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Flocculating *C. Vulgaris* with Chitosan in Alkaline Conditions

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Abstract

Using jar test techniques we measured flocculation efficiencies through the reductions in optical density of *C. vulgaris*. The optimal dose of chitosan was found to be 0.1% of the algal culture volume. The flocculation efficiency using chitosan was found to decrease in alkaline conditions, increasing only at a pH level of 13 as released polysaccharides enabled auto-flocculation to take over as the primary binding mechanism. Additionally, the optimal media and perturbation for growth were determined to be EG:JM media and stationary conditions.

Supervisors: Gavin Melaugh and Andreas Werlberger 8 Weeks

Personal Statement

This was an experimental project that has given me more familiarity with researching and working on my own. The biological and chemical background of this project was untreaded territory for me initially, but has ultimately allowed me to become more adaptable in unfamiliar environments. I participated in weekly group discussions with my supervisor's cohort which gave me experience in designing visuals and giving presentations.

My first few weeks were spent researching background literature in order to determine the experimental setup. With the aid of my supervisor, we gauged what would be feasible given our time frame and available resources. As a result of contributing to the efforts that go into planning and implementing an experiment I have gained insight into the logistics behind research and feel better equipped to make critical decisions.

Lay Summary

Flocculation is a general process where a chemical added to a liquid suspension of particles causes the particles to clump together. In this project, the chemical was a liquid solution of dissolved chitosan, which is made from shrimp exoskeletons, and the particles were an algal species called *Chlorella vulgaris*. The clumped algae would sink to the bottom of the tube, making the upper portion of the tube more clear.

We also attempted to determine the optimal conditions of algae growth by measuring the density of algae across two weeks. Looking at the algae under the microscope allowed us to examine the cell sizes and check for contamination. The healthiest algal species were then subcultured following our determination of optimal conditions for algae growth in order to grow more algae for future experiments.

Contents

1 Introduction

The growing and apparent effects of global warming have raised societal awareness about ecological issues and have subsequently increased the demand for businesses to practice ethical and environmentally conscious values. Aquaculture is a promising industry capable of producing large amounts of food, however it is fraught with several negative environmental impacts [1]. Included among these issues is the industries dependence on farmed fish: the aquaculture sector alone required roughly 70% of the worlds global fish meal supply back in 2006 [2]. One possible avenue for reducing the industries dependence on fish meal involves harvesting microalgae as a replacement food source.

Microalgae is a renewable resource with many economically viable materials [3, 4] making it an attractive alternative to fish meal. The ideal harvesting method will vary depending on the algal species investigated and the industry of application, but this paper will examine the technique of induced flocculation due to its ability to scale up [5]. Flocculation is induced by adding a flocculant to the desired culture of algae and mixing it with different stages of intensity until the algae separates into clumps of algae known as flocs. More efficient flocculation leads to larger flocs, which subsequently experience a larger gravitational force. As the flocs sediment, the initially diffuse algae becomes centralized at the bottom of the container. The most efficient flocculant and dosage can therefore be measured by comparing the optical densities (OD) following sedimentation.

Within this paper we compare the flocculation efficiencies of two extensively studied flocculants—chitosan, and NaOH—on the algal species *C. vulgaris* and *N. oculata*, as well as the growth behaviour of *C. vulgaris*. Chitosan is an organic polymer made up of crustacean exoskeletons that possesses anti-bacterial qualities [6], making it an edible and sanitary flocculant. It becomes a polyelectrolyte when its primary amino acid groups get protonated. These amino groups have a pK_a value of around 6.3, therefore they are protonated when dissolved in a slightly acidic mixture [7]. Most algal groups have a negative surface charge, thus they are attracted to the chitosan following protonation. The observed connection pattern due to electrostatic forces is known as "bridging". In bridging, microalgal cells bind partially to strands of positively charged polymers. A microalgal cell can connect to more than one polymer, hence the name bridging. Chitosan is known to flocculate *C. vulgaris* effectively [8, 9], although not as efficiently in alkaline conditions [10]. This behaviour in alkaline conditions is due to the previously mentioned amino groups, and suggests an inability to be used in conjunction with NaOH.

