^{-1}. Because of its effectiveness it was selected for a recycling experiment. Over eight weeks the influence of remaining flocculant was investigated. No adverse effect was detected although cell aggregates became visible. However the effect of the flocculant on the desired product should be investigated.

The electroflocculation experiments revealed that also other electrode materials that iron and or aluminum electrodes can be successfully applied for electroflocculation of microalgae. The chlorophyte *Scenedesmus acuminatus* was harvested using aluminum, iron, magnesium, copper, zinc and brass electrodes. The best flocculation efficiency was calculated for magnesium followed by aluminum and iron. 10 V, 20 V, 30 V and 40V were tested with the finding that higher voltages resulted in faster flocculation. For further investigation the energy consumption should be involved in the calculations considering the economic feasibility. In a media reuse experiment in all supernatants cell growth was monitored. As mentioned above, for further

application the biomass has to be investigated for metal residues an adverse effects during processing and on the product itself.

In summary all three hypothesis could be positively tested. Although a lot of research potential remains, valuable progress was made in the field of strain selection and biomass harvest. Another promising step towards an economical production of microalgal biomass was successfully made.

5 Literature

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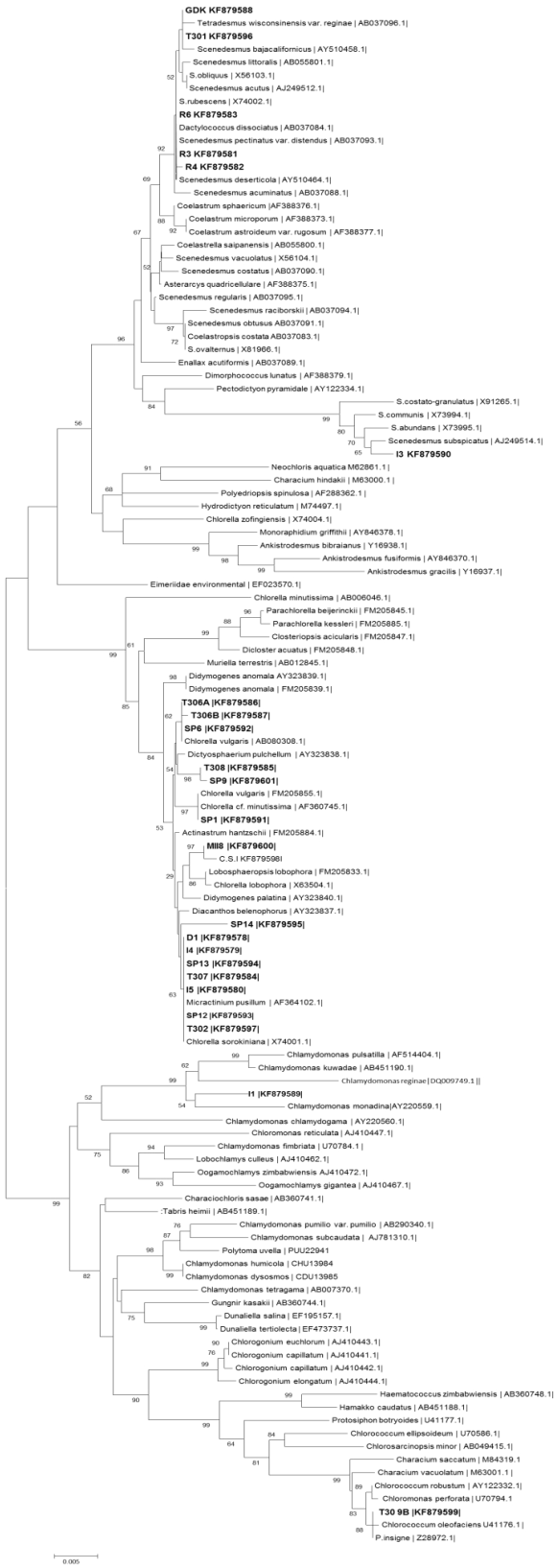
6 Appendix

1. Table S1- Supplementary data for Publication 1

    a. Table S1: GenBank accession numbers of the isolated species.

Isolate	Species	NCBI
T301	*Scenedesmus* sp.	KF879596
T302	*Chlorella* sp.	KF879597
T306	*Chlorella* sp.	KF879586
T306	*Chlorella* sp.	KF879587
T307	*Chlorella* sp.	KF879584
T308	*Chlorella* sp.	KF879585
R3	*Scenedesmus* sp.	KF879581
R4	*Scenedesmus* sp.	KF879582
R6	*Scenedesmus* sp.	KF879583
I1	*Chlamydomonas* sp.	KF879589
I3	*Scenedesmus* sp.	KF879590
I4	*Chlorella* sp.	KF879579
I5	*Chlorella* sp.	KF879580
Sp1	*Chlorella* sp.	KF879591
Sp6	*Chlorella* sp.	KF879592
Sp9	*Chlorella* sp.	KF879601
Sp12	*Chlorella* sp.	KF879593
Sp13	*Chlorella* sp.	KF879594
Sp14	*Chlorella* sp.	KF879595
CS	*Chlorella* sp.	KF879598
Ssp	*Scenedesmus* sp.	KF879599
GDK	*Scenedesmus* sp.	KF879588

b. Figure S1: Phylogenetic tree



1. **Figure S1: Phylogenetic tree comprising the new isolates. 22** Isolates were genetically identified on molecular level via 18S rDNA sequencing. The algae could be assigned to belong to four different families (Scenedesmaceae, Chlorellaceae, Chlamydomonadaceae and Chlorococcaceae) and three different orders (Chlorococcales, Chlorellales and Volvocales).

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Article

Isolation and Characterization of New Temperature Tolerant Microalgal Strains for Biomass Production

Franziska Bleeke [1,2], Vincent M. Rwehumbiza [2], Dominik Winckelmann [2] and Gerd Klöck [2,*]

[1] School of Engineering and Science, Jacobs University, 28759 Bremen, Germany; E-Mail: franziska.bleeke@hs-bremen.de

[2] Institute of Environmental Biology and Biotechnology, University of Applied Sciences Bremen, 28199 Bremen, Germany; E-Mails: v.rwehumbiza@gmail.com (V.M.R.); dominik.winckelmann@hs-bremen.de (D.W.)

* Author to whom correspondence should be addressed; E-Mail: gerd.kloeck@hs-bremen.de; Tel.: +49-421-5905-4266, Fax: +49-421-5905-4245.

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Abstract: Microalgae exhibit great potential for biomass production. Although microalgae display an enormous biodiversity, surprisingly only 15 species are used for large scale production processes worldwide. The implementation of new production strains with good process-oriented properties, especially fast growth rate and heat resistance, could improve production efficiency and reduce costs. In this study 130 environmental samples collected in Germany, Spain, Italy and Portugal were investigated for fast growing thermotolerant photosynthetic species. Isolates were characterized and identified on a molecular level. In total 21 of the isolated freshwater strains were able to grow at 40 °C. Additionally, 13 of those 21 strains are able to grow at 45 °C. The highest growth rate at room temperature was 1.16 per day (isolate T306A), compared to 0.053 per day at 45 °C (isolate Sp13). In three thermotolerant strains pigment production was induced. Molecular identification by 18S rDNA sequencing revealed that the isolates were all chlorophytes belonging to four different families.

Keywords: screening; high temperature tolerance; biomass production; freshwater microalgae; pigments

1. Introduction

Microalgae are microscopically small photosynthetically active eukaryotic organisms with an increasing importance in the biotechnology sector. Compared to higher plants they have several advantages, such as faster growth, the ability to be grown on non-arable land and the capacity to accumulate valuable products such as lipids or pigments [1,2].

There are between 200,000 and several million microalgal species [3,4]. Even though microalgae display an enormous biotechnological potential due to their biodiversity and versatility, the industry is currently using only about 15 species for large scale production [5]. Due to the increasing demand for microalgal biomass, a process-oriented strain selection is essential in order to make large scale production economical. Process-oriented strain selection supports the use of either indigenous strains from the respective production site, or strains adapted to stress, which due to fast growth rates are able to outcompete predators or weed algae. Besides light exposure and nutrient availability, the temperature influences growth efficiency significantly. Due to high and favorable illumination the summer months usually lead to high biomass productivities [6]. The optimal growth temperature for common laboratory strains of microalgae varies among different species, but is usually stated to be between 20 and 30 °C [6–11].

Higher temperature conditions in greenhouses or outdoor cultivation settings during summer months may negatively affect growth of many microalgal species [6,8,12]. In greenhouses temperatures can reach up to 55 °C, resulting in maximum culture temperatures exceeding 35 °C [13]. For outdoor cultivation similar temperatures surpassing 35 °C and even 40 °C were reported [14–18]. Whereas temperatures below the optimum lead to a retained biomass production, temperatures above the optimum results in a steep decrease in productivity and possibly the total loss of the culture. The degradation and inactivation of enzymes involved in the photosynthetic process caused by heat stress results in the inhibition of growth or even programmed cell death [1,12,19]. Construction of temperature controlled environments for cultivation of microalgae would be ideal, but has been proven to not be sustainable due to high initial investment and operation costs. Therefore, acquiring microalgae strains with the ability to grow and propagate in these severe heat conditions, especially during summer temperatures, is of utmost importance.

In the present study a simple screening protocol for environmental algal samples was developed. Within this screening the main focus was put on thermotolerant freshwater species with a high growth rate. 130 samples were collected in Germany, Italy, Spain, and Portugal and later analyzed. After an initial temperature tolerance test, pure cultures were obtained and characterized.

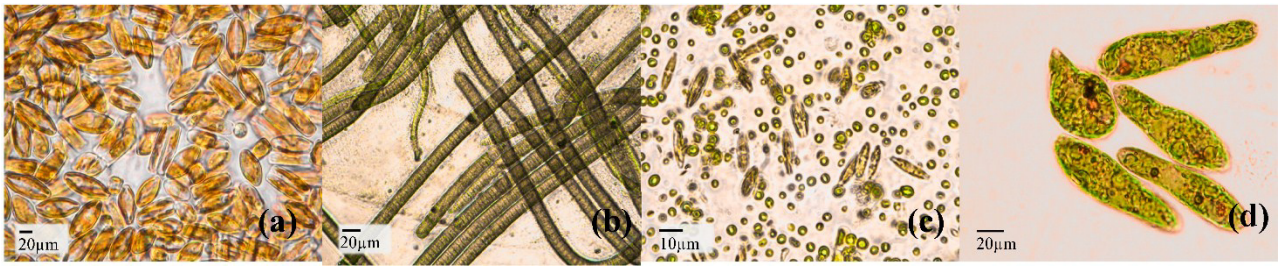
2. Results and Discussion

2.1. Sampling and Enrichment

One hundred and thirty samples from Germany, Italy, Spain and Portugal were collected and cultivated in test tubes containing Wuxal liquid medium (WM) [20]. The incubation resulted in algal growth in about 50% of the test tubes, leaving 66 samples for the screening procedure. Figure 1 shows a variety of cell types and shapes that could be observed in the test tubes using a Zeiss Axiostar Plus

light microscope (Carl Zeiss, Oberkochen, Germany) investigated in Hellfeld mode at 40-fold magnification. The images were taken by a FinePix E550 digital camera (Fujifilm, Tokyo, Japan).

Figure 1. Morphological diversity of the mixed cultures in the test tubes. (**a**) mixed culture of pennate gold-brown single cells; (**b**) intense green filamentous microalgae; (**c**) mixed culture of coccoid and elongated single cells; and (**d**) flagellated protists with red eyespots visible.



