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Harvesting *Chaetoceros gracilis* by flocculation using Chitosan

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Abstract Flocculation is a method of harvesting microalgae by separating cells from the culture medium using flocculating agents. Ferric Chloride and alum are conventional flocculants that have the disadvantages of toxic residue. In this study, chitosan, a non-toxic flocculating agent is tested on *Chaetoceros gracilis* to determine the effect of culture conditions (salinity and cell density) towards the flocculation efficiency (FE). Marine diatom *C. gracilis* is cultured using Walne's Media for 8 days in a temperature controlled room and illuminated for 12 hours. Four different salinities (20, 25, 30, 35 ppt), four different cell densities ($0.5, 2, 4, 6 \times 10^6$ cells mL⁻¹), and six chitosan concentration (0, 15, 30, 45, 60, 75 ppm) are the parameters tested in this study. Measuring cylinder test is used to measure FE using spectrophotometer (OD₇₅₀). From the results, salinity and cell density affect significantly the FE of the diatom. In 20 ppt culture, the FE was 89% after 50 minutes using 75 ppm of chitosan at a density of 4×10^6 cells mL⁻¹. Growth conditions of the diatom culture have strong influences on the flocculation performances. The chitosan can be an option for non-toxic flocculants for concentrating live feeds in the aquaculture industry.

1. Introduction

Microalgae as live feeds are widely used in the aquaculture industry as feed especially in the larval rearing and juvenile stage. Microalgae are an important supplement to the overall growth and survival of the larvae rearing stage [1]. Small size, high nutritional properties, and availability have made microalgae to be one of the choices for feed the first-feeding period in aquaculture. In preparing for this, usually, mass cultivation of microalgae is set up. Harvesting and concentrating a mass volume of microalgae using centrifugation and filtering are usually hard labor work. Flocculation, on the other hand, seems to be an alternative to a fast and efficient way of harvesting microalgae. Flocculation is a method of coagulation and separation of suspended microalgae from the medium into aggregation by adding flocculating agents. There are a few methods of flocculation such as using inorganic hydrolyzing metal salts (aluminum sulfate and ferric chloride) and organic flocculants that include cationic and anionic polyelectrolytes. Other than that there are also non-ionic polymers and biopolymers [2]. Although the conventional and traditional methods of flocculation are proved inefficiency of a large mass of wastewater treatment, however ferric chloride and alum in the environment can cause a hazard to human health and aquatic organism because of their toxicity. On



the other hand, biopolymers are environmentally friendly, non-toxic and biodegradable. One of the known biopolymers is chitosan. Chitosan is derived from chitin through the process of deacetylation and chitin is mainly from the exoskeleton on crustacean (crabs and shrimps) [3].

The efficiency of flocculation depends on many factors, one of it is the pH and salinity of media, cell densities and concentration of flocculating agent used. The zeta potential measures the surface charge cells in suspension in conductive media. In this present study, three separate tests were done. The first test was to evaluate the efficiency of flocculation at different levels of pH and chitosan concentration. The second experiment was to determine the effect of culture salinity on the flocculation efficiency using a different concentration of chitosan and lastly to test the effect of cell density on flocculation efficiency using a different concentration of chitosan.

2. Materials and method

2.1 Microalgae culture method

Microalgae strain was obtained from Live Feed Laboratory, Borneo Marine Research Institute, University of Malaysia Sabah, Malaysia. *Chaetoceros gracilis* was cultured in 3 L cylindrical glass enriched with Walne's media [4] containing the following component: nutrient solution consist of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.3 g L^{-1}); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.36 g L^{-1}); H_3BO_3 (33.6 g L^{-1}); EDTA (45 g L^{-1}); $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (20 g L^{-1}); NaNO_3 (100 g L^{-1}) and 1 mL of trace metal solution consist of: ZnCl_2 (21 g L^{-1}); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (20 g L^{-1}); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (9 g L^{-1}); $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (20 g L^{-1}). Vitamin solution consist of Vitamin B12 (Cyanocobalamin) (100 mg L^{-1}); Vitamin B1 (Thiamine.HCl) (100 mg L^{-1}); Vitamin H (Biotin) (20 mg L^{-1}). Additional of $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ (40 g L^{-1}) was added to marine diatom. All solution added into filtered and autoclaved seawater with the measurement of 1 mL L^{-1} , 100 mL L^{-1} and 2 mL L^{-1} respectively.