It is worth noting that chitosan is an expensive flocculant. The costs of microalgae harvesting demand a cheaper alternative in order to make the industry profitable. For this reason we are also investigating NaOH. Sodium hydroxide increases the pH of the liquid, subsequently agitating the algae. In response, the algae releases carbohydrates called polysaccharides. Polysaccharides coat the algal cells and lower the surface charge, enabling the algal cells to floc together. Algae cells naturally produce polysaccharides in the stationary phase of their life cycle, so this form of flocculation is known as auto-flocculation.

NaOH has proven to be effective flocculants across several studies [11, 12]. The problems of increasing the pH levels are that the polysaccharides released by *C. vulgaris* may have a negative impact on flocculation efficiency that becomes prevalent only when large concentrations are present [13]. For this reason alkaline flocculation has been argued

to be less efficient with longer cultivation times or if reusing media [14, 15]. In addition, auto-flocculation experiments carried out by Blue Planet Ecosystems found large variance in their results: sometimes they achieved 90% flocculation efficiency, other times only 50%. They performed their experiments by raising the pH to 12 and then adding Ca^{2+}. Determining a method of using NaOH that is repeatable would be of great interest as it would save costs tremendously.

2 Methods

2.1 Microbial Cultures

We used *Chlorella Vulgaris* (CC18211/11b) from the Culture Collection of Algae and Protozoa, Scotland. The cultures had grown in *Euglena gracilis* medium (EG:JM). A second strain of *C. vulgaris* and a culture of *Nannochloropsis Oculata* were provided by Blue Planet Ecosystems, Austria, grown in Phytobloom media. We also performed preliminary testing on two bacteria species, *Escherichia coli* and *Comamonas*, which were grown from cryogenic samples.

2.2 Algae Growth

Algae can survive for two to three weeks in test tubes before it enters its exponential death phase. After this, it needs to be reinoculated in order to survive. Additionally, algae releases more polysaccharides near the end of its life due to environmental stresses. These polysaccharides can lead to auto-flocculation, causing the algae to sediment within the tubes, which may impair the efficacy of chitosan flocculation. For these reasons we sought to find the optimal conditions of algal growth so that we could repopulate and upscale our stock of algae.

Container	**Dosage Ratio**	**Medium**	**RPM**
U1	1:5	EG:JM	200
U2	1:5	EG:JM	0
U3	1:5	BBM	200
U4	1:5	BBM	0
U5	1:10	EG:JM	200
U6	1:10	EG:JM	0
U7	1:10	BBM	200
U8	1:10	BBM	0

Table 1: Different procedures of algae growth. The dosage ratio refers to the mL of culture deposited into the mL of medium, i.e a ratio of 1:5 refers to an initial container of 2 mL algae and 10 mL medium.

In order to determine the optimal conditions for algal growth we subcultured the *C. vulgaris* from the CCAP in eight different 20 mL tubes. Various conditions such as the dosage ratio, the medium used, and the revolution speed were varied. The conditions for each container are shown in Table 1. The dosage ratio was varied as the CCAP recommends a 1:5 ratio for non-optimal cultures and a 1:10 ratio for ideal cultures. The mediums used were either EG:JM or bold basil medium (BBM). The CCAP recommends

EG:JM for axenic species of *C. vulgaris* and 3N-BBM+V for non-axenic species, which is just BBM with added vitamins and 3-fold Nitrogen. The work produced by Wong et al. (2017) found that BBM was the optimal growth medium for *C. vulgaris* [16], however their trials did not include EG:JM or 3N-BBM+V. We decided to compare the growth between EG:JM and BBM in order to determine the ideal medium, omitting 3N-BBM+V due to a lack of resources. Lastly, the algae cultures were either left to stand or revolved at 200 rpm. This was done to determine the impact of different revolution speeds. A previous paper, Allagutova et al. (2019) [17], found that spinning at 100 rpm was roughly 30% larger growth rate than being stationary, therefore it would be interesting to look at the behaviour at twice the rpm. To monitor the progress of growth, the optical densities were measured each day around the same time, except on weekends. Two blank cuvettes were prepared with each respective medium (1 mL of EG:JM and BBM), while eight cuvettes were prepared for each sample (0.9 mL respective medium, 0.1 mL culture). This was done in order to get more accurate measurements[1] and to minimize our loss of culture.

