Synergistic effect of dual flocculation between inorganic salts and chitosan on harvesting microalgae *Chlorella vulgaris*

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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^{TM} 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-

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

132 2.2 Flocculation experiment

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

140 In the individual flocculation experiments, a dose-response relationship protocol was used
141 to define the optimal flocculant dose. Ferric chloride and aluminium sulphate were dosed at a
142 concentration of 40 to 180 g per litre of algal suspension. This corresponds to 112 to 504 mg
143 flocculant/g dry biomass. Flopam^{TM} was dosed at 10 to 100 mg per litre of algal suspension (i.e.
144 28 to 280 mg polymer/g dry biomass). While chitosan dose was 40 to 200 mg per litre of algal
145 suspension equivalent to 112 to 560 mg chitosan/g dry biomass.

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

153 2.3 Analytical methods

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

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

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

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

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

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

172 **3 Results and discussion**

173 3.1 Optimal doses for ferric chloride and aluminium sulphate flocculants

174 A dose-response relationship can be observed when ferric chloride and aluminium sulphate
175 were used individually as the flocculant (Fig. 1). The flocculation efficiency was less than 40%
176 OD_{680} removal at 120 mg flocculant per litre of algal suspension (i.e. 336 mg flocculant/g dry
177 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.

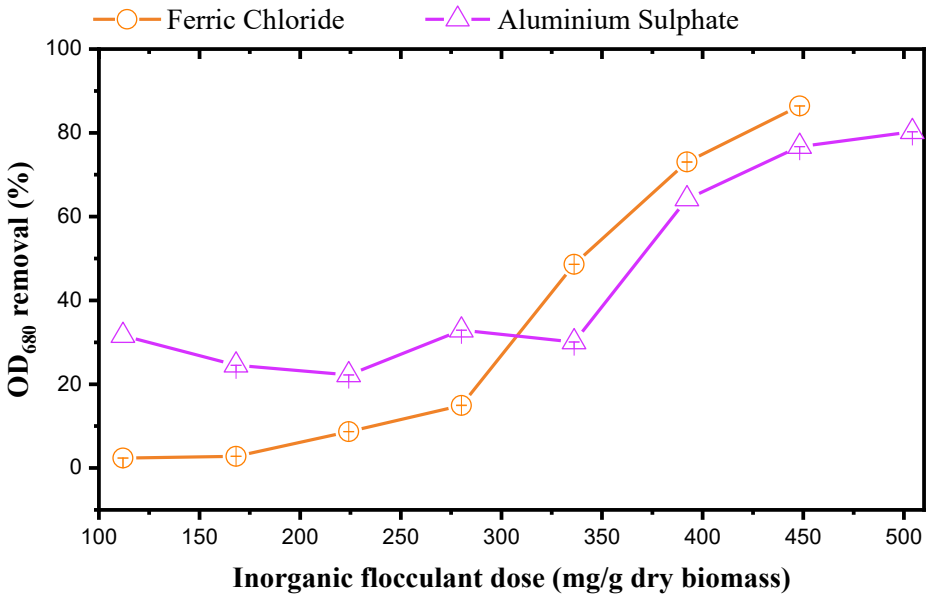


Figure 1: The *C. vulgaris* flocculation efficiency indicated by optical density removal at λ = 680nm for inorganic flocculants (a) ferric chloride and (b) aluminium sulphate at different doses. Value and error bars represent mean and standard deviation ($n = 3$).

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

195 aluminium sulphate requires high dose (Table 1). The variation in the microalgal culture and
196 growth conditions might be accountable for the difference in optimal doses among these studies.