2.2. Temperature Tolerance and Growth Kinetics

After incubation at 25 °C for 14 days, 7 (of 66) test tubes showed a decrease in coloration leaving 59 test tubes for further screening. In the following step the cultures were transferred to conical flasks (volume 100 mL) and exposed to 35 °C for seven days. This step left 40 samples for further investigations. After this initial enrichment of temperature tolerant samples, unialgal isolates were obtained by microbiological methods. These isolates were subjected to further temperature tolerance tests. When the growth temperature was further increased, 21 out of those 40 isolates were able to grow at 40 °C (Table 1), 13 of them were even growing at 45 °C. None of the isolates grew at 47 °C (Table 1, Figure 2).

Figure 2. Temperature tolerance test of purified isolates. Thirteen algae strains tolerated incubation at 45 °C. The highest growth could be observed for isolates Sp13, Sp14, I4, I5 and CS after a 48 h lag phase. A further temperature increase to 47 °C led to a decline in optical density after 48 h. Data points are the mean value of three replicates. Error bars represent standard deviation.

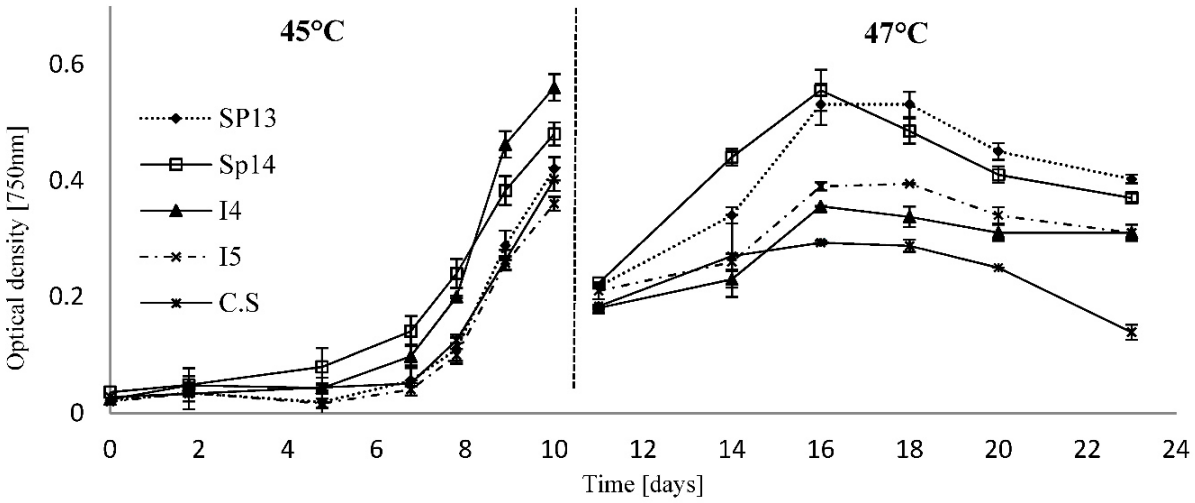


Table 1. Growth rates and the maximal temperature tolerated for biomass production of 22 unialgal isolates obtained after initial enrichment.

Isolate	μ day^{-1}	Maximal Temperature [°C]
T306A	1.163	45
Sp1	1.101	40
CS	0.943	45
T301	0.890	45
I5	0.832	45
Sp12	0.827	45
Sp13	0.766	45
Sp14	0.683	45
T308	0.667	40
Sp9	0.646	45
GDK	0.625	40
T302	0.585	45
T306B	0.564	45
T307	0.525	40
I4	0.469	45
Sp6	0.455	40
R6	0.423	45
R4	0.382	40
Ssp	0.371	40
R3	0.328	45
I1	0.144	35
I3	0.105	40

During incubation at 45 °C all isolates experienced a lag phase in the first 48 h. This lag phase was extended to 48 h for isolate I4, I5 and Sp13 and to 120 h for isolate CS. For most samples, the optical density measured at 750 nm wavelength started to rise after 48 h. The highest values were attained by I4 and Sp14 after 10 days of incubation. Determination of the cell number revealed the same trend (data not shown). Although isolate Sp13 showed the highest growth rate (Table 1), the final OD 750 value of 0.42 was lower compared to isolates I4 and Sp14 of 0.56 and 0.48, respectively. Five days of incubation showed an increase in cell number for all tested isolates. Comparing the growth rates of the isolates grown at room temperature and 45 °C, the effects of thermal induced stress were evident. The growth rates for CS, I4, I5, Sp13 and Sp14 at 45 °C were approximately 50% of the values determined at room temperature (Table 2). It was also noted that the samples cultivated at room temperature were light green while the ones grown at 45 °C were dark green.

Table 2. Comparison of growth rates at room temperature (22 ± 1 °C) and 45 °C.

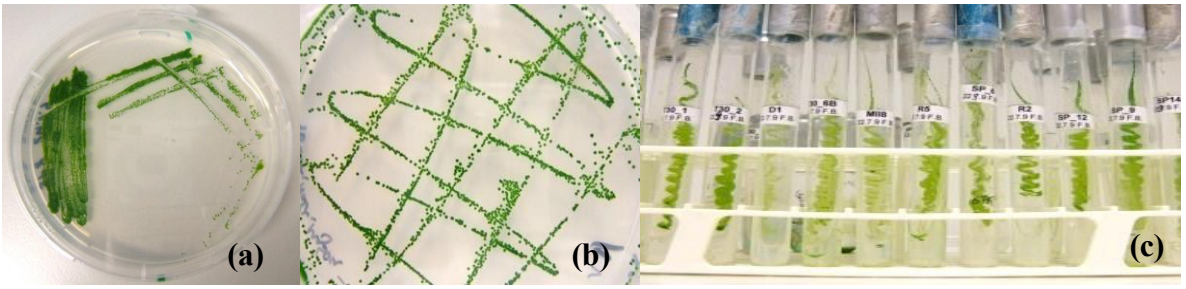
Isolate	μ day^{-1} (22 ± 1 °C)	μ day^{-1} (45 °C)
Sp13	0.766	0.53
CS	0.943	0.42
I4	0.469	0.4
I5	0.832	0.39
Sp14	0.683	0.37

Organisms inhabiting places in warmer regions are usually exposed to a wide variety of temperature conditions. Their capability to adapt to rapid changes in environmental stresses, such as temperature shifts, is an essential requirement for survival. In this study, the analyzed microalgae species responded to thermal stress exposure in various ways. The results of the temperature sensitivity tests revealed that 21 isolates out of 130 samples were able to grow at temperatures of 40 °C or even higher. The aim of this study was to obtain a greater biodiversity of thermotolerant strains and surprisingly 13 of the identified strains were genetically identified to belong to the family Chlorellaceae (Figure S1). It is well known that some *Chlorella* species tolerate a broad temperature range and have been used as model organisms for physiological studies on broad temperature tolerance of microorganisms [6,8,19,21]. This study confirms the reported ability of *Chlorella* strains to adapt and grow at high temperatures. Species belonging to genus *Chlorella* seems to be suitable for cultivation at high temperatures in outdoor settings or greenhouses.

2.3. Establishment of Unialgal Cultures

Twenty three monoalgal cultures were attained by using the thirteen-streak-method (Figure 3) [22]. The morphological features of the isolates were analyzed using a Zeiss Axiostar Plus light microscope (Figure 4). The samples were investigated in Hellfeld mode at 40-fold magnification. The images were taken using a Fujifilm FinePix E550 digital camera with macro function. Bacterial contamination occurred frequently during isolation making it necessary to repeat the procedure several times on agar media supplemented with antibiotics. No cyanobacteria were isolated as a result and fast growing colonies were selected and purified. This step makes it possible to adapt the screening procedure to other desired algae properties or products such as pigments.

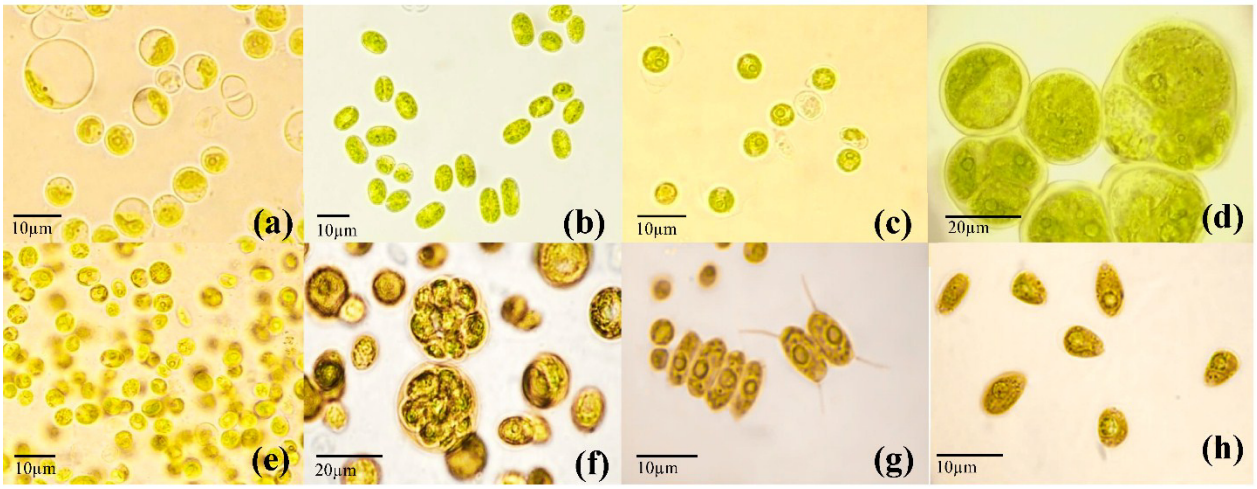
Figure 3. Establishment of pure cultures. (**a**) After purifying the environmental samples by using the thirteen-streak-method; (**b**) monocultures were obtained; (**c**) and kept in collection.



2.4. Molecular Identification

This work established the phylogenetic relationships of microalgae strains that could survive and/or grow between 35 and 45 °C relative to known microalgal genera and species. Twenty two isolates were genetically identified on molecular level via 18S rDNA sequencing and could be assigned to four different families (Scenedesmaceae, Chlorellaceae, Chlamydomonadaceae and Chlorococcaceae) and three different orders (Chlorococcales, Chlorellales and Volvocales) (Figure S1: Phylogenetic tree, Table S1: Accesion numbers).

Figure 4. Microscopic images of isolated species. (**a**) Sp14 (Chlorellaceae): Spherical cells with large vacuoles; (**b**) Sp1 (Chlorellaceae): elongated cylindrical cells; (**c**) T301 (Chlorellaceae): spherical single cells; (**d**) T309 (Chlorococcaceae): large coccoid cells containing zoospores; (**e**) R3 (Scenedesmaceae): spherical and ellipsoidal cells; (**f**) GDK (Scenedesmaceae): single cells and parental cells containing spores; (**g**) I3 (Scenedesmaceae): two and four cell cenobia with spines visible; (**h**) I1 (Chlamydomonadaceae): motile green ovoid cells with flagella.



The SSU rDNA (18S rDNA) is usually investigated for relationships between eukaryotic cells as the gene showns a slow evolutionary rate, bears well-conserved as well as rapidly evolving regions and is available in high copy numbers [23,24]. Using for example single gene analysis the small subunit ribosomal DNA (SSU rDNA) gene provides the basic structure for phylogenetic topologies but might be misleading in some aspects. Additional gene sequences could be investigated to improve the resolution of the tree, although the possibility of systematic errors increases with the number of genes and species involved [25].

3. Experimental Section

3.1. Sample Collection and Incubation

Samples were taken by using commercial cotton buds. After sampling, the cotton buds were wrapped into film to maintain moisture and then sent to the Laboratory of the University of Applied Sciences Bremen. The microalgae were inoculated by gently stirring the cotton buds into test tubes containing Wuxal liquid medium (WM) [20]. Test tubes were kept at 25 °C for two weeks in a light incubator to allow microalgal growth and any test tubes showing no algal growth after two weeks were removed.