Microalgae were cultured for 8 days in a fixed condition in different salinities (20, 25, 30, 35 ppt), under photoperiod 12:12 LDC at $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ illumination and temperature of 24°C . The initial inoculate was 20% of the working volume. Due to evaporation, the salinity of the culture was maintained throughout the culture by addition of seawater of respective salinity.

2.1.1 Measurement of growth.

Cell densities were counted microscopically using a Neubauer haemocytometer counting chamber every day and the optical density (OD) of the cell was also taken using spectrophotometer at 750 nm wavelength (OD_{750}). The growth performance of the culture was determined using these equations;

$$\text{Specific growth rate, } r = \frac{\ln (\text{Nt}-\text{No})}{\Delta t}$$

$$\text{Division per day, } k = \frac{r}{0.6931}$$

$$\text{Doubling time, } T_2 = \frac{0.6931}{r}$$

Where, Nt -the cell density at the end of exponential phase

No -the cell density at the beginning of the exponential phase

Δt -the days of the exponential phase

2.2 Preparation of chitosan solution

Chitosan was prepared in 1000 ppm of concentration. One gram of chitosan was diluted in 0.1 M acetic acid and was stirred using magnetic stirrer until fully dissolved. Sodium hydroxide (1 N) and Hydrochloric acid (1 N) were also prepared for pH alteration.

2.3 Effect of pH and chitosan concentration on flocculation efficiency

The determination of flocculation efficiencies was done using the measuring cylinder test [5]. This experiment was performed using a 100 mL measuring cylinder. Approximately 2.1 L of microalgae was distributed evenly into 21 measuring cylinders. For each treatment, a volume of microalgae was taken out so that the chitosan final concentration is exact. Seven chitosan concentrations were

experimented on this test (0, 5, 10, 20, 35, 50, 70 ppm). Microalgae from each treatment were poured in a 500 mL beaker and were stirred using a magnetic stirrer for 2 minutes. Chitosan was slowly added and pH was adjusted using 1 N NaOH and 1 N HCl. The microalgae were poured into the measuring cylinder and let settle for 50 minutes. A sample of microalgae were taken 2 cm from the surface before adding a flocculating agent and after 50 minutes of settling to measure OD₇₅₀. Flocculation efficiency of *C. gracilis* was calculated using the following formula:

$$\text{Flocculation efficiency, \%} = \frac{(A - B)}{A} \times 100$$

Where,

A -Initial OD₇₅₀

B -Final OD₇₅₀

2.4 Effect of culture salinity and chitosan concentration on flocculation efficiency

Using the findings in the previous experiment, the use of pH 9 was considered. For this test, the cell density used was approximately 4×10^6 cells mL⁻¹ for all the salinity. Six chitosan concentrations (0, 15, 30, 45, 60, 75 ppm) were used in this flocculation test. Two liters of *C. gracilis* was pre-adjusted to pH 9 using 1 N NaOH or 1 N HCl. Measuring cylinder test was also used in this experiment.

2.5 Effect of different cell concentration and chitosan concentration on flocculation efficiency.

For this test, three cell concentrations were evaluated; $0.5, 2, 6 \times 10^6$ cells mL⁻¹. The pH of media was maintained at pH 9. Similar to the previous test, the measuring cylinder was also used. The initial and final OD₇₅₀ were determined using a spectrophotometer.

2.6 Zeta potential analysis

Zeta potential of *C. gracilis* was measured using Malvern Zetasizer 90 (Malvern Instruments Ltd. USA). Their measurement was taken before and after the addition of flocculants. The measurement was taken in triplicate at 20°C.

2.7 Statistical analysis

Statistical analysis was done by using analysis of variance (ANOVA) and post-hoc tests (Tukey). Experiments were done in triplicate and data were expressed as mean \pm standard deviation. Statistical analyses were carried out using SPSS version 20 with the level of confidence of 95%.

