

Effective flocculation of *Chlorella vulgaris* using chitosan with zeta potential measurement

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Abstract. Microalgae are considered as one promising source of third-generation biofuels due to their fast growth rates, potentially higher yield rates and wide ranges of growth conditions. However, the extremely low biomass concentration in microalgae cultures presents a great challenge to the harvesting of microalgae because a large volume of water needs to be removed to obtain dry microalgal cells for the subsequent oil extraction process. In this study, the fresh water microalgae *Chlorella vulgaris* (*C. vulgaris*) was effectively harvested using both low molecular weight (MW) and high MW chitosan flocculants. The flocculation efficiency was evaluated by physical appearance, supernatant absorbance, zeta potential and solids content after centrifugal dewatering. High flocculation efficiency of 98.0-99.0% was achieved at the optimal dosage of 30-40 mg/g with formation of large microalgae flocs. This study suggests that the polymer bridging mechanism was governing the flocculation behaviour of *C. vulgaris* using high MW chitosan. Besides, charge patch neutralisation mechanism prevailed at low MW chitosan where lower dosage was sufficient to reach near-zero zeta potential compared with the high MW chitosan. The amount of chitosan polymer present in the culture may also affect the mechanism of flocculation.

1. Introduction

Harvesting of algal biomass from dilute culture medium is a major bottleneck for economical biofuel production using microalgae as feedstock. It is estimated that the cost of recovery process contributes to 20-30% of the total cost of biomass production, which accounts for a total of about 50% to the final cost of oil production [1]. Conventional harvesting techniques such as filtration, flotation, and centrifugation do not meet the economic requirements for commercialisation of algal biofuels, as compared with the other high-value, low-volume products such as β -carotene derived from microalgae. In particular, the centrifugation method manages to achieve only 50% energy efficiency on the basis of available energy in the recovered biomass relative to the energy input [1]. Therefore, one major challenge to the commercialisation of algal biofuels is to develop a harvesting technique of high efficiency, low energy demand and hence low cost.

Although microalgae are not yet produced at large scale industrial processing, it is believed that advances in research and development of microalgae present vast opportunities for sustainable development of this potential biofuel feedstock within the next 10 to 15 years [2]. Recent studies have proposed several novel microalgae harvesting techniques such as electro-flotation [3], bio-flocculation [4] and membrane separation [5]. It is noteworthy that most studies adopt some form of coagulation and/or flocculation as the sole or supplementary treatment to concentrate and harvest microalgae [3, 6].



Flocculation is able to concentrate a dilute algal suspension by about 20-100 times into a thick slurry; then centrifugation dewateres the slurry into algal paste of about 25% dry matter content [7].

The effectiveness of chitosan as a flocculant for microalgae harvesting is well known. However, the feasibility of this natural biopolymer for effective microalgae harvesting is yet to be further explored and understood in order to optimise its performance. It is also found that many studies express chitosan dosage in mg/L which confounds the comparison and interpretation of effective flocculant dosage because the biomass concentrations usually differ from one another. This study presents an experimental investigation using low molecular weight (MW) and high MW chitosan flocculants to harvest *C. vulgaris* in order to achieve favourable solids content after centrifugal dewatering. Instead of using the common solvent—acetic acid, hydrochloric acid (HCl) was used as the solvent because chitosan is highly soluble in HCl and the solution is also found to give high separation efficiency [8]. The chitosan dosage is expressed as the weight of flocculant over the weight of biomass solids in the culture, in mg/g, in order to facilitate comparison of performance regardless of its biomass concentration. The study involved two steps of harvesting, with flocculation by chitosan first as the pre-concentrating step, followed by centrifugal dewatering step. Mechanisms involved in the flocculation process were studied and justified based on the evaluation of physical appearance, supernatant absorbance, zeta potential and solids content after dewatering.

