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

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25

26 **Abstract**

27 The flocculation efficiency of microalgae *Chlorella vulgaris* for subsequent harvesting was
28 investigated using single flocculants of inorganic salts, synthetic polymer, chitosan and dual
29 flocculants of inorganic salts and chitosan. Synthetic polymer (Flopan™) could achieve over
30 90% optical density removal (OD₆₈₀ removal) at a low flocculant dose (20 to 40 mg polymer
31 per litre of algal suspension) through the bridging mechanism and charge neutralisation.
32 Inorganic salts (i.e. ferric chloride and aluminium sulphate) and chitosan individually resulted
33 in low flocculation efficiency (<90%) despite high dose (i.e. 160 to 200 mg per litre of algal
34 suspension). The dual flocculation combining ferric chloride or aluminium sulphate with
35 chitosan induced synergistic effects, resulting in >80% flocculation efficiency, significantly
36 higher than the sum of each individual flocculation. The improvement in flocculation efficiency
37 was 57 and 24% respectively for ferric chloride/chitosan and aluminium sulphate/chitosan.
38 Charge neutralisation of microalgal cells by ferric chloride or aluminium sulphate combined
39 with bridging by chitosan produced the synergy.

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

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50 **1 Introduction**

51 Microalgae are among the most important organisms in ecological evolution and history of
52 the Earth. They have the potential to shape our future with a wide range of promising
53 applications that tackle worldwide issues. The global fossil fuel supply is depleted and has
54 caused destructive environmental effects over its life cycle. There is growing interest in
55 microalgal biomass as renewable and environmental-friendly feedstock for third-generation
56 biofuel [1, 2]. The nutritive value of microalgal biomass for human as well as their versatile
57 biochemical features have allowed for the production of health supplements, bioactive
58 compounds, food additives and biotechnology applications, although there are still several
59 hurdles in terms of socio-economic aspects [3-5]. In particular, harvesting has been a major
60 technical and economic bottleneck in microalgal biomass production due to low cell
61 concentrations in cultures (0.5 to 5 g/L), small cell size ($< 30 \mu\text{m}$), the stability of cell
62 suspension and variation in culture medium [6-9]. Currently, microalgal harvesting is the most
63 expensive step (i.e. 20-30% of total cost) in the process of microalgal biomass production [6,
64 10].

65 The microalgal harvesting techniques include coagulation, flocculation, flotation, membrane
66 filtration and centrifuge [6, 11, 12]. Amongst them, flocculation has received significant
67 attention for its simple operation and relatively low-cost approach, but efficiency is dependent
68 on flocculant type [9, 11, 13]. Available chemical flocculants for microalgal harvesting can be
69 grouped into three categories: (i) inorganic flocculants such iron and aluminium salts, (ii)
70 synthetic polymer such as polyacrylamide and polyelectrolyte and (iii) natural organic polymers
71 such as chitosan and cationic starch [9, 13]. Synthetic polymers often provide high harvesting
72 efficiency at low dose [14]. However, these polymers are expensive. Inorganic flocculants such
73 as ferric chloride and aluminium sulphate are less expensive but require a higher dose.
74 Contamination and/or discolouration of microalgal biomass are possible concerns when using
75 inorganic salts. The presence of these salts in the harvested biomass hinders its applications for

76 biofuel and pigment extraction [11]. These issues with the quality of the harvested biomass can
77 be avoided by using natural polymers like chitosan. Chitosan is a promising flocculant due to
78 its advantages (e.g. natural product, biodegradation and non-toxic) [11, 15]. It has been
79 demonstrated that chitosan residual in the culture media (i.e. after biomass harvesting) is non-
80 toxic to microalgae. This feature enhances the reusability of the culture media, which is a
81 potential option to reduce cost [15]. However, the expensive cost around 20 to 50 USD/kg of
82 chitosan (depending on the purity) sets back its large-scale application [11, 16].