3. Results and discussions

3.1 The growth of microalgae cultured in different salinity

The growth curves of *C. gracilis* cultured in four salinities are shown in Figure 1. The four treatments showed similar growth. On day 3 of cultivation, the decrease in cell density was due to the addition of seawater for salinity maintenance. The final cell density, growth rate, division per day and doubling time of all treatments are shown in Table 1. Although cultured in different salinities of media, there were no significant differences ($p > 0.05$) on the final cell density at day 8. Similar to the finding by [5], *C. calcitrans* cultured with Walne's media shows no sign in growth and cell densities of microalgae cultured in salinities 25 and 35 ppt. Generally, the growth rate of microalgae depends on the culture conditions and the properties of the media used. Based on [6], although salinity is one of the important parameters for growth of microalgae but when other parameters are optimized such as the growth medium and culture conditions (temperature and pH), salinity does not give much effect.

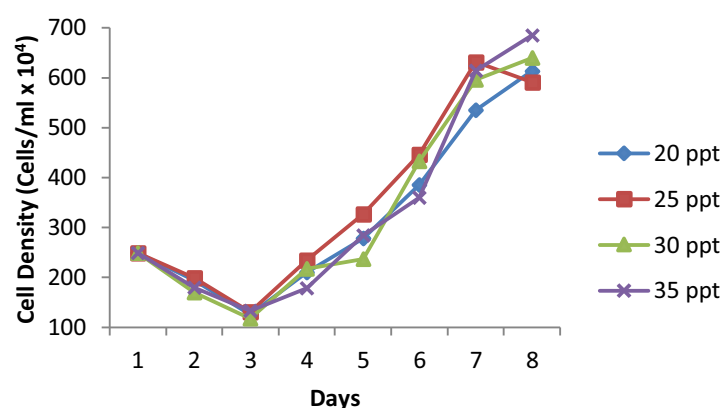


Figure 1. Growth curve of *C. gracilis* cultivated in different salinities 20 – 35 ppt.

Table 1. The final cell density (FCD), specific growth rate (SGR), division per day, and doubling time of *C. gracilis* cultured in salinities 20, 25, 30, 35 ppt.

Salinity, ppt	20	25	30	35
FCD, $\times 10^6$ cells mL^{-1}	$6.135^a \pm 0.48$	$5.915^a \pm 1.38$	$6.395^a \pm 1.21$	$6.850^a \pm 0.26$
SGR, r	$0.366^a \pm 0.04$	$0.390^a \pm 0.12$	$0.408^a \pm 0.04$	$0.413^a \pm 0.04$
Div. per day, K	$0.528^a \pm 0.06$	$0.563^a \pm 0.17$	$0.588^a \pm 0.06$	$0.596^a \pm 0.05$
Doubling time, T ₂	$1.910^a \pm 0.18$	$1.877^a \pm 0.49$	$1.702^a \pm 0.04$	$1.689^a \pm 0.18$

3.2 Effect of pH and chitosan concentration on flocculation efficiency

Media pH plays an important role in flocculation. The FE of *C. gracilis* was significantly ($P < 0.05$) affected by pH and chitosan concentration. The highest FE yielded was at pH 9 with chitosan concentration of 70 ppm (FE = 97%), but there was no significant difference when chitosan concentration was increased from 35 ppm to 70 ppm. Treatment of pH 8 also reached >80% FE starting at chitosan concentration of 20 ppm and above. The results showed that flocculation did occur at neutral condition (pH 7) and the FE was >80% at chitosan concentrations of 20, 50 and 70 ppm. Findings by [7] obtained highest FE (>90%) when flocculating the same diatom at pH 9 with chitosan concentration 80 mg L^{-1} which is comparable to the findings in this test. Test on pH ranges lower than 7 was not done a few studies had proven that chitosan flocculation does not occur in lower ranges of pH. This is because pH-induced flocculation occurred when an increase in pH caused the precipitation of magnesium ions of the culture media. The magnesium hydroxides produced by precipitation interacted with microalgae cells and caused flocculation through charge neutralization [8-9]. However, when chitosan is added as a flocculant, the mechanism of flocculation changed. Magnesium hydroxide no longer becomes the factor of flocculation but chitosan does. As reported in [9], flocculation still occurred with absent of the metal hydroxide when chitosan is added at pH 7.5 – 10 and in this case, chitosan loses its charge at high pH and precipitated.