2. Experimental procedure

2.1. Cultivation of *C. vulgaris*

Pure strain of *C. vulgaris* was purchased from Algaetech International Sdn. Bhd., Malaysia. *C. vulgaris* inoculum was cultivated in modified Bold's Basal Medium (BBM) at room temperature, with a photoperiod of 16:8 h light and dark cycle [9], and 15-min aeration every hour during the light cycle. The first batch of cultivation (labelled as Culture A) was cultured for 10 weeks while the second batch (labelled as Culture B) was cultured for 8 weeks, keeping all cultivation conditions unchanged. The modified BBM formulation with each component's concentration in the final medium is given as follows: NaNO₃ [25 g/L], CaCl₂·2H₂O [2.5 g/L], MgSO₄·7H₂O [7.5 g/L], K₂HPO₄ [7.5 g/L], KH₂PO₄ [17.5 g/L], NaCl [2.5 g/L], EDTA [50 g/L], KOH [31 g/L], FeSO₄·7H₂O [4.98 g/L], H₂SO₄ [4.98 g/L], H₃BO₃ [11.42 g/L], ZnO₄·7H₂O [8.82 g/L], MnCl₂·2H₂O [1.44 g/L], CuSO₄·5H₂O [1.57 g/L], Co(NO₃)₂·6H₂O [0.49 g/L], and 2.5 g sucrose in 1 L of the medium [10]. The final medium was sterilised by autoclave before use.

2.2. Characterisation

After 8–10 weeks of cultivation, mature culture of *C. vulgaris* was sampled for measurement of pH, zeta potential and biomass concentration. Zeta potential of the culture supernatant after 20-min settling was measured by Zetasizer Nano ZS (Malvern) using Laser Doppler Electrophoresis method. To determine the algal biomass concentration, 20-mL microalgae sample was taken from a well-mixed culture suspension and dried in oven at 60°C overnight or until constant weight was obtained. Table 1 shows the characteristics of *C. vulgaris* in both Culture A and Culture B.

Table 1. Characterisation of *C. vulgaris*.

Culture	A	B
Cultivation period	10 weeks	8 weeks
pH	7.80	7.64
Zeta potential (mV)	-35.2	-28.8
Biomass concentration (g/L)	1.79	2.09

2.3. Flocculation

Low MW chitosan (50,000–190,000 Da, Aldrich) and high MW chitosan (600,000–800,000 Da, Acros Organics) were prepared as flocculant solutions by dissolving 0.1 g chitosan in 100 mL of 0.1 M hydrochloric acid (HCl) at 40°C under gentle mixing for about an hour or until it was completely dissolved [11]. The solutions were freshly prepared prior to the commencement of experiments to avoid degradation of chitosan polymer in solvent [12]. The flocculant was added into the 100-mL *C. vulgaris* sample at the predetermined dosage, ranging from 0 mg/g to 60 mg/g. The culture medium was rapidly mixed at 500 rpm for 1 min to allow coagulation, followed by slow mixing at 100 rpm for 5 min to promote flocculation. The suspension was then left to settle under quiescent condition for 20 min and the supernatant was sampled at 1-cm depth from the suspension surface for zeta potential measurement [13].

Lambda 25 UV/Visible Spectrometer (Perkin Elmer) was used to measure the absorbance of light by *C. vulgaris*. As shown in figure 1, the characteristic peak wavelength for *C. vulgaris* was found close to 680 nm which was used for the determination of absorbance value as an indirect measurement of cell concentration and flocculation efficiency. The absorbance values were calibrated against cell concentrations for the calculation of flocculation efficiency in equation (1):

$$\text{Flocculation Efficiency (\%)} = 100 \times \left(1 - \frac{C_{\text{supernatant}}}{C_{\text{initial}}} \right) \quad (1)$$

where $C_{\text{supernatant}}$ is the concentration of the algal supernatant with flocculant and C_{initial} the initial concentration of the algal supernatant without flocculant (control).

