Inhibition of alkaline flocculation by algal organic matter for *Chlorella vulgaris*

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Abstract

Alkaline flocculation is a promising strategy for the concentration of microalgae for bulk biomass production. However, previous studies have shown that biological changes during the cultivation negatively affect flocculation efficiency. The influence of changes in cell properties and in the quality and composition of algal organic matter (AOM) were studied using *Chlorella vulgaris* as a model species. In batch cultivation, flocculation was increasingly inhibited over time and mainly influenced by changes in medium composition, rather than biological changes at the cell surface. Total carbohydrate content of the organic matter fraction sized bigger than 3 kDa increased over time and this fraction was shown to be mainly responsible for the inhibition of alkaline flocculation. The monosaccharide identification of this fraction mainly showed the presence of neutral and anionic monosaccharides. The addition of 30–50 mg L⁻¹ alginic acid, as a model for anionic carbohydrate polymers containing uronic acids, resulted in a complete inhibition of flocculation. These results suggest that inhibition of alkaline flocculation was caused by interaction of anionic polysaccharides leading to an increased flocculant demand over time.

Keywords: extracellular polymeric substances; dissolved organic matter; coagulation; harvesting; microalgae; biofuels

Introduction

Microalgae are a promising new source of biomass that can complement agricultural biomass for the production of food, feed, energy, or feed stocks for the chemical industry (Wijffels and Barbosa, 2010). However, large-scale production of microalgae is commercially hampered by inefficiencies in the harvesting process and the sustainable recycling of water and nutrients (Beuckels et al., 2015; Chisti, 2013; Markou et al., 2014). The energy required for harvesting represents a substantial part of the total energy demand of the production process. Because of the small size of microalgal cells (3–20 μm) and their low concentration in culture (0.5–5 g L^{-1}), the use of energy-intensive centrifugation or filtration processes for direct harvesting makes the production of commodity biomass economically unfeasible (Greenwell et al., 2010; Milledge and Heaven, 2012; Vandamme et al., 2013). The energy demand for dewatering using centrifugation or filtration could be significantly reduced if individual microalgal cells could be grouped into larger aggregates by means of flocculation (Molina Grima et al., 2003; Sheehan et al., 1998; Vandamme et al., 2013). Although flocculation has been widely used for decades in several industries such as mining or wastewater treatment, existing flocculation methods cannot simply be implemented in the microalgae harvesting process because they are either too expensive or result in toxic contamination of the biomass (e.g., by metal coagulants or synthetic polymer flocculants).

One flocculation technology that holds potential for application in microalgae harvesting is alkaline flocculation (Schlesinger et al., 2012; Vandamme et al., 2012a). When the pH of the medium exceeds 10.5, magnesium precipitates as magnesium hydroxide. These hydroxides are positively charged up to pH 12 and interact with the negative surface charge of microalgal cells (Hadjoudja et al., 2010). As such, magnesium hydroxides cause flocculation by charge neutralization and/or by a sweeping mechanism. Due to the high specific density of the magnesium hydroxide minerals, this flocculation mechanism has also been referred to as

ballasted flocculation (Besson and Guiraud, 2013). Magnesium hydroxide flocculation is cheap in comparison to other flocculation methods. Because the concentration of magnesium in most waters is sufficiently high for alkaline flocculation to occur, the only cost involved is that of the base needed to increase the pH. Magnesium hydroxide is a non-toxic material and, therefore, it does not contaminate the harvested biomass. We recently showed that magnesium hydroxide can be recycled from the microalgal biomass after harvesting by simple re-acidification (Vandamme et al., 2015).

When evaluating the potential of flocculation for harvesting microalgae, it is important to understand which parameters influence the dosage of flocculant required to achieve flocculation. In case of alkaline flocculation, the flocculant dose corresponds to the amount of base that is added to achieve a flocculation efficiency of 90% (García-Pérez et al., 2014). However, this dosage depends on the biomass concentration and on the culture age and phase. As a culture progresses through the exponential phase and the biomass concentration increases, the required flocculant dose increases (e.g., Wu et al., 2012). This has important implications for microalgal biofuel production because stationary phase cultivation is often required to increase the lipid content in the microalgal biomass (Laurens et al., 2014).