2.3 Flocculation Setup

Chitosan solutions of low and medium molecular weight (LMW/MMW) were prepared by dissolving 1 gram of chitosan in 100 mL of 2% acetic acid. The LMW solution mixed well on a magnetic stirrer, but the MMW solution remained viscous despite being left overnight on a magnetic hotplate. The high viscosity of our MMW was problematic as it did not pipette well—particularly at smaller volumes. Our preliminary testing began by administering varying doses of LMW and MMW to 10 mL cultures of *Comamonas*. The tubes were then transferred to the incubator where they spun at 200rpm for one minute, and then to the slow roller where they spun at 40rpm for 3 minutes. They were then left to sediment for three hours, during which time the phase separation was monitored. We found that MMW chitosan was more effective, which agrees with Low and Lau (2017) [18] in that the heavier molecular chitosan was more effective in flocculating microorganisms. Due to this, we continued the tests using only MMW solutions.

The success of *Comamonas* may have been due to it being a self-floccing species of bacteria. In order to ensure that chitosan was a successful flocculant, we examined chitosan's effect on a non-auto-flocculating species, *E. coli*. Rather than looking for macroscopic phase separation, we now wanted to measure the differences in flocculation efficiency. We measured the flocculation efficiency, η, by using an equation from Vandamme et al. (2012) [11]:

$$\eta(\%) = \frac{OD_i - OD_f}{OD_i} \times 100 \tag{1}$$

where OD_i is the optical density of the control treatment and OD_f is the optical density of the tubes with chitosan added.

We began by growing 100 mL of *E. coli* overnight. We found its OD to be 1.35 following measurement at 600 nm. The culture was partitioned into 9 different tubes and given doses ranging from 0.1 to 1.7 mL in steps of 0.2 mL. Note the exclusion of a 0 mL

[1] The spectrophotometer becomes less accurate when the OD is greater than 0.5.

dose: that was a mistake. In order to remedy this, we took the initial OD in Equation 1 to be 1.35, and measured the optical densities after 120 minutes of sedimentation. Obvious phase separation was not observed in the tubes. We thought that the starting OD had potentially been too large, so we repeated the experiment by closely following the work of Strand et al. (2001) [19]. The *E. coli* was centrifuged and washed prior to flocculation[2]. In the second *E. coli* experiment, all tubes contained roughly 4 mL of culture. The chitosan dosages ranged from 2% to 6% (v/v). A percentage dose here refers to the amount of flocculant relative to the volume of liquid bacterial culture. For a 10 mL tube of *E. coli*, a 10% percentage dose is 1 mL of chitosan. The tubes were mixed at 200 rpm for 10 minutes, gently mixed at 40 rpm for 20 minutes, and then left to sediment for 80 minutes.

Following the *E.coli* experiments, we began algae flocculation on the *C. vulgaris* sample provided by BPE. The starting OD was measured to be 0.61[3]. The culture was divided into 8 different universal tubes of 5 mL each. The tubes were given percentage doses of MMW chitosan ranging from 0.01% to 15% (v/v). Following flocculant administration, the tubes were intensively mixed at 200 rpm for 3 minute, gently mixed at 40 rpm for 5 minutes, and then pipetted into cuvettes where they were left to settle for 20 minutes. The sedimentation was monitored over the period of 200 minutes in order to identify the time of phase separation. The optical densities of the sample centres were measured at 750 nm at 20 minutes, 80 minutes, and 200 minutes. The results are shown in Figure 2.

A similar protocol was carried out for *N. oculata*. The only differences are the range of percentage doses (0.1% to 0.5%) and the time of OD measurements (30 and 80 minutes).

3 Results & Discussion

3.1 Effective Chitosan Dosages for *E. Coli*

The results of both *E. coli* flocculation are shown in Figure 1. The results of the first experiment are skewed to be more effective due to our initial optical density. As mentioned previously, we used the initial optical density prior to flocculation as OD_i in Equation 1 because we forget to prepare a blank cuvette. This is tremendously flawed because the optical density should go down with time as natural sedimentation occurs. The efficiency percentages in this experiment are likely much lower than seen in Figure 1. Although this is an incorrect method of measuring accurate efficiencies, it allowed us to determine 5% as being the most effective dose relative to the group.

The second experiment failed to produce a flocculation efficiency greater than 40%. This is more likely to reflect the faliures of our experiment rather than chitosans efficacy for a few reasons. We may have overdosed our samples as Strand et al. (2001) [19] had prepared a weaker chitosan solution. Their chitosan had a density of 0.05 - 0.2 g/100mL, while ours was 1 g/100mL. The buffer we used prevented sedimentation from taking

[2] Following centrifugation, it was difficult to get equal amounts of culture into each cuvette. Additionally, the buffer prevented sedimentation from taking place. This led to unequal starting OD's and little to no variance in the OD's measured at different times.

