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Abstract

The flocculation efficiency of microalgae *Chlorella vulgaris* for subsequent harvesting was investigated using single flocculants of inorganic salts, synthetic polymer, chitosan and dual flocculants of inorganic salts and chitosan. Synthetic polymer (Flopam^{TM}) could achieve over 90% optical density removal (OD_{680} removal) at a low flocculant dose (20 to 40 mg polymer per litre of algal suspension) through the bridging mechanism and charge neutralisation. Inorganic salts (i.e. ferric chloride and aluminium sulphate) and chitosan individually resulted in low flocculation efficiency (<90%) despite high dose (i.e. 160 to 200 mg per litre of algal suspension). The dual flocculation combining ferric chloride or aluminium sulphate with chitosan induced synergistic effects, resulting in >80% flocculation efficiency, significantly higher than the sum of each individual flocculation. The improvement in flocculation efficiency was 57 and 24% respectively for ferric chloride/chitosan and aluminium sulphate/chitosan. Charge neutralisation of microalgal cells by ferric chloride or aluminium sulphate combined with bridging by chitosan produced the synergy.

Keywords: Ferric Chloride; Aluminium sulphate; Charge neutralisation; Bridging; Dual flocculation; Polyacrylamide.

1 Introduction

Microalgae are among the most important organisms in ecological evolution and history of the Earth. They have the potential to shape our future with a wide range of promising applications that tackle worldwide issues. The global fossil fuel supply is depleted and has caused destructive environmental effects over its life cycle. There is growing interest in microalgal biomass as renewable and environmental-friendly feedstock for third-generation biofuel [1, 2]. The nutritive value of microalgal biomass for human as well as their versatile biochemical features have allowed for the production of health supplements, bioactive compounds, food additives and biotechnology applications, although there are still several hurdles in terms of socio-economic aspects [3-5]. In particular, harvesting has been a major technical and economic bottleneck in microalgal biomass production due to low cell concentrations in cultures (0.5 to 5 g/L), small cell size (< 30 µm), the stability of cell suspension and variation in culture medium [6-9]. Currently, microalgal harvesting is the most expensive step (i.e. 20-30% of total cost) in the process of microalgal biomass production [6, 10].

The microalgal harvesting techniques include coagulation, flocculation, flotation, membrane filtration and centrifuge [6, 11, 12]. Amongst them, flocculation has received significant attention for its simple operation and relatively low-cost approach, but efficiency is dependent on flocculant type [9, 11, 13]. Available chemical flocculants for microalgal harvesting can be grouped into three categories: (i) inorganic flocculants such iron and aluminium salts, (ii) synthetic polymer such as polyacrylamide and polyelectrolyte and (iii) natural organic polymers such as chitosan and cationic starch [9, 13]. Synthetic polymers often provide high harvesting efficiency at low dose [14]. However, these polymers are expensive. Inorganic flocculants such as ferric chloride and aluminium sulphate are less expensive but require a higher dose. Contamination and/or discolouration of microalgal biomass are possible concerns when using inorganic salts. The presence of these salts in the harvested biomass hinders its applications for biofuel and pigment extraction [11]. These issues with the quality of the harvested biomass can be avoided by using natural polymers like chitosan. Chitosan is a promising flocculant due to its advantages (e.g. natural product, biodegradation and non-toxic) [11, 15]. It has been demonstrated that chitosan residual in the culture media (i.e. after biomass harvesting) is non-toxic to microalgae. This feature enhances the reusability of the culture media, which is a potential option to reduce cost [15]. However, the expensive cost around 20 to 50 USD/kg of chitosan (depending on the purity) sets back its large-scale application [11, 16].

Inorganic salts provide flocculation through neutralising microalgal cell charge while chitosan flocculates microalgal biomass through bridging [11]. Therefore, it is hypothesized that the combination of these two mechanisms can enhance flocculation efficiency or harvesting efficiency. Loganathan et al. (2018) reported that a combination of alum and chitosan as flocculant aid induced a synergistic impact on harvesting seawater microalgae [17]. The author indicated that a reduction of 20 mg flocculants per litre of algal suspension was achieved while maintaining the harvesting efficiency over 95% [17]. However, there has yet been any studies on freshwater *Chlorella vulgaris* harvesting using this type of flocculant combination. The most similar approach combining ferric chloride and polyethylene was conducted by Gorin et al. [18]. They reported an increase from 60% to 90% flocculation efficiency of *Chlorella vulgaris* using dual flocculation. However, the dose of ferric chloride was very high at 500 mg/L, which may cause unfavourable effects on algal cells. Given the benefits (e.g. biological and pharmaceutical properties, nutrient contents for human health) of microalgae *Chlorella vulgaris* [19], effective harvesting of its biomass without compromising the cell quality will be a stepping stone to mass production of microalgal based products.