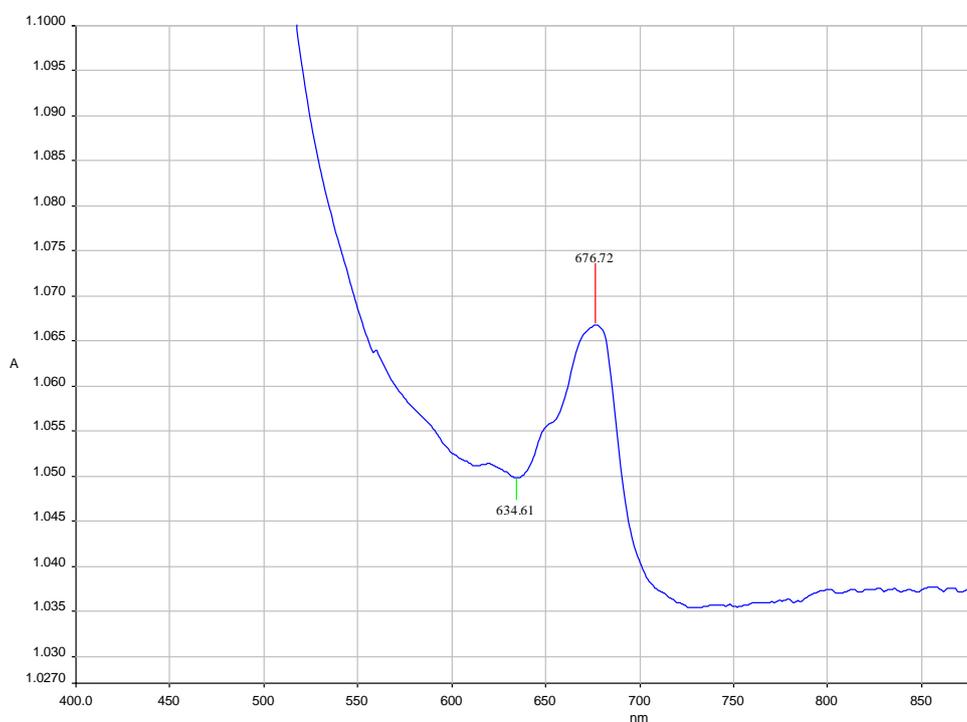


Figure 1. Absorbance spectrum for *C. vulgaris*.

2.4. Centrifugation

After flocculation, 90% of the supernatant was decanted leaving 10-mL algal flocs which were collected and centrifuged at 2000 rpm for 10 min. The concentrated algal paste was then transferred to a pre-weighed filter paper and dried in the oven at 60°C for 12 hours or until constant weight was obtained. The solids content of the dewatered algal flocs was determined using equation (2), where M_w is the weight of the wet algal paste and filter paper, M_D the weight of the dried algal paste and filter paper, and M_0 the weight of the empty filter paper, all expressed in g.

$$\text{Solids Content (\%)} = \frac{M_D - M_0}{M_w - M_0} \times 100 \quad (2)$$

3. Results

3.1. Effect of molecular weight on flocculation efficiency

From figure 2, it can be seen that high MW chitosan gave high flocculation efficiency of 97% at the dosage of 20 mg/g while low MW chitosan can only achieve 49% at the same dosage in Culture A. Additional 10 g/mg was required to reach optimum flocculation using low MW chitosan. For Culture B, the effect of molecular weight became less significant where both low and high MW chitosans gave high flocculation efficiency of 98-99% at 20 mg/g. However, at lower dosage, high MW chitosan still showed better flocculation of *C. vulgaris* than low MW chitosan. Nevertheless, no visible algal flocs were formed at lower dosage. The microalgae appeared as discrete tiny sediments at the bottom of the suspension which were easily disturbed when external force was exerted. The supernatant was not translucent (or partially translucent) at this stage. This is regarded as a phenomenon of under-dose of chitosan flocculant [7].

3.2. Zeta potential and flocculation efficiency

Flocculation efficiency as a result of charge neutralisation can be examined by correlating zeta potential with the dosage of chitosan flocculant. As the chitosan dosage increased, figure 3 shows the increase of zeta potential from -35.5 mV (Culture A) and -28.2 mV (Culture B) of the control to near-zero value approaching the dosage of 40 mg/g with good flocculation performance, except for Culture B with high MW chitosan. Charge reversal was only noticed for Culture A when flocculated with high MW chitosan at dosage above 50 mg/g. On the other hand, Culture B showed a much gradual increase in zeta potential upon addition of high MW chitosan and no charge reversal was recorded within the range of dosage in this study.

Theoretically, optimal flocculation results from charge neutralisation when zeta potential is near-zero [14]. Upon achieving zero zeta potential, the negative charges on the *C. vulgaris* cell surface are said to be fully neutralised by the cationic amino groups of the chitosan flocculant. Increase in chitosan dosage increases the amount of amino groups and improves the aggregation with formation of larger flocs as observed later in Section 3.3. However, at very high dosage of chitosan, when all available cell surfaces are fully neutralised by the amino groups, repulsion occurs among amino groups especially of long polymer chains, leading to re-stabilisation of flocs and reduced flocculation efficiency [8].

