Harvesting microalgae by bio-flocculation and autoflocculation

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Harvesting microalgae by bio-flocculation and autoflocculation

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1 General Introduction

Microalgae are considered a promising resource for different biobased commodities. Both biodiesel production (de Boer et al., 2012) and production of food commodities (Draaisma, et al., 2013) from microalgae received considerable attention in recent years. This is not without reason. For example, oil yield per area of microalgae cultures exceeds the yields obtained by the best terrestrial oilseed crops. Moreover, microalgae require less water than terrestrial crops and can be cultivated on non-arable land, minimizing associated environmental impacts (Rodolfi et al., 2009; Brennan and Owende, 2010). Despite its potential, microalgal production requires high energy inputs for water pumping, mixing and for harvesting the microalgal biomass (Schenk et al., 2008; Norsker et al., 2011; Draaisma, et al. 2013). The energy needed e.g. for harvesting of microalgae from a 0.3 $gDW^.L^{-1}$ microalgal suspension via centrifugation was calculated to be 13.8 $MJ.kgDW^{-1}$, while the combustion energy of the oleaginous microalgae was estimated to be 26.2 $MJ.kgDW^{-1}$ (Norsker et al., 2011).

The energy needed for harvesting can be reduced considerably by pre-concentration of the microalgae prior to further dewatering. A suitable method to pre-concentrate microalgae should be effective, highly reliable, easily manageable with low capital and operational costs and energy demand when applied at large scale. Preferably, the method should allow for the reuse of the medium, as this makes the overall production process more sustainable. Regarding the characteristics of microalgal cells (small size, similar density of cell and surrounding culture medium, negative charge and morphology) and the low concentration of biomass in culture systems, a pre-concentration step for harvesting microalgae is likely to consist of flocculation accompanied by either flotation or sedimentation. In this thesis, the focus is on the development of a controlled pre-concentration step in which bio-flocculation or autoflocculation using oleaginous microalgae is developed and combined with gravity sedimentation. This technology will be evaluated in terms of sustainability of the process and the energy demand for harvesting microalgae will be compared with existing harvesting methods. The prevailing harvesting methods will be discussed first, before elaborating on the bio-flocculation and autoflocculation of microalgae.

1.1. Harvesting

Harvesting microalgae is challenging due to the nature of microalgal cells (size, cell density, charge and morphology) and the concentration of biomass in culture systems which is generally low (0.2-10 gDW^-L^{-1}). Despite the fact that a wide range of solid-liquid separation techniques is available and many of them have been tested for harvesting of microalgae, the energy related to harvesting microalgae is still high (Golueke and Oswald, 1965; Shelef et al., 1984; Borowitzka, 1999; Molina Grima et al., 2003; Brennan and Owende, 2010; Uduman et al., 2010; de Boer 2012; Pahl et al., 2013).

Currently, harvesting of microalgae is mainly performed in a single centrifugation step, but to minimize the energy demand an integrated multiple step approach is needed. In the pre-concentration step, the initial concentration of microalgal suspension should be increased before further dewatering of microalgae. However, the concentration factor is not the only requirement for an efficient pre-concentration. It is also important to achieve a high recovery. The recovery is defined as the amount of microalgal biomass harvested from the microalgal biomass present in the initial microalgal suspension, while the concentration factor is the factor that provided information on the volume reduction of the microalgal suspension in time (Salim et al., 2012). Usually pre-concentration is done by flocculation after which the formed flocs are separated by either flotation or sedimentation. During flocculation the microalgal cells aggregate to larger flocs which can be easily separated by sedimentation. It is expected that higher sedimentation rates result in lower overall energy demand for harvesting. The goal of this thesis is development of a pre-concentrations step in which aggregation of the microalgal cells by flocculation will induce formation of large dispersed microalgal aggregates which not only concentrates the cells but also increases the speed at which they will settle.

1.2. Pre-concentration of microalgae

1.2.1. Methods for flocculating microalgae

Flocculation can be induced in different ways. Most flocculation methods are based on reduction and shielding of the negative charge on the cell surface of the microalgae. At natural water pH (around neutral pH), the functional groups at the surface of the microalgal cell are dissociated (Shelef et al., 1984). Particularly the carboxyl groups that are present in peptides in the cell wall (Northcote et al., 1958) or in extracellular polymeric substances (EPS) attached to the cell surface (Bernhardt et al., 1985) render a negative ζ-potential which is usually within the range of -10 to -35 mV (Henderson et al., 2008). The magnitude of ζ-potential is dependent on pH and ionic strength of the medium.

To reduce or shield the negative charge of microalgal cells, inorganic or organic flocculants can be added (McGarry, 1970; Lee at al., 1998; Papazi et al., 2010). This induced chemical flocculation technique is already extensively applied at industrial scale, especially in wastewater treatment plants (De la Nouë et al., 1992). Although induced chemical flocculation is an easy and effective method, this will not be an appropriate method for cheap and sustainable harvesting of microalgae in large scale microalgae production plants. The cationic flocculants may complicate further downstream processing of microalgae. On top of that, any excess on flocculant needs to be removed from the medium before the medium can be reused and this leads to extra operational energy (Schenk et al., 2008).

Other flocculation methods that are based on reduction or shielding the negative charge of microalgal cell make use of a change in the culture conditions. For example, extreme pH or nutrient depletion can be applied or, temperature changes can be applied, but again, these flocculation methods are not preferred for pre-concentration of microalgae at large scale, as they require treatment of the medium before reuse. Moreover, the latter methods result mainly in uncontrolled flocculation and they may induce undesired changes in cell composition (Benemann and Oswald, 1996).

Flocculation of microalgae can also be induced by extracellular polymers originating from other microorganisms. These polymeric substances can be excreted in the suspension or they remain attached to the microorganism. This biologically induced flocculation or bio-flocculation of the microalgae has shown to be successful with bacteria (Lee et al., 2009) and fungi (Zhou et al., 2012), however, it demands an additional substrate and energy source for bacterial or fungal growth, which will evoke undesirable bacterial or fungal contamination of the microalgal production plant.

Another method is induced flocculation using electric forces. Active collision of microalgal cells is induced in a generated ultrasound wave node or in an electrostatic field. Ultrasound induced flocculation was presented by Bosma et al. (2003) as a successful method however the energy demand of this method is too high to justify its use for harvesting microalgae for biodiesel. However, the National Alliance for Advanced Biofuels and Bio-products (NAABB) consortium is currently developing ultrasonic induced flocculation with simultaneous extraction which could reduce the energy demand. Recently, Vandamme et al. (2011) presented electroflocculation of microalgae as promising and effective flocculation method, but they indicated that contamination of the recovered biomass and medium with metal salts from the sacrificial anode occurred and that high energy use was associated with the anode replacement and the formation of an oxide layer on the cathode.

In this thesis, bio-flocculation of non-flocculating microalgae with autoflocculating microalgae is presented as a promising pre-concentration step for harvesting of microalgae. The use of these autoflocculating algae of the oleaginous microalgal strains does not require addition of extra medium compounds or chemicals to induce the flocculation, which makes reuse of the medium without further treatment possible after flocculation. The effectiveness of this pre-concentration method has been investigated and the energy needed when bio-flocculation is used as the pre-concentration step for harvesting different strains, has been determined. Some of the auto-flocculating microalgae showed accumulation of lipid when growing under nitrogen depletion. Due to their autoflocculation properties, these strains are very promising candidates for lipids production. Therefore the potential of using these

autoflocculating oleaginous strains were investigated as well and the energy needed for culturing and harvesting was calculated and compared with currently used production and harvesting methods.

1.2.2. Sedimentation of the formed flocs

After flocculation of the microalgae, the microalgal flocs can be subjected to sedimentation. Effective sedimentation not only requires increased particle size but also a density difference between the microalgal flocs and the surrounding medium. Settling of the flocs in a sedimentation tank requires low energy input, low design costs and low requirement for skilled operators. Sedimentation can be done in gravity thickeners, rectangular or circular in shape. The retention time depends on the dimensions of the thickener and the sedimentation rate of microalgae. To decrease the retention time in a settling tank inclined channels, plates or tubes are installed. For example, lamellar settlers which contain inclined plates to enhance sedimentation rates have been used for microalgal harvesting (Nakamura et al., 2005). Most recently a multi-channel, bottom-fed lamellar settler has been tested by Smith and Davis (2013) for harvesting microalgae. They reached 70% faster clarification rate at 8º in comparison with standard 55 º and achieved a concentration factor of 80 for unflocculated microalgae.

In this thesis, the focus will be on the combined bio-flocullation and autoflocculation with sedimentation, as this is expected to result in an energy-efficient and sustainable technology for harvesting oleaginous microalgae for lipids production.

1.3. Aim and outline of the thesis

Bio-flocculation of non-flocculating oleaginous microalgae with autoflocculating microalgae are presented as a promising pre-concentration step in harvesting of microalgae in **Chapter 2**. The presented bio-flocculation method enables the harvesting of microalgae without addition of chemical flocculants. In **Chapter 3**, the effect of the ratio between autoflocculating and target microalgae applied in bio-flocculation was studied with emphasis on the recovery, sedimentation rate and energy demand for harvesting the target

microalgae. From different microalgal strains tested, *E. texensis* showed to be the most promising candidate regarding settling and autoflocculation characteristics as well as high growth rate combined with high lipid content and therefore the effect of the growth phase on recovery, sedimentation and autoflocculation behaviour of *E. texensis* was investigated in **Chapter 4** and the lipid content of *E. texensis* was determined during the subsequent growth phases to define the optimum harvesting time of *E. texensis*. To reveal the mechanism involved in autoflocculation of *E. texensis*, this strain was compared with the non-flocculating *Chlorella vulgaris* on the cell surface charge and extracellular polymeric substances (EPS) attached to the cell surface in **Chapter 5**. Furthermore, the possible role of EPS attached to *E. texensis* cells in capturing *C. vulgaris* cells during bio-flocculation was investigated. A mathematical model for flocculation and sedimentation was developed and presented in **Chapter 6** which predicts the time needed to reach a desired concentration of microalgal suspension and describes the concentration of the particles as function of time and position of the particle in a sedimentation tank. This model was validated with experimental data using *E. texensis*. In addition, the model was used to predict the overall effect of flocculation and sedimentation on large scale harvesting of microalgae by calculating the concentration factor and the biomass recovery in a given settling tank. Based on the achieved concentration factor, the energy needed for further dewatering of the microalgae in a centrifuge could be estimated. In **Chapter 7**, the overall results of this thesis were evaluated based on the energy balance of microalgal production, the energy needed for pre-concentration of microalgae and parameters which have an influence on that. Advantages and disadvantages of bio-flocculation were compared with chemical flocculation. Finally, the future of bio-flocculation and autoflocculation as promising pre-concentration step in harvesting microalgae at industrial scale was discussed.

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2 Harvesting of microalgae by bio-flocculation

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Abstract

The high energy input for harvesting biomass makes current commercial microalgal biodiesel production economically unfeasible. A novel harvesting method is presented as a cost and energy efficient alternative: the bio-flocculation by using one flocculating microalga to concentrate the non-flocculating microalga of interest. Three flocculating microalgae, tested for harvesting of microalgae from different habitats, improved the sedimentation rate of the accompanying microalga and increased the recovery of biomass. The advantages of this method are that no addition of chemical flocculants is required and that similar cultivation conditions can be used for the flocculating microalgae as for the microalgae of interest that accumulate lipids. This method is as easy and effective as chemical flocculation which is applied at industrial scale, however in contrast it is sustainable and cost-effective as no costs are involved for pre-treatment of the biomass for oil extraction and for pre-treatment of the medium before it can be re-used.

2.1. Introduction

Oil-accumulating microalgae are a promising feedstock for biodiesel production (Benemann et al., 1977; Lee et al., 2009). Commercial microalgal biodiesel production is not economically feasible yet, mainly due to the high energy inputs required for water pumping, mixing and for harvesting the microalgal biomass combined with large investment costs (Schenk et al., 2008).

Harvesting in commercial microalgae production plants is generally done by centrifugation. Different studies showed a contribution of the costs for harvesting to more than 30% of the total cost in case of algal production in open ponds (Zittelli et al., 2006). These high costs can only be justified in case of microalgal production for high value products. For low-value bulk products both the investment as well as the operational costs should drastically decrease to make commercial production feasible (Wijffels and Barbosa, 2010).

To minimize the energy consumption of harvesting microalgae, an integrated approach is needed (Benemann, 1997). Evaluation of several harvesting methods showed that flocculation combined with flotation or sedimentation and subsequent further dewatering by centrifugation or filtration is the most promising cost and energy efficient alternative (Schenk et al., 2008). During flocculation the dispersed microalgal cells aggregate and form larger particles with higher sedimentation rate.

Flocculation can be induced in different ways. Induced chemical flocculation using Zn^{2+}, Al^{3+}, Fe^{3+} or other chemical flocculants has been studied extensively (McGarry, 1970; Lee at al., 1998; Papazi et al., 2010) and some of them are applied at industrial scale, especially in wastewater treatment plants (De la Nouë et al., 1992). Although this is an easy and effective method, this is not an appropriate method for cheap and sustainable harvesting of

microalgae in large-scale microalgae production plants because excess cationic flocculant needs to be removed from the medium before it can be re-used and this leads to extra operational costs (Schenk et al., 2008). Flocculation can also be induced by changing the culture conditions by applying extreme pH, nutrient depletion, temperature changes and changes of the level of dissolved O_2. For pre-harvesting of microalgae at large-scale these flocculation methods are not preferred. Most of the latter methods can not be applied for controlled flocculation and they may induce undesired changes in cell composition (Benemann and Oswald, 1996). All of them again require treatment of the medium to be re-used (Schenk et al., 2008). The third method that has been proposed for induced flocculation of microalgae is biologically induced flocculation with bacteria as has been applied successfully in wastewater treatment (Lee et al., 2009). Bio-flocculation of microalgae with bacteria, however, demands additional substrate as well as an extra energy source for bacterial growth and this will evoke undesirable bacterial contamination of the algal production plant. Recently, the naturally flocculating diatom *Skeletonema* was used to form flocs of *Nannochloropsis* (Schenk et al., 2008). As diatoms have a silica-based cell wall, they require different medium composition than most of microalgal strains used for biodiesel production which leads to additional cultivation costs.

In this paper, bio-flocculation of a non-flocculating microalga with another autoflocculating microalga has been evaluated as a promising alternative effective method for harvesting of microalgae. The presented bio-flocculation method enables the harvesting of microalgae without addition of chemical flocculants and allows for re-use of the cultivation medium without any additional treatment. Another advantage of this method in comparison with other applied flocculating microorganisms (bacteria, diatoms) is that it does not require different cultivation conditions and therefore avoids additional costs and prevents undesired contaminations. Furthermore the lipid content of the strains used as the flocculating and non-flocculating microalgae in this study is on average more than 25% of the dry weight biomass (Table 2.1.). The presence of the flocculating microalgae in the final biomass concentrate does thus not interfere with further downstream processing of the lipids into

biodiesel. Unfortunately, the overall lower lipid productivity of these flocculating microalgae makes them as such less attractive for biodiesel production than the faster growing non-flocculating microalgae (Griffiths and Harrison, 2008).

The bio-flocculation method will be compared with the chemically induced flocculation, in terms of recovery efficiency and time needed for sedimentation.

Table 2.1. Maximum and minimum reported lipid contents for the three flocculating microalgal strains used in this study and for the two non-flocculating microalgae.

Strain	Habitat	Lipid content (% DW)*
Flocculating microalga		
A. falcatus	freshwater	28 - 37
S. obliquus	freshwater	21 - 42
T. suecica	marine	18 - 26
Non-flocculating microalga		
C. vulgaris	freshwater	25 - 42
N. oleoabundans	marine	36 - 42

* The data are adapted from Griffiths and Harrison (2008)

2.2. Materials and Methods

2.2.1. Microalgal strains

Chlorella vulgaris (211-11b) and *Scenedesmus obliquus* (276-3a) were obtained from University of Göttingen, DE (SAG), *Neochloris oleoabundans* (1185) from University of Texas, Austin, US (UTEX), *Tetraselmis suecica* (66/38) from SAMS, UK (CCAP) and *Ankistrodesmus falcatus* (211) from the Center of Phycology, Třeboň, CZ (CCALA).

2.2.2. Culture conditions

The marine medium contained NaCl (27.00 $g^.L^{-1}$), $MgSO_4^.7H_2O$ (6.60 $g^.L^{-1}$), $MgCl_2^.6H_2O$ (5.60 $g^.L^{-1}$), $CaCl_2^.2H_2O$ (1.50 $g^.L^{-1}$), KNO_3 (1.45 $g^.L^{-1}$), $NaHCO_3$ (0.04 $g^.L^{-1}$), TRIS (hydroxymethyl) aminomethane (3.94 $g^.L^{-1}$), EDTA-Na_2 (95 $µg^.L^{-1}$), $ZnSO_4^.7H_2O$ (11 $µg^.L^{-1}$), $CoCl_2^.6H_2O$ (5 $µg^.L^{-1}$), $MnCl_2^.4H_2O$ (90 $µg^.L^{-1}$), $Na_2MoO_4^.2H_2O$ (30 $µg^.L^{-1}$) and $CuSO_4 5H_2O$ (5 $µg^.L^{-1}$) dissolved in demineralized water. For the freshwater medium KNO_3 (3 $g^.L^{-1}$), $NaH_2PO_4^.2H_2O$ (0.26 $g^.L^{-1}$), KH_2PO_4 (0.74 $g^.L^{-1}$), HEPES (2.38 $g^.L^{-1}$), H_3BO_3 (61.80 $µg^.L^{-1}$), EDTA-Fe(III)-Na, (0.11 $g^.L^{-1}$), EDTA-Na_2 (37 $mg^.L^{-1}$), $ZnSO_4^.7H_2O$ (3.20 $mg^.L^{-1}$), $MnCl_2^.4H_2O$ (13 $mg^.L^{-1}$) and $CuSO_4^.5H_2O$ (1.83 $mg^.L^{-1}$) were added to demineralized water. The pH of the solution was set at 6.8 using 4M HCl. 100 mL of this medium was dispersed into 300 mL-Erlenmeyer flasks, sealed with cotton and an aluminum cap and autoclaved for 20 minutes at 121 °C. After cooling the marine medium, K_2HPO_4 (100 $mg^.L^{-1}$), KH_2PO_4 (2 $mg^.L^{-1}$), EDTA-Fe(III)-Na (1.36 $mg^.L^{-1}$),vitamin B12 (1 $µg^.L^{-1}$), d-Biotin (1 $µg^.L^{-1}$) and Thiamine-HCl (200 $µg^.L^{-1}$) were added using a 0.2 µm non-pyrogenic sterile filter (Sartorius Stedim Biotech, FR). For the freshwater medium $MgSO_4^.7H_2O$ (0.4 $g^.L^{-1}$), $CaCl_2^.2H_2O$ (13 $mg^.L^{-1}$), vitamin B12 (1 $µg^.L^{-1}$), d-Biotin (1 $µg^.L^{-1}$) and Thiamine-HCl (200 $µg^.L^{-1}$) were added after cooling. The microalgae were grown in a light and climate controlled shaking incubator (SANYO, JP) at 100 RPM and 25 °C with a 2% CO_2 enriched airflow (3 $L^.min^{-1}$), illuminated using fluorescent light (50 $µmol^.m^{-2}s^{-1}$) with a 16 h/8 h light/dark cycle.

2.2.3. Turbidity measurements

Cell concentration was measured as the optical density at 750 nm (OD_{750}) with an Ultraspec 2000 spectrophotometer (Pharmacia Biotech Ltd. UK) equipped with a temperature controlled carousel cell holder with 6 positions. Demineralised water served as reference. The microalgal samples were diluted in a 10x10x45 mm polystyrene cuvette (Sarstedt, DE) using filter-sterilized tap water for the freshwater microalgae and with 0.46 $mol^.L^{-1}$ NaCl

solution (in demineralized water) for the marine strains (similar ionic strength as the medium applied for the marine strains) to achieve an OD_{750} value below 1.

2.2.4. Sedimentation kinetics

Samples of the microalgal suspensions were taken and diluted in a cuvette. After mixing the suspension was left to settle at 27 °C in the dark in a spectrophotometer. The temperature and pH of all samples were measured in the beginning and at the end of the sedimentation period and they were constant respectively at 27 °C and pH 7. During the settling period, turbidity of the sample was measured at 750 nm at the same height in the cuvette to determine the recovery. The microalgal recovery (microalgal removal percentage) was calculated with:

$$\text{recovery } (\%) = \frac{OD_{750}(t_0) - OD_{750}(t)}{OD_{750}(t_0)} \cdot 100 \qquad (2.1.)$$

where $OD_{750}(t_0)$ is the turbidity of sample taken at time zero and $OD_{750}(t)$ is the turbidity of the sample taken at time t (Fig. 2.1.). This was done for the suspension of non-flocculating microalga with and without addition of the bio-flocculating microalga. The sedimentation kinetics were measured in cuvettes instead of in conventional jar tests (Vandamme et al., 2010) or recently used cylindrical glass tubes (Papazi et al., 2010). Similar to the conventional tests, the recovery percentage is measured in the top part of the cuvette, where individual cells and formed flocs independently sink.
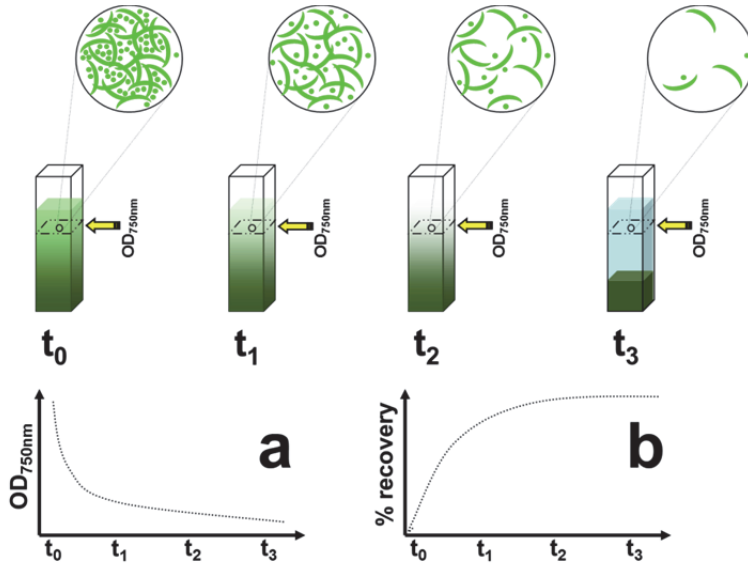
Fig. 2.1. Recovery of microalgal biomass and sedimentation kinetics calculation. **a** Schematic overview of the microalgal sedimentation test in time. **b** Recovery (%) of the microalgae from the suspension in time.

To compare different strains on their ability to be applied as flocculating microalgae, the recovery efficiency is defined as the recovery of the non-flocculating microalga in the presence of the flocculating microalga divided by the recovery of the non-flocculating microalga without flocculating microalga present. The recovery efficiency (adapted from Papazi et al., 2010 and Buelna et al., 1990) was calculated with:

$$\text{recovery efficiency (\%)} = \left[1 - \frac{\dfrac{OD_{a750}(t)}{OD_{a750}(t_0)}}{\dfrac{OD_{b750}(t)}{OD_{b750}(t_0)}} \right] \cdot 100 \qquad (2.2.)$$

where $OD_{a750}(t_0)$ and $OD_{a750}(t)$ are the turbidities of samples of non-flocculating microalga with flocculating microalga taken at time zero and at time t, respectively. $OD_{b750}(t_0)$ is the turbidity of sample of non-flocculating microalga taken at time zero and $OD_{b750}(t)$ is the turbidity of the same sample taken at time t.

Three different flocculating microalgae were tested on their ability to improve the recovery efficiency and the rate of harvesting of the non-flocculating microalga. The freshwater microalgae *A. falcatus* and *S. obliquus* were used for harvesting of *C. vulgaris*. The marine microalga *T. suecica* was used to harvest the non-flocculating marine microalga *N. oleoabundans*. For each of the three tested combinations of flocculating and the non-flocculating microalga, four sedimentation experiments were performed: (1) the flocculating microalga, (2) the non-flocculating microalga, (3) the non-flocculating microalga with low concentration of added flocculating microalga and (4) the non-flocculating microalga with high concentration of added flocculating microalga (Table 2.2.). Each of these experiments was performed in duplicate. At the end of sedimentation experiment, samples were taken from the bottom of cuvettes in order to make microscopic pictures of the formed microalgal flocs.

Table 2.2. Optical densities ($OD_{750}(t_0)$) of flocculating and non-flocculating microalgae added into the cuvettes for four combinations of three experiments.