Because microalgae are living organisms, not only the biomass concentration, but also its properties change as the culture goes from exponential to stationary phase. We previously showed that also for alkaline flocculation, the required flocculant dosage increases with the microalgal biomass concentration over the course of cultivation (García-Pérez et al., 2014). This may be due to changes in cell surface properties. For instance, the cell surface charge of the microalga *Chlorella* differs between the exponential and stationary growth phase, and these changes have an influence on the flocculant dosage (Zhang et al., 2012). These changes in surface properties may be related to the fact that *Chlorella* forms a protective layer at the outer cell wall during late cultivation stages (Gerken et al., 2013). Microalgae also excrete

large amounts of dissolved organic matter into the culture medium, often referred to as algal organic matter or AOM (Lewin, 1956). The concentration of AOM in the culture medium gradually increases with culture age and can reach up to 60–70 mg L⁻¹ C in photobioreactor systems with high biomass concentrations, corresponding to 17% of the total fixed carbon (Hulatt and Thomas, 2010). AOM is composed of a wide range of compounds such as neutral and charged polysaccharides, proteins, nucleic acids, lipids, and small molecules, and has been shown to differ between species and cultivation conditions (Henderson et al., 2008; Nicolau et al., 2015; Villacorte et al., 2015). AOM interferes with flocculation and its presence results in a strong increase in flocculant demand (Chen et al., 2008; Vandamme et al., 2012b). However, the mechanisms by which AOM inhibits flocculation or the nature of the organic compounds that are responsible for this effect on flocculation are unclear. AOM interference with flocculation has been studied in the context of drinking water production. AOM concentrations in algal cultures are typically an order of magnitude higher than in drinking water production systems (~60 mg C L⁻¹ vs. 0.1–1.5 mg C L⁻¹), which enhances any inhibitory effect and contributes to the cost of biomass production. It was found that large polysaccharides (Henderson et al., 2010), some uronic acids (Bernhardt et al., 1989), and also proteins (Pivokonsky et al., 2012) can all interfere with flocculation. At this point, not much information is available on AOM composition or how it may affect alkaline flocculation.

Therefore, the goal of this study was to investigate how the efficiency of alkaline flocculation changes with culture age in the model microalga *Chlorella vulgaris*. In addition, we wanted to investigate to what extent such changes in flocculation efficiency are due to changes in cell properties or in the quantity and composition of AOM in the culture medium. We did an initial characterization of isolated AOM and, using a combination of model compounds, we investigated which components of the AOM contributed the most to flocculation inhibition.

Materials and methods

Cultivation and culture monitoring of Chlorella vulgaris

The strain *Chlorella vulgaris* SAG 211-11b was used as a model species. *Chlorella* was cultivated in a 30-L bubble column photobioreactor (20 cm diameter) to provide a sufficient amount of culture for flocculation experiments. The medium was based on Wright's cryptophyte (WC) medium prepared in dechlorinated demineralized water (Vandamme et al., 2012a). The magnesium concentration in the medium was increased to 2 mM to ensure that alkaline flocculation was not limited by the presence of magnesium. The reactor was sparged with 0.2- μm -filtered air (5 L min⁻¹). The pH was controlled at pH 8 by addition of pure CO₂. The culture was monitored for 14 days and 2 L of culture was removed on days 3, 5, 7, 9, 10, 12, and 14 to conduct flocculation experiments.

The biomass concentration in the culture was monitored daily by measuring absorbance at 750 nm. On each sampling occasion, the biomass concentration was also determined gravimetrically by filtering a known volume of culture on a pre-weighed GF/F filter (Moheimani and Borowitzka, 2013). The concentration of dissolved organic carbon in the culture medium was measured on each sampling occasion using a Shimadzu TOC analyzer. Inorganic carbon was removed prior to analysis by acidification to pH 2 and sparging with oxygen. The concentration of dissolved carbohydrates was measured using the phenol-sulphuric acid method with glucose as standard (Dubois et al., 1956).

