Factors governing microalgae harvesting efficiency by flocculation using cationic polymers

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Abstract

This study aims to elucidate the mechanisms governing the harvesting efficiency of *Chlorella vulgaris* by flocculation using a cationic polymer. Flocculation efficiency increased as microalgae culture matured (i.e. 35–45, 75, and >97% efficiency at early, late exponential, and stationary phase, respectively. Unlike the negative impact of phosphate on flocculation in traditional wastewater treatment; here, phosphorous residue did not influence the flocculation efficiency of *C. vulgaris*. The observed dependency of flocculation efficiency on growth phase was driven by changes in microalgal cell properties. Microalgal extracellular polymeric substances (EPS) in both bound and free forms at stationary phase were two and three times higher than those at late exponential and early phase, respectively. Microalgae cells also became more negatively charged as they matured. Negatively charged and high EPS content together with the addition of high molecular weight and positively charged polymer could facilitate effective flocculation via charge neutralisation and bridging.

Keywords: Algal extracellular polymeric substances; *Chlorella vulgaris*; Growth phase; Phosphorous; Zeta potential.

1. Introduction

Microalgae are an emerging feedstock for third-generation biofuel, which can address the imminent depletion of fossil fuel and the increasing threat of global warming (Nagarajan et al., 2020; Rajesh Banu et al., 2020). The first-generation (i.e. food crops) and second-generation (i.e. lignocellulosic biomass) biofuel are more environmentally friendly than fossil fuel, but they also have inherent drawbacks especially as they compete with food security and have low conversion efficiency (Nagarajan et al., 2020; Rajesh Banu et al., 2020). As phytoplankton, microalgae are fast-growing photosynthesizing microscopic organisms that can be cultivated without any requirement for arable land and with minimal input of resources. Large-scale microalgae production has been demonstrated in the desert or even on the ocean surface. Microalgae are rich in carbohydrates, proteins, and lipids. These compounds are valuable substrates for the production of renewable fuel such as biodiesel, biomethane, and green hydrogen (Rajesh Banu et al., 2020).

The harvesting process remains a major challenge in the microalgae supply chain. The current high cost of harvesting reduces the competitiveness of large-scale biofuel production from microalgae (Khoo et al., 2020; Yin et al., 2020). Microalgae harvesting is the process of recovering a concentrated algal slurry (10 – 25% dry biomass) from the diluted algal suspension (0.02 – 0.05% dry biomass) and reuse the cultivation solution for subsequent algae production. In the current microalgae industry, the harvesting process accounts for 20 to 30% of the total algal biomass production cost (Singh & Patidar, 2018). Current microalgae harvesting methods include centrifugation, filtration, flocculation, flotation, electrocoagulation, bioflocculation, and magnetic separation (Ananthi et al., 2021; Yin et al., 2020). Comprehensive reviews of the pros and cons of these methods have highlighted flocculation as the most promising technology for low-cost harvesting of microalgae biomass for biofuel production (Ummalyma et al., 2017; Yin et al., 2020).

Microalgae flocculation using synthetic cationic polymer is a promising technique to overcome the current constraints of algal harvesting. It has been shown to effectively flocculate over 90% of freshwater and seawater microalgae at low doses with a simple and fast operation (Gerchman et al., 2017; Nguyen et al., 2019; Udom et al., 2013; Vu et al., 2020a; Vu et al., 2021). Charge neutralisation and bridging effects have been shown to be the mechanisms behind cationic polymer flocculation, although there are still questions as to how these mechanisms and the flocculation efficiency may be influenced under different algal culture conditions. Labeeuw et al. (2021) reported that the growth phases (i.e. early exponential, late exponential, and stationary) of microalgae influenced the flocculation efficiency using a highly charged cationic polymer. Three algal species (cyanobacteria *Synechocystis sp.*, freshwater *Chlorella vulgaris*, and marine diatom *Phaeodactylum tricornutum*) showed different responses to polymer flocculation at three growth phases. Flocculation by cationic polymer addition was 98% effective at flocculating *Synechocystis sp.* regardless of the growth phase, whereas it was 50% less effective for *C. vulgaris* and *P. tricornutum* at early stationary phase (Labeeuw et al., 2021). This variation may be attributed to differences in biomass concentration and algal biochemical composition at each growth phase. Thus, it is necessary to delineate the factors that may affect the polymer flocculation of microalgae at different growth phases. This will help to gain further knowledge of algal flocculation and identify the strategies to optimise algal harvesting using cationic polymer.