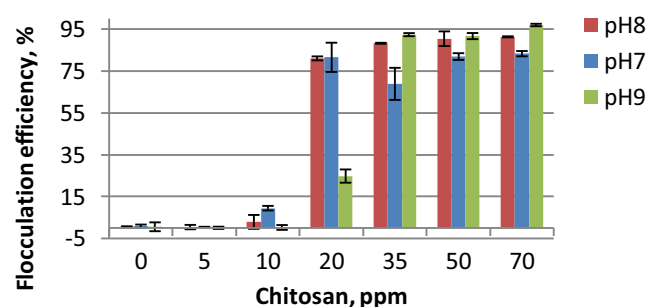


Figure 2. Flocculation efficiencies of *C. gracilis* tested with different concentration of chitosan (ppm) at pH 7, 8, 9.

3.3 Effect of salinity and chitosan concentration on flocculation efficiency

Considering the results obtained in the previous test, pH 9 was used in this and the next test. FE of *C. gracilis* in different salinities and chitosan concentration is shown in Figure 3. FE was significantly affected by media salinity and chitosan concentrations ($P < 0.05$). Flocculation process in freshwater is different in compared to flocculation in seawater as the chemical composition are different. Flocculation is more efficient in freshwater and requires less flocculant concentration compared to that in seawater [9]. This explains the results obtained in this study that the medium with salinity 20 ppt had initiated flocculation at chitosan concentration as low as 30 ppm with >40% of FE and this increased with the increasing of chitosan concentration. Even though FE in medium salinity 25, 30, and 35 ppt also increased with the increasing chitosan concentration however FE in 20 ppt at chitosan concentration 30 – 75 ppm are higher. Thus, this shows that the flocculation efficiency of marine microalgae is dependent on the salinity of media. In line with a previous study by [10], higher flocculation efficiency was observed at lower salinity (10 ppt) using pH 11 and above after 1 hour of settlement. Freshwater microalgae *Chlorella vulgaris* that was used in the study required lesser concentration of flocculant compared to marine microalgae *Chlorella stigmatophora* and *Isochrysis galbana*. According to [11], the properties makeup of marine waters can cause difficulties in flocculation as salts in seawater interfere with the process of flocculation.

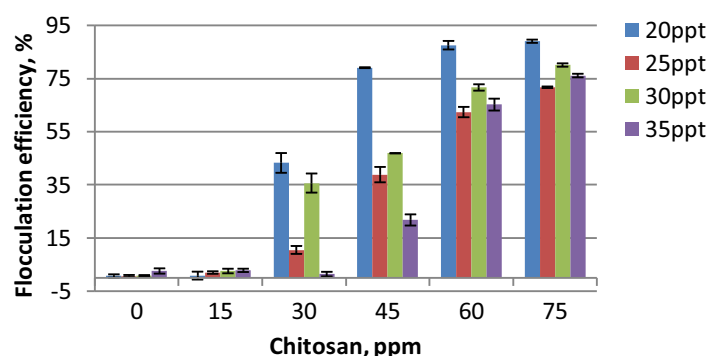


Figure 3. Effect of culture salinity and chitosan concentration to the flocculation efficiency of *C. gracilis*.

3.4 Effect of cell density and chitosan concentration on flocculation efficiency

FE was significantly affected by both cell density and chitosan concentration ($P < 0.05$). Based on Figure 4, optimum FE of *C. gracilis* was observed at a cell density of 4×10^6 cells mL^{-1} where the FE increased with the increase of chitosan concentration and reached an optimum value at chitosan concentration of 75 ppm (FE = 89.04%). On the contrary, cell densities of 0.5×10^6 cells mL^{-1} and 2×10^6 yielded lower FE than cell density of 4×10^6 cells mL^{-1} which are <60% and <85% respectively. The FE of cell density 0.5×10^6 cells mL^{-1} at 75 ppm of chitosan concentration were observed to be lower than 60 ppm chitosan concentration. This could be there were excess charges from chitosan precipitation in suspension causing stabilization when interacted with a small number of cells [12]. On the other hand, the cell density of 6×10^6 cells mL^{-1} yielded <40% FE which is the lowest. This result is comparable to a study by [8] where high cell density in suspension resulted in lower FE in both freshwater and seawater microalgae. This could be because of not enough charges to interact with the cells of microalgae causing leftover microalgae cells not be able to coagulate.