This study aims to compare the performance of four types of flocculants including two metal salts ferric chloride and aluminium sulphate, polyacrylamide polymer Flopam™ and organic polymer chitosan on *Chlorella vulgaris* harvesting. From the results of these single flocculation tests, dual flocculation tests using inorganic salt followed by chitosan addition were conducted to determine to what extent this strategy can improve the efficiency and reduce flocculant dose of the process. Optical density removal, turbidity and zeta potential were measured to evaluate flocculation efficiency and mechanisms. The results from this study is expected to contribute to the greater research on optimising microalgae harvesting, particularly using flocculation process.

2 Materials and methods

2.1 Microalgal suspension and materials

Microalgal suspension sample was prepared using the freshwater species *Chlorella vulgaris* (CS-41) (Australian National Algae Culture Collection, CSIRO Microalgae Research, Hobart, TAS). This species was grown in the MLA medium (Algaboost; Wallaroo, SA, Australia) to its mid-stationary phase following the previous protocol [14]. Its growth phase was monitored daily by measuring the optical density of the solution at wavelengths of 680 nm.

Microalgal suspensions at a mid-stationary growth phase were used for harvesting experiments (Section 2.2). The mid-stationary growth phase was selected because of its peak in biomass production. In the microalgal growth cycle, the mid-stationary phase occurs right after their population increased exponentially. At the mid-stationary phase, cell divisions had slowed down significantly due to high cell density thus the decrease in feeding factors (e.g. nutrients, light, pH and carbon dioxide). Thus, harvesting microalgae at mid-stationary phase is a common protocol.

Anhydrous ferric chloride powder (> 98% purity) was supplied by Chem-Supply (Australia). Aluminium sulphate hydrate (54 – 59% assay) was purchased from Sigma-Aldrich (Australia). Cationic polyacrylamide polymer Flopam ™ (model no. FO4808) with very high molecular weight was obtained from SNF Australia. Stock solutions of 2 g/L were prepared for each of these flocculants in 200 mL of Milli-Q water and mixed at 100 rpm for one hour. Cationic polyacrylamide polymer (2 g/L) was used within one hour of preparation to avoid polymer hydrolysis. Chitosan (originated from chitin shells of crustaceans) was purchased from Sigma-

Aldrich (Australia). Since chitosan is insoluble in water, 0.4 g of chitosan was dissolved in 10 mL of 0.1% HCl solution, followed by the dilution with 190 mL of Milli-Q water to obtain the desired 2 g/L stock concentration. The stock solutions were stored in room temperature and used within two days of preparation.

2.2 Flocculation experiment

A 4G Platypus Jar Tester (Australia Scientific, Kotara NSW) was used in flocculation experiments. Samples of 200 mL microalgal suspension were added to 500 mL beakers. Flocculant was introduced to each beaker to obtain a predetermined dose. The microalgal suspension was rapidly mixed at 200 rpm for one minute followed by 15 minutes of slow mixing at 50 rpm. The flocculated microalgal suspension was allowed to settle for one hour. A supernatant sample of 15 mL was pipetted from the suspension at between one- and two-third from the bottom for measurement of the flocculation efficiency.

In the individual flocculation experiments, a dose-response relationship protocol was used to define the optimal flocculant dose. Ferric chloride and aluminium sulphate were dosed at a concentration of 40 to 180 g per litre of algal suspension. This corresponds to 112 to 504 mg flocculant/g dry biomass. Flopam^{TM} was dosed at 10 to 100 mg per litre of algal suspension (i.e. 28 to 280 mg polymer/g dry biomass). While chitosan dose was 40 to 200 mg per litre of algal suspension equivalent to 112 to 560 mg chitosan/g dry biomass.