Phosphorous is an essential nutrient for microalgal growth, especially for the synthesis of biomolecules such as phospholipids, adenosine triphosphate, and deoxyribonucleic acids in the algal cells. Phosphorus is present in the algal medium as orthophosphates and decreases in concentration gradually with culture age. Residual phosphorous has been reported to impact coagulation and flocculation in wastewater treatment (Liu & Liss, 2007; Morgan, 1958; Park et al., 2016). The presence of phosphorous as phosphates in wastewater hindered

the flocculation and sedimentation processes (Morgan, 1958). A higher flocculant dose and a longer settling time were required to overcome the interference caused by phosphate compounds (Morgan, 1958; Park et al., 2016). Conversely, the gravitational settling velocity of sludge flocs was enhanced for wastewater with reduced phosphorous concentration (Liu & Liss, 2007). This improvement was attributed to larger and more compact flocs formed under phosphorous limited conditions. In other words, a lower phosphorous concentration can lead to a higher flocculation efficiency in a wastewater matrix. However, it is still unknown if these findings are also applicable to a microalgal culture with a very different matrix compared to wastewater.

As microalgae grow, they also secrete metabolites (e.g. carbohydrates and proteins) that surround the cells, known as algal extracellular organic matter, or extracellular polymeric substances (EPS). EPS can influence the surface properties of algal cells as well as promoting or inhibiting floc formation (Henderson et al., 2010; Roselet et al., 2017; Sano et al., 2011; Vandamme et al., 2012; Zhang et al., 2012). Algal EPS can act as a polymer aid at low concentration, as it frequently contains biopolymers that can bridge the cells and/or with hydroxide precipitates to form large flocs (Gonzalez-Torres et al., 2017). On the other hand, EPS can decrease the efficiency of the coagulation-floatation process as EPS can form complexes with the coagulant and thereby increasing the required coagulant dose to floc algal cells (Bernhardt et al., 1985; Roselet et al., 2017; Vandamme et al., 2012). Given these effects of EPS on algal harvesting, the concentration and composition of algal EPS would likely influence the flocculation efficiency using cationic polymer at different growth phases.

This study aims to elucidate the underlying factors affecting the flocculation efficiency of *Chlorella vulgaris* at different growth phases (i.e. early exponential, late exponential and stationary phase). Factors including residual phosphorus concentration, surface charge of microalgal cells, and cell EPS content are examined at each growth phase. Results presented

here are useful for further optimisation of microalgae harvesting by flocculation using organic polymers.

2. Materials and methods

2.1. *Chlorella vulgaris* cultivation

The freshwater microalgae *Chlorella vulgaris* (CS-41) was obtained from Australian National Algae Culture Collection at CSIRO Microalgae Research (Hobart, TAS, Australia). The stock culture was maintained in 0.22 µm filtered autoclaved freshwater MLA medium (Algaboost; Wallaroo, SA, Australia). The main nutrient composition of this MLA medium includes approximately 49 mg/L of $MgSO_4.7H_2O$, 170 mg/L of $NaNO_3$, 35 mg/L of K_2HPO_4, and 2 mg/L of H_3BO_3, respectively (Bolch & Blackburn, 1996).

C. vulgaris culture was prepared in three steps from 1 L bottle to 350 L photobioreactor (Supplementary data Fig. 1S). The stock culture was first cultivated in a 1 L bottle, then transferred to a 10 L bottle for further cultivation until the early stationary phase. Finally, the 10 L culture was used to inoculate two identical 350 L photobioreactors (i.e. two biological replicates). The 350 L photobioreactors were maintained at 25 °C, 100-400 µmol photons/m^2/s light in a 16:8 light:dark cycle, and air supply through air lines. These photobioreactors were also sparged with 100% CO_2 for 1 min/day to provide carbon and maintain the pH below 9.3. Microalgal growth was monitored daily by optical density measurement. Microalgae suspensions from these two photobioreactors were extracted at the same time of the day for flocculation and determination of extracellular polymeric substances (EPS).