Combination of flocculating and non-flocculating microalgae	$OD_{750}(t_0)$			
	1*	2*	3*	4*
A. falcatus	0.7	0.7	0.4	0.0
C. vulgaris	0.0	0.9	0.9	0.9
S. obliquus	0.8	0.8	0.4	0.0
C. vulgaris	0.0	0.3	0.3	0.3
T. suecica	1.1	1.1	0.5	0.0
N. oleoabundans	0.0	0.2	0.2	0.2

1* the flocculating microalga

2* the non-flocculating microalga

3* the non-flocculating microalga with low concentration of added flocculating microalga

4* the non-flocculating microalga with high concentration of added flocculating microalga

2.2.5. Morphological analysis

At the end of sedimentation experiment, samples were taken from the bottom of cuvettes in order to make microscopic pictures of the formed flocs of the microalgal cells, using a C-3030 zoom 5 mega pixel Olympus camera (Olympus, JP) connected to a CK40 Olympus microscope (Olympus, JP) with a SK20-SLP phase contrast filter and a T6 objective (40x magnification) and a NCWHK 18L ocular lens (10x magnification).

2.3. Results

Three different autoflocculating microalgae were identified; the freshwater microalgae *Ankistrodesmus falcatus* (*A. falcatus*), and *Scenedesmus obliquus* (*S. obliquus*) and the marine microalga *Tetraselmis suecica* (*T. suecica*) (Fig. 2.2. d, e and f, respectively). The freshwater microalgae were used to flocculate the strain *Chlorella vulgaris* (*C. vulgaris*) as non-flocculating microalga (Fig. 2.2. a and b), while the marine microalgal strain was used to flocculate *Neochloris oleoabundans* (*N. oleoabundans*, Fig. 2.2. c). *C. vulgaris* and *N. oleobundans* show both relatively high growth rates in comparison with the autoflocculating microalgae, but all five microalgae are reported to show relatively high lipid content (Table 2.1.).
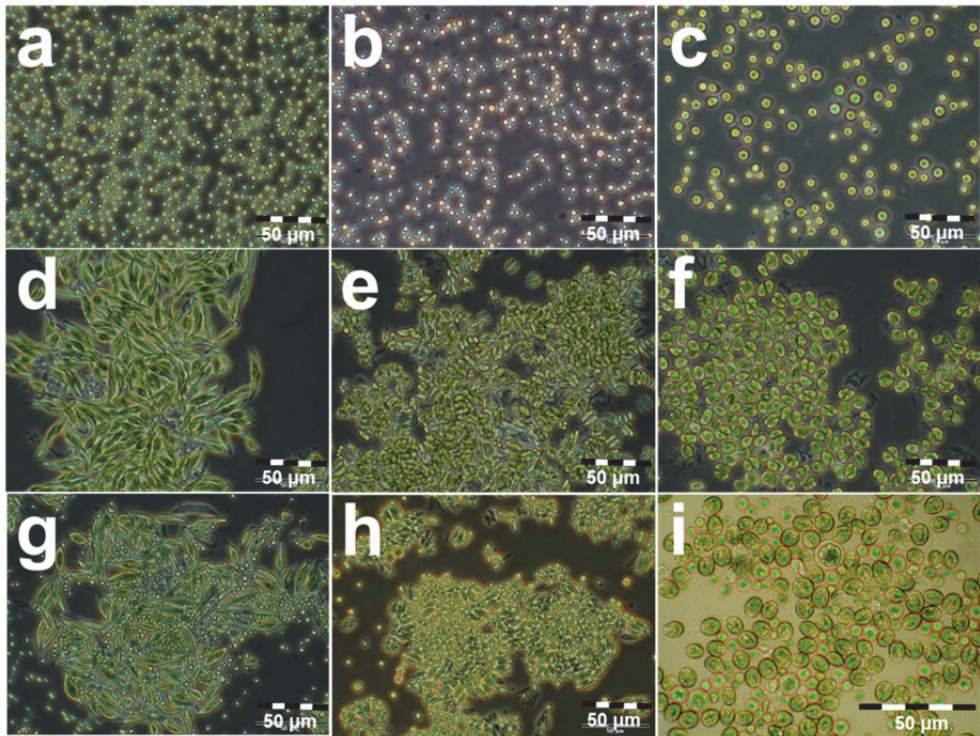
Fig. 2.2. Microscopic picture of individual and flocculated microalgal cells. The non-flocculating microalgae (**a** and **b** *C. vulgaris* and **c** *N. oleoabundans*), the flocculating microalgae (**d** *A. falcatus*, **e** *S. obliquus* and **f** *T. suecica*) and the flocs of the non-flocculating microalgae after the addition of accompanying flocculating microalga (**g** *C. vulgaris* with *A. falcatus*, **h** *C. vulgaris* with *S. obliquus* and **i** *N. oleoabundans* with *T. suecica*). For more details on the morphological analysis and sample preparation see materials and methods.

2.3.1. Microscopic analysis

Figure 2.2. shows pictures of the non-flocculating microalgae *N. oleoabundans* (Fig 2.2. c) and *C. vulgaris* (Fig 2.2. a and b). The microalgae are present as single cells and no floc

formation is observed. In the sediments of all three flocculating microalgae large flocs can be observed (Fig. 2.2. d, e and f). If the three flocculating microalgae are added to the non-flocculating microalgae (Fig. 2.2. g, h and i) the microscopic pictures show that the majority of the non-flocculating microalgae are trapped in flocs formed by the flocculating microalgae and almost no loose cells of non-flocculating microalgae remain in the suspension after the addition of flocculating microalgae. The comparison of the pictures in Fig. 2.2. a, b and c respectively with Fig. 2.2. g, h and i confirms that the addition of flocculating microalgae from different habitats (marine and freshwater) improves the recovery of various non-flocculating microalgae.

2.3.2. Sedimentation kinetics of various flocculating and non-flocculating microalgae

The sedimentation of the microalgal suspensions was monitored for eight hours and the percentage of microalgal recovery was determined over time. The sedimentation rate of the microalgae in suspension was calculated by linear regression of data in the curves of the recovery percentage in time and use of the slope of the linear regression.

The initial sedimentation rates of the flocculating microalgae measured over the first two hours of the test, are higher than those of the non-flocculating microalgae (Table 2.3.). Mixing of the flocculating microalga with the non-flocculating microalga increases the initial sedimentation rate considerably. The large flocs formed by flocculating microalgae seem to trap the non-flocculating microalgae (Fig 2.2. g, h and i) and sediment faster than individual non-flocculating microalgal cells. Furthermore, an increase in the ratio of the bio-flocculating microalga and the non-flocculating microalga leads to higher sedimentation rates. These observations again confirm that the total recovery as well as the rate of sedimentation of various non-flocculating microalgae improve upon addition of different flocculating microalgae.

Table 2.3. Initial sedimentation rate.

Combination of flocculating and non-flocculating microalgae	Initial sedimentation rate (% recovery·h^{-1})			
	1*	2*	3*	4*
A. falcatus and *C. vulgaris*	41.1	13.6	10.4	6.8
S. obliquus and *C. vulgaris*	37.0	20.4	18.7	10.2
T. suecica and *N. oleoabundans*	46.2	39.9	37.5	18.7

Details for the calculation of these initial sedimentation rates can be found in the main text and materials and methods.

1* the flocculating microalga

2* the non-flocculating microalga

3* the non-flocculating microalga with low concentration of added flocculating microalga

4* the non-flocculating microalga with high concentration of added flocculating microalga

2.3.3. Efficiency of various flocculating microalgae

The improvement in the recovery of the non-flocculating microalgae was evaluated for the three flocculating microalgae by calculation of the recovery efficiency percentage. For calculation of the recovery efficiency percentage (Equation 2.2. in Materials and Methods), the average turbidity of duplicate measurements was used. The standard deviation in measured values for sedimentation rate and recovery percentage for all tested samples was less than 3.5%. The recovery efficiency percentage of three flocculating microalgae added at low and high concentration is presented in Fig. 2.3.
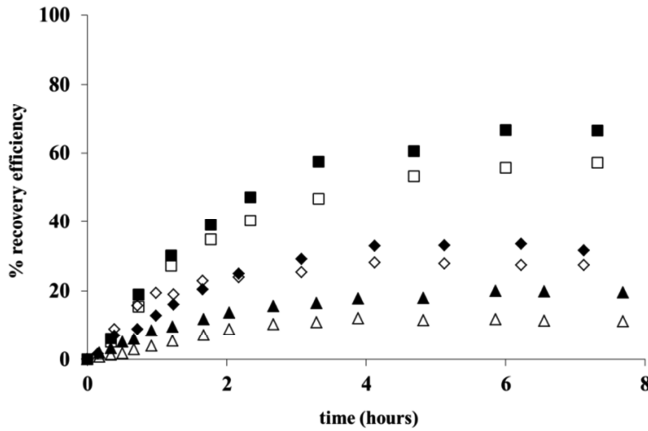
Fig. 2.3. Recovery efficiency percentage of different flocculating microalgae at two different concentrations. ■: high concentration *T. suecica*, □: low concentration *T. suecica*, ♦: high concentration *S. obliquus*, ◊: low concentration *S. obliquus*, ▲: high concentration *A. falcatus*, ∆: low concentration *A. falcatus*. The standard deviation in measured values for sedimentation rate and recovery percentage for all the tested samples was less than 3.5%. Details for calculation of these recovery efficiency percentages can be found in materials and methods.

All three flocculating microalgae show higher recovery efficiency when they are applied at higher concentration, although doubling of concentration of the flocculating microalga does not necessarily result in two times higher recovery efficiency of the non-flocculating microalga.

2.4. Discussion

The results show that addition of autoflocculating microalgae induce faster sedimentation of non-flocculating microalgae and also increase the harvesting efficiency. Similar positive

effects on sedimentation rates and harvesting efficiencies are observed with bio-flocculation of non-flocculating microorganisms with bacteria (Lee et al., 2009). In literature, adsorption of cationic polymers (Lewin, 1956; Tilton et al., 1972) excreted by the microorganisms is proposed to explain the mechanism involved in bio-flocculation. Polymer induced flocculation can be divided in two sub-mechanisms called bridging and patching (Fig. 2.4.). The positively charged polymers bind partly or completely to microalgal cells. If the polymers bind partly, the unoccupied part of the polymers can bind to other microalgal cells, thereby bridging them and resulting a network of polymers and microalgal cells. If the polymers bind the microalgal cells completely because they are too short to bind others as well, they adsorb (patch) to the surface and can create positive charges locally. These charges attract other microalgal cells and also result in flocculation of the cells.
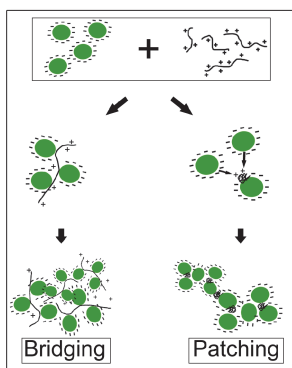

Fig. 2.4. Schematic view of possible mechanisms involved in polymer induced flocculation; bridging and patching.

Our microscopic observations suggest that bridging is the mechanism behind the floc formation by *A. falcatus* (Fig. 2.2. d) as a large network of microalgal cells is formed. Patching can be the mechanism behind the flocculation of *T. suecica* (Fig. 2.2. f) and *S.*

obliquus (Fig. 2.2. e) as they seem to be connected more locally. Based on these observations, our hypothesis is that the extracellular polysaccharides excreted by *A. falcatus* itself bind partly to the surface of *A. falcatus* and positively charged tails of these polysaccharides can bind to the other *A. falcatus* cells. During the formation of the flocs *C. vulgaris* cells are trapped in this large network of *A. falcatus* cells (Fig. 2.5.).
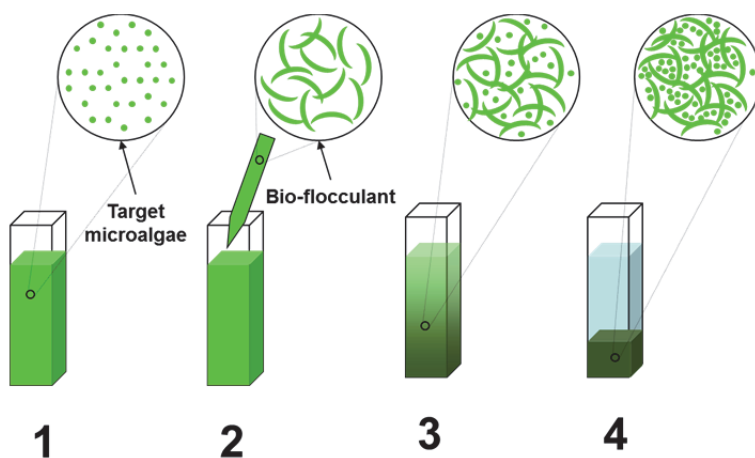

Fig. 2.5. Schematic view of the proposed mechanism involved in bio-flocculation using *A. falcatus* as the flocculating microalga.

The recovery efficiencies and time needed for sedimentation observed here using bio-flocculation are in the same range as the recovery efficiencies found by Papazi et al. (2010) applying chemically induced flocculation for separation of the microalgal biomass. They showed a recovery efficiency of 60% for harvesting *Chlorella minutissima* by addition of 1 $g \cdot L^{-1}$ of $Al_2(SO_4)_3$ and $ZnCl_2$ in respectively one and a half and six hours. The density of microalgal culture (OD_{750}) used by Papazi et al. (2010) was 2.4 which is comparable with the density of cultures used in this study. Other studies using chemical flocculation reported other concentrations and recovery efficiencies, e.g. Lee et al. (1998) and McGarry (1970)

used up to respectively 300 and 125 mg$\cdot$L^{-1} of Al^{3+}. However the microalgal density of the samples used in these studies are not mentioned and the recovery efficiencies are calculated on a different way and therefore can not be compared with results of the current study.

2.5. Future perspectives of sustainable microalgal harvesting

We presented in this study that all three chosen flocculating microalgae improved the recovery efficiency of the accompanying non-flocculating microalga. It can be concluded that the bio-flocculation by using one flocculating microalga for harvesting of another oil-accumulating microalga can be applied as the controlled and reliable pre-concentration step in harvesting of the oil-accumulating microalgae, although large scale experiments are still needed to prove the feasibility and cost efficiency of this method at industrial scale. Further it was shown in this study that different flocculating microalgal strains are available for application of bio-flocculation in marine as well as in freshwater environment. Using bio-flocculation followed by sedimentation as the pre-concentration step decreases the recovery time of the non-flocculating microalga. The amount of flocculating microalgae used is still relatively high in comparison with the non-flocculating microalgae (Table 2.2.). A decrease in the amount of flocculating microalga by half did not show any major effects on the recovery efficiency and time needed for sedimentation of the non-flocculating microlaga. This indicates that this method is indeed promising and further optimization of the ratio of the bio-flocculating microalga and the non-flocculating microalga should be done to reveal if large scale utilisation of this technique will indeed result in considerable decrease of harvesting costs and energy.

To summarize, this harvesting method is as easy and effective as chemically induced flocculation which is applied at industrial scale, however in contrast to induced chemical flocculation, this method is sustainable. Although the cultivation of flocculating microalgae requires some extra nutrients and energy, the flocculating microalgae do not require an

additional set of nutrients for cultivation in comparison with the microalgae of interest. In the economical analysis of large scale application of this promising harvesting method the additional costs for a separate cultivation system for cultivation of the flocculating microalga should also be taken into account. In addition, the flocculating microalgae accumulates lipids and no extra operational and investment costs are involved for treatment of the sediment (microalgal biomass) for further down stream processing towards biodiesel or for pre-treatment of the medium before it can be re-used.

Acknowledgements

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3 Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation

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Abstract

The effect of ratio between autoflocculating and target microalgae in bio-flocculation was studied with emphasis on the recovery, sedimentation rate and energy demand for harvesting the target microalgae. When the autoflocculating microalgae *Ettlia texensis*, *Ankistrodesmus falcatus* and *Scenedesmus obliquus* were added to *Chlorella vulgaris* at a ratio of 0.25, the recovery of *C. vulgaris* increased from 25% to respectively 40, 36 and 31%. The sedimentation rate increased as well. Addition of *Tetraselmis suecica* to *Neochloris oleoabundans* at a ratio of 0.25 increased the recovery from 40% to 50%. Application of bio-flocculation at a ratio of 0.25, followed by centrifugation reduces the energy demand for harvesting of the target microalgae from 13.8 MJ·kgDW^{-1} if only centrifugation is used to 0.24, 0.24, 0.17 and 0.13 MJ·kgDW^{-1} respectively using *T. suecica*, *E. texensis*, *A. falcatus* and *S. obliquus* and 3 hours sedimentation before centrifugation.

3.1. Introduction

Microalgae are regarded as one of the most promising feedstocks for biofuel production from lipids, but a significant reduction in the energy costs for production of the microalgal biomass should be realized to make microalgal biofuel production economically feasible (Wijffels and Barbosa, 2010). Current harvesting costs of microalgae are high (Uduman et al., 2010; Christenson and Sims, 2011; Schlesinger et al., 2012). The energy needed for harvesting of microalgae from a 0.3 gDW$^.$L^{-1} microalgal suspension via centrifugation was calculated to be 13.8 MJ.kgDW^{-1}, while the combustion energy of the oleaginous microalgae was estimated to be 26.2 MJ.kgDW^{-1} (Norsker et al., 2011). With such high energy demand for harvesting, it is obvious that cost-efficient methods for harvesting microalgae should be developed (Molina Grima et al. 2003; Shelef et al. 1984) with emphasis on pre-concentration of microalgal biomass prior to centrifugation (Vandamme et al., 2012).

Uduman et al. (2010) postulated that in an ideal pre-concentration step, the dilute microalgal suspension (typically 0.2-10 gDW$^.$L^{-1}) should be concentrated to a microalgal slurry of 20-70 gDW$^.$L^{-1} and for this step it is not preferred to add chemical flocculants to the medium as it ends up in the final microalgal product and might complicate the reuse of the medium without further treatment.

Bio-flocculation of a non-flocculating fast-growing oleaginous microalga with a second autoflocculating microalga has been presented as a promising pre-concentration step in harvesting of microalgae (Salim et al., 2011). The recovery efficiencies and the time needed for sedimentation observed in this study proved to be in the same range as for chemically induced flocculation (Papazi et al., 2010; Lee at al., 1998). The major advantage of bio-flocculation is that the energy required for harvesting will be reduced, while no extra chemicals are needed. Autoflocculating bacteria (Lee et al., 2009) and diatoms (Schenk et

al., 2008) can also be used as bio-flocculant. However the production of these bio-flocculants requires different cultivation conditions which acquire additional medium costs and increases the risk of microbial contamination of the medium. In the case of bio-flocculation with autoflocculating microalgae that grow at similar conditions as the oleaginous microalgae, the risk of contamination is reduced. Furthermore, the presence of the flocculating microalgae in the final biomass concentrate does not necessarily interfere with further downstream processing of the microalgal lipids into biofuels and co-products. As the proposed autoflocculating microalgae may contain up to 25% (w/w) lipids (Salim et al., 2011), they may even contribute to the overall biofuel production.

Salim et al. (2011) showed that flocculating microalgae (*Ankistrodesmus falcatus*, *Scenedesmus obliquus* and *Tetraselmis suecica*) improved the recovery efficiency of the accompanying non-flocculating microalga and induced faster sedimentation. The ratio in concentration of the flocculating and the non-flocculating microalgae (R_{fnf}) used in this study, however, was quite high and a 50% decrease in R_{fnf} caused only minor changes in the recovery efficiency and in the time needed for sedimentation of the non-flocculating microalgae. As the overall growth rate of these flocculating microalgae is lower than the non-flocculating microalgae, it is important to find the minimal concentration ratio needed for effective bio-flocculation and to calculate the overall energy costs to find out if bio-flocculation using autoflocculating microalgae at this ratio can indeed be used to make the overall process of production and harvesting of the microalgae energy-efficient.

In this paper, the effect of the ratio R_{fnf} on the recovery and sedimentation kinetics of the non-flocculating microalga is studied and the resulting reduction in energy demand of the centrifuge for harvesting the microalgae is calculated. In the energy analysis, the energy for production of the flocculating microalgae is taken into account. As the basis for this study, the energy for microalgal production in open ponds is calculated assuming that the microalgae are harvested at a biomass concentration of 0.3 gDW$\cdot$L^{-1} (Norsker et al., 2011).

3.2. Materials and Methods

3.2.1. Microalgal strains and cultivation conditions

Chlorella vulgaris (SAG211-11b), *Scenedesmus obliquus* (SAG276-3a), *Ankistrodesmus falcatus* (SAG202-9) and *Ettlia texensis* (SAG79.80) were obtained from the University of Göttingen, DE, *Neochloris oleoabundans* (UTEX1185) from the University of Texas, Austin, US, *Tetraselmis suecica* (CCAP66/4) from SAMS, UK. The composition of the marine and freshwater medium and the medium preparation protocol were described by Salim et al. (2011).

The microalgae were grown in 300 mL Erlenmeyer flasks (filled upto 100 ml with the medium), sealed with cotton and an aluminum cap, which were placed in a light- and climate-controlled shaking incubator (SANYO, JP) at 100 RPM and 25 °C with a 2% CO_2 enriched airflow (3 $L^.min^{-1}$), illuminated using fluorescent light (50 $\mu mol^.m^{-2}s^{-1}$) with a 16 h/8 h light/dark cycle. Microalgal cells were harvested at OD_{750} of 1 for all sedimentation experiments.

3.2.2. Sedimentation kinetics and recovery

Four different flocculating microalgae were tested for their ability to improve the recovery and sedimentation rate of the non-flocculating microalga. The freshwater microalgae *A. falcatus*, *S. obliquus* and *E. texensis* were used for harvesting of *C. vulgaris*. The marine microalga *T. suecica* was used to harvest the non-flocculating marine microalga *N. oleoabundans*. For each of the four tested combinations of flocculating and non-flocculating microalgae, different concentration ratios of the flocculating to non-flocculating microalgae (R_{fnf} between 0.1 and 1) were tested. Each of these experiments was performed in triplicate (n=3). The turbidity of the sample was measured at 750 nm

(OD$_{750}$) with a DU730 spectrophotometer (Beckman Coulter Inc. US) equipped with a carousel cell holder with 6 positions. 4 ml of the microalgal suspension were diluted in a cuvette (filled upto 40 mm) to an OD$_{750}$ of 0.5 for all sedimentation experiments. To determine the recovery during the settling period, the turbidity of the samples was measured at the same height in the cuvette (light beam falling between 5 and 12 mm from the bottom of the cuvette) and demineralised water was used as reference. The recovery was calculated with:

$$\text{recovery} = \frac{OD_{750}(t_0) - OD_{750}(t)}{OD_{750}(t_0)} \cdot 100\,\%$$ (3.1.)

where OD$_{750}$(t$_0$) is the turbidity of sample taken at time zero and OD$_{750}$(t) is the turbidity of the sample taken at time *t*. To compare different strains for their ability to be applied as flocculating microalgae and the effect of concentration ratio of flocculating to non-flocculating microalgae, the recovery efficiency was calculated with:

$$\text{recovery efficiency} = \left[1 - \frac{\dfrac{OD_{a750}(t)}{OD_{a750}(t_0)}}{\dfrac{OD_{b750}(t)}{OD_{b750}(t_0)}} \right] \cdot 100 \qquad \%$$ (3.2.)

where OD$_{a750}$(t$_0$) and OD$_{a750}$(t) represent the turbidity of samples of non-flocculating microalga with flocculating microalga taken at time zero and at time *t,* respectively. OD$_{b750}$(t$_0$) is the turbidity of sample of non-flocculating microalgae taken at time zero and OD$_{b750}$(t) is the turbidity of the same sample taken at time *t*.

For each R_{fnf}, sedimentation of the microalgal suspension was followed for three hours and the recovery was monitored over time. The initial sedimentation rate at various ratios was calculated from the slope of the recovery curves during the first 20 minutes using linear regression.

3.2.3. Concentration factor and recovery based on monitoring settled cells

The application of bio-flocculation will lead to reduction of the total volume of the microalgal suspension that needs to be further concentrated by centrifugation. The concentration factor achieved was determined in triplicate (n=3) for four flocculating microalgae at concentration ratios (R_{fnf} 0.11, 0.25, 0.67 and 1.00) in 15 ml tubes filled with 10 ml of the microalgal suspension at OD_{750} of 1. The volume of the microalgal suspension was determined by weighing the sample on a balance with 10 µg accuracy (Sartorius, US). After three hours the supernatant was removed from the settled cells and both the supernatant and the remaining settled cells were weighed. The settled cells were resuspended and the optical density of the settled cells was measured to determine the biomass concentration in the settled cells and the recovery using:

$$\text{recovery}_{sed} = \frac{OD_{750}(\text{sed})}{OD_{750}(t_0)} \cdot 100\,\% \qquad (3.3.)$$

where $OD_{750}(t_0)$ is the turbidity of sample taken at time zero and $OD_{750}(\text{sed})$ is the turbidity of the settled cells.

3.2.5 Microscopic analysis

At the end of sedimentation experiment, samples were taken from the bottom of tubes to make microscopic pictures of the formed microalgal flocs, as described by Salim et al. (2011).