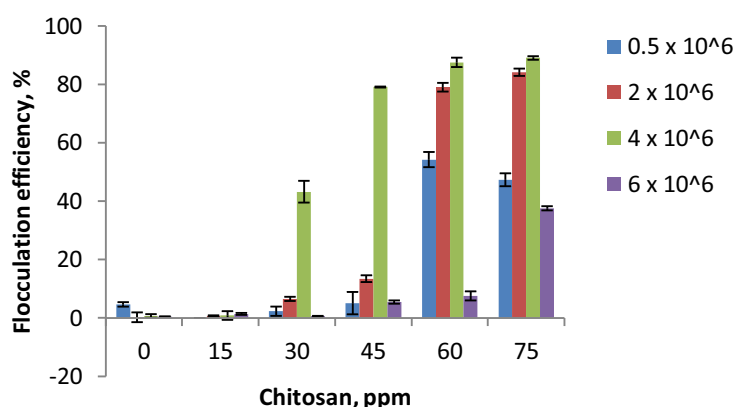


Figure 4. Effect of cell density and chitosan concentration to the flocculation efficiency of *C. gracilis*.

3.5 Zeta potential

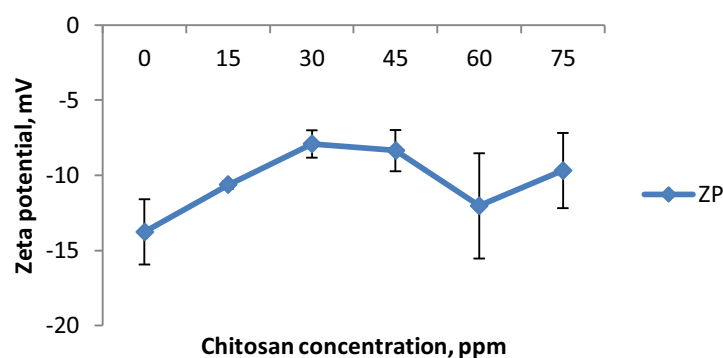


Figure 5. The change in zeta potential (ZP) of *C. gracilis* flocculation in 20 ppt and cell density of 4×10^6 cells mL^{-1} at pH 9 in different chitosan concentrations.

Chitosan concentration of 0 ppm is denoted as the condition of microalgae before adding flocculant. The zeta potential of the initial *C. gracilis* is in the range of -5 to -15 mV. As chitosan concentration increased from 0 to 30 ppm, zeta potential increased from -13.77 ± 2.17 mV to -7.91 ± 0.91 mV. Further increase in chitosan concentration to 60 ppm has decreased the zeta potential to -12.03 ± 3.5 mV. However, at 75 ppm of chitosan, the zeta potential increases its value to -9.68 ± 2.5 mV. The results obtained are comparable to that of [13] where an increase in chitosan concentration from 60 ppm to 120 ppm increased the zeta potential to positive values. In this study, although the chitosan concentration was not tested above 75 ppm and the species used is a marine microalga, however, the trend in the value of zeta potential in high concentrations of chitosan is similar.

3.6 Two-way ANOVA summary table

Table 2 shows the summary of the significant probability of main factors and the interaction between factors in each experiment. From the statistical run, the results showed the critical significance of the main factors and the interaction, except for experiment 1 where the main factor (chitosan concentration) had a p-value of 0.027.

Table 2. Summary of Two-way ANOVA significant probability (P).

Experiment 1	P - value
pH	0.0001
Chitosan	0.0270
pH x chitosan	0.0001
Experiment 2	
Salinity	0.0001
Chitosan	0.0001
Salinity x chitosan	0.0001
Experiment 3	
Cell Density	0.0001
Chitosan	0.0001
Cell Density x chitosan	0.0001

4. Conclusion

The optimum condition for harvesting *Chaeroceros gracilis* by flocculation was identified. Chitosan performed better in alkaline conditions (pH 9) as the amine groups in the chitosan dissociates and interacted with the microalgae cells to form aggregates. Lower salinity (20 ppt) media is ideal for *C. gracilis* flocculation as the salts in high salinity interferes with the flocculation process and in addition, the growth rate of *C. gracilis* in 20 ppt is comparable to the cultures in higher salinities. The cell density of around 4×10^6 cells mL⁻¹ demonstrated to be optimum for flocculation when chitosan at a concentration of 60 - 75 ppm is used. Harvesting microalgae using chitosan is not only eco-friendly but also fast and efficient in concentrating microalgae and was recommended for aquaculture uses.

5. References

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