2.2. Microalgae flocculation

2.2.1. Materials

A cationic polyacrylamide polymer (FO3801) was purchased from SNF (SNF Pty Ltd; Corio, VIC, Australia). The polymer is highly charged (75 mV by zeta potential) with a high molecular weight (over 15 MDa) and a charge density of 80%. A stock polymer solution (2 g/L) was prepared in Milli-Q water under mixing for 60 min using a magnetic stirrer. The stock solution was used for the flocculation experiment within one day to avoid any polymer hydrolysis during long-term storage.

C. vulgaris suspension (10 L) at early exponential, late exponential and stationary phase was collected from the two 350 L photobioreactors (section 2.1) for the flocculation experiment.

To investigate the impact of residual phosphorous in the algal culture on flocculation efficiency, dipotassium phosphate (K_2HPO_4) was added to *C. vulgaris* suspensions at stationary phase to achieve the phosphate (PO_4^{3-}) concentration of 10, 20, 30, and 40 mg/L. *C. vulgaris* suspension at stationary phase without K_2HPO_4 addition was used as the control. The phosphate concentration of this control suspension was 3.7 mg/L. After K_2HPO_4 was completely dissolved in the suspensions, algae flocculation was performed with FO 3801 at 35 mg polymer/g dry biomass.

2.2.2. *Experimental protocols*

Flocculation test took place in 250 mL glass beakers containing 100 mL of microalgal culture. Polymer solution was dosed at 35 mg/g dry algal biomass. This dose was the optimal dose for *C. vulgaris* flocculation as reported in a previous study (Labeeuw et al., 2021). After polymer dosing, the microalgae suspensions were rapidly mixed for 1 minute at 200 rpm, followed by slow mixing for 5 minutes at 50 rpm. The suspensions were allowed to settle for

10 minutes. Then 10 mL aliquot was pipetted at a height between one- and two-third from the bottom of the beaker. Optical density of this aliquot sample was measured to determine the flocculation efficiency (Section 2.3.3).

2.3. Analytical methods

2.3.1. Microalgae growth analysis

Two samples of 100 mL (i.e. two technical replicates) are taken from each of the two 350 L photobioreactors every second day for measurements of dry weight, optical density, pH, residual phosphorous concentration, and zeta potential.

The dry weight of *C. vulgaris* culture (i.e. dry biomass concentration) was determined gravimetrically by filtering 100 mL solution through a 1.1 µm pre-weighed glass fibre filter paper. After 12 h of oven drying at 60 °C, the weight of the filter paper with retained biomass was used to calculate the dry algal biomass concentration.

The optical density of the microalgal culture was measured by a spectrophotometer (Shimadzu UV 6000) at a wavelength of 680 nm. The residual phosphorous concentration in the algal culture was determined using Phosphorous TNTplus Vial Test high range (1.5-15.0 mg/L PO_4^{3-}) and a spectrometer (DR3900, Hach Pacific, Australia). Samples of the algal culture were filtered through 0.45 µm Nylon syringe filters to remove microalgal cells before applying the vial test to the supernatant. The zeta potential of the algal culture was measured using the zeta instrument (Zetasizer Nano ZS Zen 3600, Malvern, UK).

2.3.2. Flocculation efficiency

The optical density and zeta potential of the microalgal culture before and after polymer flocculation was measured as outlined in Section 2.3.1. The flocculation efficiency was determined using Equation 1:

$$Flocculation\ efficiency\ (\%) = \left(\frac{OD_i - OD_f}{OD_i}\right) \times 100 \qquad \text{(Equation 1)}$$

where OD_i and OD_f imply the optical density of the microalgal culture before and after flocculation.