83 Inorganic salts provide flocculation through neutralising microalgal cell charge while
84 chitosan flocculates microalgal biomass through bridging [11]. Therefore, it is hypothesized
85 that the combination of these two mechanisms can enhance flocculation efficiency or harvesting
86 efficiency. Loganathan et al. (2018) reported that a combination of alum and chitosan as
87 flocculant aid induced a synergistic impact on harvesting seawater microalgae [17]. The author
88 indicated that a reduction of 20 mg flocculants per litre of algal suspension was achieved while
89 maintaining the harvesting efficiency over 95% [17]. However, there has yet been any studies
90 on freshwater *Chlorella vulgaris* harvesting using this type of flocculant combination. The most
91 similar approach combining ferric chloride and polyethylene was conducted by Gorin et al.
92 [18]. They reported an increase from 60% to 90% flocculation efficiency of *Chlorella vulgaris*
93 using dual flocculation. However, the dose of ferric chloride was very high at 500 mg/L, which
94 may cause unfavourable effects on algal cells. Given the benefits (e.g. biological and
95 pharmaceutical properties, nutrient contents for human health) of microalgae *Chlorella vulgaris*
96 [19], effective harvesting of its biomass without compromising the cell quality will be a
97 stepping stone to mass production of microalgal based products.

98 This study aims to compare the performance of four types of flocculants including two metal
99 salts ferric chloride and aluminium sulphate, polyacrylamide polymer FlopamTM and organic
100 polymer chitosan on *Chlorella vulgaris* harvesting. From the results of these single flocculation
101 tests, dual flocculation tests using inorganic salt followed by chitosan addition were conducted

102 to determine to what extent this strategy can improve the efficiency and reduce flocculant dose
103 of the process. Optical density removal, turbidity and zeta potential were measured to evaluate
104 flocculation efficiency and mechanisms. The results from this study is expected to contribute
105 to the greater research on optimising microalgae harvesting, particularly using flocculation
106 process.

107 **2 Materials and methods**

108 2.1 Microalgal suspension and materials

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

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

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

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

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.

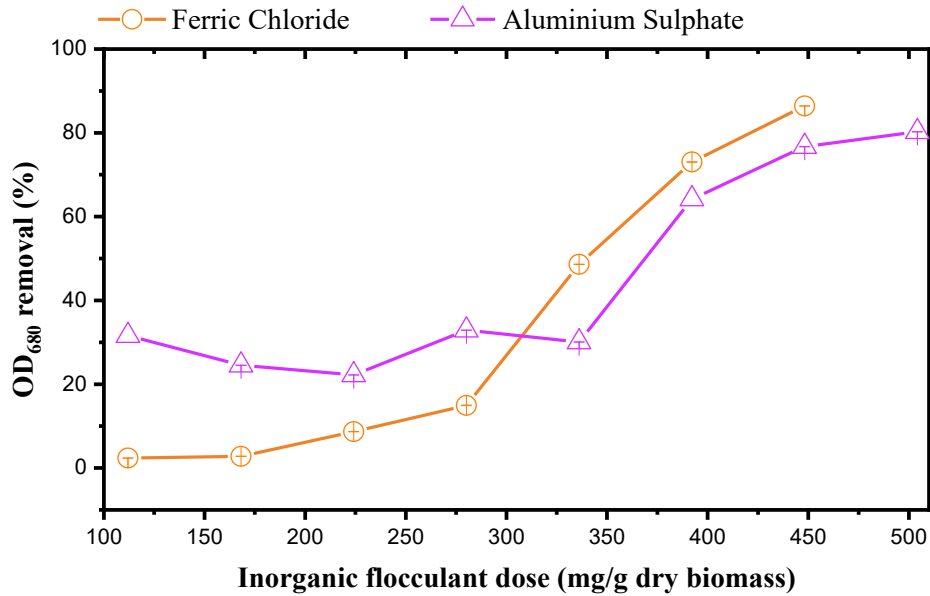
167 The solution pH was measured using a pH/conductivity meter (Orion 4-Star Plus Thermo
168 Scientific; Waltham, MA, USA). Turbidity of the microalgae solution before and after
169 flocculation was measured using a portable turbidity meter kit (Apera TN400; Columbus, OH,
170 USA) with accuracy $\pm 1\%$ 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

178 flocculation efficiency was achieved as 86% and 77% at 160 mg ferric chloride per litre of algal
179 suspension (i.e. 448 mg/g dry biomass) and 180 mg aluminium sulphate per litre of algal
180 suspension (i.e. 504 mg/g dry biomass) respectively.