In the dual flocculation experiments, ferric chloride or aluminium sulphate was added at a fixed 40 mg per litre algal suspension during the rapid mixing stage (200 rpm). This concentration was selected as it was the lowest dose tested in the single flocculation experiments, thus emphasise the purposes of dual flocculation i.e. limiting the number of metal salts in harvested biomass and minimising potential contamination of algal cells. Chitosan was then added at doses of 0 to 80 mg per litre of algal suspension (i.e. 0 to 224 mg/g dry biomass) during the slow mixing period (50 rpm).

2.3 Analytical methods

The optical density of *C. vulgaris* solution before and after flocculation was measured at a wavelength of 680 nm using the UV spectrophotometer (UV 6000 Shimadzu; Ermington, NSW, Australia). The flocculation efficiency was then calculated using these values as below:

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

Where OD_i and OD_f are the optical density of the culture before and after flocculant addition. Each flocculant was repeated three times for individual and dual flocculation experiments.

A volume of 150 mL of microalgae cell suspension was filtered through a 1.1 μm pre-weighed glass fibre filter paper. The biomass concentration of the microalgae culture was then obtained gravimetrically by drying the sample on the filter paper overnight at 60°C to a constant weight. The weight of the final filter paper was used to determine the dry microalgal biomass.

The Zetasizer nano instrument (Nano ZS Zen 3600; Malvern, UK) was used to measure the zeta potential of the microalgae solutions using the 15 mL aliquots taken before and after flocculation.

The solution pH was measured using a pH/conductivity meter (Orion 4-Star Plus Thermo Scientific; Waltham, MA, USA). Turbidity of the microalgae solution before and after flocculation was measured using a portable turbidity meter kit (Apera TN400; Colombus, OH, USA) with accuracy ±1% or 0.02 NTU. Statistical analysis was performed using Student's unpaired *t*-Test, with a two-tailed distribution.

3 Results and discussion

3.1 Optimal doses for ferric chloride and aluminium sulphate flocculants

A dose-response relationship can be observed when ferric chloride and aluminium sulphate were used individually as the flocculant (Fig. 1). The flocculation efficiency was less than 40% OD_{680} removal at 120 mg flocculant per litre of algal suspension (i.e. 336 mg flocculant/g dry biomass), after which the flocculation efficiency steadily increased (Fig.1). A higher flocculation efficiency was achieved as 86% and 77% at 160 mg ferric chloride per litre of algal suspension (i.e. 448 mg/g dry biomass) and 180 mg aluminium sulphate per litre of algal suspension (i.e. 504 mg/g dry biomass) respectively.

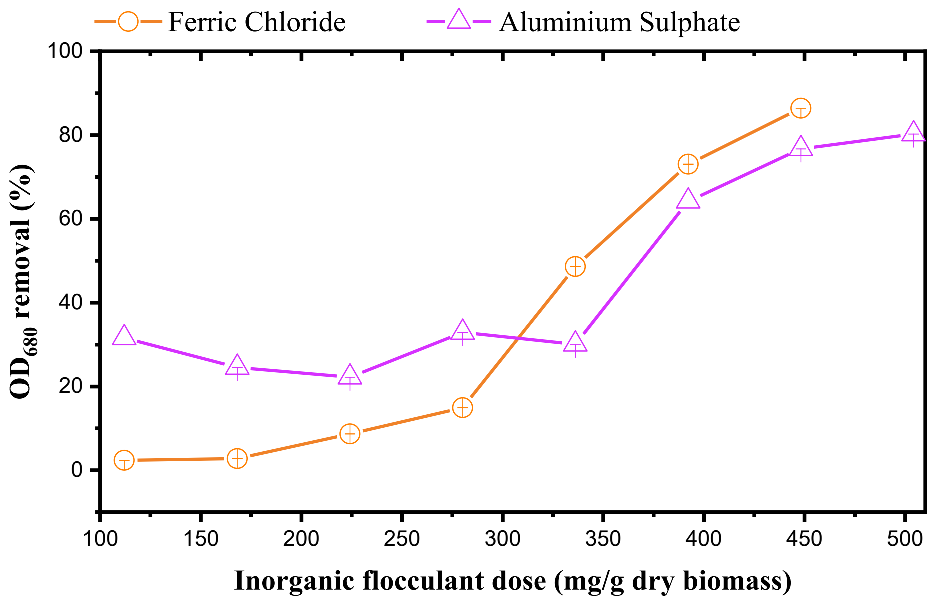

Figure 1: The *C. vulgaris* flocculation efficiency indicated by optical density removal at λ = 680nm for inorganic flocculants (a) ferric chloride and (b) aluminium sulphate at different doses. Value and error bars represent mean and standard deviation ($n = 3$).