3.3. Results and discussion

3.3.1. Effect of R_{fnf} on the sedimentation kinetics

Non-flocculating *C. vulgaris* and *N. oleoabundans* cells have an initial sedimentation rate of 7 and 15% recovery·h^{-1}, respectively (Fig. 3.1.). Addition of all four types of flocculating microalgae increased the initial sedimentation rate of the non-flocculating microalgae. *E. texensis* cells induced a 6.3-fold increase in initial sedimentation rate of the non-flocculating microalga *C. vulgaris* when applied at R_{fnf} of 0.37. *A. falcatus* and *S. obliquus* cells were less effective as bio-flocculant and show a maximum increase in initial sedimentation rate of *C. vulgaris* cells of 2.5 and 1.4 times, respectively, at the same R_{fnf}. Using the marine microalga *T. suecica* as bio-flocculant for flocculating the marine microalga *N. oleoabundans* at R_{fnf} of 0.39 resulted in a 1.8-fold increased initial sedimentation rate of this target microalga.
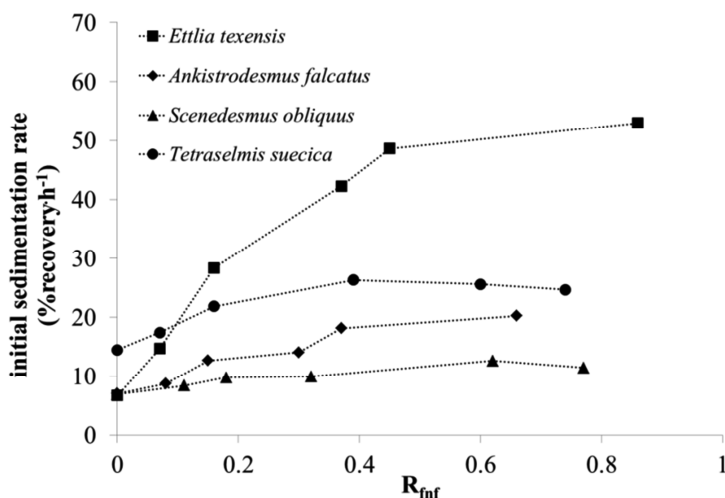
Fig. 3.1. Initial sedimentation rate of different ratios (Rfnf) of the flocculating (*E. texensis*, *A. falcatus* and *S. obliquus*) to non-flocculating microalgae (*C. vulgaris*) and of the flocculating (*T. suecica*) to non-flocculating microalgae (*N. oleoabundans*). The standard deviation (n=3) of measured values for initial sedimentation rates is too low to be visible in the figure. Details for calculation of the initial sedimentation rates can be found in materials and methods.

Further increase of the R_{fnf} hardly induces higher initial sedimentation rates of the non-flocculating microalgae for three of the strains tested in this study (Fig. 3.1.). Addition of *A. falcatus* and *S. obliquus* cells to the *C. vulgaris* culture increased the initial sedimentation rate of *C. vulgaris* to a maximum of 20 and 12% recovery·h^{-1}, respectively. The initial sedimentation rate of *N. oleoabundans* increased to a maximum of 26% recovery·h^{-1} of *T. suecica* cells. Unlike the other flocculating microalgae, *E. texensis* did not show a clear threshold value for R_{fnf}; the initial sedimentation rate of *C. vulgaris* increased to 53% recovery·h^{-1} upon increasing the R_{fnf} value to 0.86. Fig. 3.1. shows, however, that the increase of the initial sedimentation rate of *C. vulgaris* with *E. texensis* using R_{fnf} values higher than 0.45 was significantly less than the increase observed at lower R_{fnf}.

3.3.2. Effect of R_{fnf} on the recovery of the microalgae

To quantify the increase in recovery due to addition of the flocculating microalgae to the target microalgae, the recovery efficiency at various R_{fnf} was calculated for the different flocculating microalgae, using equation 3.2. Fig. 3.2. shows that addition of more flocculating microalgae led to higher recovery efficiency of the non-flocculating microalgae for all four tested flocculating microalgae. In general, the recovery efficiency of the non-flocculating microalgae increased in time, and major improvement in recovery efficiency was observed at low R_{fnf} ratio. From this figure it is obvious that *E. texensis* is a very effective bio-flocculating microalga. More than 30% improvement of the recovery efficiency of *C. vulgaris* is observed after 40 minutes at R_{fnf} of 0.37 and the recovery efficiency reached is even higher than for other bio-flocculants tested at the same R_{fnf} after 180 minutes.
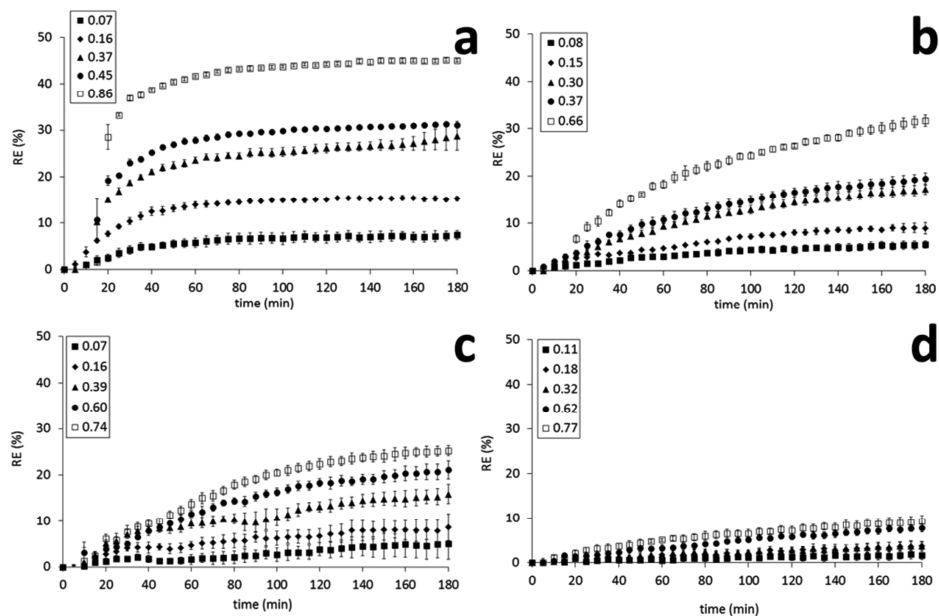
Fig. 3.2. Recovery efficiency RE(%) of the non-flocculating microalgae (*C. vulgaris* (a, b and d) and *N. oleoabundans* (c)) after addition of the flocculating microalgae (*E. texensis* (a), *A. falcatus* (b), *S. obliquus* (d) and *T. suecica* (c)) at different R_{fnf}. Different symbols represent various ratios of the flocculating to non-flocculating microalgae (R_{fnf}) as presented in the legend. The standard deviation (n=3) of measured values for recovery efficiency is presented in the figure. Details for calculation of the recovery efficiencies can be found in materials and methods.

To compare the effectiveness of the various bio-flocculants at different R_{fnf} the recovery after three hours of sedimentation were compared (Fig. 3.3.). At that time, only 25 and 40% of *C. vulgaris* and *N. oleoabundans*, respectively, were recovered. Addition of three accompanying flocculating microalgae (*E. texensis*, *A. falcatus* and *T. suecica*) significantly increased the amount of biomass harvested. Increasing the R_{fnf} led to higher recovery of the non-flocculating microalgae for all four tested flocculating microalgae. For bio-flocculation

of *C. vulgaris*, *E. texensis* showed the highest improvement of recovery (up to 60% at R_{fnf} of 0.86), followed by *A. falcatus* and *S. obliquus*. Despite the small improvement in recovery of *C. vulgaris* measured by addition of *S. obliquus* (from 25% to 31% at R_{fnf} of 0.77), it is obvious that *S. obliquus* is not an effective bio-flocculant. This is also the case for *T. suecica* as addition of *T. suecica* at R_{fnf} upto 0.74 to *N. oleoabundans* only increased the recovery from 40% to 55%.
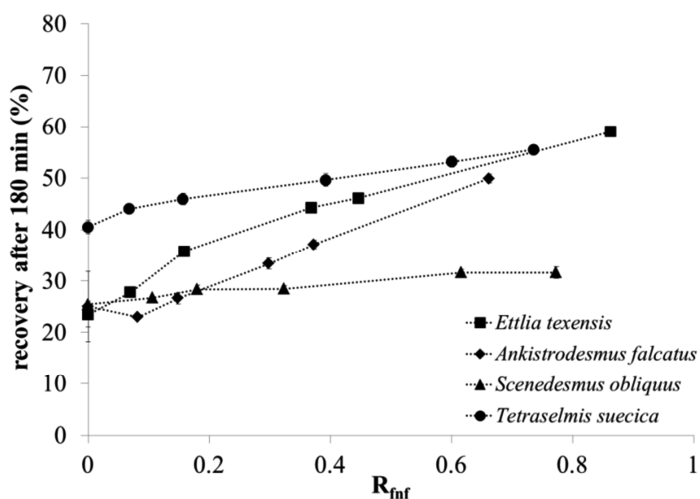
Fig. 3.3. Recovery of the biomass removed from the microalgal suspension after 180 minutes of the non-flocculating microalgae (*C. vulgaris*) at different ratios (R_{fnf}) of the flocculating microalgae (*E. texensis*, *A. falcatus* and *S. obliquus*) and of the non-flocculating microalgae (*N. oleoabundans*) at different ratios (R_{fnf}) of the flocculating microalgae (*T. suecica*). The standard deviation (n=3) of measured values for recovery is too low to be visible in the figure. Details for calculation of the recovery can be found in materials and methods.

In the previous part the recovery was based on the disappearance of biomass from the microalgal suspension during sedimentation of the microalgae, and this was monitored by measuring the decrease in optical density of the microalgal suspension. The recovery was also calculated based on the increase of microalgal biomass that reached the bottom of a sedimentation tube (Fig. 3.4.).
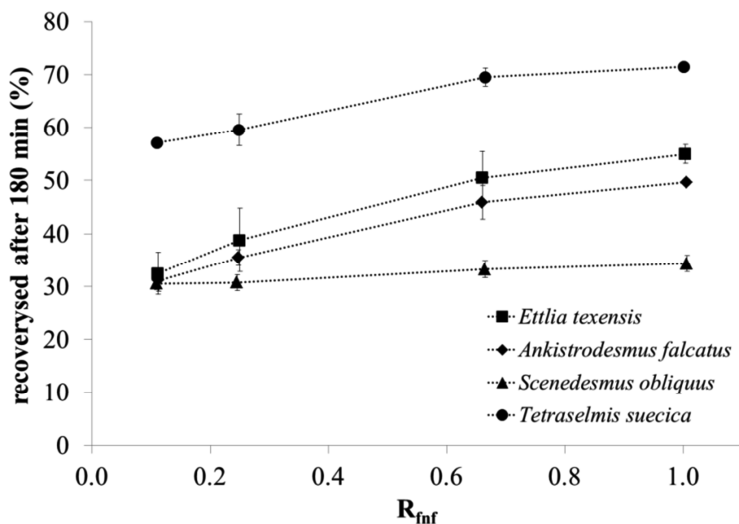

Fig. 3.4. Recovery$_{sed}$ of the biomass after 3 hours of sedimentation, collected in the pellet (paste) of the non-flocculating microalgae (*C. vulgaris*) at different ratios (R_{fnf}) of the flocculating microalgae (*E. texensis*, *A. falcatus* and *S. obliquus*) and of the non-flocculating microalgae (*N. oleoabundans*) at different ratios (R_{fnf}) of the flocculating microalgae (*T. suecica*). The standard deviation (n=3) of measured values for recovery$_{sed}$ is presented in the figure. Details for calculation of these values can be found in materials and methods.

The recovery of the non-flocculating microalgae determined after three hours of sedimentation using flocculating microalgae increased with higher R_{fnf}, and at R_{fnf} of 1 a

recovery of 72, 55, 50 and 34% was found using *T. suecica*, *E. texensis*, *A. falcatus* and *S. obliquus*, respectively, as bio-flocculant. The recoveries from Fig. 3.3. and the recoveries based on biomass collected in the pellet (Fig. 3.4.) are similar for the different ratio R_{fnf} of the flocculating microalgae *E. texensis*, *A. falcatus* and *S. obliquus*. The recoveries found for *N. oleoabundans* after addition of *T. suecica*, however, deviated; while a recovery of 53% was found at R_{fnf} of 0.6 based on the measurement of the change in cell concentration from the medium (Fig. 3.3.), a recovery of 70% was found, based on the increase of the cell concentration in the pellet (Fig. 3.4.). This difference can be explained by the porosity (density) of the microalgal flocs formed. The density of the formed flocs defines the concentration factor after removal of water. The dry weight of the collected pellet (paste) of the non-flocculating microalgae (*C. vulgaris* and *N. oleoabundans*) at different R_{fnf} of the flocculating microalgae (*E. texensis*, *A. falcatus*, *S. obliquus* and *T. suecica*) was dependent on the type of microalgae, the floc density, and the concentration factor and varied between 30 and 120 $gDW^.L^{-1}$.

The recoveries observed in the current study are in the same range as the recoveries found by Papazi et al. (2010) applying chemically induced flocculation for separation of the microalgal biomass. They showed a recovery of 60% for harvesting *Chlorella minutissima* upon addition of 1 $g^.L^{-1}$ of $Al_2(SO_4)_3$ and $ZnCl_2$ in one and a half and six hours, respectively. However, the initial density of microalgal culture (OD_{750}) used by Papazi et al. (2010) was 2.4, which is higher than used in the current study. Other studies using lower concentration of chemical flocculant, e.g., Lee et al. (1998) and McGarry (1970), used up to 300 and 125 $mg^.L^{-1}$ of Al^{3+}, respectively. The microalgal density of the samples used in their study is not mentioned and the recoveries are calculated in a different way and therefore cannot be compared with results of the current study. Vandamme et al. (2012) and Wu et al. (2012) showed that harvesting *C. vulgaris* at comparable initial concentration (respectively 0.68 and 0.5 $gDW^.L^{-1}$) by induced flocculation due to the increase of pH is only possible at pH higher than 8.6. Vandamme et al. (2012) presented a recovery of 75% at pH 11 after 30 minutes, while Wu et al. (2012) showed 90% recovery at pH 10.6 after 10

minutes. Another study of Vandamme et al. (2011) showed that under optimal conditions a recovery of 90% can be achieved at pH 8 with initial microalgal densities of 0.3-0.6 gDW$^.$L^{-1} using electro-coagulation-flocculation.

Although the recoveries achieved by bio-flocculation are lower than some of the pre-concentration methods presented above, bio-flocculation is one of the few energy-efficient and sustainable pre-concentration methods under mild pH and conductivity. Excess of cationic flocculants needs to be removed from the medium before it can be re-used and this leads to extra operational costs and energy (Schenk et al., 2008). Applying extreme pH or high current intensities for pre-concentration of microalgae at large-scale are not preferred as they cannot be applied for controlled flocculation. They may also induce undesired changes in cell composition (Benemann and Oswald, 1996) and they require treatment of the medium to be re-used (Schenk et al., 2008). Bio-flocculation presented in the current study as the pre-concentration method of choice involves no extra operational costs and energy for treatment of the microalgal biomass for further downstream processing of the microalgal biomass or for pre-treatment of the medium before it can be re-used. Furthermore the microalgal suspension with a density of approximately 0.5 gDW$^.$L^{-1} was concentrated to a slurry with densities between 30 and 120 gDW$^.$L^{-1}. This shows that the current pre-concentration method can concentrate the dilute microalgal suspension to 100 times as was suggested by Uduman et al. (2010) for an ideal two-step concentration method for harvesting and dewatering of microalgae for biofuel production. The remaining biomass in supernatant theoretically can be transferred back to the cultivation system without pre-treatment as the medium is not contaminated. The latter should, however, still be tested.

3.3.3. Microscopic analysis

The microscopic pictures of the non-flocculating microalgae *N. oleoabundans* and *C. vulgaris* did not show any floc formation at all and only single cells were observed.

Different floc sizes were observed in the sediments of four flocculating microalgae. The microscopic observations reveal that addition of four flocculating microalgae to the non-flocculating microalgae caused entrapment of the non-flocculating microalgae in the flocs formed by the flocculating microalgae. At higher R_{fnf} it can be observed that fewer loose cells of non-flocculating microalgae remained in the suspension. The comparison of pictures at similar R_{fnf} ratio shows that flocs of *E. texensis* trapped most of the single cells of *C. vulgaris*, followed by flocs of *A. falcatus* and *T. suecica* (trapping *N. oleoabundans* cells). Flocs of *S. obliquus* hardly trapped any of the individual cells of *C. vulgaris*.

This entrapment in larger flocs explains the faster sedimentation rates and the improved recovery of the non-flocculating microalgal cells after addition of the flocculating microalgae. As explained before, the faster sedimentation rate could also be caused by a change in density of the microalgal flocs formed.

3.3.4. Reduction of energy for centrifugation

The concentration factor achieved after sedimentation of the microalgal cells at four R_{fnf} was calculated to estimate the reduction in energy for harvesting the microalga of interest if bio-flocculation would be applied as pre-concentration step. The capacity of a centrifuge Q [$m^3 \cdot s^{-1}$] depends on the characteristics of the centrifuge, which are often described by the sigma factor $\sum$ [m^2] and by the sedimentation rate of the particles in suspension u_g [$m \cdot s^{-1}$].

$$Q = \Sigma * u_g \qquad (3.4.)$$

The sigma factor derived for a disk-stack bowl centrifuge (Ambler, 1952) is:

$$\Sigma = \frac{2 \cdot \pi \cdot \omega^2 \cdot (N-1)}{3 \cdot g \cdot \tan(\theta)} \cdot \left(r_o^3 - r_i^3 \right) \tag{3.5.}$$

where ω is rotation speed [rad·s^{-1}], N is number of disks in the stack, r_o and r_i are respectively outer and inner radius of the discs [m] and θ is the half-cone angle of the discs. The sigma factor enables us to estimate the decrease in required rotation speed of the centrifuge if less volume of cell suspension needs to be treated in the same centrifuge. As a worst case scenario, the flocs of the cells are assumed to settle at the same rate as the individual cells. At those conditions of similar u_g, the ratio between the capacity of the centrifuge needed to harvest the microalgal suspension after bio-flocculation Q_1 and to harvest the microalgae directly Q_2, is:

$$\frac{Q_1}{Q_2} = \frac{\omega_1^2}{\omega_2^2} \tag{3.6.}$$

Equation 6 shows that the ratio in capacity is related to the ratio of the square of the rotation speeds. The rotational kinetic energy of centrifuge E [J] is linearly related to the rotation speed:

$$E = \frac{1}{2} \cdot I \cdot \omega^2 \tag{3.7.}$$

where I is moment of inertia [kg·m^{-2}]. Using equation 3.7., the reduction of energy of a disk-stack bowl centrifuge can be estimated, since the energy demand is linearly related to the required rotation speed:

$$\frac{Q_1}{Q_2} = \frac{E_1}{E_2} \tag{3.8.}$$

The biomass harvesting energy was calculated for a raceway pond cultivation system at 100 ha plant scale (Norsker et al., 2011). The reduction in energy was calculated for harvesting the target microalga if bio-flocculation is applied as the pre-concentration step in combination with centrifugation as the post-concentration step (data from Norsker et al., 2011). This number was then compared with the base case scenario for centrifugation when the bio-flocculation was not applied as the pre-concentration step (13.8 MJ·kgDW^{-1}).
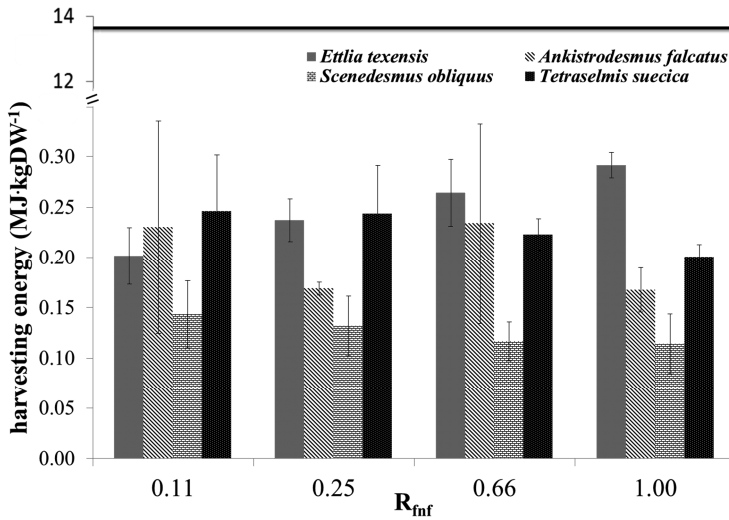
Fig. 3.5. Energy for microalgal biomass harvesting in raceway ponds at 100 ha plant scale using a disk-stack bowl centrifuge (post-concentration step). Energy of using bio-flocculation (pre-concentration step) after 180 minutes of the non-flocculating microalgae (*C. vulgaris*) at different ratios (R_{fnf}) of the flocculating microalgae (*E. texensis*, *A. falcatus* and *S. obliquus*) and the non-flocculating microalgae (*N. oleoabundans*) at different ratios (R_{fnf}) of the flocculating microalgae (*T. suecica*) compared with energy needed for the base case scenario for centrifugation (13.8 MJ·kgDW^{-1}) when the bio-flocculation was not applied as the pre-concentration step (data from Norsker et al., 2011). The standard deviation (n=3) of measured values for harvesting energy is presented in the figure. Details for calculation of these energies can be found in materials and methods.

The centrifugation energy for harvesting of the non-flocculating microalgae (*C. vulgaris* and *N. oleoabundans*) decreased significantly when bio-flocculation combined with sedimentation was applied (Fig. 3.5.). The energy of a disk-stack bowl centrifuge after applying a R_{fnf} of approximately 0.25 of *T. suecica*, *E. texensis*, *A. falcatus* and *S. obliquus* and 3 hours sedimentation decreased from 13.8 (base case scenario without 3 hours of sedimentation) to 0.24, 0.24, 0.17 and 0.13 MJ$^.$kgDW^{-1}, respectively. Increasing the R_{fnf} hardly changed the energy for centrifugation since the amount of supernatant that was removed after three hours was more or less the same, despite the fact that the amount of biomass harvested in the pellet after three hours was different for different flocculating microalgae at different R_{fnf} (Fig. 3.4.). After 3 hours of sedimentation, only 25 and 40% of respectively *C. vulgaris* and *N. oleoabundans* were recovered without addition of any bio-flocculant (Fig. 3.3.). If the target microalgae were allowed to settle without addition of any bio-flocculant, the amount of supernatant that was removed after 3 hours of settling was more or less the same as if a bio-flocculant was added at a R_{fnf} of 0.25 to 1. Therefore, the harvesting energy of *C. vulgaris* and *N. oleoabundans* after 3 hours of settling without addition of any bio-flocculant will be comparable with the results presented in Fig. 3.5. One should realize, however, that the higher recovery found in case of bio-flocculant application results in higher concentration of the settled microalgal suspension. This is highly desirable to facilitate further down-stream processing of the microalgal biomass.

The significance of energy reduction of centrifuge for harvesting microalgae of interest if bio-flocculation is applied as the pre-concentration step was also compared with the total energy needed for production of the flocculating microalgae as extra biomass was added (including power consumption for paddle wheel, medium preparation and centrifuge; data from Norsker *et al*., 2011). The extra production energy at R_{fnf} of the flocculating microalgae 0.11, 0.25, 0.66 and 1.00 was around 0.8, 1.8, 4.8 and 7.3 MJ$^.$kgDW^{-1}, respectively. The reduction of total biomass production and harvesting energy is approximately the same when different microalgal strains are used as bio-flocculant. This significant energy reduction is due to the effect of 3 hours settling of a dilute microalgal

suspension to a concentrated slurry. However, the R_{fnf} has considerable effect on the initial sedimentation rate and recovery of the target microalgae (Fig. 3.1. and Fig. 3.3.). Higher sedimentation rate and recovery of the target microalgae achieved in this study by application of bio-flocculation can reduce the harvesting cost due to reduction of the residence time for sedimentation and the harvesting energy during the post-concentration step (e.g., centrifugation). Therefore both effects of R_{fnf} on the reduction of total biomass production and harvesting energy and the initial sedimentation rate and harvesting efficiency of the target microalgae should be taken into account.

The calculation of harvesting energy of microalgae was based on the worst case scenario. The reduction of centrifuge energy due to bio-flocculation was underestimated as it was assumed that the size of particles does not change when bio-flocculation as the pre-concentration step is used. The sedimentation kinetics measurement (Fig. 3.1.) showed that the sedimentation rate of non-flocculating microalgae significantly increased due to bio-flocculation. The change in energy of a disk-stack bowl centrifuge not only depends on the ratio in volumetric capacity of the centrifuge, but also on the sedimentation rate of the particles involved:

$$\frac{E_1}{E_2} = \frac{Q_1}{Q_2} \cdot \frac{u_{g2}}{u_{g1}} \tag{3.9.}$$

The sedimentation rate of the flocs formed after flocculation u_{g2} is higher than that of single non-flocculating cells u_{g1}, while the centrifugal capacity Q_2 needed after bio-flocculation is lower than the capacity needed when no bio-flocculation is applied Q_1. Hence the reduction of centrifuge energy due to bio-flocculation will be higher than estimated in the current study.