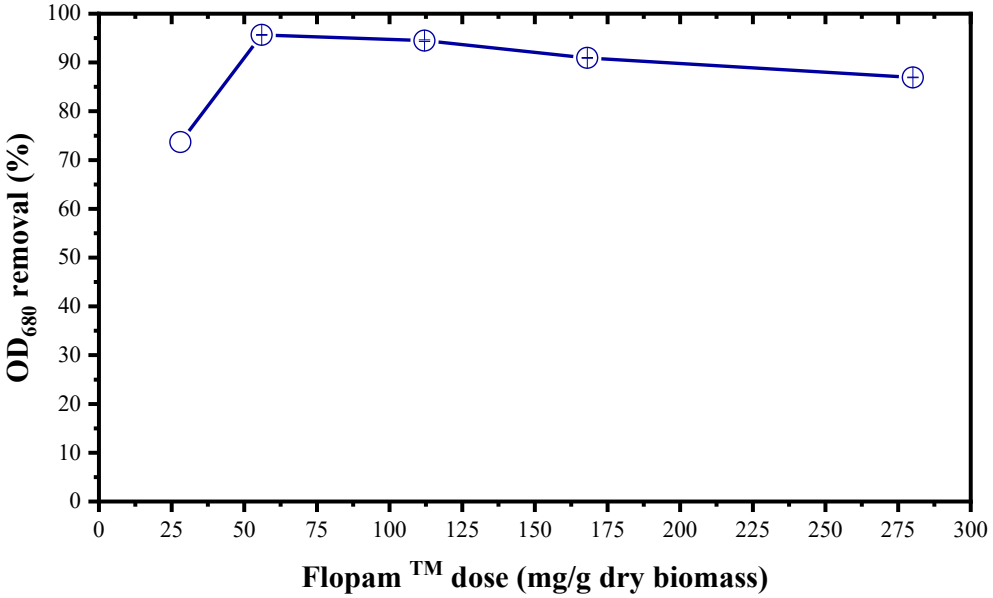
197 **Table 1:** Summary of literature on the flocculation of *Chlorella* genus using aluminium
198 sulphate and ferric chloride compared to the results from this study.

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]

199 3.2 Flocculation performance by organic polymers

200 *3.2.1 Synthetic polyacrylamide polymers*

201 Synthetic cationic polymer Flopam^{TM} showed the highest OD_{680} removal of 96% at 20 mg
202 polymer per litre of algal suspension (i.e. 56 mg polymer/g dry biomass) (Fig. 2). A further
203 increase in its dose up to 100 mg per litre of algal suspension (i.e. 280 mg/g dry biomass) caused
204 the flocculation performance to decrease gradually. Results in Fig. 2 suggest that polymer over-
205 dosing can be counterproductive. This observation is in good agreement with the literature [14].
206 Flopam^{TM} is a high molecule weight and highly charged cationic polymer. Thus, charge
207 neutralisation is the first step of flocculation, followed by entanglement and bridging of algal
208 cells and the polymer [25, 26]. As this process continues, more and more microalgae cells are
209 bridged or connected to each another, forming bigger flocs. A combination of mechanisms
210 performed by synthetic cation polymer enhances its flocculation efficiency.



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212 **Figure 2**: The flocculation performance of Flopam^{TM} indicated by its optical density removal
213 efficiency at $\lambda = 680$ nm. Value and error bars are mean and standard deviation ($n = 3$).

214 *3.2.2 Natural polymer Chitosan*

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

223 Flocculation using chitosan works presumably based on a small degree of charge
224 neutralisation and mostly bridging mechanism, similar to the synthetic cationic polymers made
225 from polyacrylamide in section 3.2.1 [27, 28]. pH plays a key role in the efficiency of chitosan
226 flocculation since at both acidic and very alkaline condition, the performance is decreased [27,
227 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.