Charge neutralisation is the main flocculation mechanism by inorganic flocculants [6, 11]. Small microalgae cells are very stable in suspension due to the repulsive force caused by their negatively charged surface (- 20.2 mV for *C. vulgaris* in this study). Thus, positively charged ferric or alum ions are required for charge neutralisation to overcome this electrostatic stabilisation through neutralising the charge of microalgae cells [20]. This was demonstrated by the plateau region below 350 mg flocculant/g dry biomass (Fig. 1) where the OD_{680} removal value remained quite low, < 35% for ferric chloride and < 20% for aluminium sulphate. Although the optimal flocculation efficiency was acceptable, it was achieved at very high doses of ferric chloride and aluminium sulphate. This aligns with the literature results in which improved flocculation performance (> 90%) of inorganic flocculants like ferric chloride and aluminium sulphate requires high dose (Table 1). The variation in the microalgal culture and growth conditions might be accountable for the difference in optimal doses among these studies.

Table 1: Summary of literature on the flocculation of *Chlorella* genus using aluminium sulphate and ferric chloride compared to the results from this study.

Microalgae culture (dry biomass g/L)	**Flocculant**	**Optimal dose (mg/g dry biomass)**	**Efficiency (%)**	**References**
Chlorella vulgaris (0.36)	Aluminium sulphate	504	77	This study
	Ferric chloride	448	86	
Chlorella vulgaris (1.2)	Aluminium sulphate	2083	> 90	[21]
	Chitosan	208		
Chlorella sp. (0.12)	Aluminium sulphate	1266	> 90	[22]
	Ferric chloride	1191		
Chlorella vulgaris (freshwater) (1.0)	Aluminium sulphate	350	> 95	[23]
	Ferric chloride	300		
Chlorella vulgaris (0.25)	Aluminium sulphate	600	> 95	[24]

3.2 Flocculation performance by organic polymers

3.2.1 Synthetic polyacrylamide polymers

Synthetic cationic polymer Flopam™ showed the highest OD_{680} removal of 96% at 20 mg polymer per litre of algal suspension (i.e. 56 mg polymer/g dry biomass) (Fig. 2). A further increase in its dose up to 100 mg per litre of algal suspension (i.e. 280 mg/g dry biomass) caused the flocculation performance to decrease gradually. Results in Fig. 2 suggest that polymer over-dosing can be counterproductive. This observation is in good agreement with the literature [14].

Flopam™ is a high molecule weight and highly charged cationic polymer. Thus, charge neutralisation is the first step of flocculation, followed by entanglement and bridging of algal cells and the polymer [25, 26]. As this process continues, more and more microalgae cells are bridged or connected to each another, forming bigger flocs. A combination of mechanisms performed by synthetic cation polymer enhances its flocculation efficiency.

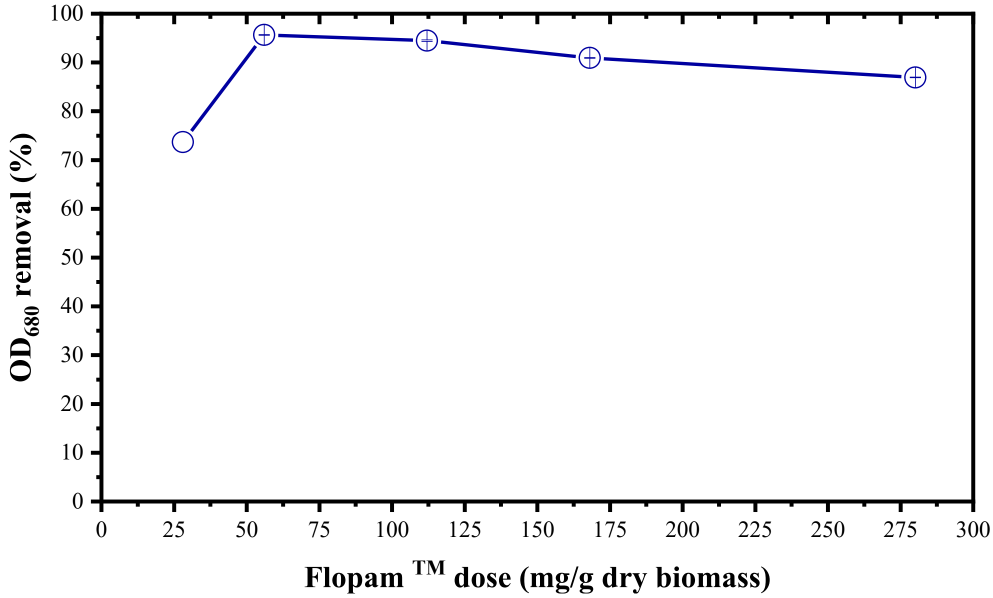

Figure 2: The flocculation performance of Flopam™ indicated by its optical density removal efficiency at λ = 680 nm. Value and error bars are mean and standard deviation (n = 3).