[3] To be clear on the steps, I diluted the algae in tap water in order to get a more accurate measurement. Tap water was used instead of medium because we didn't have the medium at this point. So, 0.1 mL of culture was put in a cuvette with 0.9 mL of tap water.

place, as the measured optical densities were exactly the same despite there being two hours between measurements. Additionally, it was difficult to get the *E. coli* into tubes following centrifugation: the cultures had an uneven starting OD.

Both experiments required the same dosage for highest relative flocculation despite the noise: a 5% dosage of chitosan. There are several studies on *E. coli* flocculation [19, 20] where efficiency was close to 100%, but we were unable to achieve similar results.

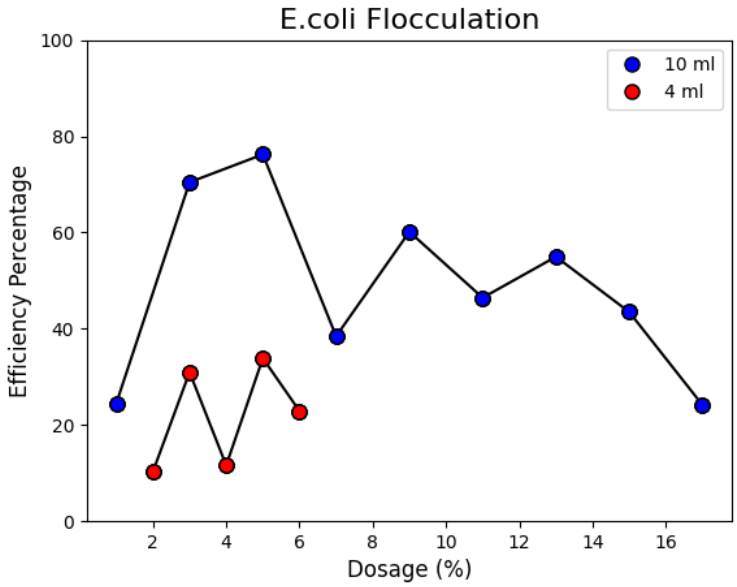

Figure 1: Efficiency of *E. coli* flocculation measured against the percentage dose of chitosan. Blue data points are from the first experiment (no blank cuvette), red data points are from the second experiment (has blank cuvette, was washed and centrifuged). All measurements were taken 120 minutes after sedimentation began.

3.2 Effective Chitosan Dosages for *C. Vulgaris* and *N. Oculata*

The results of our algae chemical flocculation experiment are shown in Figure 2. The lowest dose (0.005 mL / 0.01%) was most effective. The flocculation efficiency increased with time in all cases due to there being more time for sedimentation, however it should be noted that the rate of change for optical density decreases with time. A full list of the dosages and results are available in Table A1. All dosages greater than 1% of the algae volume were unable to induce flocculation with efficiency greater than 25%. While larger doses of chitosan become less efficient, they are still more effective than no dose.

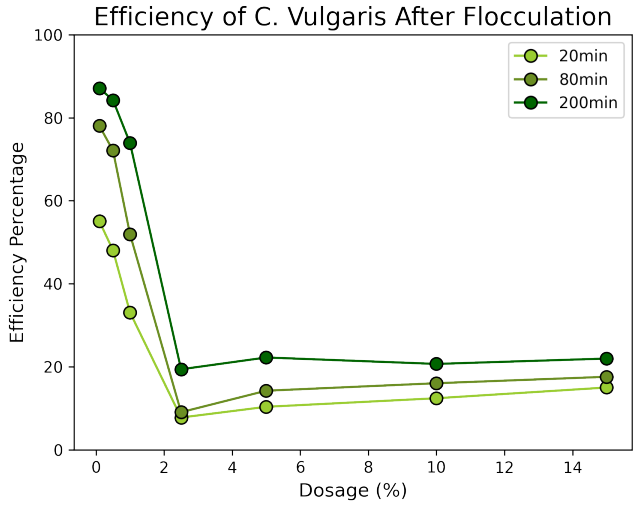

Figure 2: Efficiency of *C. vulgaris* flocculation measured against the dosage of chitosan. Light green line represents efficiencies at 20 minutes of sedimentation, green line is 80 minutes of sedimentation, and dark green line is 200 minutes of sedimentation.