2.3.3. EPS extraction and determination

EPS consists of soluble EPS and bound EPS. Microalgal suspension of 35 mL was centrifuged at 3,500 *g* and 4 °C for 30 min. The supernatant was then filtered through a 0.45 µm Nylon syringe filter to obtain soluble EPS. The algal pellet was re-suspended to a volume of 35 mL in a phosphate buffer solution (10 mM NaCl, 1.2 mM KH_2PO_4, and 6 mM Na_2HPO_4). The re-suspended algal suspension was subjected to low-strength sonication for 40 s. The sample was centrifuged again at 9,000 *g* and 4 °C for 15 min. Filtered supernatant contained bound EPS. Carbohydrate and protein concentration of the soluble and bound EPS were determined using the phenol-sulfuric acid method (Nielsen, 2010) and Lowry method (Lowry et al., 1951), respectively.

2.3.4. Statistical analysis

Statistical analysis of flocculation efficiency and biomass quality measurements was performed using Student's *t*-test (OriginPro 2019). Appropriate assumptions (i.e. data sets are normally distributed and have equal variances) were checked before statistical analysis.

3. Results and Discussion

3.1. Biomass production and nutrient profile in pilot-scale photobioreactors

Batch autotrophic cultivation of *Chlorella vulgaris* in the 350 L pilot-scale photobioreactor showed a typical S-shape growth curve with three distinctive phases (Fig. 1) similar to that reported in the literature (e.g. Do et al. (2020); dos Santos et al. (2016); Klin et al. (2020)). The duration of each algal growth phase in this study is similar to the growth of *C. vulgaris* in a previous study under the same condition (Labeeuw et al., 2021). In the early

exponential growth phase (day zero to six), cells were adapting to the new environment. Once fully adapted, algal cells started to rapidly multiply. The culture entered the exponential phase at day seven. At the end of the exponential phase (day 18), cell growth reached its limit as defined by the availability of nutrients, light, and carbon source. The culture entered the stationary phase when the production of new cells was gradually offset by cell death. Samples were taken on day seven (i.e. early exponential), day 18 (i.e. late exponential), and day 28 (i.e. stationary) for subsequent flocculation experiments.

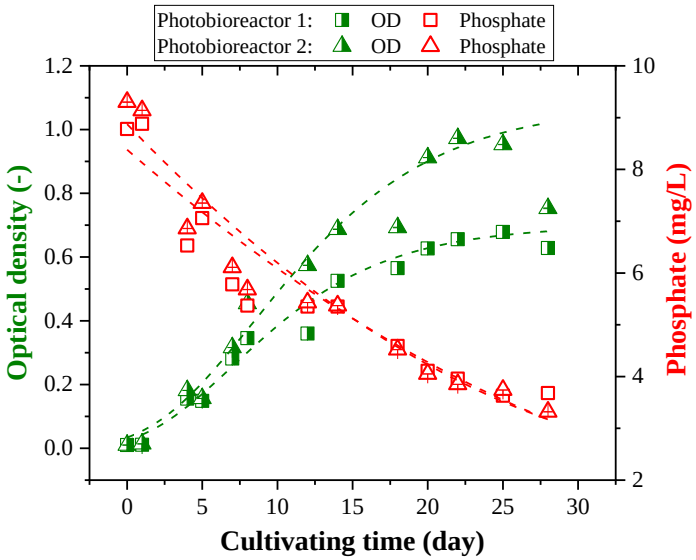


Figure 1: Change in optical density and phosphate concentration of *C. vulgaris* culture during 28-day cultivation. Values and error bars represent mean and standard deviation from two technical replicate measurements (n = 2), respectively.

As microalgal biomass was produced (i.e. increase in optical density and dry weight), phosphorous content in the culture decreased over time (Figs. 1 and 2). Microalgal cells uptake nutrients for growth and synthesis of intracellular proteins, lipids, and carbohydrates (Anto et al., 2019; Chu et al., 2013). Over 28 days of cultivation, phosphorous concentration decreased from 9.0 mg/L PO_4^{3-} (day zero) to 5.8, 4.6, and 3.7 mg/L PO_4^{3-} for early exponential, late exponential and stationary phase, respectively. This represents a final 60%

reduction in phosphorous availability during *C. vulgaris* growth (Fig. 1). The phosphorous reduction is low in this study compared to previous studies whose aim is to remove phosphorous from wastewater (Vu et al., 2020b). However, our data (optical density, dry algal biomass concentration, phosphate depletion, and zeta potential) are consistent between the two biological replicates (i.e. two photobioreactors) (*t*-test, $p > 0.05$), indicating the experimental reproducibility. The low phosphorous uptake was probably due to both light and carbon source limitations in our photobioreactor cultivation system. Nevertheless, the change in residual phosphorous will facilitate the investigation regarding its impact on microalgal harvesting (Section 3.3).