3.2. Temperature Tolerance Test

An initial experiment increased the temperature to 35 °C for seven days. Test tubes showing a decrease in growth (macroscopically evaluated) were removed. In the following temperature increase experiment the mix cultures were transferred into 100 mL conical flasks and fresh WM was added. The flasks were incubated in a light incubator with a light/dark cycle of 12:12 h and exposed to higher

temperatures for six hours per day. The cultures were illuminated with 105 µmol photons $m^{-2} \cdot s^{-1}$. Temperature pattern as well as light intensities during incubation were recorded using HOBO Data Logger (Onset, Linnich, Germany). For each sample a control was incubated at room temperature (22 ± 1 °C). Starting at 40 °C for seven days, the temperature was then increased daily by 1 °C increments up to 45 °C for ten days. Any cultures showing growth at 45 °C after ten days were exposed to 47 °C for another twelve days.

3.3. Establishment of Pure Cultures

Pure cultures were established by using the thirteen-streak-method [22]. Agar (15 g) was added to the liquid media before autoclaving to prepare solid WM. To inhibit bacterial growth an antibiotic cocktail was added to the agar by mixing penicillin (1.0 g), streptomycin (0.5 g) and chloramphenicol (0.1 g) with deionized water (16 mL), so that the final concentration of the antibiotic mix in the growth medium was 0.01% *v*/*v*. Single colonies were picked and recultured first on solid media and then in liquid media. The microalgae were cultivated at room temperature under sterile conditions in 50 mL WM liquid media in 100 mL conical flasks at 100 rpm. Fresh medium was added every 14 days. If no pure culture could be established after five purification cycles (thirteen-streak-method with subsequent plating), the sample was excluded from the screening.

3.4. Growth Kinetics

In order to compare the growth of the microalgae, the measured cell count data was standardized by calculating growth rates. Microalgae cultures were grown in batch cultures at an initial cell count of 1×10^6–1×10^7 cells mL^{-1}. These cultures were incubated at room temperature for approximately two weeks, until stationary phase was reached. Sampling was performed every two or three days by taking 1 mL of each culture in triplicate and measuring the optical density at 750 nm and to determine the cell count using the Thoma counting chamber (Paul Marienfeld GmbH + Co. KG, Lauda-Königshofen, Germany).

3.5. Molecular Identification

DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). The 18S rDNA amplification was performed in a T3 thermocycler (Biometra, Göttingen, Germany) with the primer pair EukA and EukB and additional internal primers (Table 3). The amplified products were purified using the MiniElute PCR Purification kit (Qiagen GmbH) then sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Residual nucleotides and PCR reagents were washed and removed by DyeEx Spin Kit (Qiagen) and sequencing was performed on an ABI 3100 Avant Sequencer (Applied Biosystems).

Signals generated by the capillary sequencer were investigated using the Sequencing Analysis Software v5.4 (Applied Biosystems). Single sequences were assembled to contigs using DNAStar (Madison, WI, USA) and consensus sequences were calculated. Using the BLAST (Basic Local Alignment Search Tool) algorithm (National Library of Medicine, Bethesda, MD, USA), related sequences were collected from the GenBank and a multiple sequence alignment was carried out using ClustalX (Conway Institute, UCD, Dublin, Ireland). The phylogenetic tree was constructed using

Mega 4.1 (Center for Evolutionary Functional Genomics, Tempe, AZ, USA) with the neighbor joining clustering method based on the maximum likelihood model with 1000 bootstrap replicates.

Table 3. The oligonucleotide primers used for amplification and sequencing of 18S rDNA of selected microalgae strains.

Primer	Sequence	Reference
EukA	5'-AAC CTG GTT GAT CCT GCC AGT-3'	[26], no polylinker
EukB	5'-TGA TCC TTC TGC AGG TTC ACC TAC-3'	
1055f	5'-GGT GGT GCA TGG CCG TTC TT-3'	[27]
1055r	5'-ACG GCC ATG CAC CAC CAC CCA T-3'	

4. Conclusions

The screening for new thermotolerant microalgal strains for biomass production was successful. Out of 130 environmental samples, 22 freshwater strains were isolated, cultivated and identified on a molecular level. It was found that the maximum temperature tolerated was 40 °C for eight strains and 45 °C for 13 strains, respectively. Although the results are promising, further experiments on biomass and secondary metabolite production must be conducted to give a more accurate prediction on the suitability of the isolates for industrial application. Process oriented strain selection is an important factor in making microalgal cultivation economical on an industrial scale. This study identifies new thermotolerant chlorophytes allowing cultivation under high or extreme temperature conditions in greenhouses or outdoor settings.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1996-1073/7/12/7847/s1.

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Author Contributions

Franziska Bleeke contributed to experimental design, data acquisition, result analysis and manuscript preparation. Vincent M. Rwehumbiza contributed to result analysis and manuscript preparation. Dominik Winckelmann contributed to experimental design and data acquisition. Gerd Klöck contributed to experimental design, result analysis and manuscript preparation.

Conflicts of Interest

The authors declare no conflict of interest.

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ORIGINAL RESEARCH

Optimization of freshwater microalgal biomass harvest using polymeric flocculants

Franziska Bleeke · Malgorzata Milas · Dominik Winckelmann · Gerd Klöck

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Abstract Although microalgae show a great potential in the biotechnology sector, high production costs have limited industrial applications. Biomass harvest is one of the major bottlenecks in microalgae cultivation due to high energy inputs which are needed to separate the cells from the surrounding media. Chemical flocculation is considered to be a reliable resource to improve cost-effectiveness in the downstreaming processing. Flocculation efficiency is dependent on several factors such as the polymer type and charge as well as on the microalgae species. In the present study, 15 polyelectrolytes were tested for their potential to harvest algal biomass. Cationic, anionic and nonionic flocculants were tested in different amounts at varying incubation times to determine the adequate conditions needed. By testing the three chlorophytes, *Chlorella* sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii*, the influence of different sizes, morphologies and motilities of the flocculation efficiency was verified. Furthermore, the biocompatibility of an efficient flocculant was tested in a recycling experiment over a period of 8 weeks.

Keywords Microalgae · Biomass harvest · Chemical flocculation · Polyelectrolytes · Microalgal biotechnology · Polymeric flocculants

Introduction

Microalgae are microscopically small photosynthetic protists with rising significance in the biotechnology sector. According to Posten and Walter (2012), microalgae have a five times higher biomass productivity per hectare compared to terrestrial crops (Posten and Walter 2012). Furthermore, they can be grown on non-arable land and produce biomass which can serve as food or feedstock or as potential substrate for biofuel production (Chisti 2007; Mata et al. 2010).

Although microalgae cultivation displays great potential, high production costs have limited industrial application. A major factor is the cell harvest, which contributes to 20–30 % of the biomass production costs (Grima et al. 2003; Mata et al. 2010; Uduman et al. 2010). Large volumes have to be processed, since the

F. Bleeke (✉)
Jacobs University, 28759 Bremen, Germany
e-mail: franziskableeke@hotmail.de

F. Bleeke · D. Winckelmann · G. Klöck
Institute of Environmental Biology and Biotechnology, University of Applied Sciences Bremen, 28199 Bremen, Germany

M. Milas
Gdansk University of Technology, 80-233 Gdansk, Poland

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concentration of cells is usually low at 0.5–2.5 g l^{-1} (Grima et al. 2003). Commonly used separation processes are a combination of filtration, flotation or flocculation, followed by a final centrifugation step. To separate the cells from the surrounding media, high-energy inputs are needed, often exceeding the energy content of the harvested biomass (Grima et al. 2003; Uduman et al. 2011; Wijffels and Barbosa 2010).

Increasing the efficiency at low-energy demands within the harvesting process is a major challenge in microalgal biotechnology. In flocculation, the cells coagulate and larger particles are produced with a higher settling velocity due to the higher density of the flocs. Flocculation can be achieved in several ways. Numerous studies have been published on the use of electrocoagulation (Lee et al. 2013; Uduman et al. 2011; Vandamme et al. 2011), pH-induced flocculation (Vandamme et al. 2012; Wu et al. 2012; Zheng et al. 2012) or bioflocculation using bacteria (Lee et al. 2009), filamentous fungi (Zhou et al. 2013) or another flocculating algae to floc a non-flocculating algae (Salim et al. 2011) for biomass harvest.

In chemical flocculation, a chemical coagulant is added to the algal suspension (Xu et al. 2010). Organic and inorganic flocculants like polyelectrolytes and metal salts were investigated. Aluminum, ferric and zinc salts were tested (Papazi et al. 2010) and compared with polyelectrolytes (Gerde et al. 2014; Granados et al. 2012; Papazi et al. 2010; Sirin et al. 2012). Comparative studies showed that higher flocculation efficiencies (FE) were achieved with polyelectrolytes than with metal salts (Gerde et al. 2014; Granados et al. 2012). Besides the lower FE, another disadvantage of the use of metal salts is the high concentration of metals in the algal biomass after harvest. Metal residues may impede the use of certain applications such as animal feed (Grima et al. 2003). Polymers are commonly used in wastewater purification processes and preferred for algal harvest, because lower amounts are needed and non-toxic and biodegradable substances are available (Granados et al. 2012). Polymeric flocculants are commercially available with cationic, anionic and nonionic charges in different charge densities, whereas cationic flocculants are considered to be the most effective for algal harvest (Granados et al. 2012; Tenney et al. 1969). The effect of different flocculants on several algae species has also been described. The results showed high variability with respect to the algae species and the nature of the flocculant (Gerde et al. 2014; Granados et al. 2012; Harith et al. 2009; Papazi et al. 2010; Rashid et al. 2013; Sirin et al. 2012).

Chlorella sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* are commonly used laboratory strains and model organisms for algal research (Harris 2001; Mandal and Mallick 2009; Safi et al. 2014; Sanchez et al. 2008). By testing these three chlorophytes the influence of different sizes, morphologies and motilities of the flocculation efficiency were analyzed.

In the present study, 15 polymeric flocculants were compared to test the following hypotheses. Are cationic flocculants more effective compared to other polymeric flocculants (H1)? Do closely related species show similar flocculation behavior (H2)? Is a reuse of the media after cell separation by flocculation applicable (H3)?

Materials and methods

Microalgae and culture conditions

The experiments were carried out using the freshwater chlorophytes *Scenedesmus acuminatus*, *Chlorella* sp. and *Chlamydomonas reinhardtii* from the culture collection of the University of Applied Sciences Bremen. The cells were grown at 22 °C ± 1 in a culture volume of 2 l in Wuxal liquid medium (WM)(Winckelmann et al. 2014) without pH control. Bottles were equipped with aeration hoses providing continuous bubbling with compressed air at a flow rate of approximately 2.3 l min^{-1}. Every 3 days, 600 ml of the culture was used for the experiments and fresh medium was added to the remaining culture. Illumination was offered 24 h per day by fluorescent lamps (OSRAM L 30 W, warm white) placed in front and behind the algae cultures (light intensity, 50 µmol photons m^{-2} s^{-1}).

Flocculants

Stock solutions of 15 polymeric flocculants with cationic, anionic and nonionic characteristics were prepared according to the manufacturer's recommendation, dissolved in water and stored at 4 °C for a maximum of 2 weeks.