181
182 **Figure 1:** The *C. vulgaris* flocculation efficiency indicated by optical density removal at $\lambda =$
183 680nm for inorganic flocculants (a) ferric chloride and (b) aluminium sulphate at different
184 doses. Value and error bars represent mean and standard deviation ($n = 3$).

185 Charge neutralisation is the main flocculation mechanism by inorganic flocculants [6, 11].
186 Small microalgae cells are very stable in suspension due to the repulsive force caused by their
187 negatively charged surface (- 20.2 mV for *C. vulgaris* in this study). Thus, positively charged
188 ferric or alum ions are required for charge neutralisation to overcome this electrostatic
189 stabilisation through neutralising the charge of microalgae cells [20]. This was demonstrated
190 by the plateau region below 350 mg flocculant/g dry biomass (Fig. 1) where the OD₆₈₀ removal
191 value remained quite low, < 35% for ferric chloride and < 20% for aluminium sulphate.
192 Although the optimal flocculation efficiency was acceptable, it was achieved at very high doses
193 of ferric chloride and aluminium sulphate. This aligns with the literature results in which
194 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.

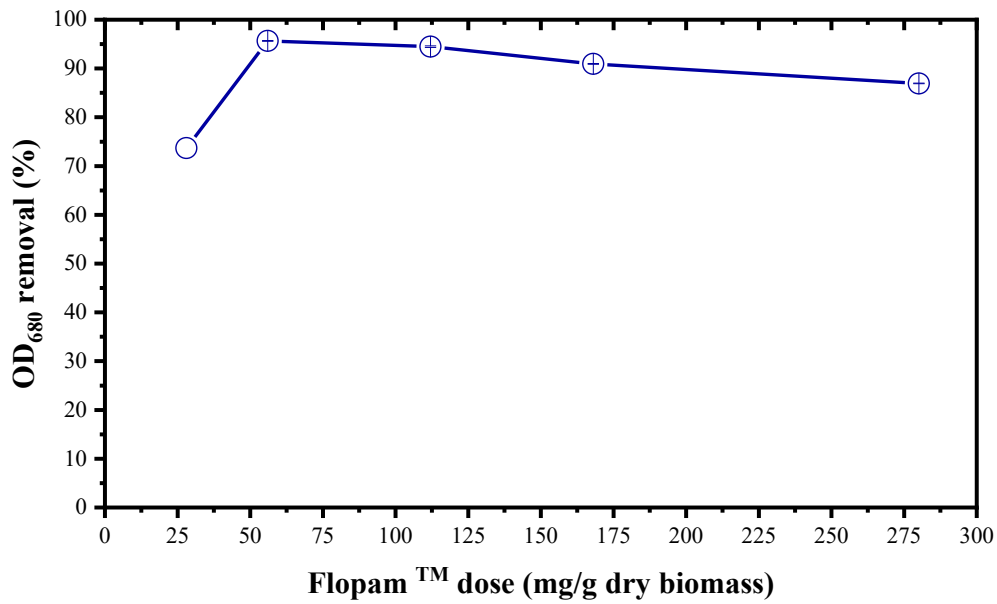
Microalgae culture (dry biomass g/L)	Flocculant	Optimal dose (mg/g dry biomass)	Efficiency (%)	References
<i>Chlorella vulgaris</i> (0.36)	Aluminium sulphate	504	77	This study
	Ferric chloride	448	86	
<i>Chlorella vulgaris</i> (1.2)	Aluminium sulphate	2083	> 90	[21]
	Chitosan	208		
<i>Chlorella</i> sp. (0.12)	Aluminium sulphate	1266	> 90	[22]
	Ferric chloride	1191		
<i>Chlorella vulgaris</i> (freshwater) (1.0)	Aluminium sulphate	350	> 95	[23]
	Ferric chloride	300		
<i>Chlorella vulgaris</i> (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™ showed the highest OD₆₈₀ 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™ 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.