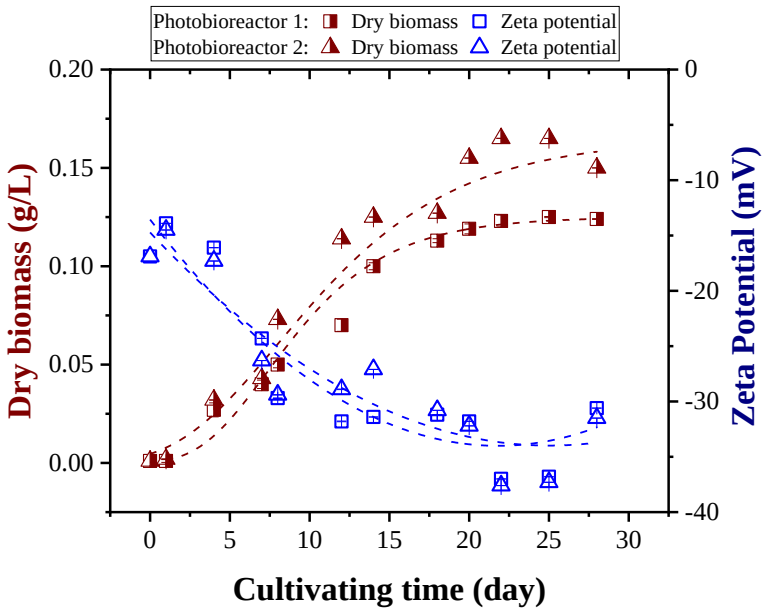


Figure 2: Change in dry biomass concentration and zeta potential of *C. vulgaris* culture during 28-day cultivation. The culture pH was fluctuating within the range of pH 8 to 9. Value and error bars represent mean and standard deviation from two technical replicate measurements ($n = 2$), respectively.

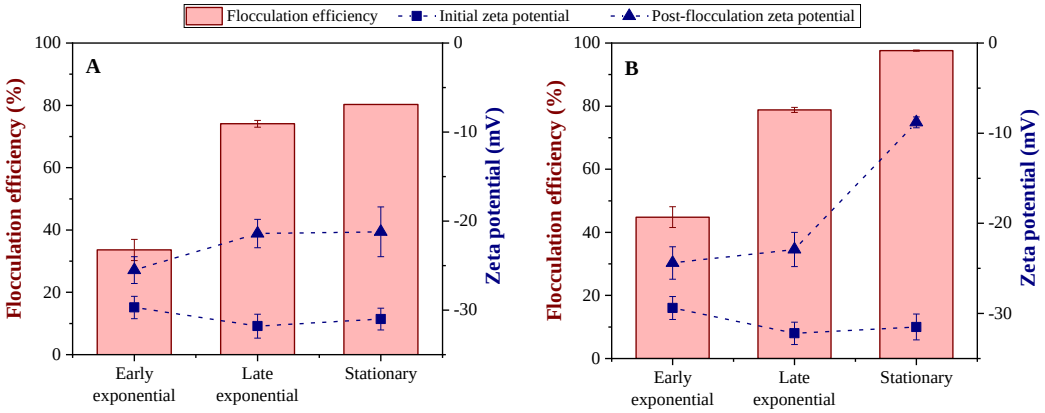
The surface charge of microalgal cells became more negative as the culture solution matured over time (Fig. 2). Microalgae cells are negatively charged so that they repel one

another by electrostatic interaction to stay dispersed in suspension. This maximises access to sunlight for photosynthesis by individual microalgal cells. The net negative charge of the cell surface is derived from the carboxylic groups on the cell membrane (Vandamme et al., 2013). In this study, the algal culture pH was slightly basic at pH 8-9, thus, these carboxylic groups dissociated to attain a negative charge for each microalgal cell. The increase in surface charge was significant within the early exponential growth phase (day zero to six) (Fig. 2). Changes in surface charge from the early exponential growth phase to the stationary phase were discernible but not statistically significant.