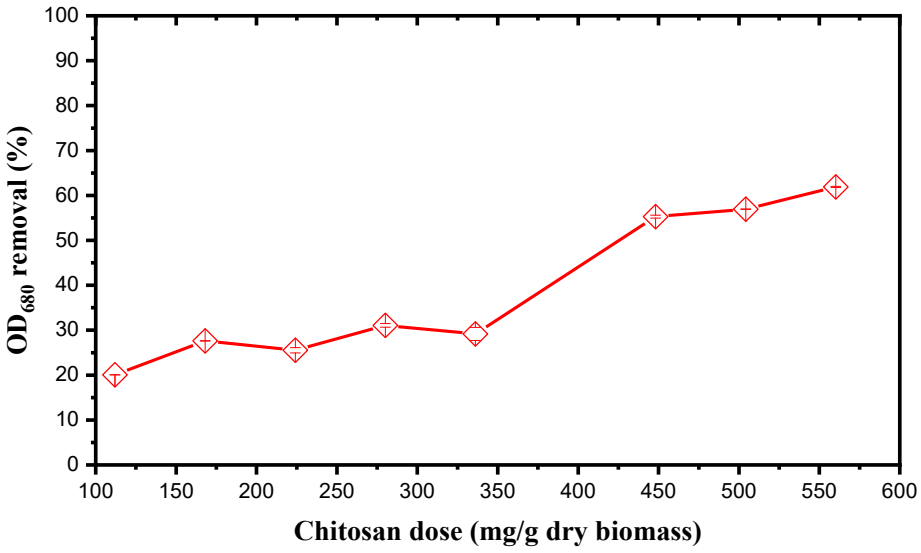


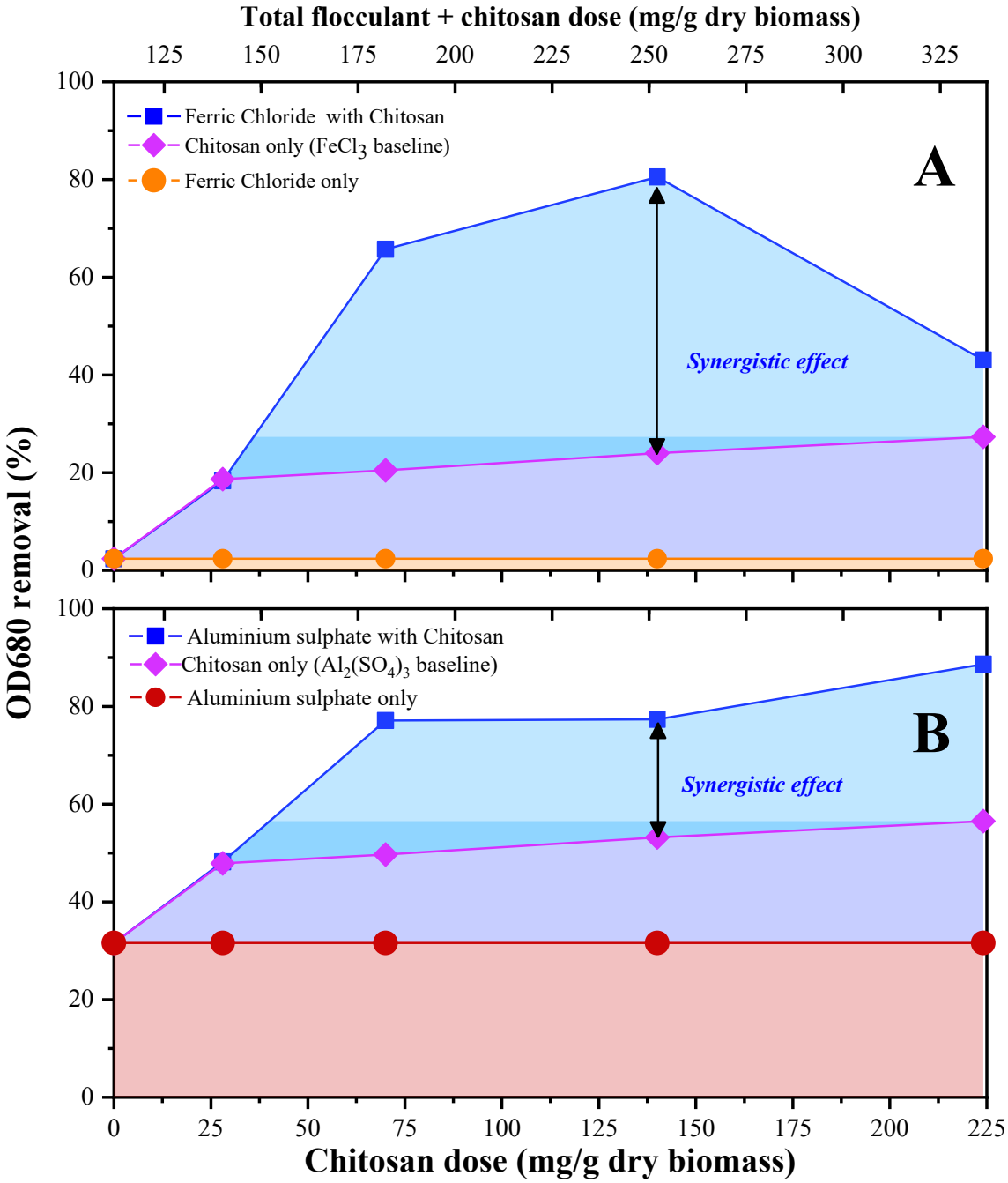
Figure 3: The effect on *C. vulgaris* flocculation using Chitosan, based on its optical density removal efficiency at λ = 680 nm. Value and error bars are mean and standard deviation (n=3).