Flocculation experiments

Before each experiment, the pH of the sample from the algae culture was carefully adjusted to 7.0 by adding 1 N HCl. The cell number was determined using a Thoma counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and subsequently adjusted to 1×10^7 cells ml^{-1} by dilution with tap water. Flocculation experiments were evaluated using jar tests (Granados et al. 2012; Hudson and Wagner 1981; Vandamme et al. 2010). 100 ml Erlenmeyer flasks were filled with 100 ml of algal suspension (Fig. 2). The flasks were stirred at 150 rpm. One type and concentration was used in each flask. Once the flocculant was added, the culture was mixed for 1 min at 250 rpm, to allow flocculant distribution. After 1 min, the mixing speed was reduced to 50 rpm for 2 min, to support floc formation, followed by a settling phase without agitation. Samples were taken directly after the flocculant distribution and after 2, 10, 30 and 60 min from 3 cm under the culture surface. Optical density was measured at 750 nm (Genesys 20, Thermo Scientific, Walthman, USA) and the flocculation efficiency (FE) was determined as follows:

$$\text{Flocculation efficiency} = \left(\frac{\text{OD}_{T0} - \text{OD}_{T1}}{\text{OD}_{T0}}\right) \times 100,$$

where OD_{T0} is the initial optical density before starting the flocculation and OD_{T1} is the optical density of the sample at a certain point of time during the process. Samples showing OD values above 0.5 were diluted with tap water for the measurements to assure linearity.

Media reuse after flocculation

A recycling experiment was conducted to investigate the potential negative impact of flocculant residues on algal growth when the media are recycled. Flocculant PK55H was chosen for the media reuse experiment. The experiment was conducted in triplicate using the microalgae *Chlamydomonas reinhardtii* under the same cultivation setup as described in "Microalgae and culture conditions". Over a period of 8 weeks, the cultures were harvested weekly by flocculation (Fig. 1a) or centrifugation (Fig. 1b), whereas the centrifugation served as positive control displaying the growth without the impact of the flocculant. The volume of the recycled media was dependent on the optical density of the culture. After cell separation, the culture supernatant was enriched with concentrated WM and led back into the culture vessel. The final optical density in the culture after media recycling was 1.5 at 750 nm.

Cell growth was monitored by optical density measurements at 750 nm, cell count determinations and dry biomass determination. For dry biomass determination, 10 ml of the algal culture was filtered through a previously balanced Whatman GF/C glass fiber filter (Whatman, Maidstone, UK). The filters were dried in an oven at 80 °C for 12 h and weighed afterward. The difference in weight was calculated and multiplied by 100 resulting in the dry biomass expressed in g l^{-1}.

Result and discussion

The flocculation process consists of different stages as displayed in Fig. 2. This shows the different phases during a flocculation process using *S.acuminatus* with the flocculant PK55H. After the addition of the polymer (a), the mixing speed was increased for 1 min to allow flocculant distribution (b). The flocculant adsorbs to the cell surface and the suspension is destabilized (c) and floc formation can be observed (d, e). The flocs grow

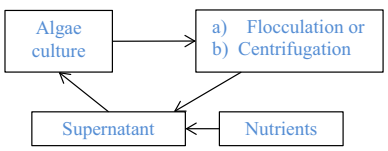

Fig. 1 Media reuse experiment. Over a period of 8 weeks, the cultures were harvested weekly by **a** flocculation or **b** centrifugation. After cell separation, the culture supernatant was enriched with concentrated WM and led back into the culture vessel

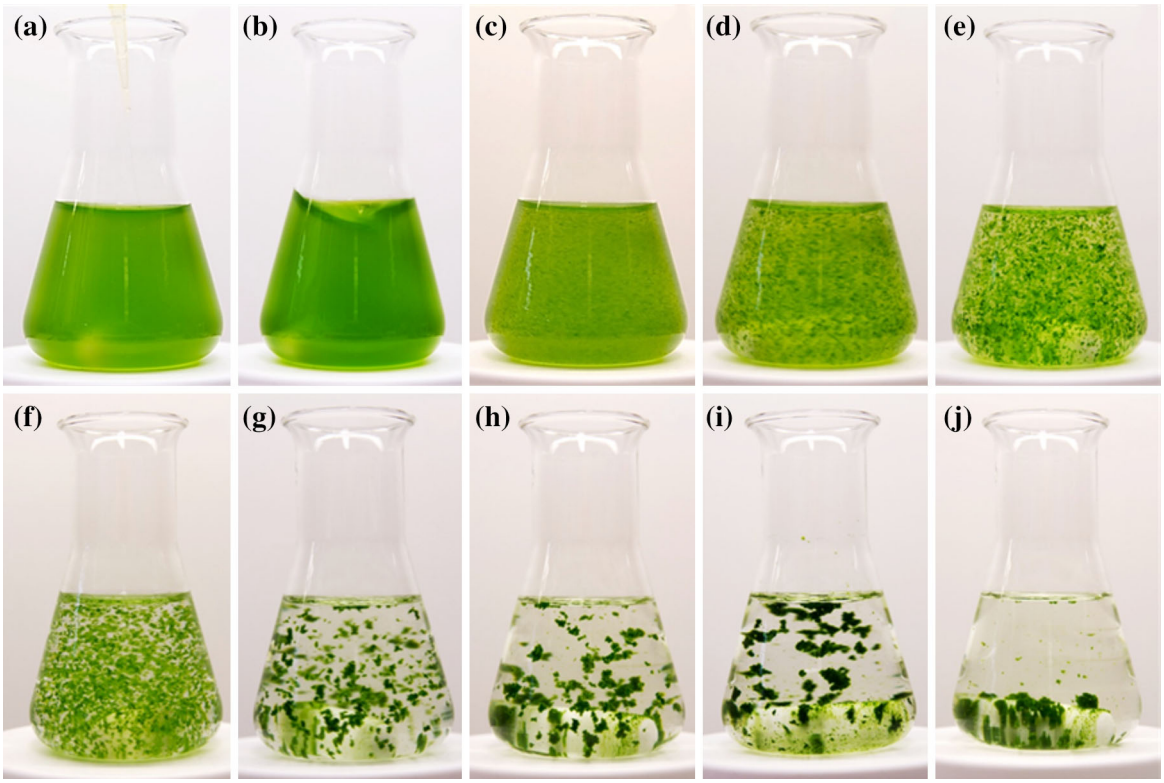

Fig. 2 Flocculation of *Scenedesmus acuminatus* using flocculant PK55H. **a** Addition of flocculant, **b** flocculant distribution, **c** destabilization of the suspension, **d**, **e** floc formation, **f–i** floc growth and **j** settling of the flocs

due to successive collisions and adsorption of microflocs (f–i). In a last phase, the agitation is stopped and the flocs are allowed to settle (j). According to the manufacturer, PK55H is a strong cationic polymer producing large dense flocs (Fig. 5i, c).

The flocculant concentration in the suspension significantly affects the FE. If a destabilization of the algal culture is not visible after flocculant addition, the amount of flocculant can be increased.

Flocculation experiments were conducted determining the FE at different dosages for all 15 flocculants investigated (Table 1). Furthermore, the optimal incubation time was identified by monitoring the FE at different time points. In each experiment, the flocculant was added and the optical density was measured after 0, 2, 10, 30 and 60 min. The settling of the microalgae was monitored as a result of flocculation. Figure 3a shows the flocculation of *Chlorella* sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* with the cationic flocculant KW100. Increasing flocculant concentration causes the FE to rise. Figure 3a shows the results for *Chlorella* sp. at concentrations between 5 and 40 mg l^{-1} of flocculant in the algae culture. Whereas 5 mg l^{-1} did not even reach a FE of 50 %, concentrations above 25 mg l^{-1} resulted in a recovery rate of 90 %. The incubation time, however, differed significantly. Increasing the amount of flocculant added reduces the incubation time needed to reach 90 % FE. 60 min was needed at 25 mg l^{-1}, 10 min at 30 mg l^{-1} and only 2 min at 35 mg l^{-1} to reach 90 %. Higher amounts were needed for the flocculation of *S.acuminatus* and *C.reinhardtii* (Fig. 3b, c). An FE of 90 % was reached after 10 and 60 min, respectively, only after adding 60 mg l^{-1}. Lower doses of 15 mg l^{-1} for *Chlorella* and 30 mg l^{-1} for *Scenedesmus* and *Chlamydomonas* resulted in insufficient algae recovery. Different behaviors in flocculation performance between the algae species were also observed in experiments displayed in Fig. 4.

Cationic, anionic and nonionic flocculants were compared with regard to the flocculation efficiency of the three algal species. First, the minimum concentration needed to achieve the highest FE was determined.

Figure 4 shows the highest FE achieved within the experiments, together with the corresponding amount of flocculant applied. The concentration is given in mg l^{-1} and is indicated on top of each bar. Whereas Fig. 4a, b shows the results of cationic flocculants, Fig. 4c shows the FE for anionic and nonionic flocculants.

Table 1 The 15 tested flocculants. Commercial name, polymer type, net charge and supplier are listed

Commercial name	Polymer type	Net charge	Stock solution [g/l]	Supplier	Price [€/kg]
Emfloc KC 750	Starch	Cationic	10	Emsland-Stärke GmbH D-49824 Emlichheim	1.25
Magnafloc LT 22S DWI	Polyacrylamide	Cationic	5	BASF Corporation Florhalm Park, USA	NA
POLY SEPAR® CFL 25	Tannin, quaternary ammonia compound	Cationic	10	Separ Chemie GmbH D-22926 Ahrensburg	2.20
POLY SEPAR® KW 100	Quaternary ammonia compound, free of polyacrylamide	Cationic	10		2.40
POLY SEPAR® KW 45	Polyacrylamide	Cationic	10		2.40
POLY SEPAR® PK 55 H	Polyacrylamide	Cationic	2		3.20
POLY SEPAR® SK 72	Starch	Cationic	10		3.00
POLY SEPAR® KW 745 H	Polyacrylamide	Cationic	10		2.40
CFL 217	Poly DADMAC	Cationic	10		2.20
CFL 229	Poly DADMAC	Cationic	10		2.20
POLY SEPAR® AN 10 TW	Polyacrylamide	Anionic	10		3.50
Magnafloc LT 27	Polyacrylamide	Anionic	5	BASF Corporation	NA
Magnafloc LT 25	Polyacrylamide	Anionic	5	Florhalm Park, USA	NA
Magnafloc LT 20	Polyacrylamide	Nonionic	5		NA
POLY SEPAR® AN20	Polyacrylamide	Nonionic	10	Separ Chemie GmbH D-22926 Ahrensburg	2.80

The prices stated are price indications subjected to daily fluctuations and dependent on the ordered quantities

NA price not available

PK55H reached the best FE at the lowest flocculant concentration. According to the manufacturers' information, PK55H is a strongly cationic polymer of high molecular weight. Even at low PK55H concentrations of 1.5 mg l^{-1} for *Chlorella* and 2- and 4 mg l^{-1} for *Scenedesmus* and *Chlamydomonas*, high FE of <95 % was attained (Fig. 4b). This flocculant is suitable for all the three cell types, resulting in large and dense flocs (Fig. 5).

The cationic charge of a flocculant is dependent on the amount of cationic charged monomers bound to the polymer chain and can vary between 0 and 100 % (Gerde et al. 2014). According to the manufacturers' information, KW 100 and KW 45 are both polymers with similar polymer structure, merely differing in the amount of cationic groups attached to the polymer chain. The polymer chain of KW100 is completely substituted with cationic groups resulting in FE <90 %. In the case of KW45, only 35 % of the available sites are covered with cationic groups showing lower FE between 40 and 80 %. This result confirms the assumption that the degree of cationic ionization affects harvesting efficiencies. A higher cationic charge of a polymer results in better FE.

CFL217, CFL 229 and CFL 25 belong to the group of polyDADMACS (Diallyldimethyl ammonium chloride). According to the manufacturer's information the molecular weight of these polymers is low with a high cationic charge. The results showed FE of 80–100 % with the best results for *Scenedesmus*. In the case of CFL25, the results for the three tested microalgae differ (Fig. 4). *Chlorella* cells were coagulated with an FE of <90 % resulting in very fine and small flocs (Fig. 5). *Chlamydomonas* cells, however, only achieved FE of 20 %. The specific surface area of the cells is another important factor to consider. The smaller size of *Chlorella* cells (2–10 µm) give them a smaller surface area compared to *Scenedesmus* and *Chlamydomonas* cells (10–30 µm). Polymers of low molecular weight can entirely absorb onto the cell surface forming regions with cationic nature. These regions can bind negatively charged regions of other algal cells. Larger cells might not be bound effectively resulting in lower FE.