Increasing surface charge leads to stronger electrostatic repulsion to prevent the agglomeration of algal cells (Zheng et al., 2019). Thus, it is useful to examine if changes in cell surface charge would affect flocculation efficiency.

3.2. Flocculation efficiency at different growth phases

The flocculation efficiency of FO 3801 was dependent upon the growth phase of *C. vulgaris* (Fig. 3). This observation agrees with previous studies (Labeeuw et al., 2021; Zhang et al., 2018). There is some variation in flocculation efficiency as well as the zeta potential of the initial and post-flocculation microalgae at the three growth phases between the two biological replicates (i.e. two independent photobioreactors) as can be seen in Fig. 3. These differences could have been due to the random biological variation of the two photobioreactors, despite the efforts to operating them in the same conditions. However, the overall pattern is same and the difference in absolute value is also small.



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254 **Figure 3:** Flocculation efficiency and the increase in zeta potential of *C. vulgaris* culture
255 at 35 mg polymer/g dry biomass of two biological replicates: (A) photobioreactor 1 and (B)
256 photobioreactor 2. Flocculation was conducted at three different growth phases: early
257 exponential, late exponential, and stationary. Values and error bars represent mean and
258 standard deviation from two technical replicate measurements (n = 2), respectively.

259 Charge neutralisation is an important flocculation mechanism and can partially, but not
260 fully explain the increase in flocculation efficiency as the microalgae culture progressed from
261 the early exponential to the stationary growth phase (Fig. 3). Results in Fig. 3 show that the
262 highly charged cationic polymer FO3801 could significantly reduce the cell surface charge.
263 However, complete charge neutralisation did not occur even at the stationary phase when the
264 highest flocculation efficiency of 97% was achieved. Previous studies have suggested that
265 complete charge neutralisation is not necessary to achieve high (>95%) flocculation
266 efficiency (Nguyen et al., 2019). It is noteworthy that in Fig. 3 the same polymer dose was
267 applied to all flocculation experiments and that the differences in the initial zeta potential
268 between the late exponential and stationary growth phase were negligible (t-test, $p > 0.05$).
269 Thus, the initial surface charge is not the only factor governing the dependency of
270 flocculation efficiency on growth phase. As the microalgae culture continued to mature, the
271 composition of the media and physiochemical properties of the algal cells also changed. The
272 possible influence of media matrix (in terms of phosphate content) and cell properties on the

effectiveness of polymer flocculation at different growth phases will be elucidated in subsequent sections.

3.3. Impact of phosphorous residue on flocculation

In this study, residual phosphorous in the algal media did not show any influence on flocculation (Fig. 4). The variation in flocculation efficiency of the control sample and the samples with added K_2HPO_4 (i.e. 10 to 40 mg/L PO_4^{3-}) was negligible (*t*-test, $p > 0.05$). In addition, there was no observed correlation between algal cell zeta potential after flocculation and phosphorous concentration. Charge neutralisation of the cultures with 10 to 40 mg/L PO_4^{3-} was comparable to that of the control culture for both photobioreactors (*t*-test, $p > 0.05$). These observations conclusively affirm that the variation in flocculation efficiency of *C. vulgaris* at different growth phases (Section 3.2) was not induced by residual phosphorous in the culture media.