211

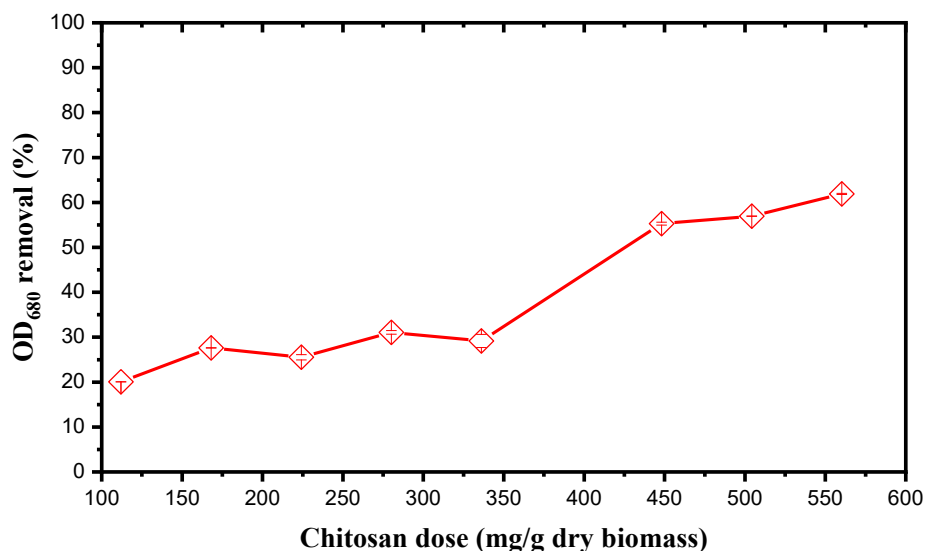
212 **Figure 2:** The flocculation performance of Flopam™ indicated by its optical density removal
 213 efficiency at $\lambda = 680$ nm. Value and error bars are mean and standard deviation ($n = 3$).

214 3.2.2 *Natural polymer Chitosan*

215 In the flocculation of *C. vulgaris* using natural polymer chitosan, the value of OD₆₈₀ 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 OD₆₈₀ 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™
 222 to achieve the same OD₆₈₀ 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

228 chain and remains dispersed due to the repulsive forces between closely placed $-NH_2$ groups
229 and $-NH_3^+$ group carrying positive charge [30]. This prevents chitosan from effectively
230 flocculate the microalgae cells. With an alkaline pH, the positive charge of chitosan is gradually
231 neutralised, thus charge neutralisation of microalgae cells becomes less efficient [29]. Optimal
232 flocculation using chitosan is obtained within a narrow pH range of approximately 6 to 8 [27].
233 In this experiment, the pH of the microalgal solution after the addition of chitosan was 8.05.
234 However, the removal efficiency reported was relatively low with high dosage, leading to the
235 subsequent study of dual flocculation using inorganic flocculants and chitosan.