We also wanted to test flocculation on the second species given to us by BPE, *N. oculata*[4]. The results are shown in Figure 3, and show that effective flocculation can be observed by using doses between 0.01% and 0.5%. The variance in efficiency at 30 minutes is likely the result of taking only one sample reading instead of averaging over several as all dosages have a similar efficiency at 80 minutes.

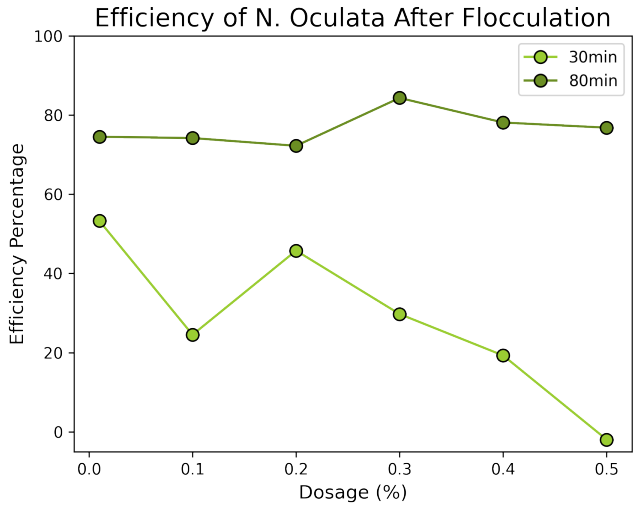

Figure 3: Efficiency of flocculation measured against the dosage of chitosan for algae species *N. oculata*. The efficencies after 30 minutes and 80 minutes are shown in green and dark green, respectively.

[4] We didn't have any medium available to reinoculate N. oculata (it grows in f/2 medium, not BBM or EG:JM), so we used all of our stock to probe dosages near the peak of *C. vulgaris* flocculation.

3.3 *C. Vulgaris* Growth

The results of monitoring *C. vulgaris* growth are shown in Figure 4. There is an obvious disconnect between the results found from using EG:JM and BBM, but the reasoning is more nuanced than one medium being explicitly better than the other. The algae culture that we used for subculturing had been growing in EG:JM media prior to reinoculation, which explains why the EG:JM-grown algae begins from the exponential growth phase and the BBM-grown algae goes through a lag phase.

The results could be improved by adding CO_2 to the system, as algae absorbs CO_2 very effectively. Further testing with improved aeration systems is necessary as the failures of our BBM are likely to be systemic issues rather than inherent ones, noting that Wong et al. (2017) [16] found success with BBM. Three of the top four performers were growing in stationary conditions which suggests that algae prefer tranquil conditions[5], however it should be noted that the overall worst performer (U8) was also in stationary conditions.

Although container U8 was the worst performer, it is important to note that it had a sporadic OD. On the final two days of measurements, the ODs were retaken as they were abnormally high (0.62 and 0.84). These were probably not signs of contamination, as otherwise the optical densities would not have decreased following a second measurement. The tubes were all sheared prior to measurement in order to break up flocs, but perhaps it was not for long enough. It seems likely that the lack of growth from container U8 is due to statistical chance.

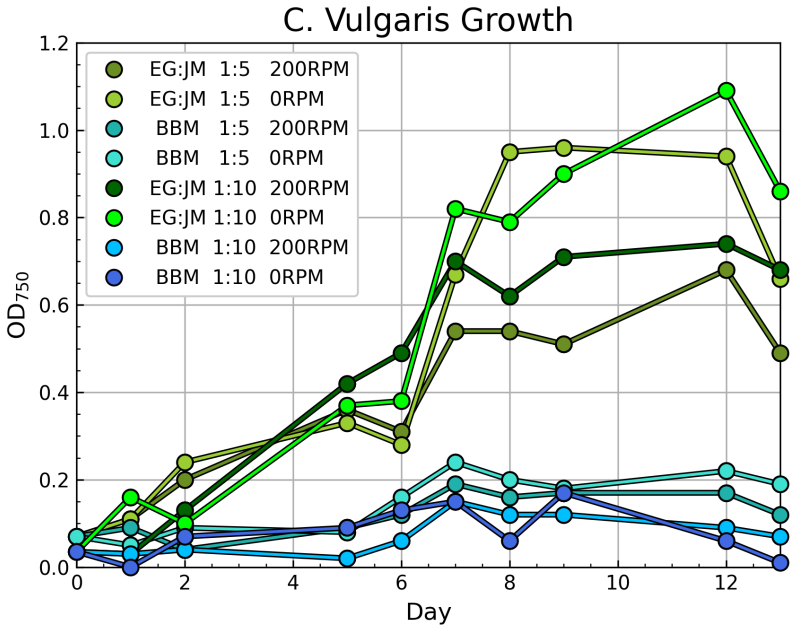

Figure 4: Measured optical density at 750 nm of eight different tubes of *C. vulgaris* measured each day for two weeks. The conditions of the containers correspond to the traits outlined in Table 1