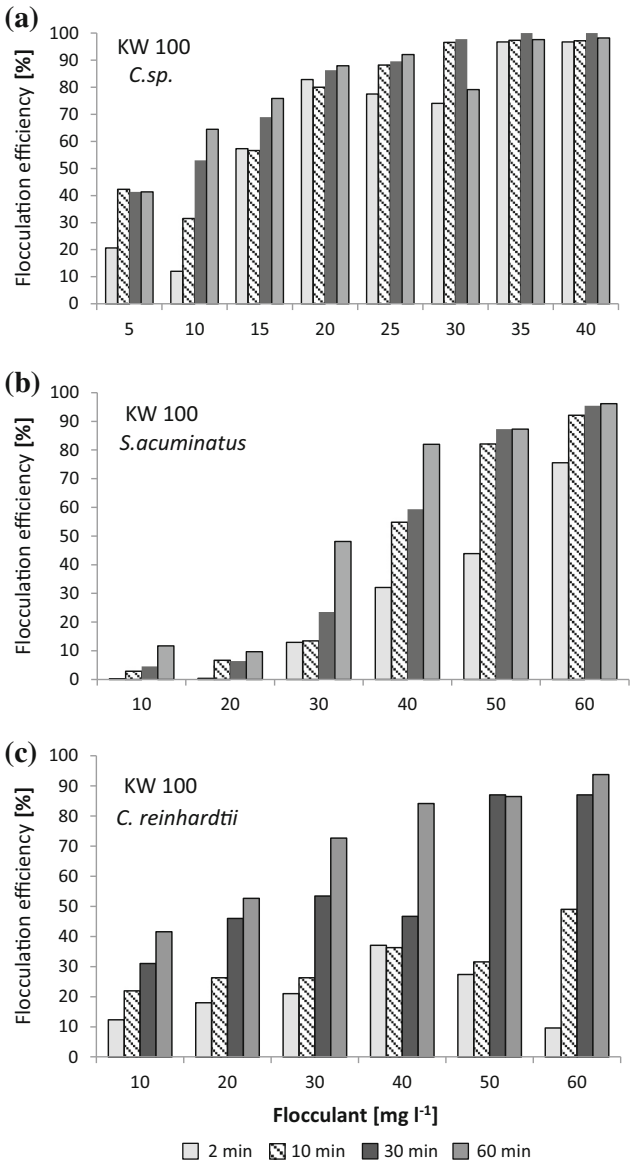

Fig. 3 Example of a flocculation experiment using the cationic flocculant KW100. Flocculation efficiencies for harvesting *Chlorella* sp. (**a**), *Scenedesmus acuminatus* (**b**) and *Chlamydomonas reinhardtii* (**c**) cultures are shown with different doses of flocculant (mg l^{-1}). Optical density measurements were conducted after 2, 10, 30 and 60 min

Several microalgae have been subjected to flocculation experiments such as *Chlorella*, *Chlamydomonas*, *Scenedesmus*, *Schizochytrium*, *Muriellopsis*, and *Phaeodactylum* resulting in different FE per milligram of flocculant (Gerde et al. 2014; Granados et al. 2012; Harith et al. 2009; Papazi et al. 2010; Rashid et al. 2013; Sirin et al. 2012). *Chlorella* sp., *Scenedesmus acuminatus* and *Chlamydomonas reinhardtii* are commonly used laboratory strains and model organisms for algal research (Harris 2001; Mandal and Mallick 2009; Safi et al. 2014; Sanchez et al. 2008). By testing these three chlorophytes, the influence of different sizes, morphologies and motilities of the flocculation efficiency should be verified. Considering the varying flocculant concentrations needed to floc the different algal species, the hypothesis that related species (Chlorophyceae) show similar flocculation behavior cannot be positively proved.

Figure 4c shows the results gained with nonionic (AN 20, LT20) and anionic flocculants (LT25, LT27 and AN10TW). No flocculation was observed for these flocculants, whichever dose was used.

At neutral pH, the cell surface of microalgae is usually negatively charged due to functional groups on the algal surface (Golueke and Oswald 1970). Therefore, generally positively charged flocculants are

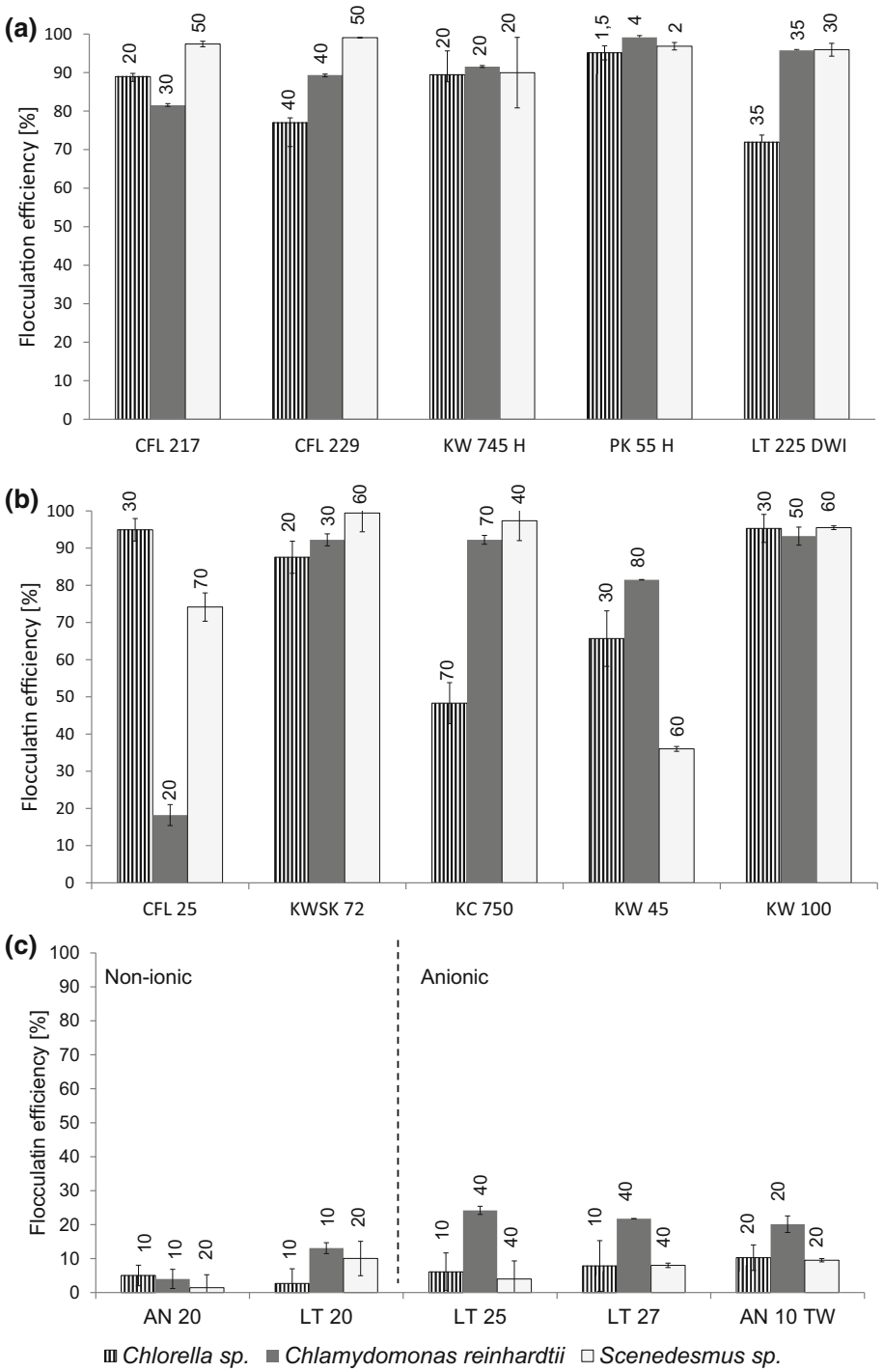

Fig. 4 Flocculation efficiencies of 15 polyelectrolytes with *Chlorella* sp., *Chlamydomonas reinhardtii* and *Scenedesmus acuminatus*. Cationic (**a**, **b**), anionic and nonionic (**c**) flocculants were tested and the most efficient concentration (mg l^{-1}) determined and displayed on *top of each bar*. The incubation time was 30 min in all experiments

recommended to neutralize the negatively charged cell surface of the microalgae (Granados et al. 2012; Tenney et al. 1969). Flocculation with polymers of the same charge can also be achieved by other mechanisms than charge neutralization, such as inter-particle bridging with long polymers. Tenney and co-workers reported the attachment of anionic polymers to the algal surface, but no floc formation in the solution was observed. Strongly anionic polymers can be effective in basic media. Harith et al. described an effective use of

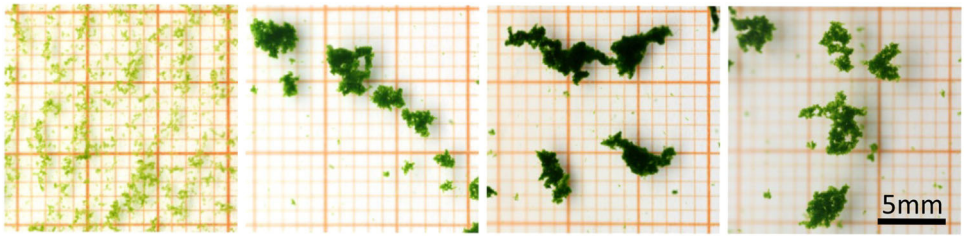

Fig. 5 Flocs produced by the flocculants CFL25, KW100, PK55H and KC750 (from *left to right*). The picture shows the result of a flocculation using *Scenedesmus acuminatus*

anionic flocculants only after algal surface charge neutralization by pH adjustments. The application of flocculant LT25 and LT 27 at a pH of 10.2 with the microalgae *Chaetoceros calcitrans* resulted in flocculation efficiencies above 90 % (Harith et al. 2009). The increase of pH before flocculation might improve FE, but negatively affect media recycling and thereby cost-effectiveness.

Based on the results, the use of anionic and nonionic flocculants cannot be recommended for harvesting of *Chlorella*, *Scenedesmus* and *Chlamydomonas* cells. The results gained positively prove the hypothesis that cationic flocculants are more effective than anionic or nonionic polymers in respects of FE.

For industrial application, the recycling of the growth media after cell harvest is an important factor in cost-effectiveness. Residues and color changes after flocculation experiments were reported, indicating that medium recycling after flocculation was not feasible (Sirin et al. 2012). It was necessary to verify if remaining flocculant particles do not negatively affect cell growth if the media are led back into the culture vessel after cell separation. A recycling experiment was conducted over a period of 8 weeks using *Chlamydomonas* cultures. The biomass was harvested weekly using the flocculant PK55H or centrifugation and the supernatant was returned into the bioreactor. Figure 6 shows the results of the growth of the microalgae by optical density (OD) measurements. After each harvesting, the optical density (750 nm) was adjusted to 1.5 to improve the light conditions for the algae. No differences between the flocculated cultures and the cultures harvested by centrifugation were visible by comparing OD values.

Although the OD values between flocculated and centrifuged cultures remained similar, the biomass concentration changed. After 10 days, the formation of aggregates was visible under the microscope and the ratio between OD and BTM changed (Table 2; Fig. 6). Although the OD values remained the same, due to the aggregates, more cells were present in the cultures, resulting in higher dry biomass values.