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

246 chitosan/g dry biomass)) achieved 89% efficiency (Fig. 4). In comparison with individual
247 flocculation (Section 3.1 & 3.2.2), an additional of 57 and 24% harvesting efficiency was
248 achieved by dual flocculation between ferric chloride/chitosan and aluminium
249 sulphate/chitosan, respectively. A synergistic effect in dual flocculation using inorganic
250 flocculants and chitosan, therefore, was present. It increased the flocculation efficiency by
251 approximately two to four times, depending on the type of inorganic salts. This synergistic
252 effect presumably was the result of multiple flocculation mechanisms (e.g. charge neutralisation
253 and bridging) used by inorganic flocculants and chitosan interacting with and assisting each
254 other. These results from the dual flocculation experiments suggest that by combining low doses
255 of inorganic flocculant and chitosan, it is possible to harvest microalgae biomass at an improved
256 efficiency with minimised cell contamination and a cheaper cost.



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258 **Figure 4**: The synergistic effect of combining inorganic flocculant (a) ferric chloride and (b)

259 aluminium sulphate with organic polymer Chitosan in flocculating C. vulgaris, indicated by the

260 optical density removal efficiency at $\lambda = 680$ nm.

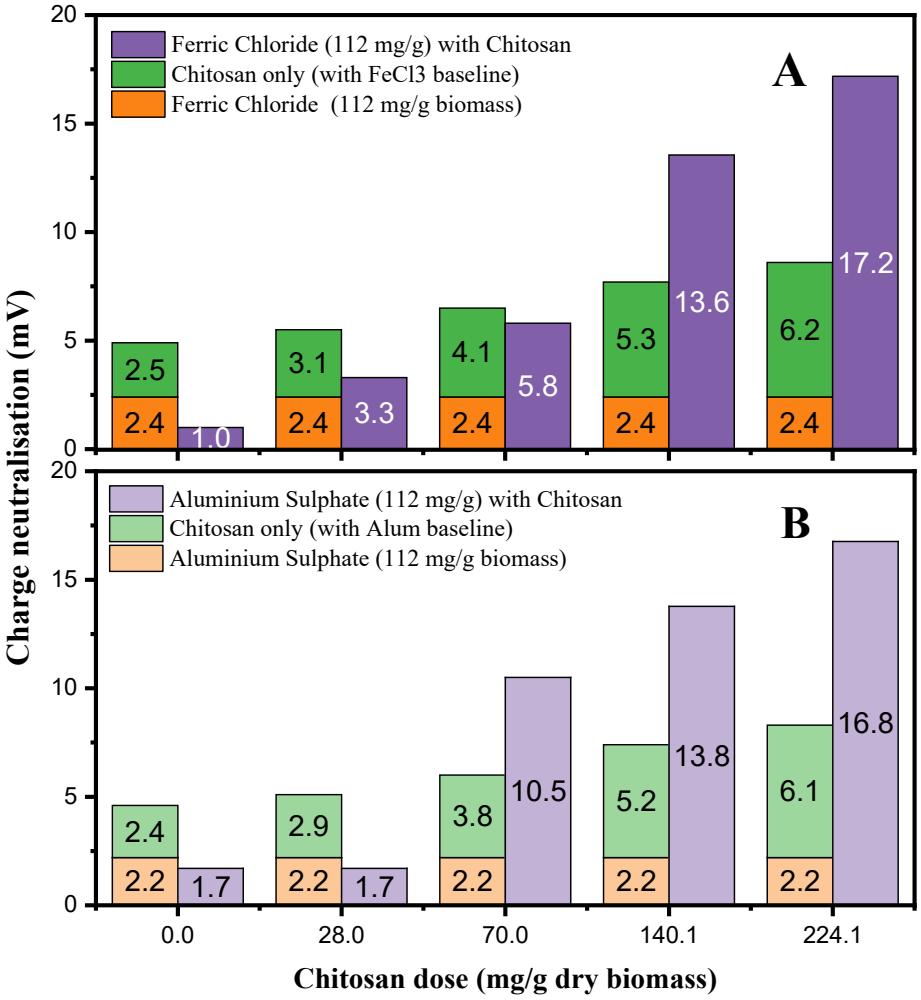
261 *3.3.2 Synergistic mechanisms of enhanced performance mechanisms*