The energy need for harvesting microalgae using the approach presented in the current study should be compared with values from other studies on pre-concentration of microalgae. Most of studies described different methods of pre-concentration of microalgal biomass, e.g., by applying chemically-induced flocculation (Papazi et al., 2010; Lee et al., 1998; McGarry, 1970), by induced flocculation due to the increasing of pH (Vandamme et al., 2012) or by addition of microbial flocculants (Zheng et al., 2012). None of these studies presented an energy analysis that takes the energy involved in the production and application of the chemical and bacterial flocculants and bases into account. Therefore it is difficult to compare the energy analysis of bio-flocculation presented in the current study with these studies. Electro-coagulation-flocculation is one of the energy-efficient harvesting methods recently presented by Vandamme et al. (2011). Power consumption of electro-coagulation-flocculation varied between 5 and 123 $MJ^.kgDW^{-1}$ for *C. vulgaris* and between 1 and 6 $MJ^.kgDW^{-1}$ for *Phaeodactylum tricornutum* using different current densities and electro-coagulation-flocculation times without taking the extra energy and costs involved in the anode hydrolysis into account. The extra energy needed for production of the flocculating microalgae when bio-flocculation is applied as the pre-concentration step using different R_{fnf} of the flocculating microalgae varied between 0.8-7.3 $MJ^.kgDW^{-1}$.

Furthermore, one should realize that it was assumed that only the target microalgae contained lipids for biofuel production. In reality, the proposed autoflocculating microalgae also contain lipids for around 25% of biomass dry weight (Salim et al. 2011) which can contribute to the conversion of the lipids into biofuel. This means that the contribution of bio-flocculation as the pre-concentration step in harvesting microalgae will improve the energy balance of the whole process to a greater extent than estimated in the current study.

3.4. Conclusions

This study confirms that the recovery as well as the sedimentation rate of various non-flocculating microalgae improves upon addition of different flocculating microalgae. Increasing the ratio of flocculating to non-flocculating microalgae (R_{fnf}) resulted in higher sedimentation rate and recovery of the non-flocculating microalgae. Furthermore, this study shows that bio-flocculation is an energy-efficient pre-concentration step in harvesting microalgae. Application of bio-flocculation combined with centrifugation can reduce the harvesting energy of the microalgal biomass of interest (with a combustion energy of 26.2 $MJ.kgDW^{-1}$) from 13.8 to at least 0.24 $MJ^.kgDW^{-1}$ at a ratio R_{fnf} of 0.25.

Acknowledgements

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4 Effect of growth phase on harvesting characteristics, autoflocculation and lipid content of *Ettlia texensis* for microalgal biodiesel production

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M. H. Vermuë

R. H. Wijffels

Abstract

The effect of growth phase on the recovery of the autoflocculating microalgae *Ettlia texensis* was studied. In the stationary phase, 90% recovery was achieved after three hours settling. Scanning electron microscopic pictures revealed that extracellular polymeric substances (EPS) on the cell surface were involved in autoflocculation. During the stationary phase an increase of the protein fraction in the EPS was observed while the total fatty acids content increased. The autoflocculating properties of *E. texensis* combined with favourite fatty acid content and composition make this microalgae an excellent candidate for biodiesel production if harvested at the end of the stationary phase.

4.1. Introduction

Large-scale cultivation of microalgae for biodiesel production is developing fast but still faces several challenges to become economically feasible (Norsker et al., 2011; Rodolfi et al., 2009). One of the main challenges is harvesting of the microalgae. Chini Zittelli et al. (2006) and Molina-Grima et al. (2003) estimated that the costs associated to microalgae harvesting represent more than 20-30% of the total costs of microalgal production. A recent LCA study has underlined that centrifugation without prior bulk harvesting contributed 92.7% to the entire energy input (Sander and Murthy, 2010). For harvesting, centrifugation is traditionally used, but this is very energy intensive as the biomass concentrations in microalgal production systems are low (e.g. 0.2-1 gDW·L^{-1} in open ponds) while the cells are small in size (2-50 µm diameter) and have a density similar to water. In addition, microalgae are generally negatively charged which gives rise to formation of stable cell suspensions (Li et al., 2008; Sukenik and Shelef, 1984). Currently, research efforts are devoted to optimize microalgal harvesting methods by addition of chemical flocculants to pre-concentrate the cells prior to centrifugation (Bilad et al., 2012; Vandamme et al., 2011), but this requires energy and costs for the addition of the flocculant and to remove traces of remaining flocculant from the harvested biomass and prior to recycling the medium. To avoid these additional costs, bio-flocculation has been proposed as pre-concentration step using autoflocculating microalgae (Salim et al., 2011).

Autoflocculating microalgae show apparent spontaneous floc formation (Sukenik and Shelef, 1984) without addition of any flocculant. This approach to pre-concentrate microalgae has been tested on laboratory scale (Rodolfi et al., 2009; Lavoie and De la Nouë, 1987) as well as for microalgae in outdoor ponds (Sukenik and Shelef, 1984). Salim et al. (2012) demonstrated that *Ettlia texensis*, *Ankistrodesmus falcatus*, *Scenedesmus obliquus* and *Tetraselmis suecica* have autoflocculating properties. *E. texensis* showed to be the most promising candidate regarding settling characteristics.

E. texensis is also a potential candidate for biodiesel production, as it is able to reach lipid contents of 35 % (w·w^{-1}) (Isleten-Hosoglu et al., 2012) to 50 % (w·w^{-1}) under nutrient

starvation conditions (Yoo et al., 2013). The lipid accumulation in microalgae is generally triggered by nitrogen limitation (Dunstan et al., 1996; Kalacheva et al., 2001) which often occurs during the stationary growth phase of the microalgae. Although the effect of nitrogen limitations on lipid productivity during microalgal cultivation has been observed in several microalgae (Dunstan et al., 1996; Kalacheva et al., 2001; Hu et al., 2008), it is not clear yet how the autoflocculation characteristics of the promising oleaginous microalgae will change when nitrogen depletion in the medium occurs.

During different growth phases, microalgal cells undergo changes in their morphology, cell wall structure and composition and cell content and this may affect flocculation and changes are also observed in extracellular polymeric substances (EPS) attached to the cell wall and surface charge during subsequent growth phases which also may induce aggregation of microalgal cells (Danquah et al., 2009). Knowledge on how these properties change with the autoflocculation behaviour during different growth phases is important to determine the optimum harvesting time. At the moment, it is not clear if autoflocculation of *E. texensis* is caused by neutralization of charged groups at the microalgal outer cell layer or by formation, excretion and binding of EPS or by a combination of both and how the cell surface properties change with the growth phase. The amount and profile of sugars of the cell wall or of groups attached to the cell wall of microalgae is growth stage dependent. This also counts for compounds such as starch and lipids that are used for internal energy storage (Takeda, 1991). Microalgae are able to convert fatty acids to polysaccharides and vice versa via the enzymes isocitrate lyase and malate synthase (Gonzalez-Fernandez and Ballesteros, 2012). This means that the accumulation of lipids can occur at the expense of polymeric substances and vice versa. Autoflocculating oleaginous microalgae like *E. texensis* may behave differently. If they show autoflocculation due to formation of extra cellular polymeric substances, they would need extra carbon and energy to form these EPS, while they use carbon and energy for the lipid accumulation. Therefore, it is important to study the effect of the growth phase on the flocculation properties (due to the EPS attached to the cell wall) and the lipid content of these oleaginous microalgae. There are several

examples of different microalgal strains that show different flocculation characteristics dependent on the growth phase. Lavoie and De la Nouë (1987) observed a low sedimentation rate of *S. obliquus* during the exponential growth. The sedimentation rate increased during the declining growth due to formation of large particles (mainly flocs) and mainly occurred during the stationary phase. Lee et al. (1998) also observed growth phase dependent flocculation properties of *Botryococcus braunii* using flocculants, but they observed flocculation in particular during the exponential growth phase. More recently, Danquah et al. (2009) showed that the dewatering of a *Tetraselmis suecica* and *Chlorococcum sp.* suspension was better at low growth rate phases (the beginning of the stationary phase) than at high growth rate phases (the exponential and linear growth phase).

In this paper, the effect of the growth phase on recovery, sedimentation and autoflocculation behaviour of *E. texensis* is studied. In addition, the lipid content of *E. texensis* is determined during the subsequent growth phases. This will provide crucial knowledge on the optimum harvesting time considering the flocculation properties and lipid content of *E. texensis*.

4.2. Materials and methods

4.2.1. Microalgal strains and cultivation conditions

Ettlia texensis (SAG79.80) was obtained from the University of Göttingen, DE. The composition of the freshwater medium and the medium preparation protocol were described by Salim et al. (2011) and some adjustments were made. The HEPES buffer for cultivation of *E. texensis* in the photobioreactor was omitted. The freshwater medium used for *E. texensis* contained KNO_3 (0.316 and 0.632 $g^.L^{-1}$ in first and second batch respectively), $NaH_2PO_4^.2H_2O$ (0.066 and 0.132 $g^.L^{-1}$ in first and second batch respectively) and KH_2PO_4 (0.034 and 0.068 $g^.L^{-1}$ in first and second batch respectively) instead of the concentrations

mentioned by Salim et al. (2011). The cultivation was performed in a stirred photobioreactor (4 L, Applikon, NL) in batch mode, which was sterilized prior to inoculation of the microalgae. The reactor was stirred at 300 RPM, the temperature was set at 26 °C and the pH was 6.5 regulated by sparging a mixture of CO_2 in N_2 (250 mL$\cdot$min^{-1}) and illuminated using fluorescent with an average incident light intensity of 330 μmol$\cdot$m^{-2}s^{-1}. Two independent batch experiments were performed and samples were taken at different growth phases to study the effect of growth phase on recovery, sedimentation and lipid content of *E. texensis*. These two batches were inoculated with inoculum which was cultivated in shake flasks with similar medium and similar cultivation conditions. Another five independent batch experiments were performed for collection of samples at different growth phases to study the effect of growth phase on mechanism behind autoflocculation of *E. texensis*. The first batch was inoculated with inoculum which was cultivated in shake flasks with similar medium and similar cultivation conditions. At the end of the first batch, the biomass left in the photobioreactor was used as inoculum for the second batch and this was repeated for the next batches. Each measurement for each individual sample from different growth phases during each batch experiments was performed in triplicate.

4.2.2. Microalgal dry weight

Whatman glass microfiber filters (Ø 55 mm, pore size 0.7 μm) were dried at 95ºC overnight and placed in a desiccator to cool to room temperature. The empty filters were weighed. Approximately 10 mg of sample (triplicate) was filtrated. The filter was rinsed twice with demineralized water to remove adhering inorganic salts. The wet filters containing the samples were dried at 95ºC overnight, allowed to cool to room temperature in a desiccator, and weighed.

4.2.3. Microalgal optical density

The optical density of the sample (triplicate) was measured at 750 nm (OD_{750nm}) with a DU730 spectrophotometer (Beckman Coulter Inc. US). Demineralized water served as

reference. The microalgal samples were diluted using demineralized to achieve an OD_{750nm} value below 1.

4.2.4. Growth phase

In the growth curve, different growth phases are defined. As the microalgal culture becomes light limited (around OD_{750nm} of 0.6) due to the light intensity applied and the diameter of the photobioreactor, the cells go from the exponential growth phase to the linear growth phase. The cells enter the lipid accumulation phase as the nitrate becomes depleted in the medium. However the cell concentration (OD_{750nm}) still increases in this phase. The final phase is the stationary phase in which the cell concentration remains constant.

4.2.5. Sedimentation and biomass recovery

To determine the recovery during the settling period, the optical density of the samples was measured (Salim et al, 2012). The recovery was calculated with:

$$\text{recovery} = \frac{OD_{750nm}(t_0) - OD_{750nm}(t)}{OD_{750nm}(t_0)} \cdot 100\%$$

(4.1.)

where $OD_{750nm}(t_0)$ is the turbidity of sample taken at time zero and $OD_{750nm}(t)$ is the turbidity of the sample taken at time t. Sedimentation of the microalgal suspension was followed for three hours and the recovery was monitored over time. The time at which 50% recovery was achieved and the recovery of microalgal cells after three hours of settling were chosen to evaluate the effect of growth phase on the tendency of the cells to flocculate.

4.2.6. ζ-potential

The ζ-potential of *E. texensis* suspension at different growth phases was measured using the Zetasizer as it was described by Salim et al. (2013).

4.2.7. Floc and cell size analysis

The size of microalgal individual cells and flocs in the suspension was measured in a Mastersizer (Malvern, AU). The samples were diluted to OD_{750nm} around 0.1 due to the measurement threshold of the Mastersizer with Milli-Q water and were placed in the dispersion unit of Mastersizer (Hydro 2000SM, Malvern, AU) which pumps the sample homogenously at 350 RPM (preventing air bubble formation) into the measurement chamber of Mastersizer. Milli-Q water was used as reference.

The volume percentage distribution in particle size of microalgal cells after Mastersizer measurements was analysed by dividing the particle distribution into three classes according to the appearance of peaks of size distribution for *E. texensis*; small single cells (3 – 6.5 µm), big single cells and/or small flocs (6.5 – 20 µm) and big flocs (> 20 µm).

4.2.8. Morphological analysis

Microscopic pictures were taken from the microalgal cells, using a C-3030 zoom 5 mega pixel Olympus camera (Olympus, JP) connected to a CK40 Olympus microscope as described by Salim et al. (2013).

4.2.9. SEM preparation

The presence of EPS at the outer surface of microalgal cells and flocs was visualized using scanning electron microscopy (SEM) as described by Salim et al. (2013).

4.2.10. EPS extraction and identification of carbohydrates and proteins in EPS

The extraction of EPS was performed following the procedure described by Frolund et al. (1996) with some modifications as described by Salim et al. (2013). The phenol-sulphuric acid method of Dubois et al. (1956) was used as a quantitative analysis for the total carbohydrates content present in EPS with glucose as standard (Salim et al., 2013). The Lowry method was used for determination of the protein concentration with Bovine serum albumin (BSA) as standard (Lowry et al., 1951) using the BIO-RAD *DC*[TM] Protein Assay (BIO-RAD, US) according to Salim et al. (2013).

4.2.11. Fatty acids analysis

Samples (approximately 10 mg) at different growth phases were centrifuged and the pellets were flushed with N_2 and stored at -80°C. The pellets were then freeze dried, grinded with a mortar and pestle and transferred to bead beating tubes (Lysing Matrix E, MP Biomedicals, US). Upon addition of a chloroform/methanol mixture (2:2.5), containing 47 µg$^.$mL^{-1} of the internal standard tripentadecanoin, complete cell disruption was achieved by bead beating for 30 min. This solution was then transferred to heat resistant glass tubes. The bead beating tubes was rinsed with a chloroform/methanol mixture to achieve complete recovery of the fatty acids in the sample. After vortexing and sonication, a 50 mM Tris-buffer containing 1 M NaCl was added to the suspension. The samples were vortexed and sonicated once more and subsequently centrifuged to separate the polar and apolar phase. The chloroform phase

was transferred to a fresh tube and the polar phase and debris were re-extracted twice with chloroform. The fatty acids present in the lipids of the dried chloroform extracts were converted into fatty acid methyl esters (FAMEs) using a solution of 5% (v/v) H_2SO_4 in methanol. After vigorous mixing the samples, they were incubated for three hours at 70°C in a block heater. The samples were then cooled to room temperature and extracted with hexane. After mixing the samples again, they were centrifuged. The hexane phase was collected and washed with water. Gas chromatography (GC) analysis was done using a HP 6890 (Hewlett Packard Inc., US) with FID detector. The next steps of GC were adapted from a protocol optimized by Lamers et al. (2010).

4.2.12. Nitrate analysis

The exponential growth phase is characterized as the growth phase in which the microalgae do not experience any light or nutrient limitation and an exponential increase in biomass concentration is monitored. During the linear growth phase, the microalgae experience light limitation resulting in a linear increase of the biomass, while sufficient amounts of nitrate is still available. During the late linear phase, nitrate starts to be depleted until the microalgae reach the stationary growth phase. To determine when the stationary phase was reached, the nitrate concentration in the medium was measured. The samples were filtered using a 0.20 µm, sterilized single use filter (Minisart, Sartorius AG, DE) to remove the biomass. Samples with a concentration higher than 20 $mg^.L^{-1}$ were diluted with MilliQ water. The nitrate concentration in the samples were measured by use of an Ion Chromatograph (Metrohm Compact IC 761, Metrohm AG, CH).

4.3. Results and discussion

4.3.1. Growth

Fig. 4.1. shows the growth curves of both batch experiments of *Ettlia texensis* and residual nitrate concentration in the medium. *E. texensis* did not experience a lag phase as it was pre-cultivated in similar medium and hardly needed to adapt to the new cultivation conditions. Furthermore, *E. texensis* showed a short exponential phase, followed by a linear phase. Due to the nitrate depletion in *E. texensis*, the stationary phase was observed. Although the nitrate was depleted around OD_{750nm} of 0.55 ($\pm$ 0.00) and 0.97 ($\pm$ 0.01) in the first and second batch respectively, *E. texensis* cells entered the stationary phase around OD_{750nm} of 1.26 ($\pm$ 0.02) and 2.49 ($\pm$ 0.01) in the first and second batch respectively. The final cell concentration (OD_{750nm}) reached by *E. texensis* cells were 1.55 ($\pm$ 0.02) and 2.93 ($\pm$ 0.01) in first and second batch respectively. The final cell concentration reached in the first batch was approximately twice lower than the second batch because the initial nitrate concentration in the medium was also two times lower in the first batch.

The second batch showed a longer linear growth phase than the first one; 10 days (between OD_{750nm} of 0.77 and 2.49) and 3 days (between OD_{750nm} of 0.55 and 1.26) respectively. This was expected as both batch cultures were light limited around OD_{750nm} of 0.6 due to the light intensity applied and the diameter of the photobioreactor, but nitrate in the first batch was depleted earlier than in the second batch.
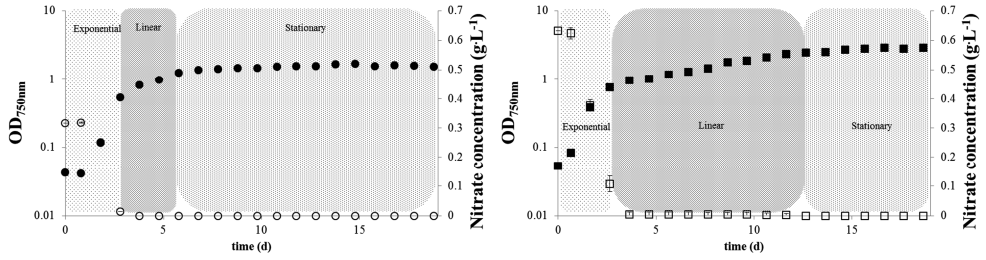
Fig. 4.1. Growth curve of *E. texensis* in the first (●) and second (■) batch and residual nitrate concentration in the medium in the first (○) and second (□) batch. The standard deviation of triplicate measurements for the cell concentration and residual nitrate concentration were too low to be visible in this figure.

4.3.2. Biomass recovery

In Fig. 4.2., the biomass recovery in two batch experiments is presented. The difference between growth phases on biomass recovery is represented by OD_{750nm} (cell concentration). The time needed for 50% biomass recovery of *E. texensis* varied over the exponential growth phase (Fig 4.2.). In the beginning, middle and end of the exponential phase in the first batch 50% recovery was observed after 22 (± 1), 9 (± 1) and 43 (± 2) minutes respectively and in the middle and end of the exponential phase in the second batch 10 (± 0) and 47 (± 1) minutes respectively. The 50% biomass recovery time did not change during linear and stationary growth phases in the first batch and stayed around 40 minutes. In the second batch the 50% biomass recovery increased in the linear phase from 40 to around 60 minutes and stayed around 60 minutes, also in the stationary growth phase.
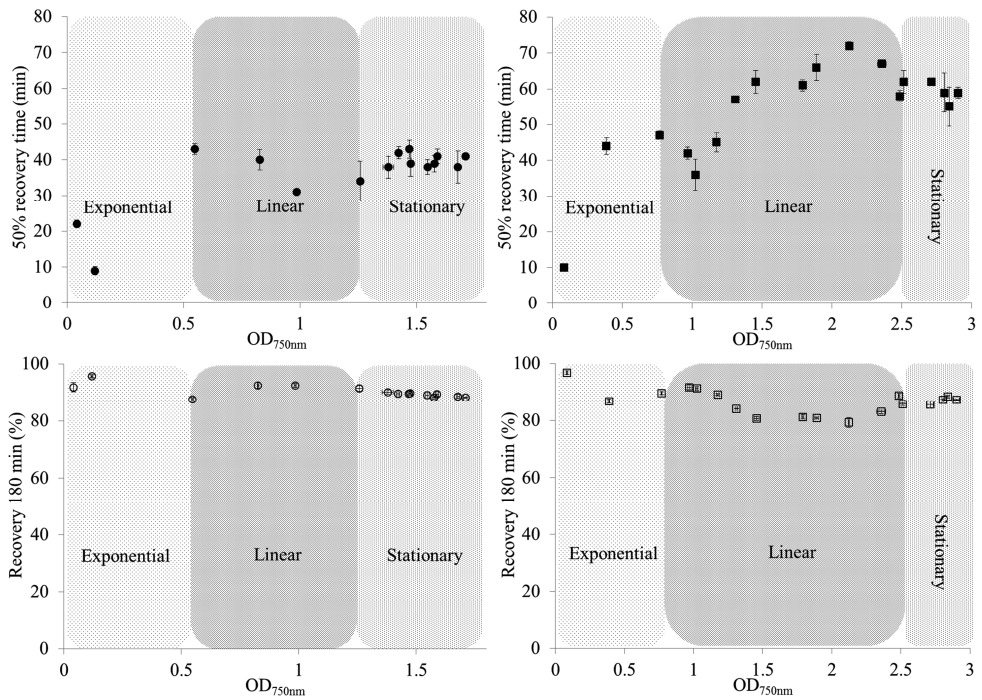
Fig. 4.2. 50% biomass recovery time in the first (●) and second (■) batch and biomass recovery after three hours in the first (○) and second (□) batch of *E. texensis* at different growth phases represented by OD_{750nm}

The recovery of *E. texensis* after three hours was constant around 90% during the growth in the first and second batch experiment (Fig. 4.2.).

4.3.3. Particle properties

4.3.3.1. ζ-potential

The ζ-potential of *E. texensis* cells slightly increased with the growth phase from -18.9 (± 1.2) mV at the beginning of the exponential phase to -13.3 (± 0.5) mV in the linear growth phase and stayed constant around -12.1 (± 0.9) mV in the linear and stationary phase. An increase in ζ-potential of microalgal cells leads to reduction of the repulsive electrostatic forces between the individual microalgal cells. In case of *E. texensis* cells, attractive van der Waals forces seem to dominate and cause the microalgae to form flocs. The small variation in ζ-potential at different growth phases was also measured for other microalgal species such as diatom *Nitzschia linearis* which had a ζ-potential of -30, -35 and -28 mV in the exponential, linear and stationary phase respectively (Konno, 1993). As the variation in ζ-potential of *E. texensis* at different growth phases is small, it is most likely that EPS dominate the autoflocculation behaviour of *E. texensis*. To prove this, the presence of EPS on the surface of *E. texensis* cells was investigated and the amount of EPS attached to the cell surface of *E. texensis* was quantified to find out if variation in amounts of EPS present at the surface coincided with variation in autoflocculation properties of *E. texensis* during the subsequent growth phases.