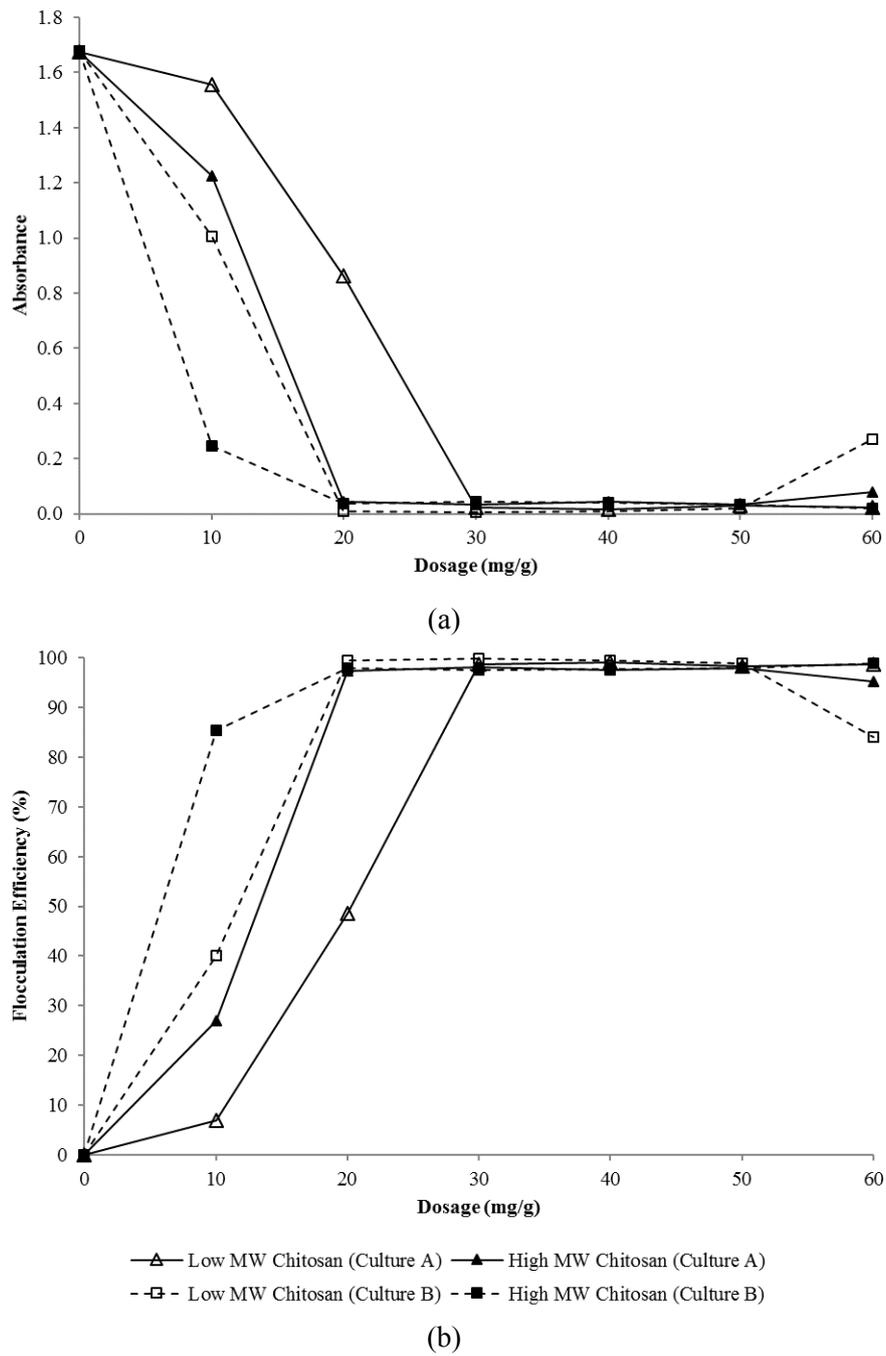


Figure 2. Effect of molecular weight of chitosan on (a) absorbance measurement, and (b) flocculation efficiency.