At day 7 and 14, biomass samples were collected, centrifuged (4,000 $\times$ g; 15 min), freeze-dried, and stored at -80°C. Fatty acid methyl ester (FAME) content was analyzed using a direct transesterification method (Cho et al., 2013). In short, 2 mL of extraction reagent (chloroform:methanol 2:1) was added to 5 mg of freeze-dried microalgae and vortexed for 10

min, followed by addition of 1 mL of chloroform including icosanoic acid (C20:0) as an internal standard. Additionally, 1 mL of methanol and 300 μ L of H₂SO₄ was added and the mixture was vortexed for 5 min and heated at 100°C for 10 min in a water bath. After cooling down, samples were centrifuged (8,500 $\times$ g; 10 min) followed by separation and drying (using Na₂SO₄) of the organic phase. The extract was dried down using N₂ and re-dissolved in hexane. The obtained FAMES were separated by gas chromatography (GC) with cold on-column injection and flame ionisation detection (FID) (Thermo Scientific Trace GC Ultra) using a Grace EC Wax column (length, 30 m; ID, 0.32 mm; film, 0.25 μ m). The used time-temperature program was 70–180°C (5°C/min), 180–235°C (2°C/min), and 235°C (9.5 min). Fatty acids were identified using standards containing a total of 35 different FAMES (Nu-check, Elysian USA). Peak areas were quantified and normalized against an internal standard with Chromcard software (Interscience).

Differentiating cellular effects from medium property effects

Flocculation was investigated at different stages during batch cultivation to test whether the dosage of flocculant changed during transition from exponential to stationary phase. In the case of alkaline flocculation, the flocculant dosage corresponds to the amount of base that is added to achieve a flocculation efficiency of 95% (García-Pérez et al., 2014). To avoid that the base dosage is influenced by changes in biomass concentration during different cultivation phases, the biomass concentration was standardized in all experiments to 300 mg L⁻¹. To distinguish between changes in cell properties and changes in AOM in the medium, we separated the microalgal cells from the cultivation medium containing the AOM using centrifugation (4,000 $\times$ g, 15 min.). Flocculation was evaluated for three different scenarios. In a first treatment ('medium + cells'), microalgae were harvested at different time points (days 3, 6, 9, 12, and 14) and resuspended in their supernatant at a fixed biomass concentration (300 mg L⁻¹) to investigate how both changes in cell properties and medium

composition influence the dosage of base required to induce alkaline flocculation. In a second treatment ('cells'), the cells were harvested at different time points (days 3, 6, 9, 12, and 14) and resuspended in fresh WC medium to investigate the flocculation behavior of cells with different properties in standardized medium. For the third treatment ('medium'), the cell-free supernatant containing AOM was collected at different time points (days 3, 6, 7, 10, 12, and 14) as described above and stored in a refrigerator at 4°C for a maximum of 12 days.

Chlorella cells, harvested using centrifugation ($4,000 \times g$; 15 min) from a culture in exponential growth (day 7), were resuspended in the collected supernatant to evaluate the effect of different concentrations and composition of AOM on the flocculation behavior of standardized cells.

The effect of AOM size and composition on flocculation was studied by fractionation using cross-flow membrane filtration with a pore size of 3 kDa. At day 3, 7, and 14, 3 L of *Chlorella* culture was harvested using centrifugation ($4,000 \times g$, 15 min) and filtered. The permeate at day 14 was used to evaluate the effect of AOM size on flocculation. *Chlorella* was resuspended in the permeate (containing AOM < 3 kDa) of that same day. Flocculation behavior in these samples was evaluated versus resuspended *Chlorella* cells in their original medium (containing all AOM). The retentate at day 3, 7, and 14 (containing AOM > 3 kDa) was dialyzed with demineralized water until a constant conductivity of $100 \mu\text{S cm}^{-1}$ was achieved. Retentate samples were stored at -20°C prior to analysis. Total carbohydrate levels were analyzed using the phenol-sulphuric acid method with glucose as standard (Dubois et al., 1956) and protein levels using a Lowry assay with bovine albumin as standard (Lowry et al., 1951). The monosaccharide composition of isolated AOM from the cultures was studied after hydrolysis of the large MW retentate fraction. A total of 1 mL of the retentate was dried down using N_2 prior to hydrolysis with 1 mL 4 N TFA at 100°C for 4 h (De Ruiter et al., 1992). The treatment yielded a partial hydrolysis of the residue and the resulting monosaccharides were derivatized with 30 μL N,O-Bistrifluoroacetamide (BSTFA) + 30 μL