[5] I had the opportunity to visit Blue Planet Ecosystems' facility in Austria, and we left three algae cultures overnight in Phytobloom medium (Phytobloom is a brand). The two cultures left stationary had a higher OD than the one left on a magnetic stirrer, which provides merit to our results.

3.4 Flocculation in Alkaline Conditions

The final part of the experiment was to monitor the behavoiur of chitosan flocculation and auto-flocculation in alkaline conditions. The two largest and optically dense cultures, U5 (0.66) and U6 (0.74), were partitioned into eight tubes of 5 mL each. We found the inital pH level to be around 9 for all cultures, and added doses of NaOH in order to raise the pH levels further[6]. A full list of our data values can be seen in A2 of the Appendix. The initial pH value of our cultures was unexpected. A typical pH value should have been around 7, making our value of 9 slightly irregular. The algae was likely growing in more alkaline medium due to its growth conditions. Algae absorbs CO_2 from its medium during daylight and releases it at night. Over the course of two weeks of algae growth this would add up to a large pH difference as it spends much more time in light conditions than dark ones.

Following pH adjustment, chitosan was administered to half of the universal tubes. The tubes were incubated at 200 rpm for 10 minutes, then spun at 40 rpm for 20 minutes, and then left to sediment in cuvettes for 30 minutes. The optical densities were then measured and plotted in Figure 5. Four of the containers were made using U5 and the other four were made using U6, so the difference in initial optical density was subtracted from U6 in order to bring its results inline with U5[7].

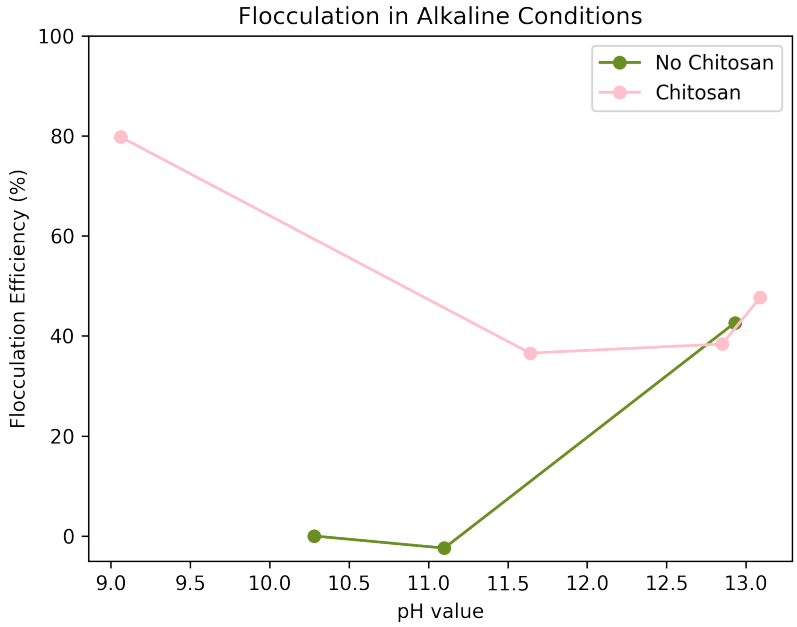

Figure 5: Flocculation efficiencies of *C. vulgaris* at different pH levels. Pink line denotes flocculation including chitosan dosage, while green line is solely due to the addition of NaOH.

All chitosan dosages were 0.1% of the tube volume, so the results of chitosan flocculation at a pH of 9 match reasonably with the previous test. There are two trends

[6] We initially wanted to examine 7, 10, 11, and 12, however the amount of NaOH required to raise the pH was much less than what we started with. Let it be known that it would be very useful to have test cultures on hand purely for testing the amount of NaOH needed to get the desired pH.