3.2.2 Natural polymer Chitosan

In the flocculation of *C. vulgaris* using natural polymer chitosan, the value of OD_{680} removal improved with the increasing doses (Fig. 3), suggesting a proportional relationship between flocculation efficiency and chitosan dose. At the lowest dose of 40 mg chitosan per litre of algal suspension (i.e. 112 mg chitosan/g dry biomass), the OD_{680} removal was 20%. This was increased to 62% when using 200 mg chitosan per litre of algal suspension (i.e. 560 mg chitosan/g dry biomass). Flocculation efficiency of chitosan in this study is not only much lower, but it also required a dose twenty times that of the synthetic cationic polymer Flopam™ to achieve the same OD_{680} removal around 60%.

Flocculation using chitosan works presumably based on a small degree of charge neutralisation and mostly bridging mechanism, similar to the synthetic cationic polymers made from polyacrylamide in section 3.2.1 [27, 28]. pH plays a key role in the efficiency of chitosan flocculation since at both acidic and very alkaline condition, the performance is decreased [27, 29]. Gualteri et al., 1988 explained that in an acidic environment, chitosan exists as a linear chain and remains dispersed due to the repulsive forces between closely placed $-NH_2$ groups and $-NH^{3+}$ group carrying positive charge [30]. This prevents chitosan from effectively flocculate the microalgae cells. With an alkaline pH, the positive charge of chitosan is gradually neutralised, thus charge neutralisation of microalgae cells becomes less efficient [29]. Optimal flocculation using chitosan is obtained within a narrow pH range of approximately 6 to 8 [27]. In this experiment, the pH of the microalgal solution after the addition of chitosan was 8.05. However, the removal efficiency reported was relatively low with high dosage, leading to the subsequent study of dual flocculation using inorganic flocculants and chitosan.

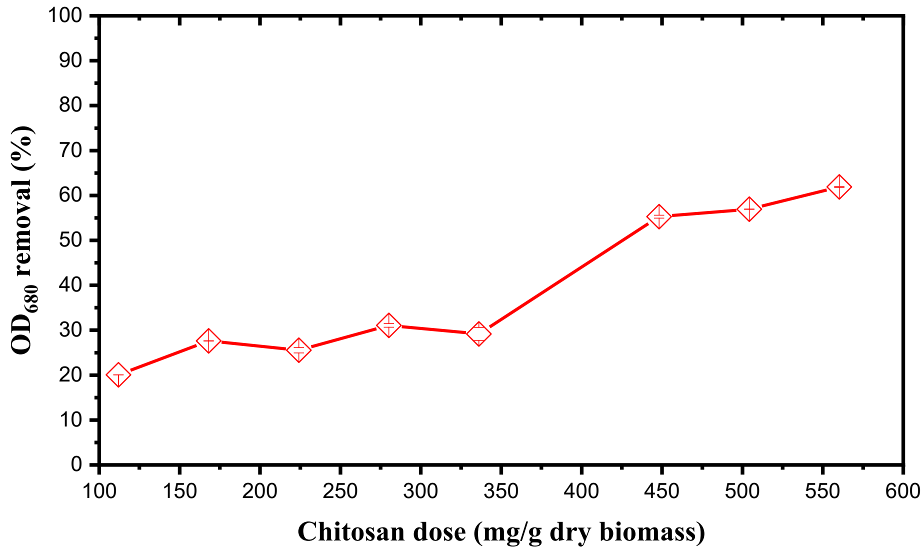

Figure 3: The effect on *C. vulgaris* flocculation using Chitosan, based on its optical density removal efficiency at $\lambda = 680$ nm. Value and error bars are mean and standard deviation (n=3).