Table 2 lists the dry biomass values measured every week before harvesting. The data from the control and the recycling experiment differed significantly. After 8 weeks of cultivation, the dry biomass of the recycling

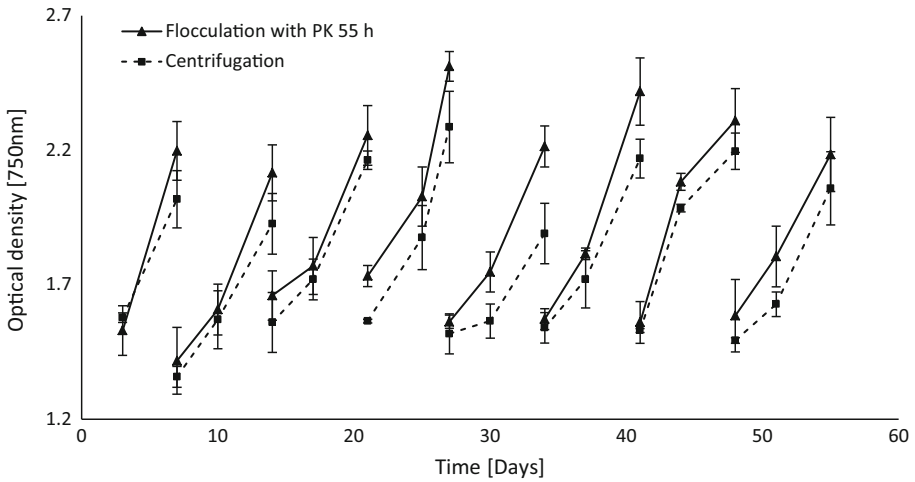

Fig. 6 Media reuse after flocculation. Over a period of 8 weeks the culture was harvested weekly by flocculation or centrifugation and the supernatant enriched with nutrients was led back into the bioreactor. The graph displays the optical density measurements registered three times per week

Table 2 Dry biomass concentration in g l^{-1}

Day	Flocculation MV ± SD	Centrifugation MV ± SD
0	1.52 ± 0	1.52 ± 0
3	1.49 ± 0.16	1.47 ± 0.04
10	2.18 ± 0.22	1.73 ± 0.01
17	2.14 ± 0.03	1.60 ± 0.18
25	2.36 ± 0.17	1.50 ± 0.11
30	2.09 ± 0.04	1.34 ± 0.10
37	2.42 ± 0.12	1.59 ± 0.04
44	2.53 ± 0.08	1.68 ± 0.22
51	2.75 ± 0.18	1.72 ± 0.14

During the media reuse growth experiment, the dry biomass was determined every week before harvesting

experiment was 1 g l^{-1} higher than that of the control cultures, although the OD values remained similar. The growth rates, however, indicate that neither the added flocculant nor a possible accumulation of algal products negatively affected cell growth.

Over a period of 8 weeks, the culture media were successfully recycled after flocculation. The hypothesis that the reuse of the media is applicable was positively proved.

Conclusion

The flocculation efficiency of microalgae for cell harvest was significantly influenced by the net charge of the tested polymer. Cationic flocculants achieved the highest flocculation efficiencies, whereas anionic and nonionic flocculants resulted in no or insufficient flocculation. The amount of flocculant needed varied among the chemicals from 1.5 to 70 mg l^{-1}, but also between the algae species (e.g., CFL25 with 30 mg l^{-1} for *Chlorella* and 70 mg l^{-1} for *Scenedesmus*). Because of high flocculation efficiencies above 95 % at low concentrations of 1.5 mg l^{-1}, the cationic polyelectrolyte PK55H was selected for recycling experiments. No limitation in culture growth and biomass production was detected, although an aggregation of the cells was visible. The results showed that chemical flocculation is a simple, efficient and inexpensive method for harvesting different types of microalgae.

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RESEARCH

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Effect of voltage and electrode material on electroflocculation of *Scenedesmus acuminatus*

Franziska Bleeke[1,2*], Gunnar Quante[2], Dominik Winckelmann[2] and Gerd Klöck[2]

Abstract

Background: Microalgae are a promising new source for biomass production. One of the major challenges in regards to cost effectiveness is the biomass harvest. High energy input is required for the separation of the small algal cells from a large volume of surrounding media. Electroflocculation is reported as a promising harvesting technique to improve cost effectiveness within the downstream process. In the present study, six electrode materials were tested for electroflocculation of *Scenedesmus acuminatus*. Besides the commonly used aluminum and iron electrodes, magnesium, copper, zinc and brass electrodes were tested for biomass harvest and compared. The influence of four different voltages (10, 20, 30 and 40 V) was investigated and evaluated.

Results: Electroflocculation was applicable with all tested electrode materials. The highest flocculation efficiency was achieved using magnesium electrodes followed by Al, Zn, Cu, Fe and brass. Using magnesium, 90% of the suspension was clarified at 40, 30, 20, and 10 V after 9.2, 12.5, 18.5, and 43 min, respectively. All electrode materials showed the fastest flocculation at 40 V and the lowest at 10 V. The pH increased from 7.5 to values between 9.3 and 11.9 during the flocculation processes. Reuse of the supernatant showed no adverse effect on algal growth. The highest cell counts after 12 days of incubation were achieved with iron at 1.86×10^7 cells ml^{-1} and the lowest with copper at 1.23×10^7 cells ml^{-1}.

Conclusion: Besides the commonly used iron and/or aluminum electrodes, other materials like magnesium, copper, zinc and brass can be successfully used for microalgal biomass harvest. For special biomass applications like food or feed additives, metals like magnesium have other advantages besides their high flocculation efficiency such as their low toxicity at high concentrations. Higher voltages increased the maximum flocculation efficiency but also increased the required energy input.

Keywords: Electroflocculation, Microalgae, Biomass harvest, *Scenedesmus acuminatus*, Flocculation

Background

Microalgae are eukaryotic, photosynthetic microorganisms that convert sunlight into chemical energy. The produced biomass can be used as food, feedstock or as potential substrate for biofuel production (Chisti 2007; Mata et al. 2010). They show a broad application in biotechnology since they grow fast at low nutritional and environmental requirements (Chisti 2007; Mallick 2002; Mata et al. 2010; Wang et al. 2008). Although they are easy to cultivate, the bottleneck which often makes microalgal cultivation uneconomical is the downstream processing which contributes to 20–30% of the biomass production costs (Grima et al. 2003; Mata et al. 2010; Uduman et al. 2010). Separation of the cells (2–10 μm) from the surrounding growth media requires high energy inputs (Grima et al. 2003). Large volumes must be processed, since the concentration of cells is very low at around 0.5–2.5 g l^{-1}. Common separation processes combine filtration or flotation with a final centrifugation step (Grima et al. 2003; Uduman et al. 2011), often exceeding the energy content of the harvested biomass

*Correspondence: franziskableeke@hotmail.de
[1] Jacobs University Bremen, Bremen, Germany
Full list of author information is available at the end of the article

(Wijffels and Barbosa 2010). Increasing the efficiency at low energy demands within the harvesting process is a major challenge. By flocculation, the cells coagulate and larger particles are produced with a higher settling velocity.

Several studies are available on the use of chemical flocculation using metal salts or polyelectrolytes (Gerde et al. 2014; Granados et al. 2012; Papazi et al. 2010; Tenney et al. 1969), pH induced flocculation (Vandamme et al. 2012; Wu et al. 2012; Zheng et al. 2012), and bioflocculation using bacteria or filamentous fungi for biomass harvest (Zhou et al. 2013). In electroflocculation, the flocculant is produced by releasing metal ions from a sacrificial electrode (Vandamme et al. 2011). Numerous studies have been published on the use of electroflocculation for algal biomass harvest (Lee et al. 2013; Uduman et al. 2011; Vandamme et al. 2011); however, these experiments used primarily aluminum electrodes (Kim et al. 2012; Lee et al. 2013; Vandamme et al. 2011; Xu et al. 2010) and/or iron electrodes (Uduman et al. 2011; Vandamme et al. 2011). Very little information can be found on the use of other electrode materials like Mg, Zn, Cu or brass for electroflocculation of microalgae.

The microalgae *Scenedesmus* is one of the most common genera found in freshwater ecosystems. These polymorphic chlorophytes show a broad application in wastewater treatment (Chinnasamy et al. 2010; Hodaifa et al. 2008; Martinez et al. 2000), biodiesel production (Mandal and Mallick 2009; Tang et al. 2011) and in the production of high value pigments like lutein (Sanchez et al. 2008). Flocculation of *Scenedesmus* by metal salts, bioflocculants, and cationic polymers has been tested in prior experiments, but little research has been done on electroflocculation of *Scenedesmus* (Mallick 2002; Uduman et al. 2010; Vandamme et al. 2011, 2012; Wang et al. 2008).

In the present study, six electrode materials were tested at four different voltages. It was verified whether metals other than aluminum and iron are suitable for the electroflocculation process (H1) and if the flocculation efficiency increases at higher voltages applied (H2).

Methods

Culture media and microalgae cultivation

All experiments were carried out using the freshwater chlorophyte *Scenedesmus acuminatus* from the culture collection of the University of Applied Sciences Bremen. The cells were grown in 2 l of liquid Wuxal medium (WM) (Winckelmann et al. 2015). Aeration was provided by compressed air, which was introduced into the culture by aeration hoses. Light was emitted by fluorescent lamps (OSRAM L 30W, warm white) placed in front and behind the algae cultures for 24 h per day.

Electroflocculation

The flocculation experiments were carried out at room temperature in 100 ml beakers filled with 90 ml of algae suspension. The initial cell concentration of the algae was 1×10^7 cells ml^{-1}. The electrode plates were cut from commercial grade metal sheets. Before use, they were mechanically polished using abrasive paper and placed parallel and vertically into the algae suspension. The distance between the cathode and anode was 2.5 cm and the depth of immersion was 4 cm resulting in a submerged area of 51.2 cm^2 (40 mm × 32 mm × 4 electrode sides) for both electrodes. All experiments were carried out with the same area immersed in water and the same electrode distance. During the flocculation process, the culture was gently stirred at 100 rpm using a magnetic stirrer.

The electrodes were connected to a DC power supply (Hewlett Packard, Model 6205b, USA) and the voltage was adjusted to 10, 20, 30 or 40 V.

To determine the flocculation efficiency (FE), 1 ml samples were taken before the flocculation and every 2.5 min during the process. Samples were taken from 3 cm below the surface. Optical densities for each sample were measured at a wavelength of 750 nm in a Genesys 20 (Thermo scientific, Walthman, USA) photometer and pH values were recorded.

FE was calculated as follows:

$$Flocculation\ efficiency = \left(\frac{OD_{t0} - OD_{t1}}{OD_{t0}}\right) \times 100$$

OD_{t0} is the initial optical density before starting the flocculation and OD_{t1} is the optical density of the sample at a certain point of time during the process.

After the flocculation, the biomass and supernatant were separated and kept at −20°C for recycling experiments. Before and after each flocculation, the electrodes were dried at 60°C for 2 h.

Iron, magnesium, aluminum, zinc, copper and brass electrodes were tested and compared. Each material served as both the anode and cathode at the same time. For comparison, a continuous function of the flocculation efficiency depending on the flocculation time, an interpolation, was used based on the following assumption:

The flocculation process can be described by a sigmoid function with an upper limit of 100%, leading to the following function:

$$Efficiency(t) = \left(\frac{100}{1 + a^{b-t}}\right)$$

The coefficients a and b were determined for each voltage and electrode material, respectively, using a numerical curve fitting tool. By determining the coefficients a and b, it was possible to solve the efficiency function for

the time when the curve reached values of 90%. Those values were used in order to compare the various flocculation experiments.