[7] Ideally there would be enough mL of algae from one culture, but this was the next best solution.

within our data: auto-flocculation begins somewhere after a pH of 11, and chitosan loses efficiency in more alkaline conditions.

The results of auto-flocculation were not quite what was expected. We expected *C. vulgaris* to auto-flocculate at pH levels of 11 and higher following the results of Vandamme et al. (2012) [11], however our results never reached the same amount of efficiency. This may be due to their addition of calcium and magnesium to supplement flocculation, however their preliminary testing found that flocculation would still occur without those. The issue may then be explained by examining Vandamme et al. (2016) [14], where it is explained that algal organic matter excreted from older cultures inhibits alkaline flocculation. Their findings found that a larger dose of NaOH is required to achieve auto-flocculation.

The reduction in chitosan efficiency matches the result of previous studies, such as those listed by Matter et al. (2019) [5]. For higher efficiency in alkaline environments, there needs to be a higher dosage of chitosan [21]. The most pressing issue within our findings is the lack of data points as it makes it difficult to investigate chitosans reduction in efficiency. Chitosan is just as effective at a pH of 11.5 as it is at a pH of 12, and even increases in efficiency at a pH of 13. The final increase may be due to auto-flocculation taking over as the primary mechanism, becoming most effective at the highest pH.

4 Conclusion

The optimal conditions for algae growth were found using EG:JM and still waters. The culture to medium ratio was not seen to have a significant impact on growth speed. Chitosan was found to flocculate algae at quite high efficiencies (80%) for both *C. vulgaris* and *N. oculata*, while NaOH was only able to reach an efficiency of around 40%. Adding Ca^{2+} to the culture after raising the pH may improve further efforts, as this was what was performed by Blue Planet Ecosystems.

Future work could focus on streamlining and improving the experiments undertaken here. Chitosan solution is typically prepared fresh and used within 24 hours, while we have been re-using one solution for a month. Aeration systems were deemed unnecessary as we were culturing algae below 150 mL, but the lack of light available to algae in the centre may become more prominent at larger scales. The lack of algae growth within BBM necessitates an investigation of 3N-BBM+V as it would be just as easy to replenish in-house. All experiments could have been improved by washing the microbes more thoroughly. This would have made flocculation efficiencies more accurate, and would have made lag-phases start at the same time when monitoring growth.

Other efforts that were unable to fit within the scope of this project included measuring size distributions, using cationic starches, and measuring zeta potentials. Size distributions can be measured by taking images with the microscope, and would allow us to probe cell health in different media. Cationic starches are an alternative to chitosan. They are cheaper and are likely to work in alkaline conditions (see Mohseni et al. (2020) [22]). As they do not come ready-made and need to be synthesized, it would be most fitting for someone with a chemical background. As most starches are incredibly cheap, they have a larger industrial demand than chitosan. It would also be interesting to compare the zeta potentials of algae and bacteria to the charge of cationic polymers. This could better determine the point of charge neutralization or charge reversal, as has been done in other studies [9, 18, 23].

5 Acknowledgements

I would like to thank Dr. Gavin Melaugh from the University of Edinburgh for his supervision and Andreas Werlberger from Blue Planet Ecosystems for both providing algal cultures and giving me the opportunity to visit their company in Vienna. The advice given by both of them was invaluable to this project. I also want to thank Tracy Scott for producing the BBM used within this project, and François De Tournemi for helping with the setup of the *E. coli* experiments.

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A Appendices

	η (%)		
Dose (mL)	**20min**	**80min**	**200min**
0.005	55	78	87
0.025	48	72	84
0.05	33	52	74
0.125	8	9	19
0.25	10	14	22
0.5	12	16	21
0.75	15	18	22

Table A1: Full table of flocculation efficiency percentages of *Chlorella vulgaris* following administration of MMW chitosan. Measurements for the optical density were made at 20, 80, and 200 minutes at 750 nm.

Container	**NaOH Dose (uL)**	**pH**
NC1	0	8.97
C2	0	9.06
NC3	100	12.93
C4	100	13.09
C5	50	12.85
NC6	2	10.28
C7	5	11.64
NC8	5	12.1

Table A2: Table listing the final pH of 5 mL *Chlorella vulgaris* containers following NaOH mixing for alkaline flocculation. NC denotes no-chitosan, while samples labeled C were dosed with chitosan. The numbers following the letters are purely for naming convention.