3.3 Synergistic effect of dual flocculation

3.3.1 Improved flocculation using a combination of inorganic flocculants and chitosan

Signifcantly better OD_{680} removal efficiency was observed for dual flocculation combining inorganic salts with chitosan, compared to that achieved by individual flocculation (Fig. 4). Dual flocculation using ferric chloride and chitosan achieved an OD_{680} removal of 81% at 80 mg chitosan per litre of algal suspension (i.e. 224 mg chitosan/g dry biomass). Likewise, aluminium sulphate (40 mg/L) and chitosan (80 g/L per litre of algal suspension (224 mg chitosan/g dry biomass)) achieved 89% efficiency (Fig. 4). In comparison with individual flocculation (Section 3.1 & 3.2.2), an additional of 57 and 24% harvesting efficiency was achieved by dual flocculation between ferric chloride/chitosan and aluminium sulphate/chitosan, respectively. A synergistic effect in dual flocculation using inorganic flocculants and chitosan, therefore, was present. It increased the flocculation efficiency by approximately two to four times, depending on the type of inorganic salts. This synergistic effect presumably was the result of multiple flocculation mechanisms (e.g. charge neutralisation and bridging) used by inorganic flocculants and chitosan interacting with and assisting each other. These results from the dual flocculation experiments suggest that by combining low doses of inorganic flocculant and chitosan, it is possible to harvest microalgae biomass at an improved efficiency with minimised cell contamination and a cheaper cost.