Trimethylsilyl chloride (TMCS) at 60°C for 120 min, followed by addition of 200 µL acetonitrile [NREL, unpublished procedure]. The silylated monosaccharides were identified by GC-MS (Agilent GC 7890A – Agilent MS 597C inert XL HSD) using the following analysis parameters: HP5 MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent), 1 µL injected, inlet at 280°C, running at constant He flow of 1 mL/min. The oven ramp program (total of 71 min) was developed for optimal resolution of the anomeric silylated monosaccharide derivatives and can be summarized as follows: 70°C for 3 min; 3°C/min to 160°C, hold for 3 min; 1.7°C/min to 175°C; 70°C/min to 130°C; 70°C/min to 195°C; 70°C/min to 165°C, hold for 1 min; 3°C/min to 174°C, hold for 2 min; 5°C/min to 200°C, hold for 3 min; and finally 10°C/min to 300°C. Integration and peak area calculations were carried out using Agilent Chemstation E.02.02 for GCMS.

Flocculation experiments

For each treatment, a series of jar test experiments was carried out to determine the optimal required dosage of base for induction of alkaline flocculation. In each jar test, a specific dose of sodium hydroxide (0.5 M) was added to a beaker containing a 100-mL microalgal suspension with a concentration of 300 mg L⁻¹. The microalgal suspension was intensively mixed (500 rpm) for 10 min during the addition of sodium hydroxide, which was followed by gentler mixing (250 rpm) for an additional 20 min. The pH was monitored for every jar test. Subsequently, the suspension was allowed to settle for 30 min. In order to estimate the flocculation efficiency η_a , a sample (3.5 mL) was collected in the middle of the clarified zone and absorbance was measured at 750 nm. The flocculation efficiency η_a was calculated according to Eq. 1:

$$\eta_a = \frac{OD_i - OD_f}{OD_i} \quad (\text{Eq. 1})$$

where OD_i is the optical density of the control suspension after 30 min sedimentation without pH adjustment, and OD_f is the optical density of the suspension after pH adjustment.

To accurately determine the required optimal dosage of sodium hydroxide for flocculation induction, 9 arbitrarily chosen doses were first added to roughly estimate the dosage at which flocculation was efficient. Subsequently, 9 additional doses were selected that were close to the inflection point.

Influence of model organic compounds on flocculation

Adipic acid, albumin, alginic acid, galacturonic acid, gluconic acid, glucose, and glycine (Sigma Aldrich) were selected as model compounds to test the influence of organic compounds on flocculation of *Chlorella*. The cells were harvested at day 7 and resuspended in fresh medium at a constant biomass concentration (300 mg L^{-1}) as previously described. The model compounds were added at a concentration of 50 mg L^{-1} during the mixing step, prior to the addition of sodium hydroxide. The effects of 10 and 30 mg L^{-1} alginic acid were also evaluated. Flocculation efficiency was determined as previously described and compared with two additional control treatments (*Chlorella* resuspended in fresh and in original medium).

Statistical analysis

An empirical fit of all dose-response curves obtained in the flocculation experiments was done using a 3-parameter logistic model based on item response theory (IRT):

$$P(\theta) = c + \frac{(1-c)}{1 + \exp[-1.7a(\theta - b)]} \quad (\text{Eq. 2})$$

where θ is the unbounded variable which deals with ability, a is the slope of the curve at the point of inflection, b is the difficulty parameter of determining dosage at the point of inflection and c is the lower dosage parameter (Harris, 1989). All 3 parameters are derived

from the empirical dose-response data with a 95% confidence interval. The model was then used to calculate the dose sodium hydroxide (mM) needed to achieve 95% flocculation efficiency (D[95%]). The model equation was implemented in the symbolic computation MAPLE software package (Maplesoft, 2014).