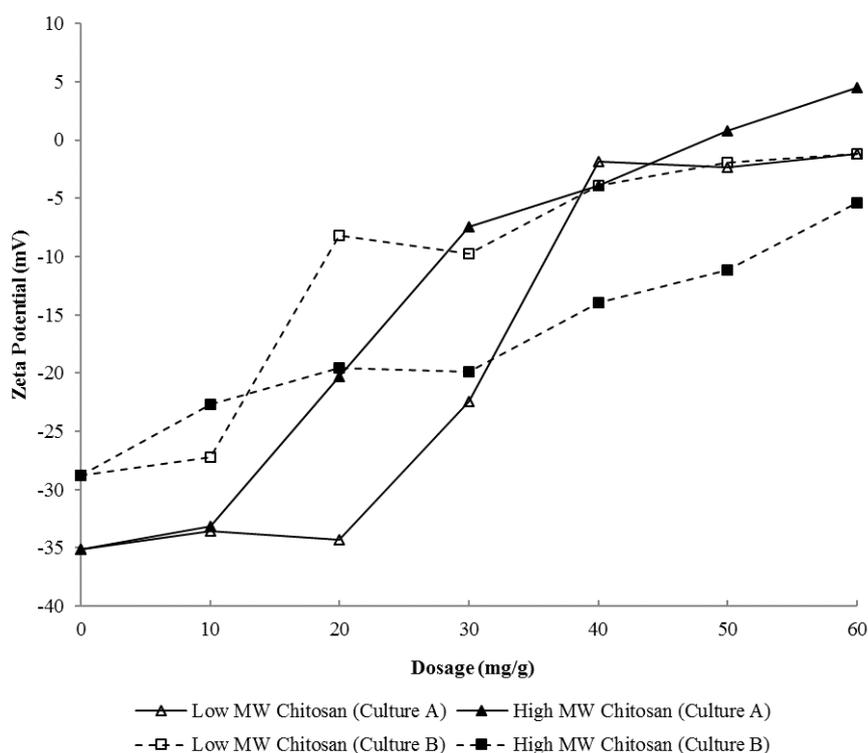


Figure 3. Effect of molecular weight of chitosan on zeta potential values.

3.3. Physical appearance of flocs

It can be seen from figures 4a and 4b that no obvious flocs were formed at the dosage of 10–20 mg/g low MW chitosan in Culture A, which is consistent with the low flocculation efficiency (less than 50%) in figures 2a and 2b. Visible flocs started to form with the addition of 30 mg/g of low MW chitosan and settled fast at the bottom of the beaker after the flocculation mixing was stopped (figure 4c). Although a flocculation efficiency of 98.6% was recorded at this dosage, the respective negative zeta potential value (-22.5 mV) indicated that significant charge repulsion still existed at this point with the formation of smaller flocs. On the other hand, large aggregates and clear supernatant were noticed at the optimum dosage of 40 mg/g with zeta potential approaching zero (i.e. -1.9 mV) (see figure 4d). Overdosing *C. vulgaris* culture at 50–60 mg/g of low MW chitosan did not cause much change to the flocculation efficiency and zeta potential, but the flocs seemed to disintegrate into smaller flocs at 60 mg/g, compared with those formed at 50 mg/g (figures 4e and 4f).

For the flocculation in Culture B, figure 5 shows that at a lower dosage of 20 mg/g low MW chitosan, visible flocs started to form and settled, leaving behind clear supernatant with high flocculation efficiency of 99.4% and zeta potential -8.4 mV which is a quicker response to dosage as compared with Culture A. The colour of *C. vulgaris* cells was found to be lighter green with shorter cultivation period of 8 weeks in Culture B in comparison with Culture A (10 weeks). Besides, the flocs formed in Culture B were generally smaller, even at optimum dosage, unlike the formation of large aggregates in Culture A at optimum dosage. At 60 mg/g, the microalgae cells in Culture B were re-suspended with reduced flocculation efficiency of 83.9% but the zeta potential value remained close to zero (i.e. -1.2 mV). Similar trend of flocculation behaviour was observed for high MW chitosan in Cultures A and B which was not shown here. It is believed that the microalgae culture age has significant effect on the flocculation efficiency and quality. Culture B was harvested at the early stationary growth phase while Culture A was harvested at the late stationary growth phase. More studies are required to understand the effect of culture age on the flocculation behaviour of *C. vulgaris* aided with chitosan flocculant.