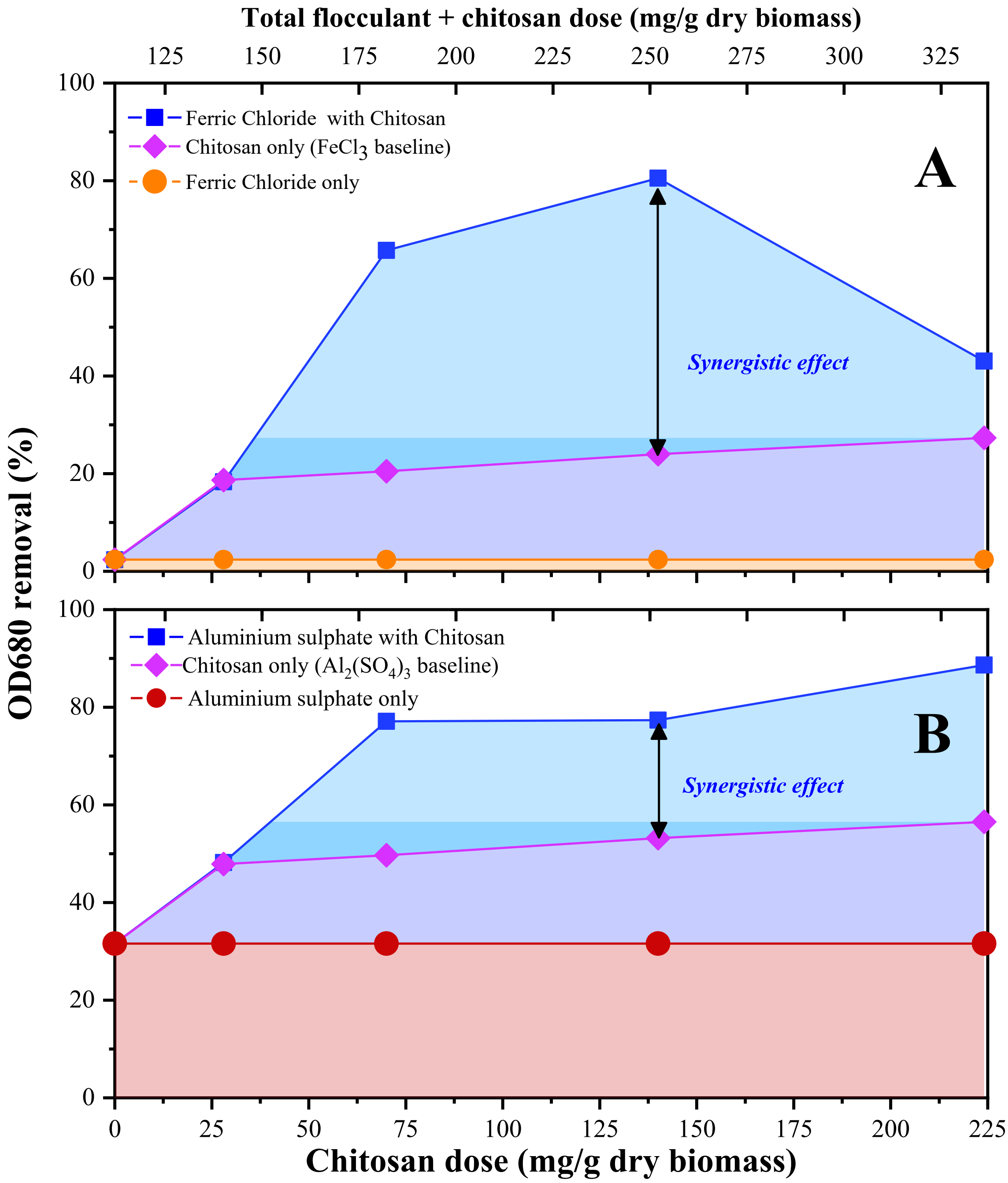

Figure 4: The synergistic effect of combining inorganic flocculant (a) ferric chloride and (b) aluminium sulphate with organic polymer Chitosan in flocculating C. vulgaris, indicated by the optical density removal efficiency at $\lambda = 680$ nm.

3.3.2 Synergistic mechanisms of enhanced performance mechanisms

The combination of charge neutralization and bridging is the main reason for the observed synergy. By adding ferric chloride or aluminium sulphate as a primary flocculant in the rapid mixing step, negatively charged *C. vulgaris* cells were neutralised to higher zeta potential and no longer remained stable in suspension (Fig. 5). Collision among cells was initiated leading to the formation of small flocs. When chitosan was slowly mixed in at this stage, particle entrapment and bridging took place [17]. Chitosan chains attached to existing microalgal-alum/ferric flocs and further agglomerated them into bigger masses (i.e. macroflocs of size >1 cm, data not shown). These combined mechanisms increased the flocculation efficiency of the dual experiment to above 80%, much greater than that achieved by solely ferric or aluminium flocculation (Section 3.3.1).

At high dose of chitosan (>70 mg/g dry biomass for ferric chloride/chitosan and >140 mg/g dry biomass for aluminium sulphate/chitosan), a synergistic effect is observed for charge neutralisation of the microalgae cells (Fig. 5). Flocculation using positively charged ferric chloride, aluminium sulphate and chitosan primarily work on the basis of neutralising negatively-charged algal cells to destabilise cells in suspension [6, 11]. Although the main mechanism of chitosan flocculation is bridging, the addition of chitosan at a higher dose in the dual flocculation still significantly increased the charge neutralisation compared to single ferric chloride or aluminium sulphate flocculation. At optimal chitosan dose, charge neutralisation was 13.8 mV for ferric chloride/chitosan flocculation and 17.2 for aluminium sulphate/ chitosan flocculation (Fig. 5). A lower dose of chitosan (< 70 mg/g dry biomass) did not induce any synergistic effect because chitosan was working mostly on the bridging mechanism and charge neutralisation had a negligible effect on the dual flocculation performance.