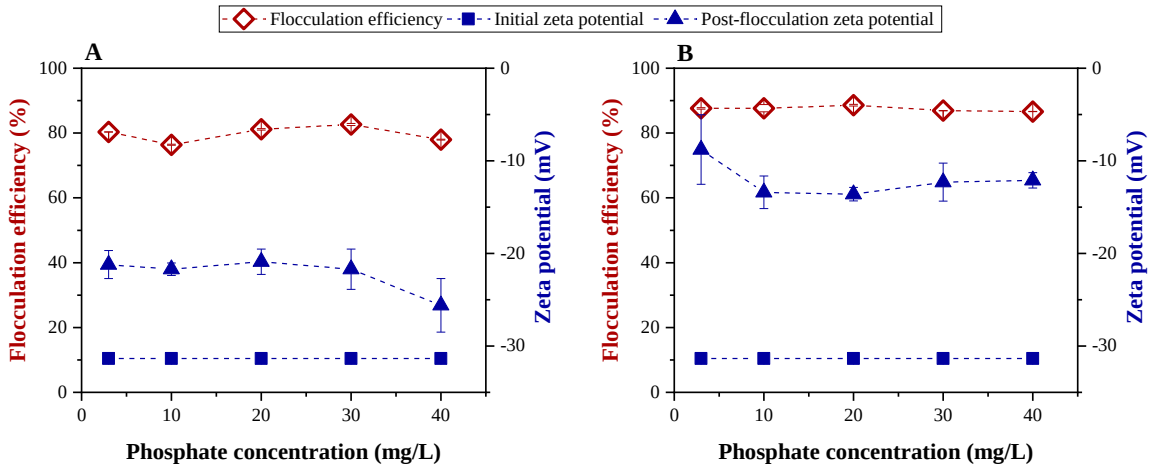

Figure 4: Flocculation efficiency and charge neutralisation of *C. vulgaris* culture at various concentrations of phosphate in the media during the stationary phase for (A) photobioreactor 1 and (B) photobioreactor 2. The polymer dose was 35 mg FO 3801/g dry biomass. Value and error bars represent mean and standard deviation from two technical replicate measurements ($n = 2$), respectively.

3.4. EPS content and impact on flocculation efficiency

Soluble and bound EPS content of *C. vulgaris* increased as the microalgal culture sequentially transited through the three growth phases (Fig. 5). The concentration of soluble EPS in terms of both carbohydrate and protein reached the highest value at the stationary phase (Fig. 5A and 5C). This value is approximately two and three times the total soluble EPS of late exponential and early exponential phase, respectively. Likewise, algal culture media at the stationary phase had the highest bound EPS content (6.5 mg/L), followed by late exponential (3.9 mg/L) and early exponential (2.5 mg/L) phase (Fig. 5B and 5D).