4.3.3.2. SEM analysis of the cell surface

To investigate the effect of the growth phase on the autoflocculation behaviour of *E. texensis*, SEM pictures of samples from a *E. texensis* culture were taken; one at the end of the exponential phase at OD_{750nm} of 0.52 (± 0.10) (Fig. 4.3. A) and one at the end of the stationary phase at OD_{750nm} of 1.86 (± 0.12) (Fig. 4.3. D) and the surface structure was compared.
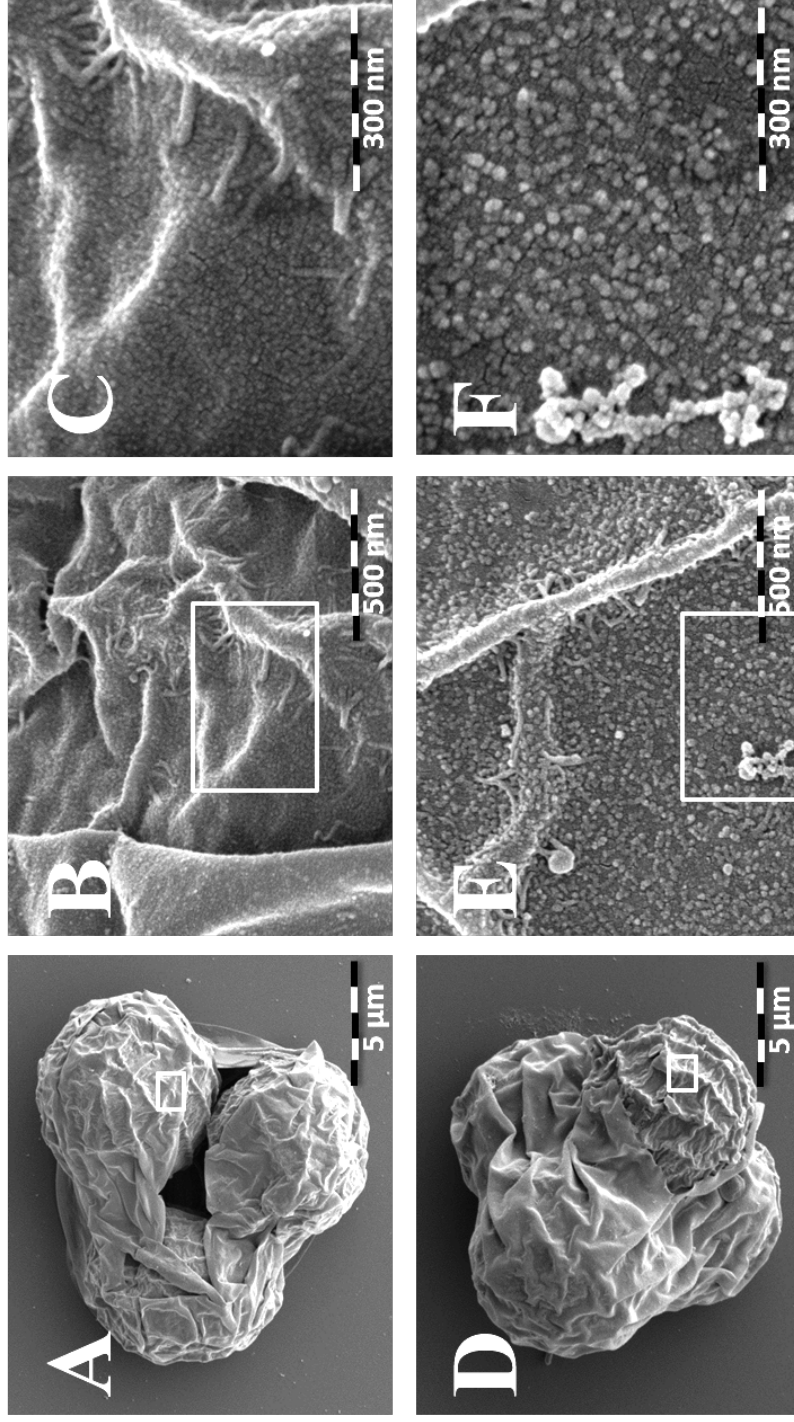
Fig. 4.3. SEM picture of *E. texensis* floc from culture at OD_{750nm} 0.52 ($\pm$ 0.10) at the end of the exponential phase (A) and the surface of *E. texensis* at this phase (B and C) and *E. texensis* floc from culture at OD_{750nm} 1.86 ($\pm$ 0.12) at the end of the stationary phase (D) and the surface of *E. texensis* at this phase (E and F)

The flocs formed in the exponential (Fig. 4.3. A) and in the stationary phase (Fig. 4.3. D) hardly show any difference in the SEM pictures. However, when zooming in on the surface of the individual *E. texensis* cells, the SEM pictures revealed that the surface was covered with EPS at the end of stationary phase (Fig. 4.3. E and F) and that less EPS were found on the surface of *E. texensis* at the end of the exponential phase (Fig. 4.3. B and C).

In literature, the growth phase dependency of the flocculation behaviour of microalgal cells has been described; *e.g.* Konno (1993) showed that the settling velocity of diatom *N. linearis* varies greatly depending on the growth phase. The settling velocity of *N. linearis* in the stationary phase was faster than in the exponential phase and the settling velocity got faster at the declining phase. SEM pictures showed that the surface of *N. linearis* in the declining phase has a very smooth surface in comparison with the very rough surface in the linear phase suggesting that EPS at the cell surface were involved. The amounts of EPS attached to the diatom surface, however, were not measured in the study by Konno (1993).

Table 4.1. Proteins and carbohydrates concentration (n = 3) in the EPS fraction extracted from the surface of *E. texensis* cells at the end of the exponential phase (OD_{750nm} of 0.57 (+/- 0.06)) and at the end of the stationary phase (OD_{750nm} of 1.86 (+/- 0.02)) and in the EPS fraction not attached to the cells (supernatant)

EPS concentration ($mg \cdot gDW^{-1}$)	Proteins cells	Proteins supernatant	Carbohydrates cells	Carbohydrates supernatant
E. texensis (exponential)	29 (± 14)	N.D.[*]	75 (± 3)	74 (± 12)
E. texensis (stationary)	233 (± 8)	N.D.[*]	96 (± 10)	35 (± 1)

[*] Not detectable

In the current study, the amounts of proteins and carbohydrates in the EPS attached and not attached to the cell surface of *E. texensis* at different growth phases was measured. While the amounts of proteins in the EPS found in the supernatant are negligible, the amounts of carbohydrates in the EPS not attached as well as attached to the cell surface of *E. texensis* did not change considerably at different growth phases (Table 4.1.). The amounts of

proteins in the EPS attached to the cell surface of *E. texensis* increased considerably from the exponential to the stationary phase (Table 4.1.). Again, these results coincide with the difference in EPS-like structures observed at the cell surface of cells harvested at the end of the exponential phase (Fig. 4.3. A-C) and at the end of the stationary phase (Fig. 4.3. D-F). However, the fraction of extracted proteins in EPS during the stationary phase is higher than in the exponential phase, but the flocs formed in the exponential (Fig. 4.3. A) and in the stationary phase (Fig. 4.3. D) hardly show any difference in the SEM pictures.

4.3.3.3. Particle size

The volume fraction of different particles sizes in two batch experiments is presented in Fig. 4.4. The difference between growth phases on volume fraction of different particles sizes is represented in this figure as well by OD_{750nm} (cell concentration). In the beginning, middle and end of the exponential phase in the first batch experiment, the volume percentage of big flocs (> 20 µm) of *E. texensis* was 83.9 (± 0.2), 87.2 (± 0.2) and 39.8 (± 0.1) % respectively and dropped in the linear phase to approximately 20% and stayed constant around 20% in the stationary phase (Fig. 4.4.). In the middle and end of the exponential phase in the second batch experiment the volume percentage of big flocs (> 20 µm) of *E. texensis* 92.5 (± 0.6) and 28.9 (± 0.1) % respectively and dropped in the linear phase to approximately 10% and stayed constant around 10% in the stationary phase (Fig. 4.4.). The opposite pattern was observed for the volume percentage of small flocs and big individual cells of *E. texensis* (6.5 – 20 µm), in the beginning, middle and end of the exponential phase in the first batch experiment, it was 13.3 (± 0.0), 11.4 (± 0.0) and 57.5 (± 0.2) % respectively and increased to 69.4 (± 0.1) % in the linear phase and stayed constant around 75% in the stationary phase (Fig. 4.4.). This gradual increase of the volume percentage of small flocs and big individual cells of *E. texensis* (6.5 – 20 µm) was also observed for the second batch experiment; in the middle and end of the exponential phase, it was 6.7 (± 0.0) and 68.3 (± 0.2) % respectively and increased to 86.4 (± 0.3) % in the linear phase and stayed constant around 85% in the stationary phase (Fig. 4.4.). The volume

percentages of different particles in *E. texensis* suspension in the first and second batch show that almost no single cells (3 – 6.5 µm) were present.
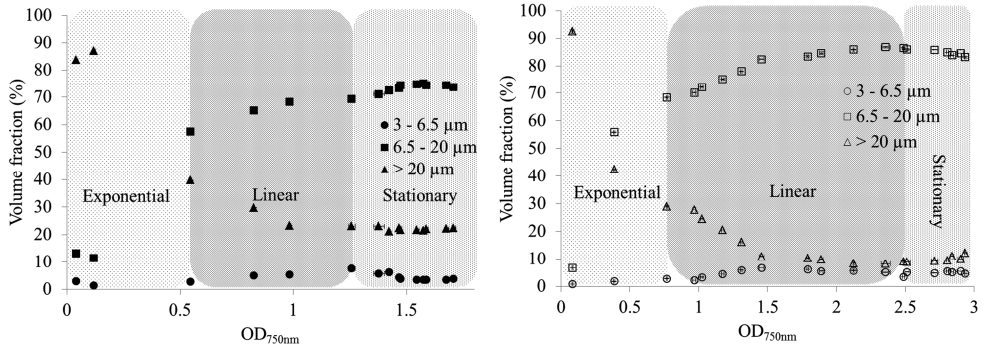

Fig. 4.4. Volume fraction of different particle sizes; small single cells (3 – 6.5 µm) in the first (●) and second (○) batch, big single cells and/or small flocs (6.5 – 20 µm) in the first (■) and second (□) batch and big flocs (> 20 µm) in the first (▲) and second (Δ) batch of *E. texensis* at different growth phases represented by OD_{750nm}

The dry weight of *E. texensis* per unit OD_{750nm} in the first batch experiment was 0.75 (± 0.01) $g·L^{-1}·OD_{750nm}^{-1}$ at the end of the exponential phase and changed between 1.01 (± 0.01) and 1.19 (± 0.02) $g·L^{-1}·OD_{750nm}^{-1}$ in the stationary phase (Fig. 4.5.). It can be concluded that the changes observed in the 50% biomass recovery time (Fig. 4.2.) in the exponential phase and between exponential, linear and stationary phases of *E. texensis* growth in the first batch experiment are due to particle size and dry weight changes respectively. In the second batch experiment, the dry weight of *E. texensis* per unit OD_{750nm} was 0.81 (± 0.00) $g·L^{-1}·OD_{750nm}^{-1}$ at the end of the exponential phase and raised to 0.89 (± 0.01) $g·L^{-1}·OD_{750nm}^{-1}$ at the end of linear phase and reached 1.04 (± 0.03) $g·L^{-1}·OD_{750nm}^{-1}$ in the stationary phase (Fig. 4.5.). The changes in the 50% biomass recovery time (Fig. 4.2.) in the exponential phase and between exponential, linear and stationary phases of *E. texensis* growth in the

second batch experiment can be also explained by changes of particle size and dry weight respectively.
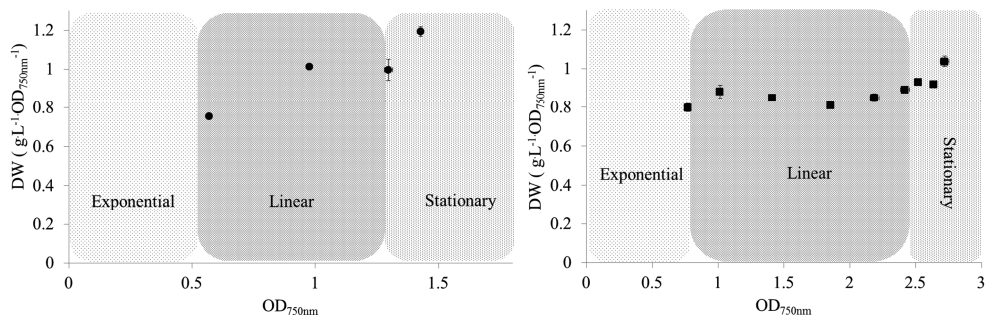

Fig. 4.5. Dry weight of *E. texensis*) in the first (●) and second (■) batch at different growth phases represented by OD_{750nm}

4.3.4. Fatty acids content and composition

The major lipid classes in microalgae are the polar lipids (mostly phospholipids and glycolipids), which are common membrane components, and the triacylglycerides (TAG), which are a reserve of fatty acids for cellular division, metabolic energy, membrane maintenance and synthesis. The TAG accumulation in microalgae usually occurs in the stationary growth phase and can be triggered by nitrogen limitation (Dunstan et al., 1996; Kalacheva et al., 2001; Hu et al., 2008). This TAG accumulation also occurs in *E. texensis* (Fig. 4.6. A and B). The total fatty acids content of *E. texensis* in the first batch experiment was 4.4 (± 0.3) % ($w^{.}w^{-1}$) in the beginning of the linear phase and then slightly increased to 5.9 (± 0.2) % ($w^{.}w^{-1}$) in the linear phase. In the stationary phase, the total fatty acids content started to increase as nitrate depletion triggered the lipid accumulation in *E. texensis*. The total fatty acids content of *E. texensis* reached 25.1 (± 0.0) % ($w^{.}w^{-1}$) in the stationary phase. The same pattern was also observed for the total fatty acids content in the second

batch experiment. It was 5.2 (± 0.3) % (w·w^{-1}) at the end of the exponential phase and increased in the linear phase to 9.2 (± 0.0) % (w·w^{-1}). The total fatty acids content of *E. texensis* in the second batch cultivation reached 12.9 (± 0.1) % (w·w^{-1}) in the stationary phase. The lower total fatty acids content of *E. texensis* in the second batch in comparison with the first batch can be explained by the fact that the lipid accumulation in *E. texensis* was less triggered by nitrate depletion in the stationary phase as the microalgal cells experienced shorter stationary phase; 6 days in the second batch versus 12 days in the first batch. The lipid content of *E. texensis* (EGEMACC-68) measured by Isleten-Hosoglu et al. (2012) was 14 – 19 % (w·w^{-1}) under non-optimized culture conditions and increased to 35 % (w·w^{-1}) under optimized culture conditions for lipid accumulation. Another study with another specie in the *Ettlia* genus, *Ettlia* sp. YC001 showed a lipid content of 50 % (w·w^{-1}) under nutrient starvation conditions (Yoo, et al. 2013). In both studies the lipid content was measured gravimetrically and it was not only the total fatty acids content as presented in the current study.
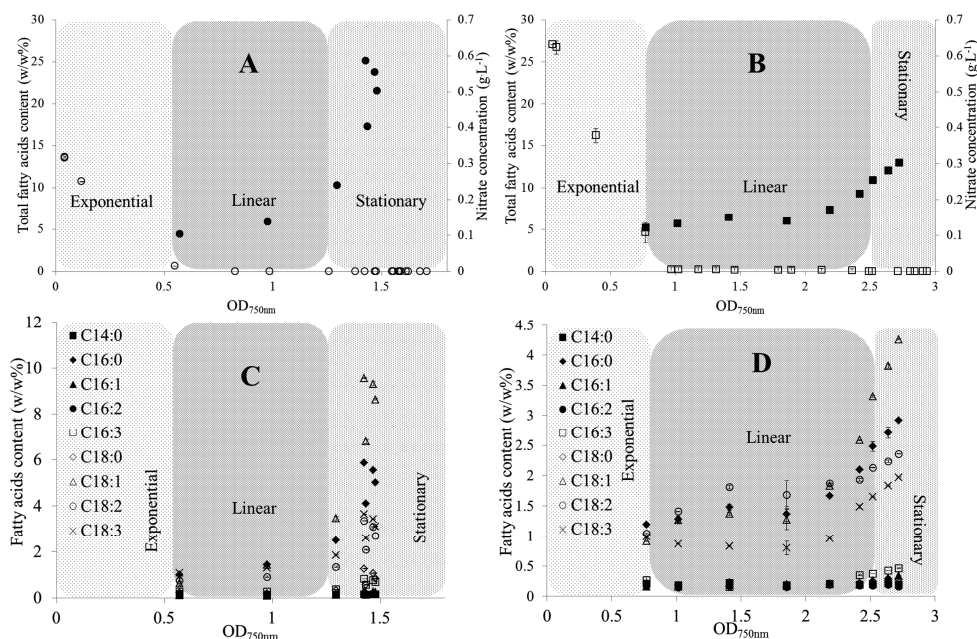
Fig. 4.6. Total fatty acids content of *E. texensis* in the first (A) (●) and second (B) (■) batch and residual nitrate concentration in the medium in the first (A) (○) and second (B) (□) batch and fatty acids profile of *E. texensis* in the first (C) and second (D) batch at different growth phases represented by OD_{750nm}

The fatty acids composition showed strong dependency on the growth phase for *E. texensis* in both batch experiments. In the beginning of the linear phase, C18:3, C18:2, C16:0 and C18:1 were the fatty acids present at highest percentages with no considerable mutual difference in fatty acid profile (Fig. 4.6. C and D). At the end of the linear phase and in the stationary phase, however, C18:1 represents the highest percentage in the fatty acids profile of *E. texensis*, followed by C16:0 and by C18:2 and C18:3. The nitrate depletion was not only the trigger for lipid accumulation but also the trigger to raise the amount of C18:1 in comparison with other fatty acids. C18:1 content increased 15 and 5 times in the first and second batch experiment respectively from the beginning of the linear phase to the stationary phase which is a desirable component of biodiesel. The shift in fatty acids

composition in favour of C18:1 in *E. texensis* was smaller in the second batch than in the first batch also because the cells experienced shorter stationary phase. Isleten-Hosoglu et al. (2012) also measured C18:1 with the highest percentage in the fatty acids profile of *E. texensis* (EGEMACC-68) followed by C16:0 C18:2 and C18:3. Yoo, et al. (2013) also found similar level of C18:3, C18:2, C16:0 and C18:1 in the beginning of cultivation in fatty acids profile of *Ettlia* sp. YC001 and the proportions of C18:1 and C16:0 increased significantly over the cultivation period.

4.3.5. Future perspectives

The current study showed the importance of the harvesting time in making biodiesel production from microalgae feasible. However the optimum harvesting time will be strain dependent and therefore more study is needed for a better insight in mechanisms involved in the lipid accumulation at different growth phases simultaneously with the effect of growth phase on harvesting and extraction of microalgae due to changes in e.g. the cell wall structure.

4.4. Conclusions

The amount of extracted EPS and SEM pictures indicate changes at the cell surface during subsequent growth phases. The growth phase has a large impact on the recovery time of *E. texensis* and the fatty acids content and composition. The results of both batch experiments confirmed this and showed that observed patterns are reproducible. The short recovery time in the stationary phase, combined with an increase of the total fatty acids content and a profile change in favour of C18:1 and C16:0 make *E. texensis* a promising candidate for biodiesel production with the optimum harvesting time in the stationary phase.

Acknowledgements

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5 Extracellular polymeric substances; the key factor in autoflocculation of *Ettlia texensis*

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Abstract

The oleaginous *Ettlia texensis* is an autoflocculating microalgae that can be used for bio-flocculation of microalgae to facilitate harvesting. In this study the mechanism behind autoflocculation of *E. texensis* was revealed by scanning electron microscopy (SEM) analysis and by characterisation of the cell surface properties. SEM analysis and extracellular polymeric substances measurement showed that autoflocculation of *E. texensis* is due to the polymers (EPS) containing mainly glycoproteins patched to the cell surface. Despite the presence of charged groups on the cell surface, they do not seem to attribute to autoflocculation of *E. texensis*. During bio-flocculation of *E. texensis* with *Chorella vulgaris* fibre like EPS structures between both microalgal species were observed. EPS thus not only play a predominant role in autoflocculation of *E. texensis* but also in bio-flocculation when using this microalgae to harvest others.

5.1. Introduction

Flocculation of microalgae is the most promising technique to substantially reduce the energy costs of harvesting microalgae (Salim et al. 2012). Preferably, autoflocculation should be used as this requires no addition of toxic or costly flocculants to the cells. Moreover, the medium can be reused without additional steps needed to remove remaining flocculants or to adjust the pH. Autoflocculation of microalgae is defined as the ability of microalgal cells to form flocs spontaneously. The majority of microalgae, however, do not form flocs spontaneously and only few microalgal species show high flocculation potential. One of them is *Ettlia texensis* (Salim et al. 2012). *Ettlia texensis* is a microalgae that combines good autoflocculation and sedimentation potential with high lipid content and this makes this particular microalgae a very promising candidate for biodiesel production (Salim et al. 2013). However, the mechanism behind the spontaneous floc formation by this species is still unknown.

Autoflocculation of microalgae is generally dictated by specific interactions between molecules at the surface of the microalgal cells and its surrounding medium or interactions between the microalgae themselves. At natural water pH (around neutral pH), the microalgal cells are negatively charged due to the dissociation of functional groups at their cell surface. Particularly the carboxyl groups that are present in peptides in the cell wall (Northcote et al. 1958) or in extracellular polymeric substances (EPS) attached to the cell surface (Bernhardt et al. 1985) render a negatively charge cell surface. When this negative charge of microalgal surface is completely or locally neutralized, autoflocculation of the microalgae is bound to occur. Surface charge is therefore mentioned as an important parameter that plays a role in the mechanism involved in autoflocculation of microalgae (Konno 1993). It can be measured by determining the ζ-potential of the cells. A drop in ζ-potential indicates a reduction in the repulsive electrostatic forces which can lead to a critical ζ-potential where the attractive van der Waals forces overcome these electrostatic forces and microalgae flocculate (Henderson et al. 2008b).

Autoflocculation can also occur due to production, excretion, adsorption and bridging of polymers originating from the microalgae. These polymeric substances can be excreted by the microalgae in suspension or they can be attached to the microalgal cells. These microalgal EPS can bind partly or completely to microalgal cells. If they bind partly, the unoccupied part of the polymers can bind to other microalgal cells, thereby bridging them and a network of polymers and microalgal cells is formed. If the microalgal polymers entirely bind to the microalgal cells because they are too short to bind others as well, they fully adsorb (patch) to the surface (Tilton et al. 1972). Compounds identified in EPS are mainly glycoproteins, polysaccharides and low molecular weight sugars (Fogg 1996). More recently, Guo et al. (2013) presented that the self-flocculation of *Scenedesmus obliquus* was mediated by cell wall-associated polysaccharides.

The objective of the current research is to reveal the mechanism involved in autoflocculation of *E. texensis*. The non-flocculating *Chlorella vulgaris* will be used as the reference for comparison with the autoflocculating *E. texensis* cells. Furthermore, the possible role of EPS released from or attached to *E. texensis* cells in capturing *C. vulgaris* cells during bio-flocculation which was presented by Salim et al. (2012) was investigated.

5.2. Materials and methods

5.2.1. Microalgal strain and cultivation conditions

Ettlia texensis (SAG79.80) and *Chlorella vulgaris* (SAG211-11b) were obtained from the University of Göttingen, DE. The composition of the freshwater medium and the medium preparation protocol were described by Salim et al. (2011). The freshwater medium used for *E. texensis* contained KNO_3 (0.316 $g^.L^{-1}$), $NaH_2PO_4^.2H_2O$ (0.066 $g^.L^{-1}$) and KH_2PO_4 (0.034 $g^.L^{-1}$) instead of the concentrations mentioned by Salim et al. (2011) and the HEPES buffer was omitted.

The cultivation was performed in a fully-controlled photobioreactor (4 L, Applikon, NL) in batch mode, which was autoclaved prior to inoculation of the microalgae. The reactor was stirred at 300 rpm, the temperature was set at 26 °C and the pH was controlled at 6.5 by CO_2 addition. A mass flow controller unit was used to control the total gas flow (being a mixture of CO_2 in N_2) at 250 mL·min^{-1}. Fluorescent lamps were used to provide an average incident light intensity of 300 μmol·m^{-2}s^{-1}. Each measurement for each individual sample was performed in triplicate.

5.2.2. Analysis of dry weight and cell concentration

Whatman glass microfiber filters (Ø 55 mm, pore size 0.7 μm) were dried at 95ºC overnight and placed in a desiccator to cool to room temperature and weighed. Approximately 10 mg of sample was filtered. The filter was rinsed twice with demineralized water to remove adhering inorganic salts. The wet filters containing the samples were dried at 95ºC overnight, allowed to cool to room temperature in a desiccator, and weighed again. As measure for the cell concentration, the optical density of the sample was measured at 750 nm (OD_{750nm}) with a DU730 spectrophotometer (Beckman Coulter Inc. US). For the measurement of the optical density, demineralized water served as reference and the samples were diluted using demineralized water if needed.

5.2.3. ζ-potential

For the assessment of the ζ-potential, a Zetasizer (Malvern, Nano ZS, Zen 3600, AU) was used. The microalgal suspension was put into a folded capillary electrophoresis cell (model DTS 1060C, Malvern, AU) with a syringe to prevent formation of air bubbles. The ζ-potential was measured 5 times for three biological replicates and the average ζ-potential was determined.

5.2.4. Particle size distribution

The particle size distribution in *E. texensis* and *C. vulgaris* suspensions was measured using the Mastersizer as it was described by Salim et al. (2013).

5.2.5. Morphological analysis

Microscopic pictures were taken from the microalgal cells, using a C-3030 zoom 5 mega pixel Olympus camera (Olympus, JP) connected to a CK40 Olympus microscope (Olympus, JP) with a SK20-SLP phase contrast filter and a T6 objective (40x magnification) and a NCWHK 18L ocular lens (10x magnification).

5.2.6. SEM preparation

The presence of EPS at the outer surface of microalgal cells and flocs was visualized using scanning electron microscopy (SEM). For this, poly-L-lysine coated microscopy cover slips were submerged in a microalgal suspension for 2.5 hours. The glass cover with attached microalgal cells were rinsed by dipping them into fresh culture medium and the cells were fixated for one hour in a 3% glutaraldehyde solution in culture medium. After 2 times washing with culture medium, the samples were post-fixated in a 1% OsO_4 solution for one hour, rinsed with demineralized water and dehydrated in acetone. Subsequently, they were lyophilized using CO_2. The cover slips with cells were fit on a sample holder using carbon adhesive tabs, and sputter-coated with a 10 nm Iridium layer. The microalgal cells were analysed at 2 kV at room temperature in a high-resolution scanning electron microscope.