Results and discussion

Influence of cell age and medium composition on alkaline flocculation

The biomass concentration in the batch culture of *Chlorella* increased rapidly between day 1 and day 5 with a specific growth rate of 0.5 day^{-1} (Fig. 1). Between day 7 and day 14, the biomass concentration continued to increase but at a lower rate (specific growth rate 0.04 day^{-1}). The final dry weight biomass concentration on day 14 was 0.47 g L^{-1} . The lipid content of the biomass (as estimated from the sum of all fatty acid methyl esters) was 19% on day 7 and increased to 34% on day 14, suggesting that culture progression is accompanied by significant metabolic rearrangements, in this case resulting in an increase in lipid content. This phenomenon has been reported before for *Chlorella* cultures (e.g., Laurens et al., 2014). The total organic carbon concentration in the culture medium increased from 9.3 mg C L^{-1} at the start, to 20 mg C L^{-1} on day 10, and remained constant afterwards. The total amount of dissolved carbohydrates in the culture medium was lower than 1.5 mg C L^{-1} at day 5, and increased to 4.5 mg C L^{-1} at day 14 (Fig. 1).

In the treatment ‘cells + medium’, *Chlorella* cells that were harvested during different cultivation phases were resuspended in their original medium at a standardized biomass concentration (Fig. 2A). Despite the fact that the biomass concentration was identical for each cultivation phase in this experiment, the required dose of sodium hydroxide to achieve a flocculation efficiency of 95% increased with the cultivation time. On day 3, only 1.27 mM of

sodium hydroxide was required to induce flocculation at pH 10.7. At day 6, this was 1.96 mM at pH 10.9, while on day 14 the dosage increased more than threefold to 4.12 mM to obtain flocculation at pH 11.1. To evaluate whether this increase in flocculant demand per unit of biomass was due to changes in cell properties, cells harvested during different cultivation phases were resuspended in fresh WC medium (treatment ‘cells’, Fig. 2B). In this freshly prepared medium lacking AOM, the dose of sodium hydroxide required to achieve a flocculation efficiency of 95% at pH 10.4 was only 0.55 mM at day 14. Nevertheless, there was an increase in dosage in function of cultivation time from 0.21 mM on day 3 to 0.41 mM on day 6 and 0.55 mM on day 14. In the ‘medium’ treatment, *Chlorella* cells that were harvested from an exponential phase culture were resuspended in cell-free medium that had accumulated AOM and that was collected from batch cultures during different cultivation phases. In this treatment, the dose of sodium hydroxide required to achieve a flocculation efficiency of 95% was more than three times higher than in the ‘cells’ treatment. The dose doubled from 1.61 mM on day 3 to 3.3 mM on day 14 (Fig. 2C). In comparison, when the same cells were resuspended in fresh culture medium lacking AOM, the dose of base required for flocculation was only 0.78 mM.

These results show that when *Chlorella* cells were resuspended in their own spent medium, 4.2 to 13.7 mmol sodium hydroxide was required to flocculate 1 g of dry weight *Chlorella* biomass. When the same cells were resuspended in fresh medium lacking AOM, however, the dosage of sodium hydroxide was only 0.7 to 1.8 mmol g⁻¹ *Chlorella* biomass, which is 6–7 times less. This implies that only a small fraction of the added sodium hydroxide to actually used to induce flocculation of the microalgal cells, while the majority appeared to be wasted on the AOM present in the culture medium. This indicates that the dose of base required to induce alkaline flocculation depends to a large extent on the quantity and/or composition of AOM in the culture medium, while the influence of the amount and properties of the microalgal cells is limited. We next monitored the pH as function of sodium hydroxide

dose in order to study the potential buffering capacity of AOM. The pH increase as function of NaOH concentration was higher when AOM was present (cells + medium) at days 3, 6, and 14 (Fig. 3). In addition, the final pH noted at the optimum flocculation point increased as function of cultivation time when AOM was present (Fig. 2). Potentially, the AOM chelating properties limited the availability of magnesium for precipitation (Kaplan et al., 1987).