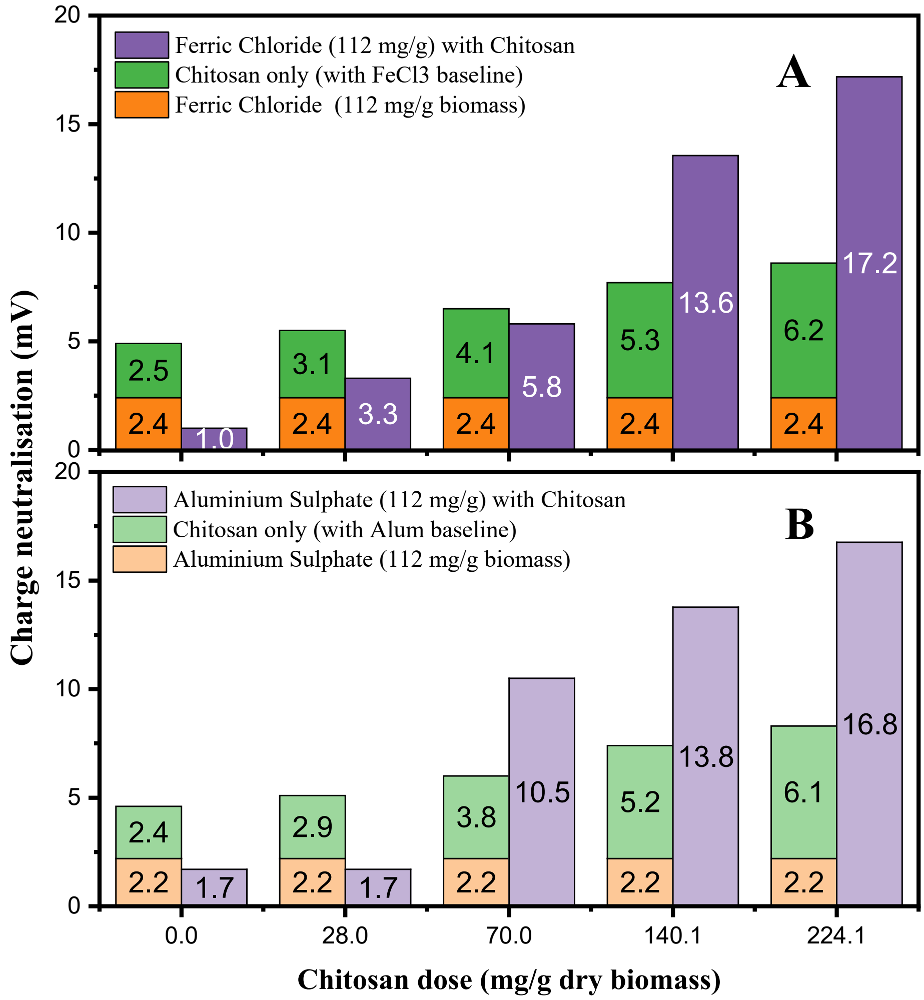

Figure 5: The synergistic effect of dual flocculation using (a) ferric chloride with chitosan and (b) aluminium sulphate with chitosan on the zeta potential of particles in *C. vulgaris* solution, demonstrated by the change in charge neutralisation.

3.4 Comparison of flocculants

An indicative cost analysis was conducted for each individual and dual flocculation to obtain an overview of the large-scale feasibility (Table 2). Flopam™ performed excellent flocculation of *C. vulgaris* cells, however, the cost per ton dry *C. vulgaris* biomass for it is estimated at 120 USD (Table 2). This value is more than the cost per ton of dry biomass for aluminium sulphate (105 USD) but less than that of ferric chloride (364 USD). Chitosan is the most expensive (i.e. 20-50 USD/kg) among all the flocculants investigated in this study. The cost to achieve >90%

flocculation efficiency per ton of dry *C. vulgaris* biomass using chitosan is approximately 7280 USD (Table 2).

For dual flocculation, the combination of aluminium sulphate and chitosan would cost 4920 USD per ton dry *C. vulgaris* biomass, while it is 7925 USD for ferric chloride and chitosan combination. This suggests that by combining aluminium sulphate and chitosan, the cost could be reduced significantly by approximately 30%. Further research into the optimisation of dual flocculation for microalgae using inorganic flocculant and chitosan (e.g. biomass quality and quantity, processing times, species specific and toxicity), there is potential for prospective applications of this method in a large-scale environment.

Table 2: Cost comparison for types of flocculants or polymers used in this study based on their current market value.

Flocculant/Polymer (s)	**Indicative cost, US$/ton**[a]	**Cost (US$) per ton dry *C. vulgaris* biomass**[b]
Single flocculation		
Flopam™ (FO 4808)[c]	2 000 – 2 300	120
Chitosan	20 000 – 50 000	7280
Aluminium Sulphate	150 – 200	105
Ferric Chloride	455 – 1 000	364
Dual Flocculation		
Aluminium sulphate + Chitosan	--	4920
Ferric chloride + Chitosan	--	7925

[a] Prices are collected from Alibaba.com
[b] Average value from indicative cost is used for calculation
[c] Price is reported by SNF Australia

4 Conclusions

A preliminary assessment of microalgal flocculation efficiency was reported in this study. Individual flocculant including ferric chloride, aluminium sulphate and polymer chitosan required a high dose to achieve a benchmark of 90% harvesting efficiency. Polymer Flopam™ can effectively harvest microalgae at a lower dose and thus lower cost. A dual flocculation method combining ferric chloride or aluminium sulphate with chitosan resulted in a synergistic effect. The synergistic effect was resulted from the interaction between charge neutralisation and bridging mechanisms. The dual flocculation method has a great potential for large-scale microalgal harvesting application.

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