5.2.7. EPS extraction

Microalgal samples were taken from the reactor and kept in the fridge (4°C). After the cells were settled, the supernatant was decanted. The extraction of EPS was performed following the procedure described by Frolund et al. (1996) with some modifications. The settled cells were resuspended in demineralized water. The resuspended cells were transferred to a beaker and were stirred with a floating magnetic stir bar (Nalgene, US) at 1300 rpm for two hours to extract the EPS from the cell wall. The cells were separated by centrifugation at 4000 rpm for five minutes.

5.2.8. Identification of carbohydrates and proteins in EPS

The phenol-sulphuric acid method of Dubois et al. (1956) was used as a quantitative analysis for the total carbohydrates content present in EPS with glucose as standard. The Lowry method was used for determination of the protein concentration with Bovine serum albumin (BSA) as standard (Lowry et al. 1951) using the BIO-RAD *DC*TM Protein Assay (BIO-RAD, US). The total carbohydrate and protein content were presented in milligrams present in the EPS per gram of the total dry weight of the harvested microalgal cells.

5.3. Results and discussion

5.3.1. Influence of ζ-potential on flocculation mechanism

The ζ-potential of a microalgal cell is typically electronegative for pH 4 - 10, ranging from -10 to -35mV (Henderson et al. 2008a). In our experiments the pH of culture medium used was set at 6.5. This pH results in a net negatively charged surface of the both cells of *Ettlia texensis* and *Chlorella vulgaris*. The ζ-potential of *C. vulgaris* did not vary with the growth phase and remained at -38.4 (± 3.5) mV. Henderson et al. (2008a) measured a similar value of -33 mV for the ζ-potential of *C. vulgaris* cells in a comparable culture medium. The ζ-

potential of *E. texensis* cells was higher than the ζ-potential measured for the non-flocculating *C. vulgaris* and slightly increased with the growth phase from -18.9 (± 1.2) mV at the beginning of the exponential phase to -13.3 (± 0.5) mV in the linear growth phase and stayed constant around -12.1 (± 0.9) mV in the linear and stationary phase.

The difference in ζ-potential of *C. vulgaris* and *E. texensis* is not due to pH or the ionic strength of the medium as both microalgae were cultivated in the same medium. The difference in ζ-potential can only be attributed to a difference in the groups attached to the cell surface. This difference in ζ-potential of *C. vulgaris* and *E. texensis* can be one of the reasons why *C. vulgaris* cells under natural conditions (neutral pH, low ionic strength) do not form flocs while *E. texensis* cells do. An increase in ζ-potential of microalgal cells leads to reduction of the repulsive electrostatic forces between the individual microalgal cells. In case of *E. texensis* cells, attractive van der Waals forces seem to dominate and cause the microalgae to form flocs.

As the variation in ζ-potential of *E. texensis* at different growth phases is small, the autoflocculation behaviour of *E. texensis* is most likely not determined by the ζ-potential. Our hypothesis is that EPS could dominate the autoflocculation behaviour of *E. texensis*. To prove this, the presence of EPS on the surface of *E. texensis* cells was investigated and the amount of EPS attached to the cell surface of *E. texensis* was quantified and compared with the non-flocculating *C. vulgaris*.

5.3.2. Microscopic analysis of the cell surface

Microscopic pictures of *C. vulgaris* and *E. texensis* cells in suspension at the end of the stationary phase show that *E. texensis* cells form large flocs (Fig. 5.1. A) while individual cells of *C. vulgaris* are homogenously distributed in the suspension (Fig. 5.1. B). When

zooming in on the cell surface structure by SEM, the cell surface of *E. texensis* cells (Fig. 5.1. C, D and E) and *C. vulgaris* (Fig. 5.1. F and G) could be analysed in more detail.
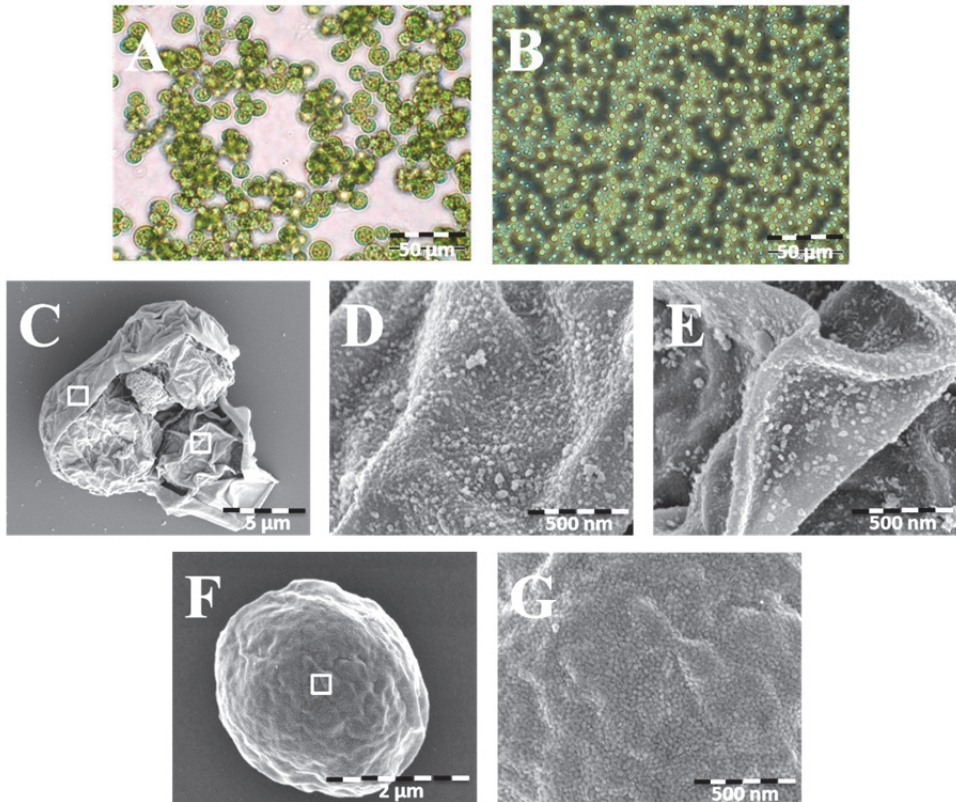

Fig. 5.1. Microscopic pictures of *E. texensis* (A) and *C. vulgaris* (B), SEM pictures of an *E. texensis* floc (C), extracellular polymeric substances (EPS) covering as an extra layer the whole *E. texensis* floc (D) and on the cell surface of the individual *E. texensis* cells (E), a *C. vulgaris* cell (F) and the cell surface of a *C. vulgaris* cell (G). The samples of *C. vulgaris* and *E. texensisi* were taken at the end of the stationary phase.

E. texensis shows matrices of extracellular polymeric substances (EPS) not only at the cell surface of the individual cells (Fig. 5.1. E) and between the individual cells in a floc (Fig. 5.1. C) but they were also present as an extra layer covering the whole floc (Fig. 5.1. D). The results of SEM analysis indicate that autoflocculation of *E. texensis* is due to the polymers attached to the cell surface. The SEM picture of *E. texensis* (Fig. 5.1. C) shows that these polymers seem to patch to the cell surface of the *E. texensis* cells and that these EPS are too short to bind other *E. texensis* cells as well. The SEM pictures of *C. vulgaris* show a smooth and equal cell surface with no polymeric structures (Fig. 5.1. F and G).

5.3.3. EPS extraction

Bernhardt and Clasen (1991) suggest that several strains of microalgae produce EPS attached to their peripheral cell walls. To verify if this was the case for *C. vulgaris* and *E. texensis* cells, the EPS were extracted from the surface of the cells, harvested at the end of the stationary phase. In addition, the EPS that were not attached to the cells, but free in suspension were measured (Table 5.1.). For both species, no proteins were found in the EPS released in the medium and the amount of carbohydrates found in the medium was comparable for both species. The amounts of proteins and carbohydrates measured in the extracted EPS from the cell surface of *E. texensis,* however, are much higher than the amounts measured in the EPS extracted from *C. vulgaris*. These results coincide with the observation of matrices of extracellular polymeric substances in the SEM pictures of *E. texensis* (Fig. 5.1. C-E) and absence of polymeric structures on the surface of *C. vulgaris* cells (Fig. 5.1. F-G).

To verify if the measured proteins and carbohydrates did not originate from microalgal cells that broke due to centrifugation and stirring during the extraction process, the release of intercellular carbohydrate and proteins was investigated. The effect of shear forces imposed on the cells during centrifugation and stirring was tested by measuring the particle size distribution in *E. texensis* and *C. vulgaris* suspensions using the Mastersizer. The number of

particles smaller than 3 µm (representing the cell debris) did not increase at different centrifugation speeds upto 14680 rpm and different stirring speeds upto 1300 rpm in comparison with the non-centrifuged and non-stirred samples respectively. These results prove that the extracted EPS did not originate from broken cells.

Table 5.1. Proteins and carbohydrates concentration (n = 3) in the EPS fraction extracted from the surface of *E. texensis* cells at the end of the stationary phase (OD_{750nm} of 1.86 (+/- 0.02)) and *C. vulgaris* cells at the end of the stationary phase (OD_{750nm} of 1.83 (+/- 0.21)) and in the EPS fraction not attached to the cells (supernatant)

EPS concentration ($mg \cdot gDW^{-1}$)	Proteins cells	Proteins supernatant	Carbohydrates cells	Carbohydrates supernatant
E. texensis	233 (± 8)	N.D.[*]	96 (± 10)	35 (± 1)
C. vulgaris	17 (± 3)	N.D.[*]	22 (± 3)	50 (± 8)

* Not detectable

The amount of proteins in the EPS attached to the surface of *E. texensis* cells is higher than the amount of carbohydrates (Table 5.1.). This indicates that glycoproteins are most probably the main compound forming the EPS attached to the cell surface of *E. texensis* cells. This corresponds well with the findings by Fogg (1996), who suggested that the compounds identified as EPS attached to the cell surface of microalgae are mainly glycoproteins and polysaccharides. As the non-flocculating *C. vulgaris* cells also excreted considerable amount of carbohydrates in the suspension (Table 5.1.), it is most likely that carbohydrates present in the EPS fraction in the supernatant are not involved in the floc formation.

5.3.4. Bio-flocculation of *C. vulgaris* with *E. texensis*

Salim et al. (2012) presented that addition of the autoflocculating microalgae *E. texensis* to non-flocculating *C. vulgaris* increases the recovery of *C. vulgaris* as well as the sedimentation rate. They suggested that this bio-flocculation is due to either entrapment or

attachment of the *C. vulgaris* by the flocs formed by the *E texensis*. SEM pictures of the bio-flocculated cell suspension of *E. texensis* and *C. vulgaris* cells (Fig. 5.2.) show that the EPS released from or attached to *E. texensis* cells are indeed involved in capturing *C. vulgaris* cells.
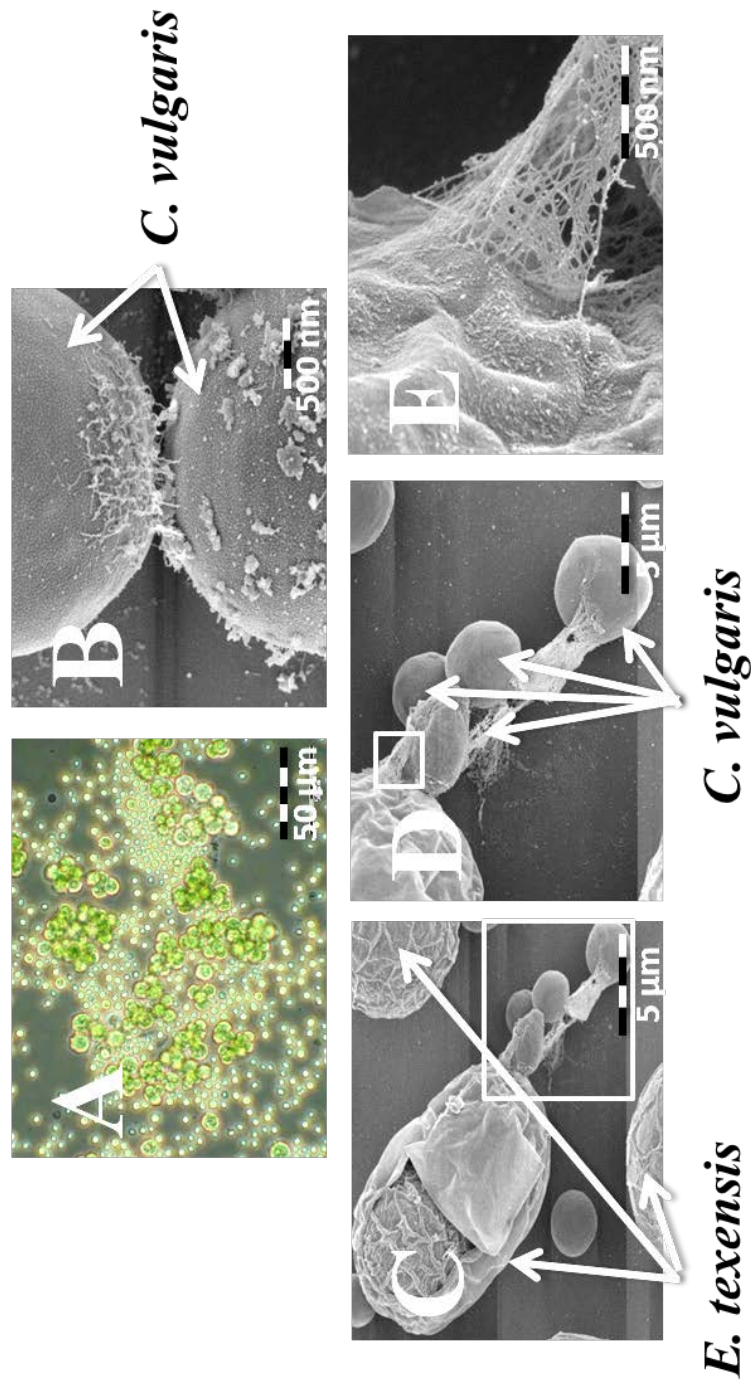
Fig. 5.2. Microscopic picture of *E. texensis* and *C. vulgaris* suspension (A), SEM picture of two *C. vulgaris* cells with EPS released from *E. texensis* in the suspension and attached to the surface of *C.vulgaris* cells (B), *E. texensis* and *C. vulgaris* suspension (C), several *C. vulgaris* cells attached to EPS from *E. texensis* (D) and EPS structure attached between *E. texensis* and *C. vulgaris* cells (E)

Microscopic picture of *E. texensis* and *C. vulgaris* suspension (Fig. 5.2. A) shows large *E. texensis* flocs with *C. vulgaris* cells trapped in between them. The SEM pictures of the same suspension show the EPS released from *E. texensis* attached two *C. vulgaris* cells together (Fig. 5.2. B). The EPS attached to an *E. texensis* floc are also attached to *C. vulgaris* cells (Fig. 5.2. C-E). These EPS show a strong fibre structure which connects autoflocculating *E. texensis* cells to non-flocculating *C. vulgaris* cells (Fig. 5.2E).

5.4. Conclusions

The combined results of SEM analysis and EPS measurement show that autoflocculation of *E. texensis* is due to the polymers; mainly glycoproteins patched to the cell surface. EPS also seem to play a predominant role in bio-flocculation. The fibre structures between both microalgal species involved were detected, but further analysis is needed to identify and characterize these compounds.

Acknowledgements

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6 Modeling microalgal flocculation and sedimentation

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Abstract

In this study, a combined flocculation and sedimentation model is developed. The model predicts the time needed to reach a desired concentration of microalgal suspension in a sedimentation tank. The concentration of the particles as function of the time and the position in the tank is described. The model was validated with experimental data for *Ettlia texensis*. The concentration changes measured in time at different heights in the sedimentation vessel corresponded well with model predictions. The model predicts that it takes 25 hours to reach a final concentration of 5.2 gDW$\cdot$L^{-1}, when the initial concentration is 0.26 gDW$\cdot$L^{-1} and the tank height is 1 m. This example illustrates the use of this model for the design of the settling tank needed for pre-concentration of microalgal biomass before further dewatering.

6.1. Introduction

Centrifugation and microfiltration are currently the most frequently used methods for harvesting microalgae because microalgal cultures are very dilute and microalgae are small. Both harvesting methods are costly and require a high energy input (Uduman et al., 2010; Norsker et al., 2011). Pre-concentration of the cells via flocculation and sedimentation can significantly reduce the energy demand of centrifugation for final dewatering. Several ways to flocculate microalgae have been presented, ranging from flocculation induced by chemicals or by an electric field, to bio-flocculation and spontaneous autoflocculation (Salim et al., 2011; Vandamme et al., 2012).

To be able to predict the time needed to reach a desired concentration of the microalgal suspension in large scale production facilities a mathematical model is developed that describes the flocculation and simultaneous settling of the microalgal biomass. In this model the flocculation model of Smoluchowski is combined with Stokes' law and the Richardson-Zaki model for sedimentation. The expanded model of Smoluchowski with a particle size depended collision frequency is used to describe flocculation of colloidal particles (Thomas et al., 1999). The sedimentation model is based on Stokes' law. In addition, hindered settling is included to account for high concentrations of particles (Quispe et al., 2000; Davis and Gecol, 1994). The combined flocculation and sedimentation model describes the concentration of the particles as function of time and position in a sedimentation tank and thus predicts the time needed to reach a desired concentration of cell dry mass. The model is validated with experimental data using the microalga *Ettlia texensis*. In addition, the model was used to predict the overall effect of flocculation and sedimentation on large scale harvesting of microalgae by calculating the concentration factor and the biomass recovery in a given settling tank. Based on the achieved concentration factor the energy needed for further dewatering of the microalgae in a centrifuge is estimated.

6.2. Materials and methods

6.2.1. Description of the model

6.2.1.1. Flocculation

Flocculation is defined as a process of contact and adhesion whereby the particles of a dispersion form larger-size clusters (flocs). Already in 1917, Smoluchowski presented a flocculation model for colloidal particles (Thomas et al., 1999). In this model, flocs of a given size can either be formed through flocculation of two other smaller particles (birth), or be lost by flocculation with another particle. This can be represented in a rate equation for particles with k cells:

$$r_k = \frac{1}{2}\alpha \sum_{f=1}^{k-1} \beta(f, k-f)C_f C_{k-f} - \alpha \sum_{i=1}^{\infty} \beta(i, k)C_i C_k \qquad (6.1.)$$

where α is the collision efficiency and β is the collision frequency between two particles. f, i and k are the numbers of cells in a floc, C_f, C_i and C_k are the concentrations of flocs with f, i and k cells, respectively. The following collision frequency (β) is used (Han et al., 2003):

$$\beta_{i,k} = \left(\frac{G}{6}\right) * (d_i + d_k)^3 \qquad (6.2.)$$

where G stands for the shear rate and d_i and d_k are the diameters of the colliding particles. The collision frequency used in the current study is valid for orthokinetic flocculation, meaning that the collisions are caused by hydrodynamic motions caused by convection or sedimentation and the particles are subject to laminar flow conditions.

Assumptions made by Smoluchowski are that the collision efficiency α is equal for all collisions, they are spherical in shape and remain so after flocculation, no breakage of flocs occurs and collisions involve only 2 particles (Thomas et al., 1999).

6.2.1.2. Sedimentation

The velocity of an individual falling particle in water can be predicted with Stokes' law (Bürger and Concha, 1998):

$$v_{\infty,i} = \frac{1}{18} * \frac{(\varDelta\rho)}{\eta} * g * d_i{}^2 \tag{6.3.}$$

where d_i is the diameter of particle, $\varDelta\rho$ is the density difference between the particle and the liquid, η is the viscosity of the liquid and v_i is the velocity of the settling particle i.

6.2.1.3. Integration of flocculation and sedimentation models

The settling tank was modelled as a cascade of ideal mixers (Fig. 6.1.) with $z = 1$ for the top mixer and $z = z_{max}$ for the bottom mixer. Particles were divided into limited number (six) of size classes $1 \leq i \leq i_{max}$, with size class 1 containing single cells of 1 to 3 µm (Table 6.1.) and size class i_{max} containing flocs of 395 to 579 µm (Table 6.1.). To prevent an indefinite growth of flocs, a maximal floc size (i_{max}) is defined. For the starting situation the division of the particles over the size classes that are present in the suspension is provided. The assumptions made here are; during the flocculation there is no net growth or loss of biomass and the collision efficiency (α) of all particles is equal to one. Multiple particle sizes are included in the model to simulate a polydisperse solution consisting of particles with equal density.
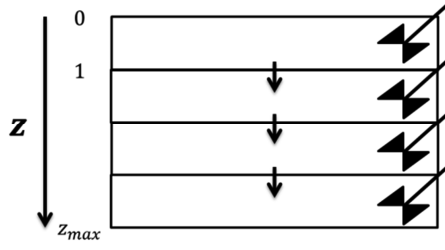
Fig. 6.1. Schematic overview of the settling tank modelled as a cascade of ideal mixtures.

The population balances were solved numerically using an Euler approximation for the time steps. The balance over mixer z for particles in size class i gives:

$$C_{i,z,t+1} = C_{i,z,t} + \left(\frac{v_{i,z-1,t} C_{i,z-1,t} - v_{i,z,t} C_{i,z,t}}{\Delta z} + r_{i,z,t} \right) \Delta t \qquad (6.4.)$$

where $C_{i,z,t}$ and $v_{i,z,t}$ are respectively the concentration and velocity of particle class i at position z at time t, v_i. The boundary conditions are $v_{i,0} = 0$ and $v_{i,z_{max}} = 0$.

The production rate of particles in size class i is calculated with Smoluchowski's model (Eq. 6.1.):

$$r_{i,z,t} = \frac{1}{2} \alpha \sum_{f=1}^{i-1} \frac{G}{6} (d_f + d_j)^3 C_{f,z,t} C_{i-f,z,t} - \alpha \sum_{j=1}^{\infty} \frac{G}{6} (d_i + d_j)^3 C_{i,z,t} C_{j,z,t} \qquad (6.5.)$$

where f, i and j are the numbers of cells in a floc, C_f, C_i and C_j are the concentrations of flocs with f, i and j cells, respectively and d_f, d_i and d_j are the diameters of the colliding particles. The first term in this equation describes formation of particles in size class i due

to collision of two smaller particles; the second term describes disappearance due to collision of particles in size class i with other particles. Particles in size class 1 cannot be formed and particles in size class i_{max} cannot disappear. The collision frequency is calculated according to Eq. 6.2. (Han et al., 2003).

The sedimentation velocity of a single particle in size class i is given by Stokes' law (Eq. 6.3.). Stokes' law is only valid for Reynolds number (*Re*) < 1. This condition is met for all (clusters of) cells.

In the current model, the assumption is made that no particles are leaving the bottom layer. However, this means that the bottom layer would get an infinite high concentration which is not realistic. In addition, the particles in the bottom layer can no longer be regarded as single falling particles. They experience hydrodynamic interactions with the surrounding particles, resulting in changes of the liquid flow around the particles, and hindered settling occurs (Johnson et al., 1996; Bürger and Concha, 1998; Quispe et al., 2000; Davis and Gecol, 1994). In a particle swarm, hindered settling has to be taken into account. We used a modified Richardson-Zaki model which takes into account the minimum external porosity in a packed layer of particles (Davis and Gecol, 1994):

$$v_{i,z,t} = v_{\infty,i} \left(\frac{\varepsilon_{ext_{z,t}} - \varepsilon_{min}}{1 - \varepsilon_{min}} \right)^{5.1} \tag{6.6.}$$

where $\varepsilon_{ext_{z,t}}$ is the external porosity of the solution in layer z, ε_{min} is the minimum external porosity of the particles in the solution and v_{∞_i} is the velocity of a spherical particle of class size i in a dilute solution, following Stokes' law with an empirical value of 5.1 suggested by Garside and Al-Dibouni (1977) for spherical particles with low *Re* in a dilute solution.

6.2.2. Validation of the model

To validate the model the autoflocculating microalgae *Ettlia texensis* was used and the values of the model parameter have been determined.

6.2.2.1. Microalgae culture

E. texensis (SAG79.80) was obtained from the University of Göttingen, DE. The composition of freshwater medium and the medium preparation protocol were described by Salim et al. (2011). The microalgae were grown in 300 mL Erlenmeyer flasks (filled up to 100 ml with the medium), sealed with cotton and an aluminium cap and placed in a light- and climate-controlled shaking incubator (SANYO, JP) at 100 RPM and 25 °C with a 2% CO_2 enriched airflow (3 $L^. min^{-1}$), illuminated using fluorescent light (50 $\mu mol^. m^{-2} s^{-1}$) with a 16 h/8 h light/dark cycle. Microalgal cells were harvested at OD_{750} of 1 for all sedimentation experiments. Each measurement for each individual sample was performed in triplicate.