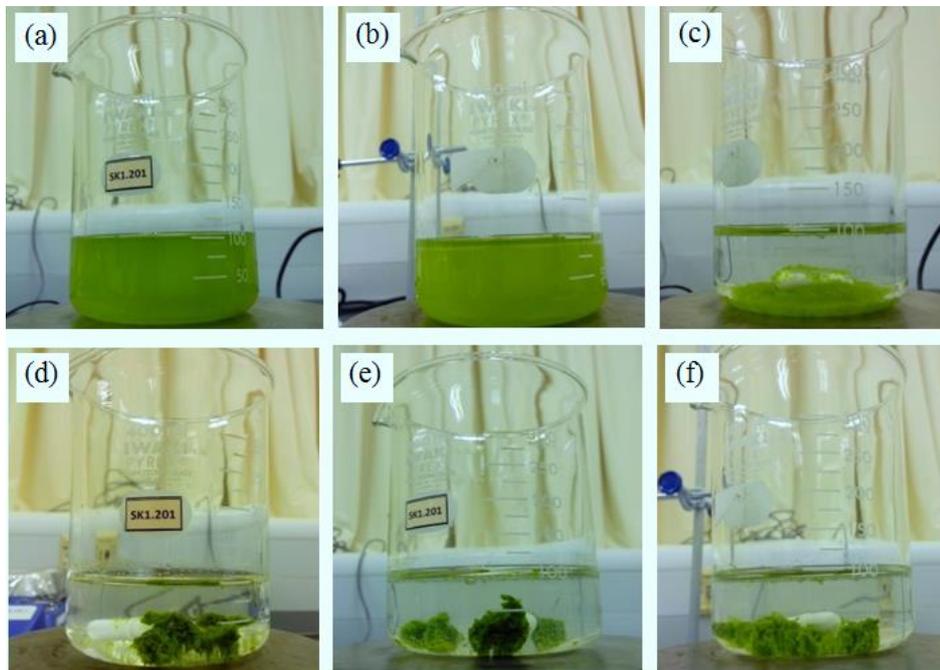


Figure 4. Sequence of flocculation at different doses of low MW chitosan in Culture A: (a) 10 mg/g (-33.6 mV); (b) 20 mg/g (-34.3 mV); (c) 30 mg/g (-22.5 mV); (d) 40 mg/g (-1.9 mV); (e) 50 mg/g (-2.3 mV), and (f) 60 mg/g (-1.2 mV).

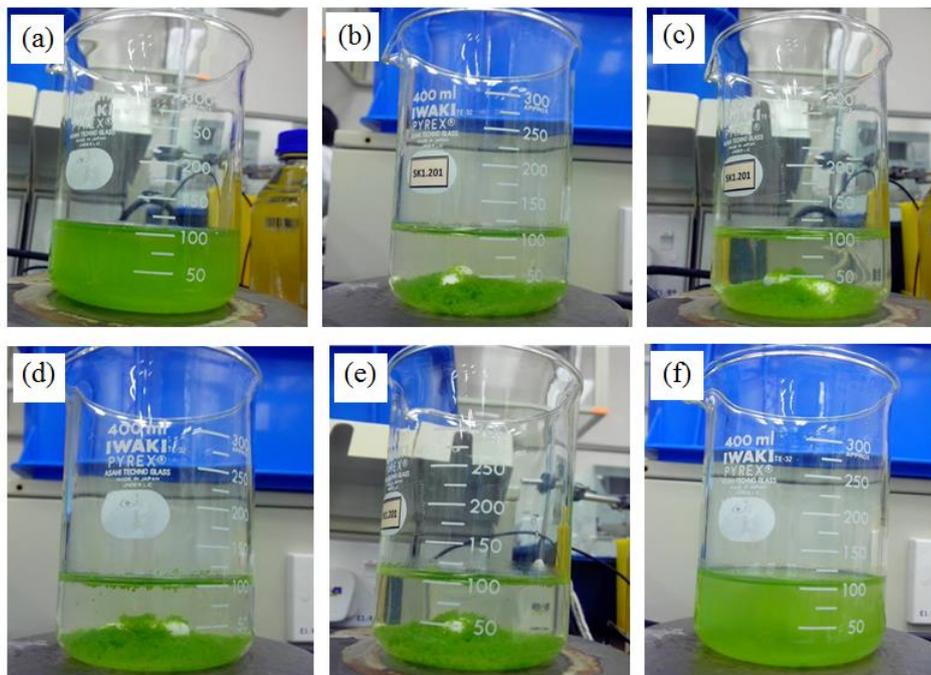


Figure 5. Sequence of flocculation at different doses of low MW chitosan in Culture B: (a) 10 mg/g (-27.2 mV); (b) 20 mg/g (-8.2 mV); (c) 30 mg/g (-9.8 mV); (d) 40 mg/g (-3.9 mV); (e) 50 mg/g (-2.0 mV), and (f) 60 mg/g (-1.2 mV).

In most of the flocculated samples, small amount of algae flocs was found floating on the surface of the medium (see figure 6). However, such phenomenon was more obvious when added with low MW chitosan flocculant. Similar experience was reported by Rashid, Rehman [8] and they attributed it to the formation of fragile flocs with large volume and low weight as a result of intra molecule repulsion among the amino groups of unreacted chitosans. Flotation of microalgae flocs was likely to cause losses of solid mass when the supernatant was decanted.

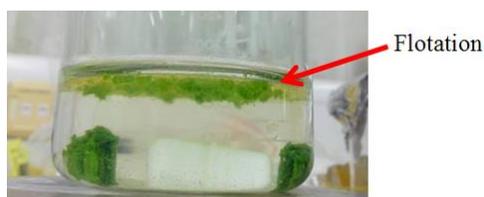


Figure 6. Flotation of microalgae flocs.