Because precipitation of magnesium hydroxide is a function of the product of the available magnesium and the hydroxide concentration, a decrease in Mg because of chelation will result in an increase in the OH concentration that is required to induce precipitation. However, further research is needed to prove this hypothesis by linking the molecular composition and potential chelating properties of AOM to the inhibition of alkaline flocculation observed in this study. Between day 7 and day 14, the lipid content of the biomass increased by 79%, but the amount of sodium hydroxide required to flocculate the same quantity of biomass more than doubled from 6.66 to 13.73 mmol g⁻¹ biomass. This has important financial implications in the context of harvesting microalgae biomass for biofuels, because an increase in lipid content, contributing to an increase in biomass feedstock value, will be partly offset by an increase in the required base dosage and, consequently, in harvesting cost.

Influence of model organic compounds on alkaline flocculation

The AOM produced by microalgae is thought to be a complex mixture of polysaccharides, proteins, lipids, and small molecules (Henderson et al., 2008; Hoyer et al., 1985; Villacorte et al., 2015). To test which of these biochemical compounds are responsible for interfering with alkaline flocculation, we studied the flocculation of *Chlorella* cells in fresh WC medium to which different organic compounds were added. These model compounds included a protein (albumin), a free amino acid (glycine), a monosaccharide (glucose), two uronic acids (gluconic acid, galacturonic acid), an organic acid (adipic acid) and an anionic polysaccharide (alginic acid), and were added at a relatively high

concentration (Suppl. Fig 1; 50 mg L⁻¹). Of all the compounds tested, only alginic acid had a strong inhibitory effect on flocculation. Addition of gluconic acid resulted in flocculation inhibition as well, but only at sodium hydroxide concentration higher than 2 mM, potentially by the formation of magnesium gluconate at higher pH (Sawyer, 1964). No flocculation could be induced in medium that contained 50 mg L⁻¹ alginic acid, even when the sodium hydroxide concentration was higher than 8 mM (Table 1). When the alginic acid concentration was reduced to 30 mg L⁻¹, the sodium hydroxide dosage needed was 5.6 mM, which is more than twice as much as in control medium containing AOM (2.58 mM). When the concentration of alginic acid was further reduced to 10 mg L⁻¹, the required dosage of base was 1.37 mM, which is still more than 4 times as much as in fresh WC medium lacking AOM (0.42 mM). Alginic acid or alginate is an anionic polysaccharide with a linear polymer structure composed of guluronate and mannuronate, with carbonyl and carboxylic acids as main functional groups. A recent study has demonstrated that magnesium ions and alginate form hydrogels in the presence of long guluronic units (Topuz et al., 2012). This suggests that anionic polysaccharides containing uronic acids may play an important role in the inhibition of alkaline flocculation.

Influence of AOM size and composition on flocculation

To confirm the role of polysaccharides in the inhibition of flocculation, we evaluated how removal of large polysaccharides from the culture medium affects alkaline flocculation. Large polysaccharides were removed from cell-free medium collected at day 14 of cultivation using a 3-kDa ultrafiltration membrane. Membrane filtration removed 47% of the polysaccharides from the culture medium. The sodium hydroxide dose required to induce alkaline flocculation was reduced from 2.8 mM prior to membrane filtration to 1.32 mM after filtration (Suppl. Fig. 2).