The influence of each electrode material was tested and the best material regarding FE at 90% was determined.

The mass loss as a function of time was assumed to be linear. This assumption allowed calculating the amount of mass lost during the time until a flocculation efficiency of 90% was reached. This mass was multiplied by the exchange prices of the corresponding electrode materials in order to estimate the costs of each flocculation process (London metal exchange 2015).

Media reuse after flocculation

A batch-recycling experiment was conducted over 12 days in 100 ml Erlenmeyer flasks, filled with 50 ml of algae cells incubated in flocculation supernatant. Cell growth was monitored every other day by optical density and cell count measurements. Cell number was determined using the a Thoma counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and adjusted to 2×10^6 cells ml^{-1} by the addition of concentrated cells to the cell-free supernatant. For positive control, the cells were diluted in fresh Wuxal Media. All experiments were carried out in triplicate.

Results and discussion

Iron, magnesium, aluminum, zinc, copper and brass electrodes were tested and their corresponding FEs compared. Figure 1 shows an example setup of the flocculation experiment. The electrodes were connected to a DC power supply (Hewlett Packard, Model 6205b, USA) and the voltage was adjusted to 10, 20, 30 or 40 V depending on the recent experiment (a, b). During the flocculation process, the suspension became milky white and gas visibly formed at the electrodes resulting in foam formation on the top of the liquid surface (c, d). The flocculation was successful when the liquid phase was clear and algal cells were located in the foam on top (d).

In Fig. 2, the performance (FE) of the different electrode materials at different voltages over time is shown. All graphs show the fastest flocculation at 40 V and the slowest at 10 V; the higher the applied voltage, the faster the maximal flocculation efficiency. Similar results were published by other authors (Alfafara et al. 2002; Poelman et al. 1997; Vandamme et al. 2011; Xu et al. 2010; Zhang et al. 2015).

The highest FE was observed when using Mg electrodes (Fig. 2a). Ninety percent of the suspension was clarified at 40 V after 9.2 min (Table 1). When the voltage was decreased to 30 V, the graph leveled slightly, and the recovery time increased. At 30 V, a FE of 90% is reached after 12.5 min, at 20 V after 18.5 min and at 10 V only after 43 min (Table 1).

During the EF process, metal ions are continuously released from the anode by electrolytic oxidation. In H_2O these ions immediately react and form metal hydroxides. These metal hydroxides represent the flocculant which reacts with the algae cells. For the magnesium electrodes, the following reaction occurs:

$$\begin{aligned} Mg &\rightarrow Mg^{2+} + 2e^- \quad 2H_2O \\ &\rightarrow 4H^+ + O_2 + 4e^- \quad \text{(Anode)} \end{aligned}$$

$$\begin{aligned} M(aq)\, n^+ + ne^- &\rightarrow M(s) \quad 2H_2O + 2e \\ &\rightarrow^- H_2 + 2OH^- \quad \text{(Cathode)} \end{aligned}$$

At higher applied voltages, more ions are produced, and consequently more flocculant is available in the algal solution (Mollah et al. 2001, 2004). The positively charged metal hydroxides react with the negatively charged cell surface of the microalgae. The potential of the algal cell

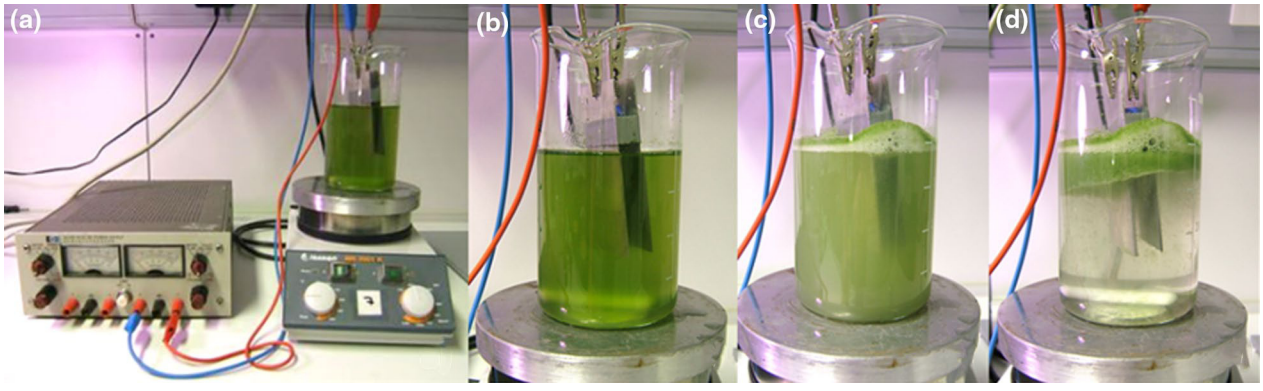

Fig. 1 Electroflocculation process. **a** Experimental setup: The flocculation experiments were carried out in 100-ml beakers filled with 90 ml of algae suspension. The electrodes were connected to a DC power supply (Hewlett Packard, Model 6205b, USA), **b** algae culture before flocculation, **c** during the flocculation process and **d** after the flocculation.

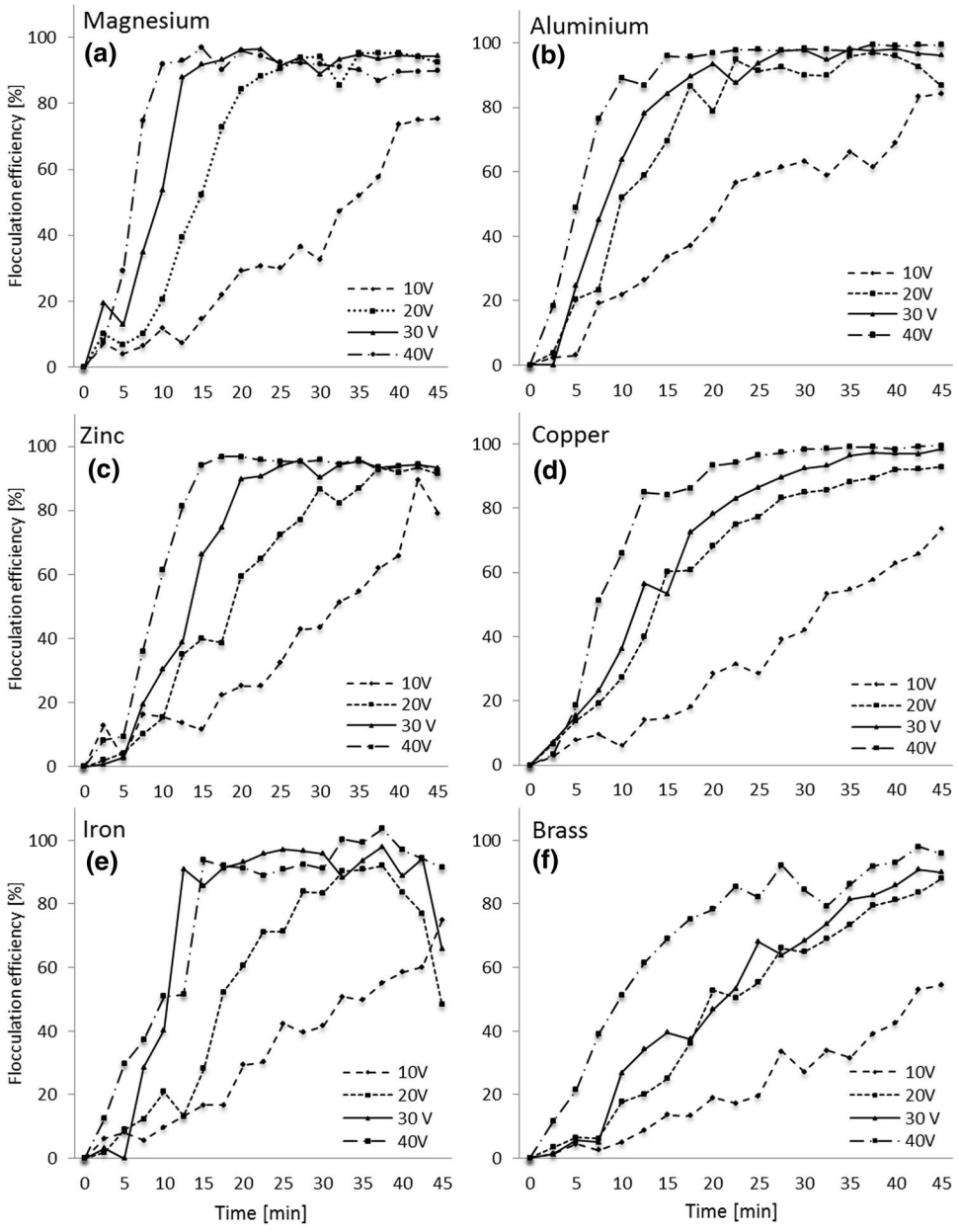

Fig. 2 Effect of electrode material and applied voltage on the recovery rate during the electroflocculation process. Flocculation experiments were conducted using different electrode materials: magnesium (**a**), aluminum (**b**), zinc (**c**), copper (**d**), iron (**e**) and brass (**f**). The graphs were arranged according to the calculated electrode efficiency from high (**a**) to low (**f**). The voltage was adjusted to 10, 20, 30 or 40 V and the flocculation efficiency was measured every 90 s.

Table 1 Calculated incubation time until a 90% flocculation efficiency is reached

	Magnesium	Aluminum	Zinc	Copper	Iron	Brass
10 V	43.2	42.1	53.6	61.0	53.7	71.1
20 V	18.5	20.6	30.6	31.3	27.9	43.9
30 V	12.5	11.4	21.7	20.3	16.0	40.7
40 V	7.3	9.0	14.2	14.6	46.9	30.9

The incubation time is given for each electrode material at 10, 20, 30 and 40 V.

is increased, and the surface charge is neutralized (Henderson et al. 2008). The suspension becomes destabilized, and flocs are formed. At 10 V a short lag phase could be detected in all experiments, which is assumed to be due to insufficient flocculent availability at the start the flocculation process. After 5–10 min, the values start to rise. The shortened lag phase present at higher voltages is due to the faster ion release from the electrodes. Magnesium is bivalent and therefore forms extra stable hydrogen bonds, resulting in an effective floc formation.

The contamination of the harvested biomass and the remaining media with metal particulates might interfere with further processing steps or the use of the biomass as food or feed additive. Here the use of magnesium shows another advantage. Accepted magnesium limits are higher when compared to aluminum, iron, copper, or brass. In the German Drinking Water Ordinance, for example the limits for magnesium and also for zinc were removed since a negative impact for human health was considered as very low, whereas concentrations of 0.2 mg l^{-1} for aluminum and iron and 2 mg l^{-1} for copper are not to be exceeded (Ordinance 2001).

Extensive research has been conducted regarding the use of aluminum electrodes (Chen et al. 2009; Kim et al. 2012; Lee et al. 2013; Vandamme et al. 2011; Xu et al. 2010) and iron electrodes (Uduman et al. 2011; Vandamme et al. 2011). Aluminum was found to have the second highest FE (Fig. 2b). Ninety percent FE was reached after 9, 11.4, 20.6 and 42 min at 40, 30, 20 and 10 V (Table 1). In literature the use of aluminum electrodes is compared to the use of iron electrodes in coagulation processes with (Vandamme et al. 2011) or without algae (Zongo et al. 2009) involved. The results of this present study agree with the data reported that the use of aluminum is more efficient than the use of iron electrodes (Fig. 2e). The lower efficiency of the iron electrode compared to aluminum might be explained by the lower current efficiency of the iron electrode (Zongo et al. 2009).