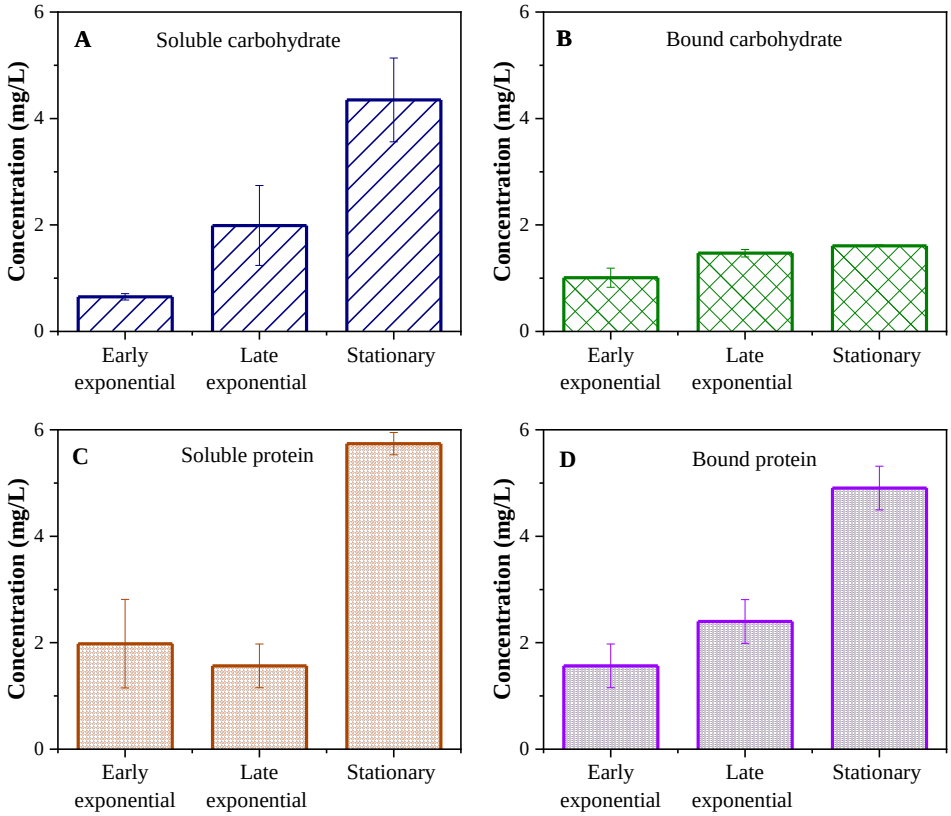

Figure 5: Accumulation of EPS in terms of (A) soluble carbohydrate, (B) bound carbohydrate, (C) soluble protein, and (D) bound protein in the *C. vulgaris* culture media at the early exponential, late exponential and stationary growth phases. Value and error bars represent mean and standard deviation from two biological replicate measurements (n = 2), respectively.

A similar trend of increasing EPS content as the microalgal culture continued to mature has been reported for other microalga species such as *Asterionella formosa, C. vulgaris, Microcystis aeruginosa,* and *Ettlia texensis* (Henderson et al., 2008; Salim et al., 2013; Zhang et al., 2018). Algal cells were actively dividing and excreting metabolites (i.e. carbohydrates and proteins) during the exponential growth phase. Thus, a higher EPS content was observed at the late exponential phase than the early exponential phase. Although growth in the stationary phase ceased, algal cells continued releasing metabolites, partly due to cell autolysis. As a result, the EPS components at the stationary phase were influenced by the algal intracellular contents (i.e. the biochemical composition). This explains the significant increase in protein content of soluble and bound EPS at stationary phase (Figs. 5C and 5D) as *C. vulgaris* is a protein-rich microalga. The solubilisation of the bound EPS fraction formed during the exponential phase also contributed to the increase in soluble EPS content of algal culture media at the stationary phase (Henderson et al., 2008). Therefore, the increase in EPS content with culture age appears to be a key factor influencing *C. vulgaris* flocculation and can elucidate its growth-phase dependent flocculation efficiency (section 3.2.1).

Microalgal EPS are dominated by hydrophobic proteins and hydrophilic carbohydrates (Henderson et al., 2008). These biopolymers can contribute to the bridging mechanism, which facilitates flocculation. In other words, these biopolymers from the algal cells and the added cationic polymer can form EPS-cell-polymer networks via electrostatic interaction to induce the flocculation process (Rao et al., 2021). As discussed in section 3.2, electrostatic attraction is expected between the negatively charged algal cell surface and the positively charged cationic FO3801 polymer to form large EPS-cell-polymer networks (i.e. flocs) via a combination of charge neutralisation and bridging effects (Gonzalez-Torres et al., 2017; Rao et al., 2021). Results from Fig. 5 are consistent with the dependence of flocculation efficiency on growth phase.

Previous studies have established the dependency of flocculation efficiency on culture maturity. Results from this study reveal for the first time underlying mechanisms governing the relationship between flocculation efficiency and culture maturity. The results are significant for optimising microalgae cultivation and harvesting. Microalgal EPS production is species dependent. Thus, further work is necessary to corroborate findings from this study to other microalgae species.

4. Conclusions

Flocculation efficiency of *Chlorella vulgaris* using cationic polymer increased with microalgae culture maturity. The highest flocculation efficiency (>97%) was achieved with the algal culture at stationary phase. Phosphorous residue in the culture did not affect *C. vulgaris* flocculation and cell surface charge, contrary to its negative impacts on flocculation in wastewater treatment application. The dependency of flocculation efficiency on growth phase was induced by changes in cell properties (e.g. EPS and surface charge). High EPS content with negative charges surrounding algal cells interacted with cationic polymer to form polymer-EPS-cell networks via charge neutralisation and bridging, thus promoting the flocculation process.

5. Acknowledgements

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6. Declarations

The authors declare that there is no conflict of interest regarding the publication of this article.

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