The inhibition of flocculation was greatly reduced when only AOM smaller than 3 kDa was present, suggesting that inhibition of alkaline flocculation for *Chlorella* was mainly caused by the fraction of AOM > 3 kDa. We determined the main composition of the AOM fraction > 3 kDa in function of the cultivation time (Table 2). Total carbohydrates increased from 0.44 mg C L⁻¹ at day 3, to 2.1 mg C L⁻¹ at day 14, while the total protein concentration remained constant. The composition of the polysaccharides was studied by GC-MS after hydrolysis and silylation of the released monosaccharides. Even though the hydrolysis was not complete, we found that the composition of the hydrolyzed monosaccharides stayed relatively constant in function of time, while the concentration increased, which is consistent with the measured increase in total carbohydrate content (Table 2). The identified monosaccharides included neutral and anionic monosaccharides, primarily glucose, ribose, arabinose, rhamnose, glucuronic acid (along with unconfirmed additional uronic acids, possibly guluronic and mannuronic), mannose, and galactose. Based on peak area calculations, we found that 10% of the total sugar content consisted of uronic acids. This high prominence of uronic acid residues is consistent with the hypothesis that a charged anionic polymer, with similar properties as alginic acid, is involved in the flocculation inhibition for the tested *Chlorella vulgaris* cultures. This result suggests that the inhibition of alkaline flocculation for *Chlorella* is caused by interaction with high-molecular-weight polymer carbohydrate structures, rather than with proteins. However, the potential involvement of the charged polysaccharide portion of the excreted glycoprotein components cannot be excluded nor confirmed at this point. Although all microalgae are known to produce AOM, the quantity and quality is species dependent. Henderson et al. (2008) showed that for *Chlorella vulgaris* 60% of the AOM consisted of molecules > 30 kDa and contributed to 84% of the total charge of the culture. However, cyanobacteria like *Microcystis aeruginosa* produce AOM that is richer in proteins compared to eukaryotic microalgae (Nicolau et al., 2015). This means that the inhibition of alkaline flocculation will depend on the composition of the AOM.

Conclusion

Alkaline flocculation is inhibited with increased cultivation time, which results in a significant increase in the dose of NaOH needed to achieve efficient flocculation. By separating the cells from the culture media, we demonstrated here that this inhibition of flocculation is primarily due to changes in the composition of the medium rather than changes at the cell surface. The concentration of carbohydrates larger than 3 kDa in the medium increased as function of cultivation time while protein concentrations remained constant. Moreover, addition of alginate, a model anionic carbohydrate polymer, resulted in complete inhibition of flocculation. These results suggest that inhibition of alkaline flocculation could be caused by anionic polysaccharides leading to an increased flocculant demand. However, the charged polysaccharide portion of excreted glycoprotein components could also contribute to the observed inhibitory effects.

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Fig 1: Biomass dry weight concentration (mg DW L^{-1}), total extracellular carbohydrates in medium (mg C L^{-1}) and total organic carbon in medium (TOC; mg C L^{-1}) as function of cultivation time for *Chlorella vulgaris*.

Fig 2: (A) Influence of cultivation time, (B) cell age, and (C) medium composition on flocculation efficiency for *Chlorella vulgaris*. Predicted NaOH doses (D[95%] in mM) and corresponding pH required to obtain 95% flocculation efficiency are shown.

Fig 3: pH as function of log NaOH added for *Chlorella* without AOM (cells) and containing AOM (cells + medium) at (A) day 3, (B) day 6 (C) day 14. Arrow indicates when flocculation efficiency of 95% was observed.

Table 1 Influence of model organic compounds on flocculation efficiency of *Chlorella vulgaris* versus fresh and original medium: predicted NaOH dose response (D[95%] in mM) to obtain 95% flocculation efficiency.

Model compound	Concentration (mg L ⁻¹)	D[95%]
Fresh medium	/	0.42
Original medium	/	2.58
Adipic acid	50	0.48
Albumin	50	0.44
Alginic acid	10	1.37
Alginic acid	30	5.6
Alginic acid	50	>8
Galacturonic acid	50	0.38
Gluconic acid	50	0.81
Glucose	50	0.53
Glycine	50	0.83

Table 2 Total carbohydrates and protein concentration in AOM >3kDa fraction as function of cultivation time for *Chlorella vulgaris*.

General composition AOM > 3kDa	D3	D7	D14
Total carbohydrates (mg C L ⁻¹)	0.44±0.01	1.64±0.03	2.1±0.1
Total proteins (mg albumin eq L ⁻¹)	2.1±0.04	2.0±0.03	2.2±0.04

Inhibition of alkaline flocculation by algal organic matter (AOM)