3.4. Solids content after centrifugation dewatering

Flocs formed from various doses of chitosan in Culture B were taken for centrifugal dewatering. Figure 7 shows the concentrated algal paste collected at the bottom of the tube after centrifugation. It can be observed that more biomass solids were captured at high dosage of chitosan.

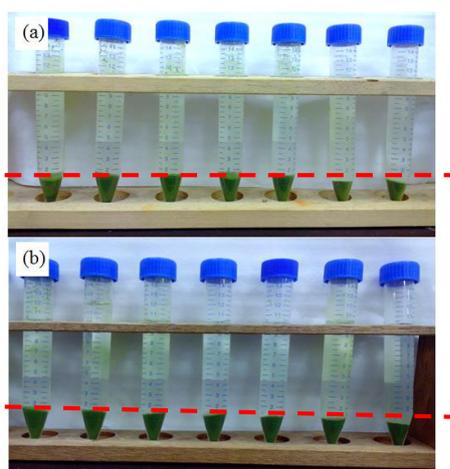


Figure 7. Biomass solids separated from centrifugation after flocculation using (a) low MW chitosan, and (b) high MW chitosan, from the dosage of 60 mg/g to 0 mg/g (left to right).

The solids content of the dewatered flocs was found to increase from 7.2% in the control to 10.8% and 11.8% for low MW and high MW chitosans respectively at the dosage of 10 mg/g (see figure 8). Further increase in flocculant dosage caused the solids content fluctuate between 11.6% and 12.0% for low MW chitosan, and between 10.3% and 11.4% for high MW chitosan. A general impression is that centrifugation effectively separated the algal biomass and chitosan flocculation enhanced the capture of biomass solids. However, a direct relationship between flocculation efficiency and dewatered solids content was not evident from this study, at least not after the centrifugal dewatering. More research effort will be required to better measure the correlation.

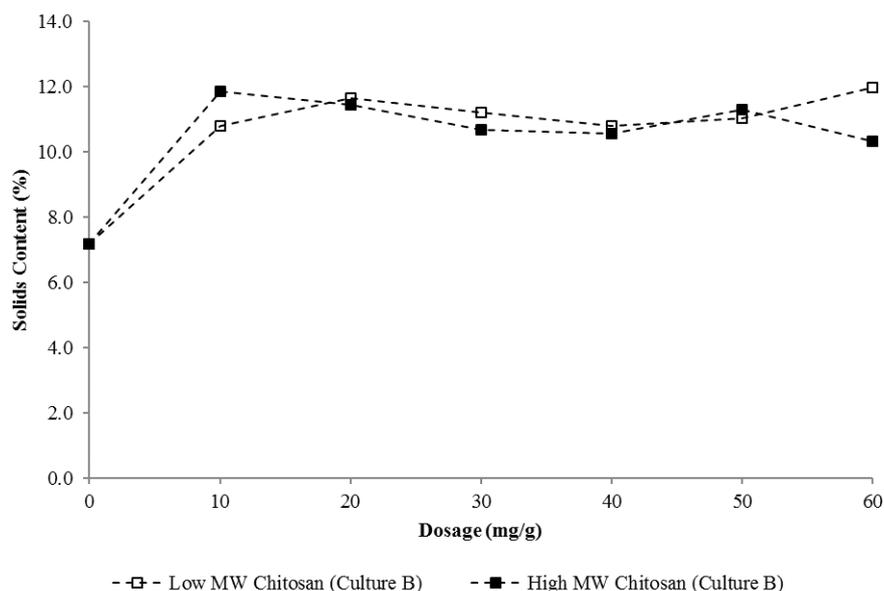


Figure 8. Effect of molecular weight of chitosan on solids content after centrifugal dewatering.

4. Discussion

4.1. Coagulation—charge patch mechanism

In this study, coagulation is the process where *C. vulgaris* cells are destabilised during the rapid mixing step which promotes collision between chitosan flocculant and the microalgae cells. During the coagulation (also known as charge neutralisation or destabilisation) process, the positively charged amino groups of chitosan, which dissociates in acidic medium, attract the negatively charged *C. vulgaris* cells. This results in reduction of electrostatic repulsion among the microalgae cells, and hence reduction in zeta potential as the negative charges on the cell surface are adsorbed or neutralised by the cationic chitosan polymer. The destabilised microalgae cells are attracted to each other and form microflocs. This mechanism is known as the charge patch or electrostatic mechanism [15] in coagulation. Van der Waals' force is responsible in the binding of microalgae cells and destabilising of the suspension [16]. The neutralised cells become capable to “stick” together and form microflocs. However, microflocs are too small to be observed by naked eyes (such as those in figures 4a and 4b).