6.2.2.2. Determination of the cell number concentration

The optical density at 750 nm (OD_{750nm}) is measured using a DU730 spectrophotometer (Beckman Coulter Inc. US). The microalgal samples are diluted in a 10x10x45 mm^3 polystyrene cuvette (Sarstedt, DE) using demineralized water to achieve an OD_{750nm} value below 1. To find the relation between the OD_{750nm} and the cell number concentration, 10 µL of different dilutions of *E. texensis* were injected on a disposable hemocytometer and the cells were counted using a microscope (400x magnification; Olympus, JP). For each dilution a minimum of 25 pictures were taken of the set squares of 100 by 100 μm^2. *E. texensis* cells formed flocs and therefore the number of single cells in each floc was counted for determination of cell number concentration. The calibration curve of number

concentration of *E. texensis* suspension versus OD_{750nm} was 4.13×10^9 ($\pm$ 2.55×10^7) $L^{-1} \cdot OD_{750nm}^{-1}$.

6.2.2.3. Determination of diameter, density and porosity of microalgal particles

The average diameter of the single cells is measured from microscopic pictures taken using a microscope (400x magnification; Olympus, JP).

Three tubes of 10 mL were filled with demineralized water and three with highly concentrated microalgal suspension (OD_{750nm} of 76.3 ($\pm$ 2.9)). The density of the wet cells was calculated from the difference between the average mass of 10 mL of microalgal suspension and the average mass of 10 mL demineralized water and the known number and volume of microalgal cells in the suspension.

To measure the minimum external porosity of microalgal cells, microalgal suspension (OD_{750nm} of 0.99 ($\pm$ 0.01)) were placed in 10ml tubes and allowed to settle for 24 hours. The OD_{750nm} of the sediment was converted to a cell number and cell volume (V_x (mL)). The volume of the pellet was calculated from the measured supernatant volume (V_s (mL)), using the density of water at 21.5 °C. The minimum external porosity (ε_{min}) is equal to the porosity of the pellet:

$$\varepsilon_{min} = \frac{10 - V_s - V_x}{10 - V_s} \tag{6.7.}$$

6.2.2.4. Particle size distribution

The initial particle size distribution is measured using a Mastersizer 2000 (Malvern Instruments Ltd, UK). The Mastersizer measures the size of the particles in microalgal suspension and counts the number of particles. The outcome is a particle size distribution in volume or number percentage.

6.2.2.5. Sedimentation experiments

The sedimentation is followed by measuring the optical density of the microalgal suspension at 750 nm (OD_{750}) at different heights in time in a 10x10x45 mm^3 cuvette (Fig. 6.2.).
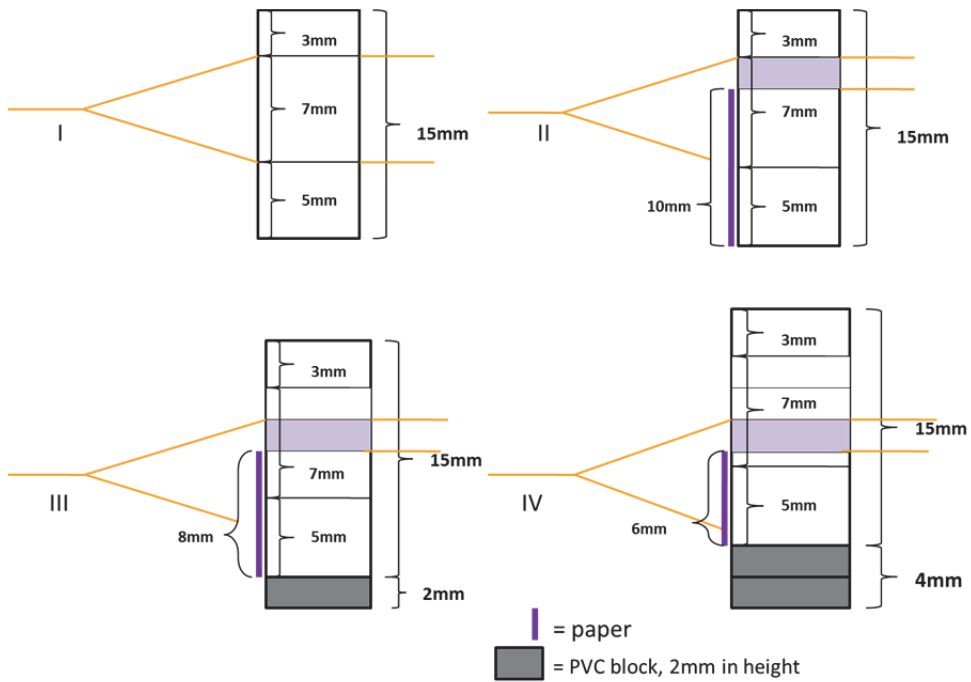
Fig. 6.2. Schematic picture of the method used to follow the sedimentation at different heights in the cuvette. The light beam of the spectrophotometer is 7 mm in height. The measuring area on the cuvette placed in the spectrophotometer is 15 mm in height. Distinction between extinction changes in time for different layers in the cuvette is poor with the light beam being 7 mm in height. Therefore dark papers of a certain size are placed in front of the cuvette in such a way that a light beam of 2 mm in height falls on the cuvette. PVC blocks of 2 mm high are placed below the cuvette to allow measurements at different heights in the cuvette.

6.3. Results and discussion

The microalga *Ettlia texensis* was used to validate the model. Model parameters such as the diameter of individual cells, the density of the cells, the initial optical density, the initial

particle size distribution and the minimum external porosity of *E. texensis* have been determined.

6.3.1. Characterisation of the cells

Microscopic pictures showed that the average diameter of a single cell of *E. texensis* was 3.5 (± 0.3) µm.. The density of *E. texensis* cells was 1029 (± 3) g·L^{-1}. The volume fraction of microalgal cells in the settled fraction of microalgal suspension of *E. texensis* was 0.01 (± 0.00) which means that the minimum external porosity ε_{min} of microalgal suspension of *E. texensis* is 0.99.

6.3.2. Initial particle size distribution

The number percentage of all particle sizes present in the microalgal suspension of *E. texensis* was measured with the Mastersizer (Fig. 6.3. A). Using the initial OD$_{750nm}$ of suspension (1.19 (± 0.01)) and the calibration curve of number concentration *E. texensis* suspension versus OD$_{750nm}$, the number concentration of single cells in the measured suspension was calculated to convert the number percentages, measured with the Mastersizer into the number concentration of different particle size classes (Fig. 3B). Different particle sizes were divided into six classes; 2.8 – 4.4, 4.7 – 6.6, 7.1 – 8.7, 9.4 – 13.2, 14.2 – 22.9 and 24.6 – 26.3 µm which correspond to particle classes 2, 5, 11, 35, 167 and 353 respectively containing 1 – 3, 4 – 6, 7 – 15, 16 – 53, 54 – 280 and 281 – 424 single cells.
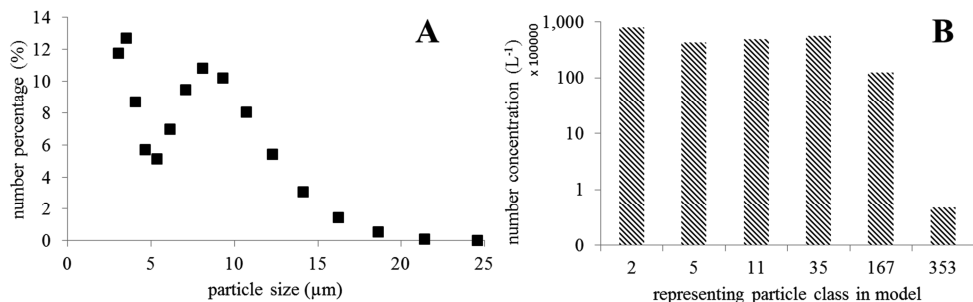
Fig. 6.3. (A) The number percentage of all particle sizes present in the microalgal suspension of *E. texensis* measured with the Mastersizer and (B) number concentration (L^{-1}) of single cells in six particle classes; 2, 5, 11, 35, 167 and 353, containing respectively 1 – 3, 4 – 6, 7 – 15, 16 – 53, 54 – 280 and 281 – 424 single cells which correspond to particle sizes; 2.8 – 4.4, 4.7 – 6.6, 7.1 – 8.7, 9.4 – 13.2, 14.2 – 22.9 and 24.6 – 26.3 µm respectively.

6.3.3. Sedimentation

The optical density OD$_{750nm}$ of the microalgal suspension of *E. texensis* was measured in triplicate in three different layers (z_{max-5}, z_{max-4} and z_{max-3}). The sedimentation was monitored every minute for half an hour. A cuvette, as explained in Fig. 6.2. filled with demineralized water was used as blank. The measured OD$_{750nm}$ of the microalgal suspension was converted into a number concentration (L^{-1}) in each layer using the calibration curve of number concentration of *E. texensis* suspension versus OD$_{750nm}$. After implementation of the measured parameters in the model (Table 6.1.), the number of particles was predicted for each layer and cell number concentration (L^{-1}) in each layer was calculated (Fig. 6.4.).

Table 6.1. Measured parameters for the combined flocculation and sedimentation model

Model parameters	Value
Collision efficiency (α)*	1
Minimum external porosity (ε_{min})	0.99
Viscosity of the liquid (η)	8.9×10^{-4} Pa·s
Density of liquid (ρ_l)	997 g·l^{-1}
Shear rate (G)**	20 s^{-1}
Density of cells (ρ)	1029 g·l^{-1}
Diameter of a single cell (d)	3.5×10^{-6} m
Particle classes	$c_2 = 7.85\times10^7$ $c_5 = 4.22\times10^7$ $c_{11} = 4.80\times10^7$ $c_{35} = 5.60\times10^7$ $c_{167} = 1.22\times10^7$ $c_{353} = 4.74\times10^4 L^{-1}$

* Collision efficiency (α) of all particles is equal to one which means that all collisions will lead to flocculation

** The parameter value used as an assumption for shear rate was based on the literature parameter measured for yeast cells in a comparable system (Han et al., 2003).
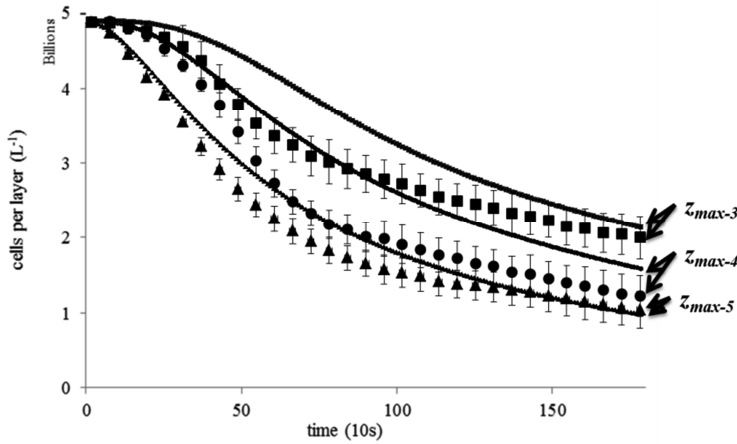
Fig. 6.4. Measured cell number concentration change in time in layers z_{max-3} (■), z_{max-4} (●) and z_{max-5} (▲) as measured in the cuvette and calculated using measured parameters for the combined flocculation and sedimentation model (Table 6.1.).

The predicted cell number concentrations agree reasonably with the measurements (Fig. 6.4.). The cell number concentrations measured in layers z_{max-3}, z_{max-4} and z_{max-5} after 30

minutes are 2.04×10^9 ($\pm$ 2.87×10^8) L^{-1}, 1.26×10^9 ($\pm$ 2.72×10^8) L^{-1} and 1.07×10^9 ($\pm$ 2.48×10^8) L^{-1}, respectively. This implies a decrease of 41.5% ($\pm$ 5.8), 25.6% ($\pm$ 5.5) and 21.9% (+/- 5.0), respectively, compared to the initial cell number concentration (4.91×10^9 ($\pm$ 4.13×10^7) L^{-1}). The model predicted the cell number concentration in layers z_{max-3}, z_{max-4} and z_{max-5} after half hour at 2.16×10^9, 1.61×10^9 and 9.90×10^8 respectively which is a decrease of 43.9%, 32.7% and 20.1% respectively.

In the model, flocculation and sedimentation occur simultaneously. However the outcome of the model shows that the effect of flocculation is negligible. As the collision frequency was low (order of 10^{-13} to 10^{-15} $m^{3 \cdot} s^{-1}$), the particle class distribution did not change drastically in time. The parameter values presented in Table 1 are measured for *E. texensis* except for the shear rate (G) which was based on the literature parameter measured for yeast cells in a comparable system (Han et al., 2003). For the validation of this model, the sample in the cuvette was not mixed during the sedimentation experiment. This means that the used low value for the shear rate is a plausible assumption. An observation of the settling sample of *E. texensis* with an Eyetech analyzer confirmed as well that the particle size distribution of *E. texensis* did not change as the cells settled.

6.3.4. Prediction of recovery and concentration factor for large scale pre-concentration

The validation results showed that the combined flocculation and sedimentation model can be used to predict the time that is needed for settling of a specific microalgae and the obtained concentration of the biomass per layer. This implies that the model can be used for the design of the pre-harvesting step. To illustrate this, the concentration factor and the biomass recovery is predicted for a microalgal suspension of *E. texensis* that is transferred in a settling tank operating under a batch mode with a diameter of 1 m and a height of 1 m (total volume of 0.78 m^3) for pre-concentration. The microalgal suspension has an OD_{750nm}

of 0.24, comparable with the optical density obtained in outdoor open pound systems and contains approximately 0.26 gDW·L^{-1} biomass. Fig. 6.5. shows the calculated percentage of recovered microalgal biomass and concentration factor obtained after 5 to 25 h, when the supernatant is removed and the initial volume of microalgal suspension is 97.5.5, 87.5, 75, 62.5 or 50% reduced, containing the settled microalgae.
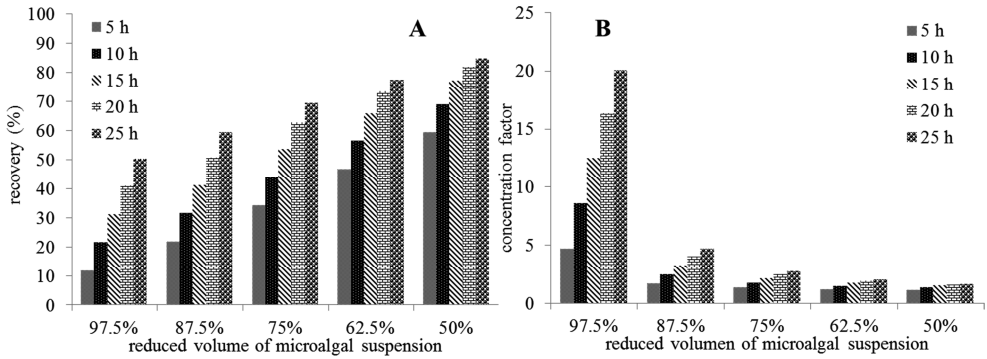

Fig. 6.5. The biomass recovery (A) and concentration factor (B) in a settling tank with a diameter of 1 m and a height of 1 m calculated for a microalgal suspension of *E. texensis* at OD$_{750nm}$ of 0.24 obtained after 5 to 25 h,   after removing the supernatant, when the supernatant is removed and the initial volume of microalgal suspension is 97.5.5, 87.5, 75, 62.5 or 50% reduced, containing the settled microalgae. The model parameters used in the combined flocculation and sedimentation model are presented in Table 6.1.

Fig. 6.5. shows that both the biomass recovery and concentration factor increase in time. When the initial volume is 97.5% reduced, the concentration factor that is achieved, differs considerably from the concentration factor reached after a volume reduction of 87.5% till 50% (Fig. 6.5. B). After a volume reduction of 97.5%, the microalgal suspension is concentrated 20 times after 25 hours of settling versus 5 times concentration after a 87.5% volume reduction (Fig. 6.5. B), while the recovery does not show such dramatic differences

(Fig 6.5. A). For a volume reduction of 87.5% after 25 h of settling a 50% recovery of the microalgae is reached, while this percentage increases only to 59% when the volume of microalgal suspension is 87.5% reduced (Fig. 6.5. A).

The concentration factor is important for the energy needed in the post-concentration step following the sedimentation of the flocculated cells, but it is also important to obtain a high recovery of the cells. The concentration factor calculated for the volume reduction of 97.5% varies from 5 to 20 after 5 and 25 h of settling of the *E. texensis* suspension, respectively. A concentration factor of 20 would be high enough to start further dewatering of the settled biomass in case a disk stack bowl centrifuge is used for further dewatering. Salim et al. (2012) showed that the energy needed for further dewatering of microalgal suspension of *E. texensis* can be also reduced by 20 times when the volume of microalgal suspension is 97.5% reduced. They also mentioned that the non-recovered microalgal cells are sent back with the medium to be reused during production. This makes the degree of recovery less relevant. However this was not tested yet and therefore needs more investigation. Furthermore the estimated time of settling of *E. texensis* is based on the chosen height of the settling tank (1 m). Most of settlers which are used at large scale e.g. in water purification plants are operating under a continuous mode and the settling distance of the particles is considerably less than 1 m. Therefore the settling time of microalgal suspension in these settlers when applied for pre-concentration of microalgae at large scale is considerably less than values presented in Fig. 6.5.

6.4. Conclusions

The flocculation and subsequent sedimentation model predicts the settling time and achieved concentration of the microalgal biomass well. The model is applicable for different microalgal strains if the parameter values dependent on the strain (density, minimum external porosity and single cell size) and on the applied conditions (initial

number concentration, initial size distribution of the particles and shear rate influencing collision frequency) are known. The outcomes; settling time and achieved concentration can be used to design a settling tank needed for the pre-concentration step and type and size of the post-concentration step for further dewatering of the microalgal biomass.

Acknowledgements

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7 General Discussion

Production of microalgae requires high energy inputs for water pumping, mixing and harvesting (Schenk et al., 2008; Norsker et al., 2011). In this chapter the energy use for harvesting will be analysed. Harvesting by a single step centrifugation will be compared with a two steps harvesting process with different pre-concentration techniques followed by centrifugation. The pre-concentration techniques that were studied in this thesis, bio-flocculation and autoflocculation combined with gravity sedimentation, will be compared with other pre-concentration techniques such as electro-coagulation-flocculation and chemical flocculation.

7.1. Energy requirement for a single step harvesting

The energy consumption of microalgal production is high and differs for the different cultivation systems used (Norsker et al., 2011). The concentration of the microalgal suspension that is obtained after production is generally low (0.2-5 $gDW^.L^{-1}$). As a consequence a lot of water needs to be removed during harvesting which makes the harvesting energy intensive. The energy needed e.g. for harvesting of microalgae from a 0.3 $gDW^.L^{-1}$ microalgal suspension using a disk stack bowl centrifuge was calculated to be 13.8 $MJ.kgDW^{-1}$ (Norsker et al., 2011). The typical oleaginous microalgae, *Chlorella vulgaris*, with an average lipid content of 30%, has a combustion enthalpy of 26.2 $MJ^.kgDW^{-1}$ (Duboc et al., 1999). The energy requirement of single step harvesting via centrifugation thus requires approximately 50% of the total energy content of the microalgal biomass. In Table 7.1., it is shown that all single step harvesting processes require relatively high amounts of energy when compared with the combustion enthalpy of microalgae. This energy can be reduced considerably if the microalgae are pre-concentrated prior to further dewatering.

Table 7.1. Energy demand for different harvesting techniques for non-flocculated microalgae.

Device	Energy requirement (MJ.kgDW^{-1})*
Disk stack centrifuge	8-16
Decanter	97
Evodos centrifuge	7
Belt filter	5
Vacuum drum filter	71
Filter press	11
Tangential flow membrane filter	37-120

*The data are adapted from Pahl (2013) and initial concentration of microalgal suspension to be harvested is assumed to be approximately 0.3 gDW.L^{-1}. Note that these numbers are extrapolated from different studies in which different initial concentration of the microalgal slurry were used and different concentration factors were achieved.

7.2. Two steps harvesting

Usually pre-concentration is done by flocculation after which the formed flocs are separated by either flotation or sedimentation. During flocculation the microalgal cells aggregate to larger flocs which can be easily separated by sedimentation. There are different ways to make microalgal cells flocculate such as electro-coagulation-flocculation, chemical flocculation and bio-flocculation. Vandamme et al. (2011) reported a power consumption of 5-123 MJ.kgDW^{-1} for *C. vulgaris* using different electro-coagulation-flocculation times at different electrical current density. In this case, the energy consumption is comparable with the energy demand of single step harvesting (Table 7.1.). This makes electro-coagulation-flocculation not a suitable pre-concentration method to replace a single step centrifugation process. For chemical flocculation of *Neochloris oleoabundans*, the energy needed for flocculation was 1.1 and 3.6 MJ.kgDW^{-1} using chitosan and ferric sulphate, respectively (Beach et al., 2012). Considering this relatively low amount of energy needed to pre-concentrate the microalgal cell suspension, chemical flocculation may indeed lead to substantial reduction of the overall energy demand for harvesting. However, the use of inorganic or organic flocculants can lead to the formation of highly porous microalgal flocs and this results in a relatively low final concentration after sedimentation. The low concentration of the microalgal suspension after the pre-concentration step, requires more energy in the post-harvesting step needed for further

dewatering. This illustrates that the concentration factor that is reached in the different pre-concentration techniques affects the energy required for final dewatering of the microalgal suspension. In contrast to chemical flocculation, flocs formed by bio-flocculation as presented in **Chapter 2** are more dense and after sedimentation a higher final concentration of microalgae is reached.

7.3. Experiment to compare the energy reduction of a two steps harvesting using bio-flocculation or chemical flocculation as the pre-concentration step

In Table 7.1. the energy for harvesting microalgae by disk stack centrifuge varies from 8 to 16 $MJ^.kgDW^{-1}$. This variation is caused by the deviation in initial concentrations of microalgae used. In order to be able to make a fair comparison of the overall energy required for harvesting the microalgal cells using chemical flocculation and using bio-flocculation, we decided to do an experiment in which we harvested the microalgae from the same suspension of *C. vulgaris* cells with initial concentration of 0.65 $gDW^.L^{-1}$ and determine the energy needed for final dewatering by centrifugation. The *C. vulgaris* cells were subjected to chemical flocculation as described by Vandamme et al. (2012) and to bio-flocculation with autoflocculating *Ettlia texensis* cells as described by Salim et al.(2012). For chemical flocculation, the *C. vulgaris* suspension was mixed with a Mg^{2+} and Ca^{2+} solution of 1.6 and 0.1 mM respectively and pH was adjusted to 11.5 by addition of 1 M sodium hydroxide to induce chemical flocculation. For bio-flocculation, *E. texensis* cells were added as autoflocculating microalgae to the non-flocculating *C. vulgaris* cells at a ratio of flocculating to non-flocculating microalgae (R_{fnf}) of 0.25 with a total concentration of 0.65 $gDW^.L^{-1}$. Both suspensions were mixed intensively (1000 rpm) for 10 min and then gently (250 rpm) for another 20 min, after which they were allowed to settle for 3 hours to determine the recovery and the final concentration of microalgal biomass.

The energy for harvesting the biomass from the layer of settled cells was calculated based on the method presented in **Chapter 3**. This result was compared with the energy needed for concentrating the microalgal suspension by using centrifugation only. In a two steps

harvesting, the energy demand of a disk stack centrifuge after applying bio-flocculation was calculated to be 0.24 MJ·kgDW^{-1}. Using chemical flocculation, it was 8.85 MJ·kgDW^{-1} (Table 7.2.), while in a single step harvesting, the energy demand of a disk stack centrifuge would be 13.8 MJ·kgDW^{-1}. The recovery when bio-flocculation was used, was similar to the recovery when using chemical flocculation; 39 and 40 %, respectively.

Table 7.2. Energy demand of single step and two steps harvesting techniques for a dilute microalgal suspension

Harvesting technique	Harvesting energy (MJ·kgDW^{-1})	Energy needed for added flocculant (MJ·kgDW^{-1})
Single step (centrifugation)	13.8	0
Two steps (bio-flocculation and sedimentation with centrifugation)	0.24	1.78
Two steps (chemical flocculation and sedimentation with centrifugation)	8.85	N/D*

* Not defined

This energy analysis shows that bio-flocculation results in considerable reduction of the energy needed for further dewatering while this is not the case for the chemical flocculation. However, for comparison of the total energy needed, the energy needed for production of the flocculants should also be accounted for. The extra energy needed which was calculated based on the method presented in **Chapter 3** for production of the autoflocculating microalgae when bio-flocculation is applied as the pre-concentration step is 1.78 MJ·kgDW^{-1} (Table 7.2.). This makes the total energy for harvesting microalgae using bio-flocculation as the pre-concentration step 2 MJ·kgDW^{-1} which is still considerably lower than energy needed for chemical flocculation even without taking the energy needed for production of the chemical flocculants into account. This shows that in comparison with a single step harvesting via centrifugation, an energy reduction of 85% for harvesting can be achieved when a two steps harvesting is applied, using bio-flocculation

combined with sedimentation as the pre-concentration step followed by centrifugation as the post-concentration step.