262 The combination of charge neutralization and bridging is the main reason for the observed

263 synergy. By adding ferric chloride or aluminium sulphate as a primary flocculant in the rapid

264 mixing step, negatively charged *C. vulgaris* cells were neutralised to higher zeta potential and
265 no longer remained stable in suspension (Fig. 5). Collision among cells was initiated leading to
266 the formation of small flocs. When chitosan was slowly mixed in at this stage, particle
267 entrapment and bridging took place [17]. Chitosan chains attached to existing microalgal-
268 alum/ferric flocs and further agglomerated them into bigger masses (i.e. macroflocs of size
269 >1 cm, data not shown). These combined mechanisms increased the flocculation efficiency of
270 the dual experiment to above 80%, much greater than that achieved by solely ferric or
271 aluminium flocculation (Section 3.3.1).

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



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

288 3.4 Comparison of flocculants

289 An indicative cost analysis was conducted for each individual and dual flocculation to obtain
290 an overview of the large-scale feasibility (Table 2). Flopam^{TM} performed excellent flocculation
291 of *C. vulgaris* cells, however, the cost per ton dry *C. vulgaris* biomass for it is estimated at 120
292 USD (Table 2). This value is more than the cost per ton of dry biomass for aluminium sulphate
293 (105 USD) but less than that of ferric chloride (364 USD). Chitosan is the most expensive (i.e.
294 20-50 USD/kg) among all the flocculants investigated in this study. The cost to achieve >90%

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

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

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

Flocculant/Polymer (s)	**Indicative cost, US$/ton[a]**	**Cost (US$) per ton dry *C. vulgaris* biomass[b]**
Single flocculation		
Flopam^{TM} (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

306 [a] Prices are collected from Alibaba.com

307 [b] Average value from indicative cost is used for calculation

308 [c] Price is reported by SNF Australia

309 **4 Conclusions**

310 A preliminary assessment of microalgal flocculation efficiency was reported in this study.
311 Individual flocculant including ferric chloride, aluminium sulphate and polymer chitosan
312 required a high dose to achieve a benchmark of 90% harvesting efficiency. Polymer Flopam^{TM}
313 can effectively harvest microalgae at a lower dose and thus lower cost. A dual flocculation

314 method combining ferric chloride or aluminium sulphate with chitosan resulted in a synergistic
315 effect. The synergistic effect was resulted from the interaction between charge neutralisation
316 and bridging mechanisms. The dual flocculation method has a great potential for large-scale
317 microalgal harvesting application.

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