4.2. Flocculation—polymer bridging mechanism

Formation of microalgae flocs is the main objective of the flocculation step. Ideally, strong flocs of *C. vulgaris* which are not reversible should be obtained, and the layer of clear transparent supernatant can be separated from the solid mass easily which then allows the medium to be recycled and reused. It is suggested that the flocculation process mainly adheres to polymer bridging mechanism. Coagulation process reduces repulsions between the destabilised *C. vulgaris* cells, followed by the flocculation step which initiates the formation of macroflocs. Theoretically, the microalgae flocs will continue to grow until zero zeta potential is achieved. It is evident from this study that large microalgae flocs were formed at zeta potential approaching zero especially in Culture A.

During formation of microalgae flocs, the long chain of chitosan polymer links two or more microflocs together with polymer bridges. The same process may continue even when coagulation is not successful. The polymer chains may bind directly to the microalgae cells and form microflocs, which later grow into macroflocs. According to Blanco, Negro [16], flocculation may produce either soft or hard flocs. Soft flocs are said to be easily break when excessive shear is applied to the flocs, especially when there is turbulence in the medium. Hard flocs formed possess significantly higher strength than

soft flocs, but will lose their strength over time. According to Norell, Johansson [17], low MW polymer often contributes to formation of soft flocs, where high MW polymer aids the formation of hard flocs. This aspect has not been assessed in this study. Nevertheless, the centrifugal separation of water from the microalgae flocs in this work did not indicate much difference in terms of solids capture for both low MW and high MW chitosans, presuming soft and hard flocs were formed respectively.

Flocculation using high MW chitosan is more prompt to polymer bridging mechanism. Higher MW chitosan possesses longer polymer chains which can bind to more microalgae cells during flocculation process. However, at high dosage, the unbound polymer chains are extended into the bulk solution where the positive charges on the polymer chains contribute to an overall positive zeta potential. This explains the charge reversal which occurred in Culture A when flocculated with high MW chitosan.

4.3. Flocculation—charge patch mechanism

Besides coagulation, charge patch mechanism also promotes the formation of microalgae flocs. During coagulation, destabilised cells are attached together to form the microflocs. For the low MW chitosan in particular, the short polymer chain may adsorb completely onto the cell surface but is not long enough to bind to another cell. It creates positive charges locally on the cell surface and attracts another cell or partially destabilised cell to bind onto itself. However, the microalgae flocs obtained from this mechanism may be smaller in size and are not as strong as those formed from polymer bridging mechanism.

5. Conclusions

Considering both the absorbance measurement for flocculation efficiency as well as zeta potential measurement, the optimal dosage of chitosan for effective flocculation of *C. vulgaris* was found to be 30 mg/g for high MW chitosan and 40 mg/g for low MW chitosan in Culture A. High flocculation efficiency of 98.0-99.0% was achieved at the optimal dosage with formation of large microalgae flocs. Near-zero zeta potential values were attained only at the higher flocculant dosage approaching 40 mg/g (except for Culture B with high MW chitosan) where charge patch neutralisation mechanism is said to be complete. The measurement of zeta potential also revealed that the flocculation of *C. vulgaris* using high MW chitosan required higher dosage to achieve near-zero zeta potential value and charge reversal was observed at increasing dosage. This suggests that the polymer bridging mechanism was governing the flocculation behaviour of *C. vulgaris* using high MW chitosan as the unbound long polymer chains may extend to the bulk microalgal suspension and cause charge reversal. Besides, charge patch neutralisation mechanism was found more significant using low MW chitosan where lower dosage was sufficient to reach near-zero zeta potential compared with the high MW chitosan. The amount of chitosan polymer present in the culture may also affect the mechanism of flocculation. It is proposed that the polymer bridging mechanism prevailed in the flocculation of *C. vulgaris* at lower flocculant dosage since effective flocculation was observed even before charge neutralisation. In addition, the age of microalgae harvested at the early and late stationary growth phase was found to affect the flocculation behaviour of *C. vulgaris*. Although the use of chitosan as a flocculant for microalgae harvesting sounds promising, it may result in microbiological contamination, which affects the downstream processing of microalgal biomass for biofuel production. Future studies should further investigate the influence of the microalgal cell properties or culture conditions on chitosan flocculation, and the respective downstream treatment. Besides, holistic cost evaluation is required to consider both the cost of flocculation as well as the influence on the entire production process.

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