One of the major disadvantages of using bio-flocculation combined with sedimentation is that it is relatively slower than chemical flocculation. This means higher investment costs due to the need for larger settling tanks. Fortunately, thee sedimentation time needed for settling of the microalgal flocs in a settling tank can be decreased by applying inclined channels, plates or tubes. For example, lamellar settlers have been also used for microalgal harvesting which contain inclined plates to decrease the time needed for sedimentation (Nakamura et al., 2005). Recently a multi-channel, bottom-fed lamellar settler has been tested by Smith and Davis (2013) for harvesting microalgae. They reached 70% faster clarification at an incline angle of 8º in comparison with standard 55 ° and achieved a concentration factor of 80 for non-flocculated microalgae. Bio-flocculation and autoflocculation combined with gravity settling at industrial scale can also be accelerated by applying a multi-channel lamellar settler. The advantages of a multi-channel lamellar settler in comparison with conventional settling tanks are the higher capacity for processing larger volumes of microalgal suspension due to cascading of multi-channels and faster sedimentation due to the shorter sedimentation distance.

7.4. Energy demand of harvesting microalgal cells from a less dilute culture

The initial concentration of microalgal suspension in the prior paragraph was 0.3-0.65 $gDW^.L^{-1}$. These are typical concentrations of a microalgal suspension that are reached in open raceway ponds. The initial concentration of the microalgae is higher when other microalgal cultivation systems are used than raceway ponds (Norsker et al., 2011). In this paragraph, we focus on the energy demand of harvesting microalgae form less dilute cultures. To harvest these less dilute cultures, the amount of energy for harvesting can also be reduced by a two steps harvesting using bio-flocculation as the pre-concentration step.

But the energy reduction of consequent centrifugation will be smaller if the initial concentration of microalgal suspension is higher. For example, the energy consumption of a disk stack centrifuge reduces from 13.8 MJ$\cdot$kgDW^{-1} at 0.3 gDW$\cdot$L^{-1} to 2.2 MJ$\cdot$kgDW^{-1} at 2 gDW$\cdot$L^{-1} initial concentration of microalgal suspension (Norsker et al., 2011). The harvesting energy can be reduced by a two steps harvesting using bio-flocculation followed by centrifugation to 0.04 MJ$\cdot$kgDW^{-1} when the initial concentration of microalgae is 2 gDW$\cdot$L^{-1} (Table 7.3.). However, considering the extra energy needed for production of the autoflocculating microalgae when bio-flocculation is applied as the pre-concentration step (1.78 MJ$\cdot$kgDW^{-1}), it can be concluded that total energy in a two steps harvesting using bio-flocculation as the pre-concentration step is not reduced in comparison with a single step using only centrifugation.

Table 7.3. Energy demand of single step and two steps harvesting for a dense microalgal suspension

Harvesting technique	Harvesting energy (MJ$\cdot$kgDW^{-1})	Energy needed for added flocculant (MJ$\cdot$kgDW^{-1})
Single step (centrifugation)	2.2	0
Two steps (bio-flocculation and sedimentation with centrifugation)	0.04	1.78

It should be mentioned that these calculation are based on the worst case scenario. The reduction of centrifuge energy due to bio-flocculation is underestimated as it is assumed that the size of the flocs remains the same during bio-flocculation. In **Chapters 4** and **6**, we showed that the flocs that are formed have an average diameter of approximately 9 µm while single microalgal cells are around 3.5 µm. The sedimentation rate of the flocs formed by bio-flocculation will be up to 7 times higher than that of single non-flocculating cells based on Stokes' law (**Chapter 6**) which means 7 times more reduction of energy of centrifugation than estimated in Table 7.2. and 7.3. This additional reduction of the energy, however would still be insufficient for harvesting the microalgae from a 2 gDW$\cdot$L^{-1} suspension, as the energy needed to produce the bio-flocculant itself, is simply too high compared with the energy needed for the centrifugation. The energy needed to produce the

bio-flocculant vanishes if autoflocculation could be applied. In that case, the two step harvesting consisting of autoflocculation combined with sedimentation and post-concentration via centrifugation would reduce the energy needed substantially also in case of harvesting the microalgae from a suspension with a higher initial concentration

7.5. Concluding remarks

In this thesis, bio-flocculation and autoflocculation combined with gravity sedimentation have been presented as a promising pre-concentration step for harvesting of microalgae to make sustainable microalgal production feasible. The comparison of the energy use of bio-flocculation and autoflocculation combined with gravity sedimentation with other pre-concentration techniques showed this method is an appropriate technology that requires substantially less energy for harvesting the microalgae. More research is needed in terms of robustness and controllability of this method and to reduce the settling time at industrial scale.

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Summary

Summary

Microalgae are considered a promising resource for different biobased commodities. However, commercial microalgal production is not economically feasible yet. This is mainly due to the high energy inputs required for water pumping, mixing and for harvesting the microalgal biomass. Harvesting in commercial microalgae production plants is generally done by centrifugation, but this requires upto about 50% of the total energy gained from the microalgae. The energy needed for harvesting can be reduced considerably by pre-concentration of the microalgae prior to further dewatering. The focus of this thesis was on development of a controlled pre-concentration step in which bio-flocculation and autoflocculation using oleaginous microalgae is applied combined with gravity sedimentation. This technology was evaluated in terms of energy demand for harvesting microalgae.

Bio-flocculation of non-flocculating oleaginous microalgae with autoflocculating microalgae was presented as a promising pre-concentration step in harvesting of microalgae in **Chapter 2**. Flocculating freshwater microalgae *Ankistrodesmus falcatus* and *Scenedesmus obliquus* and the marine microalga *Tetraselmis suecica* which were tested for harvesting of the non-flocculating freshwater microalga *Chlorella vulgaris* and the marine microalga *Neochloris oleoabundans*. Addition of the flocculating microalgae considerably improved the sedimentation rate and increased the recovery of non-flocculating microalgae. Bio-flocculation enables the harvesting of microalgae without the need to add chemical flocculants or to adjust the medium conditions for growth of the microalgae and therefore permits reuse of the medium without further treatment.

In **Chapter 3**, the effect of the ratio between autoflocculating and target microalgae applied in bio-flocculation was studied with emphasis on the recovery, sedimentation rate and energy demand for harvesting the target microalgae. When the autoflocculating microalgae *Ettlia texensis*, *A. falcatus* and *S. obliquus* were added to *C. vulgaris* at a ratio of 0.25, the recovery of *C. vulgaris* increased from 25% to respectively 40, 36 and 31%. The

sedimentation rate increased as well. Addition of *T. suecica* to *N. oleoabundans* at a ratio of 0.25 increased the recovery from 40% to 50%. Application of bio-flocculation at a ratio of 0.25, followed by centrifugation reduced the energy demand for harvesting of the target microalgae from 13.8 MJ·kgDW^{-1} if only centrifugation is used to 0.24, 0.24, 0.17 and 0.13 MJ·kgDW^{-1} respectively using *T. suecica*, *E. texensis*, *A. falcatus* and *S. obliquus* after 3 hours of combined bio-flocculation and sedimentation prior to final dewatering by centrifugation.

From the different microalgal strains tested, *E. texensis* showed to be the most promising candidate regarding settling and autoflocculation characteristics. It combines these characteristics with relative high growth rate and high lipid content. Therefore, the effect of the growth phase on recovery, sedimentation and autoflocculation behaviour of *E. texensis* was investigated in **Chapter 4** and the lipid content of *E. texensis* was determined during the subsequent growth phases to define the optimum harvesting time of *E. texensis*. The growth phase had a large impact on the recovery time and on the total fatty acids content, as well as on the fatty acids composition. Both batch experiments showed that *E. texensis* should be harvested in the stationary phase. 90% of the cells was recovered after three hours settling in both batch experiments. The total fatty acids content increased to 25 % (w·w^{-1}) in the stationary phase, with high percentage of C18:1 and C16:0. This fatty acid content combined with the autoflocculating properties of *E. texensis* makes it a very suitable candidate for the production of biodiesel.

To reveal the mechanism involved in autoflocculation of *E. texensis*, this microalga was compared with the non-flocculating microalga *C. vulgaris* by scanning electron microscopy (SEM) analysis and by characterisation of the cell surface properties such as the cell surface charge and extracellular polymeric substances (EPS) attached to the cell surface in **Chapter 5**. Furthermore, the possible role of EPS attached to *E. texensis* cells in capturing *C. vulgaris* cells during bio-flocculation was investigated. The SEM analysis and EPS

measurement showed that autoflocculation of *E. texensis* is due to the polymers (EPS) containing mainly glycoproteins patched to the cell surface. Despite the presence of charged groups on the cell surface, they do not seem to attribute to autoflocculation of *E. texensis*. During bio-flocculation of *E. texensis* with *C. vulgaris* fibre-like EPS structures between both microalgal species were observed. EPS thus not only play a predominant role in autoflocculation of *E. texensis* but also in bio-flocculation when using this microalgae to harvest others.

A mathematical model for flocculation and sedimentation was developed and presented in **Chapter 6**. This model predicts the time needed to reach a desired concentration of microalgal suspension and describes the concentration of the microalgal flocs as function of time and position of the particle in a sedimentation tank. This model was validated with experimental data using *E. texensis*. In addition, the model was used to predict the overall effect of flocculation and sedimentation on large scale harvesting of microalgae by calculating the concentration factor and the biomass recovery in a given settling tank. Based on the achieved concentration factor, the energy needed for further dewatering of the microalgae in a centrifuge could be estimated. The changes in concentration which were measured in time at different heights in a sedimentation vessel corresponded well with model predictions. The model predicts that it takes 25 hours to reach a final concentration of 5.2 $gDW^.L^{-1}$ of *E. texensis*, when the initial concentration is 0.26 $gDW^.L^{-1}$ and a sedimentation of 1 m height is used. This final concentration would be high enough to start further dewatering of the settled biomass in case a disk stack bowl centrifuge is used for further dewatering. The energy needed for further dewatering of microalgal suspension of *E. texensis* can be reduced by a factor 20 due to the concentration factor achieved after 25 hours of settling. This example illustrates that the model can be used for the design of settling tanks needed for pre-concentration of microalgal biomass.

In **Chapter 7**, the overall results of this thesis were used to evaluate the effect of autoflocculation and bio-flocculation on the overall energy use of microalgal biodiesel production. The energy needed for pre-concentration of microalgae was calculated. Advantages and disadvantages of bio-flocculation were compared with chemical flocculation. Bio-flocculation and autoflocculation combined with gravity sedimentation have been presented as a promising pre-concentration step for harvesting of microalgae to make sustainable microalgal biodiesel production feasible. Although the comparison of the energy use of bio-flocculation and autoflocculation combined with gravity sedimentation with other pre-concentration techniques showed this method is an appropriate technology that requires substantially less energy for harvesting the microalgae, but more research is needed in terms of robustness and controllability of this method and to reduce the settling time at industrial scale.

Samenvatting

Microalgen worden beschouwd als een veelbelovende bron voor verschillende biobased producten, maar commerciële productie van microalgen is nog niet economisch haalbaar. Dit is vooral te wijten aan het hoge energieverbruik; nodig voor pompen van water, mengen en voor het oogsten van de microgen. Momenteel gebeurt het oogsten van microalgen hoofdzakelijk in een centrifuge, maar dit vereist ongeveer 50% van de totale energie die een microalg bevat. Daarom is het nodig om het energieverbruik van het oogsten van microalgen te minimaliseren. Dat kan door een geïntegreerde multi-stappen benadering toe te passen. In een eerste verdikkingstap die relatief weinig energie kost, kan de initiële concentratie van microalgen aanzienlijk verhoogd worden, voordat er een verdere scheiding van de microalgen van het water plaats vindt. Hoe hoger de concentratie factor is die je bereikt tijdens de verdikkingsstap, des te minder energie je nodig hebt om het laatste water uit de microalgensuspensie te verwijderen. Maar de concentratie factor is niet het enige vereiste voor een efficiënte verdikking. Het is ook belangrijk om een hoog opbrengst aan microalgen te bereiken tijdens de verdikkingsstap. In dit proefschrift, een gecontroleerde pre-concentratie stap voor het oogsten van microalgen is onderzocht waarin bio-flocculatie en autoflocculatie met oliehoudende microalgen wordt toegepast in combinatie met sedimentatie. Het energie verbruik van deze technologie wordt ook geëvalueerd.

Bio-flocculatie van niet-vlokvormende oliehoudende microalgen met autoflocculerende microalgen is geïntroduceerd als een veelbelovende pre-concentratie in het oogsten van microalgen in **Hoofdstuk 2.** Autoflocculerende zoetwater microalgen *Ankistrodesmus falcatus* en *Scenedesmus obliquus* en de marine microalg *Tetraselmis suecica* zijn gebruikt voor het oogsten van de niet-vlokvormende zoetwater microalg *Chlorella vulgaris* en de marine microalg *Neochloris oleoabundans*. Deze autoflocculerende microalgen groeien onder dezelfde omstandigheden als de te oogsten niet-vlokvormende microalgen en er hoeft dus geen extra kweekmedium of extra chemicaliën te worden toegevoegd om de flocculatie te initiëren. Dit maakt het hergebruik van het medium mogelijk zonder verdere behandeling van het medium na flocculatie. Toevoeging van de autoflocculerende microalgen verbetert de bezinkingssnelheid van de niet-vlokvormende microalgen aanzienlijk en bovendien is de opbrengst aan niet-vlokkende microalgen hoger.

In **hoofdstuk 3** wordt het effect van de ratio autoflocculerende en niet-vlokvormende microalgen in bio-flocculatie bestudeerd met de nadruk op de opbrengst, de sedimentatiesnelheid en de nodige energie voor het oogsten van niet-vlokvormende microalg. Toevoeging van de autoflocculerende microalgen *Ettlia texensis, A. falcatus* en *S. obliquus* aan *C. vulgaris* bij 0,25 ratio verhoogt de opbrengst van *C. vulgaris* van 25% tot respectievelijk 40, 36 en 31 %. Toevoeging van *T. suecica* aan *N. oleoabundans* bij een ratio van 0,25 verhoogt de opbrengst van 40% naar 50 %. Toepassing van bio-flocculatie bij een ratio van 0,25, gevolgd door centrifugeren vermindert de energie voor het oogsten van de niet-vlokvormende microalg van 13,8 $MJ^.kgDW^{-1}$ wanneer alleen centrifuge wordt gebruikt tot 0,24 , 0,24 , 0,17 en 0,13 $MJ^.kgDW^{-1}$ respectievelijk met *T. suecica, E. texensis, A. falcatus* en *S. obliquus* na 3 uur van gecombineerde bio-flocculatie en sedimentatie vóór definitieve ontwatering.

Van de verschillende microalgen die wij hebben bestudeerd, heeft *E. texensis* beste autflocculatie en bezinking eigenschappen. De combinatie van deze eigenschappen met relatief hoge groeisnelheid en hoge vetgehalte maakt deze microalg interessant voor de biodiesel productie. Het effect van verschillende groeifases op de opbrengst, de sedimentatiesnelheid en autoflocculatie gedrag van *E. texensis* is onderzocht in **Hoofdstuk 4** en het vetgehalte van *E. texensis* is gemeten in verschillende groeifases om de optimale oogsttijdstip van *E. texensis* te kunnen bepalen. De groeifase heeft een grote invloed op opbrengst en op het totale vetzuren gehalte, evenals op de vetzuren samenstelling. Beide batch experimenten laten zien dat *E. texensis* moet worden geoogst in de stationaire fase. 90% van de cellen zijn geoogst na drie uur in beide batch experimenten. Het totale gehalte aan vetzuren is verhoogd tot 25 % ($w^.w^{-1}$) in de stationaire fase, met een hoog percentage van C18:1 en C16:0. Deze vetzuren gecombineerd met de autoflocculatie eigenschappen van *E. texensis* maken deze microalg een zeer geschikte kandidaat voor de productie van biodiesel.

Om het mechanisme achter de autoflocculatie van *E. texensis* te ontrafelen, is deze microalg vergeleken met de niet-vlokvormende microalg *C. vulgaris* met behulp van scanning elektronen microscopie (SEM) analyse en karakterisering van de cel oppervlakte-eigenschappen zoals de lading van het cel oppervlak en extracellulaire polymere substanties (EPS) verbonden aan het cel oppervlak in **Hoofdstuk 5.** Bovendien is de mogelijke rol van de EPS bij bio-flocculatie van *C. vulgaris* cellen met *E. texensis* cellen onderzocht. De SEM analyse en EPS-meting laat zien dat autoflocculatie van *E. texensis* is te wijten aan de polymeren (EPS) verbonden aan het cel oppervlak met voornamelijk glycoproteïnen. Ondanks de aanwezigheid van geladen groepen op het cel oppervlak, spelen ze geen rol in autoflocculatie van *E. texensis*. Tijdens bio-flocculatie van *E. texensis* met *C. vulgaris* zijn vezelachtige EPS structuren tussen beide microalgen waargenomen. EPS spelen dus niet alleen een dominante rol in autoflocculatie van *E. texensis* maar ook in bio-flocculatie bij gebruik van deze microalg voor het oogsten van andere microalgen.

Een wiskundig model voor flocculatie en sedimentatie is ontwikkeld en gepresenteerd in **Hoofdstuk 6.** Dit model voorspelt de tijd die nodig is om te komen tot een gewenste concentratie van microalgensuspensie en rekent de concentratie van de microalgen vlokken als functie van tijd en plaats in een bezinktank uit. Dit model is gevalideerd met gegevens uit experimenten met *E. texensis*. Bovendien, wordt het model gebruikt voor het voorspellen van het algehele effect van flocculatie en sedimentatie op grote schaal oogsten van microalgen door de concentratiefactor en de opbrengst te berekenen in een bepaalde bezinktank. Op basis van de gerealiseerde concentratiefactor, wordt de energie die nodig is voor verdere ontwatering van de microalg in een centrifuge geschat. De veranderingen in concentraties die zijn gemeten in de tijd op verschillende hoogtes komen goed overeen met de modelvoorspellingen. Het model voorspelt dat het 25 uur duurt om een uiteindelijke concentratie van 5,2 gDW$^.$L^{-1} van *E. texensis* te bereiken wanneer de initiële concentratie 0,26 gDW$^.$L^{-1} is en een bezinkingstank van 1 m hoogte wordt gebruikt. De energie die nodig is voor verdere ontwatering van microalgensuspensie van *E. texensis* kan worden gereduceerd met een factor 20 met de bereikte concentratie factor na 25 uur bezinking. Dit

voorbeeld illustreert dat het model kan worden gebruikt voor het ontwerp van bezinkingstanks nodig voor pre-concentratie van microalgen.

In **hoofdstuk 7** zijn de resultaten van dit proefschrift gebruikt voor de evaluatie van het effect van autoflocculatie en bio-flocculatie op het totale energiegebruik van microalgen biodiesel productie. De energie die nodig is voor pre-concentratie van microalgen is berekend. Voor- en nadelen van bio-flocculatie zijn vergeleken met chemische flocculatie. Bio-flocculatie en autoflocculatie gecombineerd met sedimentatie zijn gepresenteerd als een veelbelovende pre-concentratie stap voor het oogsten van microalgen voor een duurzame microalgen biodiesel productie. Hoewel de vergelijking van het energieverbruik van bio-flocculatie en autoflocculatie gecombineerd met sedimentatie met andere pre-concentratie technieken laat zien dat deze methode een geschikte technologie is die aanzienlijk minder energie nodig heeft voor het oogsten van de microalgen, maar er is meer onderzoek nodig op het gebied van robuustheid en controleerbaarheid van deze methode en het verminderen van de bezinkingtijd op industriële schaal.

چکیده فارسی

در حال حاضر جداسازی ریز جلبک های تک سلولی از محیط کشت بوسیله سانتریفیوژ انجام می شود. در این فرآیند تا حدود پنجاه درصد از کل انرژی موجود ریز جلبک های تک سلولی هدر میرود.

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About the author

Curriculum Vitae

Sina Salim was born on April the 22nd 1984 in Esfahan, Iran. After graduating from high school (Special Talent Center, Sampad, Esfahan, Iran) in 2001, he started studying B.Sc. Biotechnology in 2002 at Wageningen University, The Netherlands. During his bachelor thesis, he developed a mathematical model which resulted in co-authorship of a paper on capturing sunlight into a photobioreactor for cultivation of microalgae. Afterwards, Sina continued his M.Sc. Bioprocess Engineering at Wageningen university. During his master thesis at Bioprocess Engineering group of Wageningen University, he developed a mathematical model which describes the transport phenomena in fungal aerial mycelia. Sina left to Berkeley, California in 2006 for his internship at the Blanch Lab (UC Berkeley) which resulted in co-authorship of a paper on different approaches to enhance cultivability of bacteria associated with marine sponges. In 2007, he graduated cum laude in Bioprocess Engineering. Afterwards, he worked for a year on a project at Bioprocess Engineering group of Wageningen University entitled "Bio-alcohol production from syngas" which resulted in co-authorship of a book chapter on transportation biofuels. Sina started his PhD at Bioprocess Engineering group of Wageningen University in 2008. The results of his PhD research are presented in this thesis. Sina is working for the Dutch Democratic Party (D66) since 2010 as councilor and party chairman in the city council of Ede. He is also active member of the theme group sustainability and since 2012 he has been appointed as an expert in sustainable energies in the expert board of the online platform "Nederland Krijgt Nieuwe Energie".

List of publications

Zijffers, J-W.F., Salim, S., Janssen, M., Tramper, J., Wijffels, R.H., 2008. Capturing sunlight into a photobioreactor: Ray tracing simulations of the propagation of light from capture to distribution into the reactor. Chem. Eng. J. 145 (2), 316-327.

Hoogendoorn, A., van Kasteren, H., 2011. Transportation Biofuels: Novel Pathways for the Production of Ethanol, Biogas and Biodiesel (Bio-Alcohol production from Syngas). ISBN: 978-1-84973-043-3.

Salim, S., Bosma, R., Vermuë, M.H., Wijffels, R.H., 2011. Harvesting of microalgae by bio-flocculation. J. Appl. Phycol. 23, 849-855.

Sipkema, D., Schippers, K.J., Maalcke, W.J., Yang, Y., Salim, S., Blanch, H.W., 2011. Multiple Approaches to Enhance the Cultivability of Bacteria associated with the Marine Sponge *Haliclona* (?*gellius*) sp. App. Environ. Microbiol. 77(6), 2130-2140.

Salim, S., Vermuë, M.H., Wijffels, R.H., 2012. Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. Bioresour. Technol. 118, 49-55.

Salim, S., Shi, Z., Vermuë, M. H., Wijffels, R. H., 2013. Effect of growth phase on harvesting characteristics, autoflocculation and lipid content of *Ettlia texensis* for microalgal biodiesel production. Bioresour. Technol. 138, 214-221.

Salim, S., Gilissen, L., Rinzema, A., Vermuë, M.H., Wijffels, R.H., 2013. Modeling micoralgl flocculation and sedimentation. Bioresour. Technol. http://dx.doi.org/10.1016/j.biortech.2013.07.026.

Salim, S., Kosterink, N.N., Tchetkoua Wacka, N.D., Vermuë, M.H., Wijffels, R.H., 2013. Extracellular polymeric substances; the key factor in autoflocculation of *Ettlia texensis*. (submitted).

Salim, S., Vermuë, M. H., Wijffels, R. H., 2013. Energy requirement for harvesting microalgae. (submitted).

Overview of completed training activities

Discipline specific activities

International Algae Congress[2] (Amsterdam , The Netherlands, 2008)

NPS-9[1] (Veldhoven, The Netherlands, 2009)

Mini-symposium Current work in Algal Biotechnology[2] (San Diego, USA, 2010)

NBC-13[2] (Ede, The Netherlands, 2010)

NBC-14[2] (Ede, The Netherlands, 2012)

Young Algaeneers symposium[2] (Wageningen, TheNetherlands, 2012)

1st international symposium about microalgae biotechnology for young researchers[1] (Almeria, Spain, 2012)

Algae biomass summit[1] (Denver, USA, 2012)

General courses

PhD week VLAG (Veldhoven, The Netherlands, 2009)

Teaching and supervising thesis students (Wageningen, The Netherlands, 2009)

Scientific Writing (Wageningen, The Netherlands, 2009)

Career Assessment (Wageningen, The Netherlands, 2012)

Optionals

PhD domestic excursion Intervet (Boxmeer, The Netherlands, 2008)

PhD foreign excursion to Japan[1] (2008)

Brainstormday BioProcess Engineering (2008, 2009[1], 2010, 2011[1], 2012[1])

Simulation with Super Pro Design (INTELLIGEN Inc.) (Antwerp, Belgium, 2009)

Wetsus Internal Congress[1] (2009, 2011)

UTEX Algal Workshop (Austin, USA, 2010)

Algen Symposium[1] (Alkmaar, The Netherlands, 2010)

Algae mini-symposium[1] (Wageningen, The Netherlands, 2010)

PhD foreign excursion to USA[1,3] (2010)

Process Economics and Cost Engineering (OSPT) (Enschede, The Netherlands, 2010)

PhD foreign excursion to Spain[1] (2012)

Last Stretch of the PhD workshop (Wageningen, The Netherlands, 2013)

Mini-symposium Biorefinery[1] (Wageningen, The Netherlands, 2013)

[1]Presentation

[2]Poster

[3]Organization