The lowest recovery efficiency was found using brass electrodes (Fig. 2f). After 15 min at 40 V only approximately 70% of the biomass was recovered. Ninety percent was reached after 30 min which is believed to have been caused by the high pH value during the process (Table 2). Figure 3a shows that after 11.5 min, a pH of 11.8 was observed. It is suspected that algal recovery efficiency decreases at pH values of 12 because of algal lysis (Contreras et al. 1981; Xu et al. 2010).

Electroflocculation technologies are currently in use at wastewater treatment facilities (Mollah et al. 2001). In industrial wastewater cleaning, the initial pH is one of the most important factors influencing the EF process (Mouedhen et al. 2008). In Fig. 3, the pH evolution during the EF with different electrode materials at 40 V is shown. Figure 3a shows the data for Al, Mg and Zn while Fig. 3b shows the values monitored with Fe, Cu and brass. In all of the experiments, the pH increased during the flocculation process.

A fast pH increase up to 10–12 was monitored in all samples except for Al (Fig. 3; Table 2). The highest pH value was reached during the flocculation using brass electrodes. The lowest pH was recorded in the samples using Al electrodes. Since the pH is a measure for the hydrogen and hydroxide concentration, it is expected that the produced metal hydroxides were immediately bound and thereby not contributing to pH increase. During the flocculation process, the algae recovery rate increased in the beginning but started to decrease after 20 min of flocculation (Fig. 2e). Similar effects were observed by other authors. Xu et al. (2010) described a decrease in recovery time and efficiency from pH 7 to 11 and an increase of recovery time at pH 12 which is expected to be because of algal lysis (Contreras et al. 1981; Xu et al. 2010). The fluctuations in the Al and Fe graphs might be due to insufficient mixing during sample taking.

Figure 4 shows the mass loss and the calculated material costs until a flocculation efficiency of 90% is reached. All tested electrodes lost weight between 1.1 g for magnesium and 101 g for iron. The highest mass loss was recorded for Fe with 101 g at 40 V and for brass at 20 and 30 V. Although the mass loss of the iron electrodes is high, the costs for the flocculation with iron are comparably low. Since brass is the most expensive electrode material showing the lowest FE, it cannot be recommended for algal harvest using electro flocculation. The most economical efficient flocculation was achieved with Mg, Al and Fe.

Table 2 pH values recorded after 30 min of electroflocculation

	Aluminum	Brass	Magnesium	Zinc	Copper	Iron
10 V	9.3	10.7	11.4	11.5	11.5	11.6
20 V	10.3	11.5	11.5	11.3	11.6	11.7
30 V	9.7	11.9	11.4	11.6	11.7	11.6
40 V	10.4	11.9	11.5	11.7	11.6	11.6

The table lists the pH values gained with aluminum, brass, magnesium, zinc, copper, and iron electrodes at 10, 20, 30 and 40 V starting with the lowest values (left) to the highest (right).

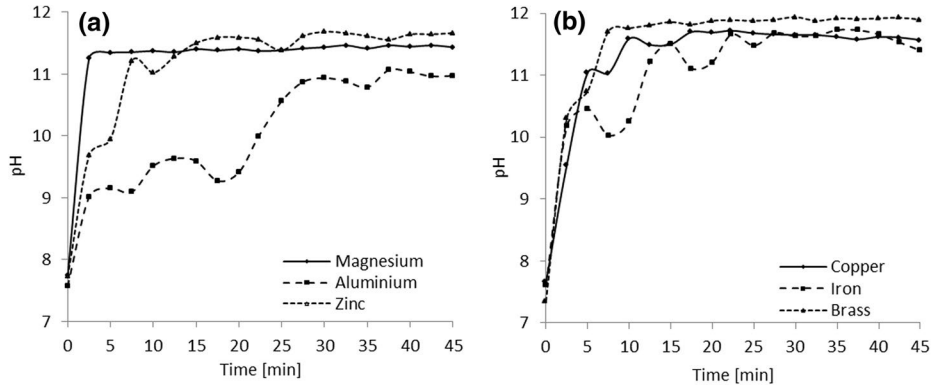

Fig. 3 Effect of electroflocculation on the pH value using aluminum, iron and brass (**a**) and zinc, copper and magnesium (**b**) for electrode material. Measurements were recorded during a flocculation at 40 V.

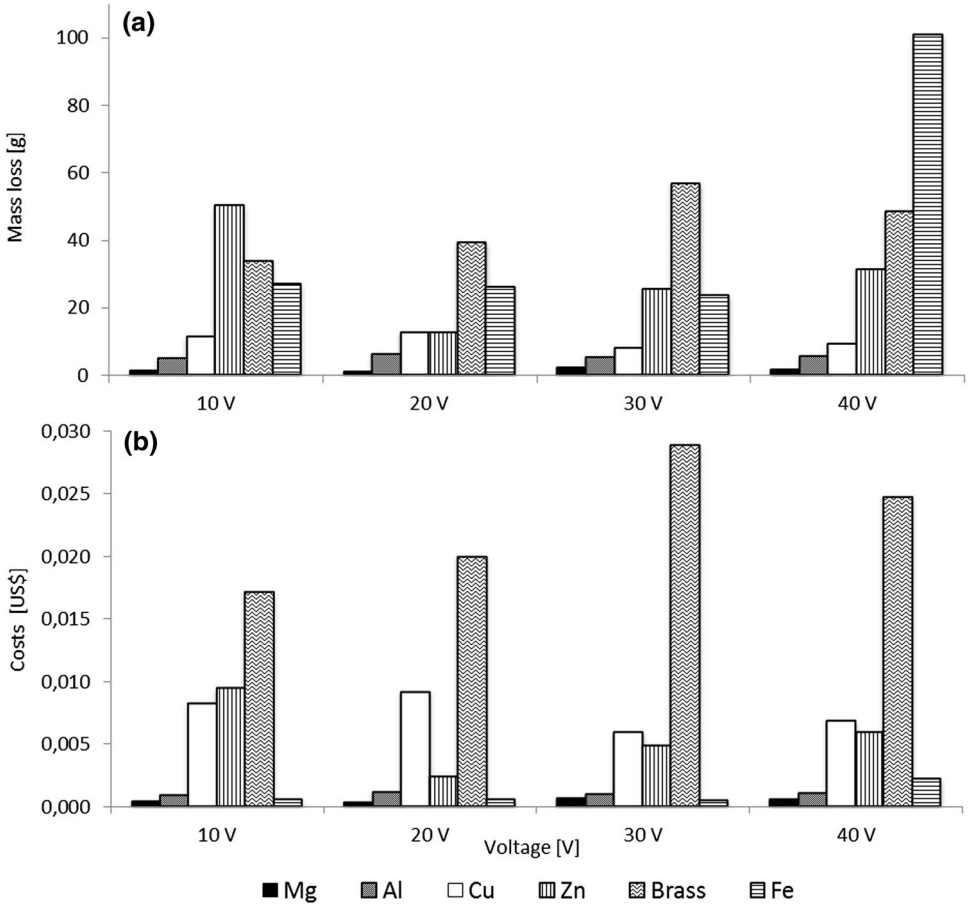

Fig. 4 Mass loss and electrode costs. Mass loss of the electrodes during a flocculation experiment (**a**) and the corresponding metal costs (**b**). The metal prices were stated by the London metal exchange and are subjected to daily fluctuations.

For industrial applications, reuse of the growth media after cell separation is an important factor regarding cost effectiveness. A recycling experiment was conducted to investigate if the high pH value and/or remaining metal residues negatively affect cell growth if the media is led back into the culture vessel after cell harvest. The growth of the *Scenedesmus* cells was monitored in a batch experiment over twelve days of cultivation.

Cell growth was monitored in all of the experiments (Fig. 5). The best result was achieved with Iron

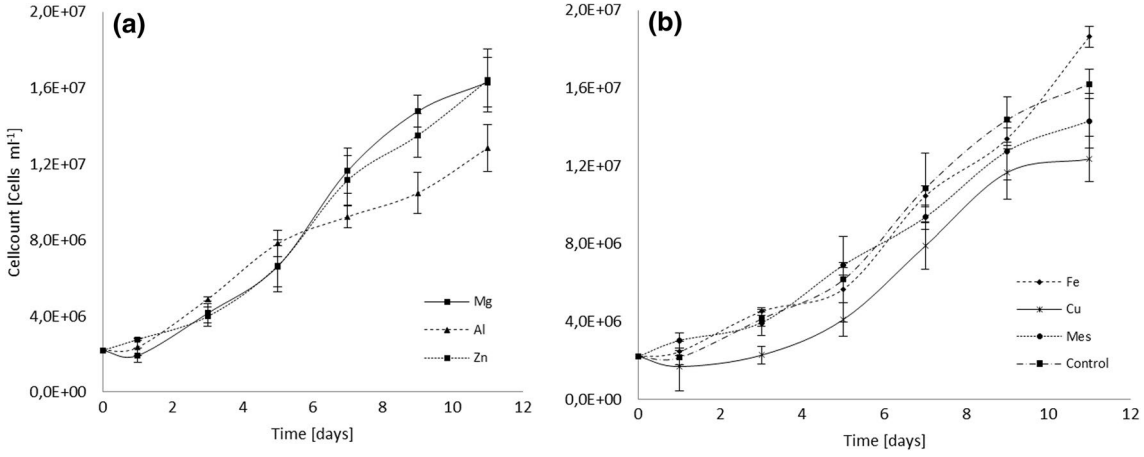

Fig. 5 Media reuse after flocculation. For 12 days *Scenedesmus* cells were incubated in magnesium, aluminum and zinc (**a**) as well as iron, copper and brass (**b**) flocculation supernatant enriched with nutrients. The graphs show the cell growth measured in cells ml^{-1} monitored every other day. For positive control the cells were incubated in WM.

supernatant and a final cell concentration of 1.86×10^7 cells ml^{-1} (Fig. 5b). The control experiments with fresh media resulted in final cell concentration of 1.62×10^7 cells ml^{-1}.The lowest cell concentration was reached with the copper supernatant with 1.23×10^7 cells ml^{-1}. Although copper is an essential micronutrient for plants, higher concentrations are known to inhibit photosynthetic reactions (Kupper et al. 2009). The reuse of all supernatants showed similar growthbehavior within the 12 days of cultivation.

Several experiments have been published on the use of electroflocculation for algal biomass harvest (Lee et al. 2013; Uduman et al. 2011; Vandamme et al. 2011) mainly using aluminum electrodes (Kim et al. 2012; Lee et al. 2013; Vandamme et al. 2011; Xu et al. 2010) and/or iron electrodes (Uduman et al. 2011; Vandamme et al. 2011). Magnesium, copper, zinc or brass electrodes have so far not been used for this purpose. This study revealed that besides iron and aluminum, magnesium shows high potential for algal harvest by electroflocculation. Besides a high FE and cost effectiveness, magnesium is non-toxic and can be utilized in a broad range of application.

Conclusion

Besides the commonly used iron and or aluminum electrodes other materials like magnesium, copper, zinc and brass can be successfully used for microalgal biomass harvest by electroflocculation. The most cost effective flocculation was achieved with Mg, Al and Fe as electrode material. For special biomass applications like food or feed additives metals like Magnesium have other advantages besides their high flocculation efficiency such as their relative harmlessness even at higher concentration. A higher voltage increased the maximum flocculation efficiency but also increased the energy input needed. Recycling of the supernatant was shown to be possible but should be repeated in a long term experiment comprising several harvesting steps.

Authors' contributions

FB contributed to experimental design, data acquisition, result analysis and manuscript preparation. GQ contributed to result analysis and manuscript preparation. DW contributed to experimental design and data acquisition. GK contributed to experimental design, result analysis and manuscript preparation. All authors read and approved the final manuscript.

Author details

[1] Jacobs University Bremen, Bremen, Germany. [2] Institute of Environmental Biology and Biotechnology, University of Applied Sciences Bremen, Bremen, Germany.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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