236

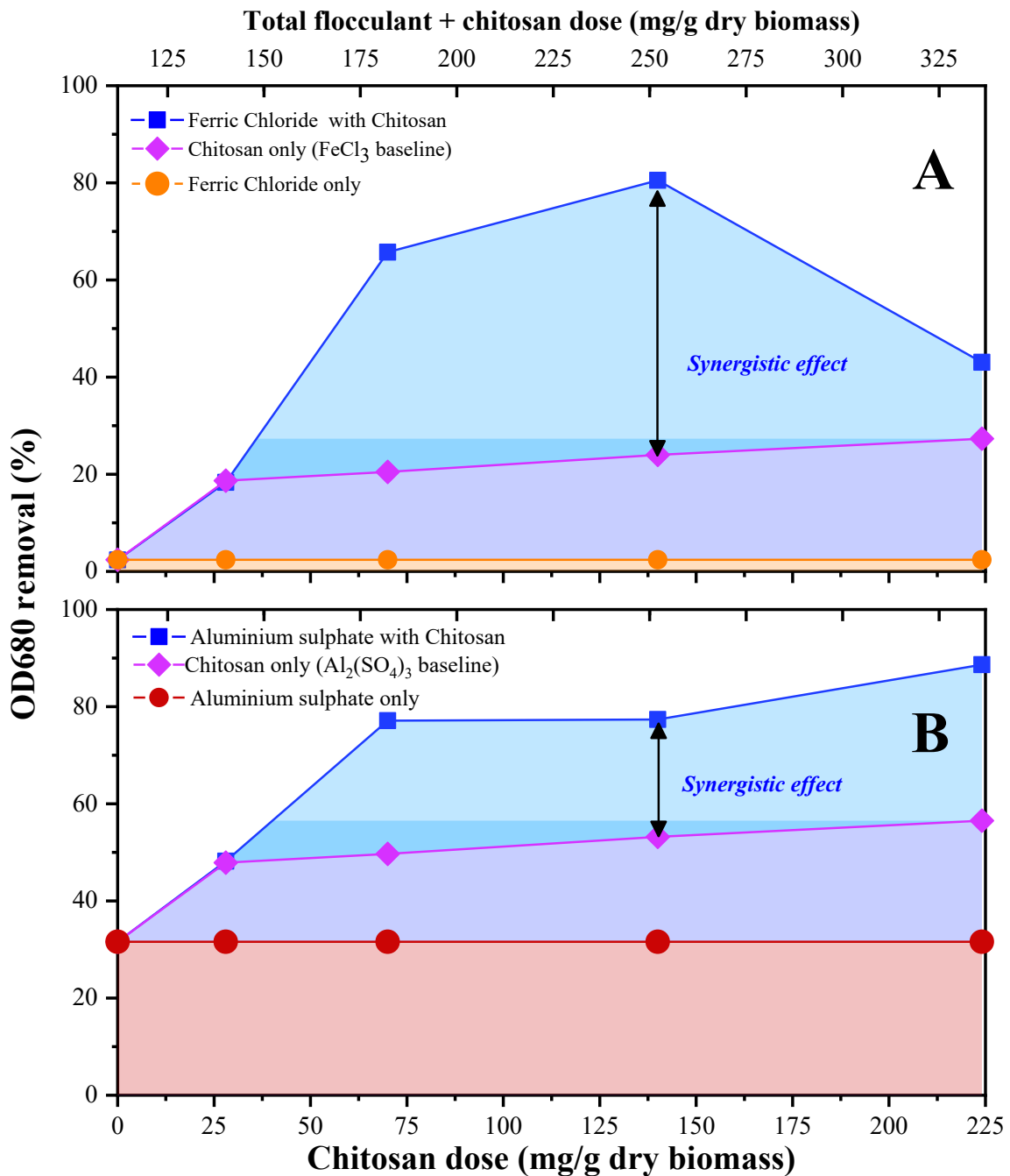
237 **Figure 3:** The effect on *C. vulgaris* flocculation using Chitosan, based on its optical density
238 removal efficiency at $\lambda = 680$ nm. Value and error bars are mean and standard deviation ($n=3$).

239 3.3 Synergistic effect of dual flocculation

240 3.3.1 Improved flocculation using a combination of inorganic flocculants and chitosan

241 Significantly better OD₆₈₀ removal efficiency was observed for dual flocculation combining
242 inorganic salts with chitosan, compared to that achieved by individual flocculation (Fig. 4).
243 Dual flocculation using ferric chloride and chitosan achieved an OD₆₈₀ removal of 81% at 80
244 mg chitosan per litre of algal suspension (i.e. 224 mg chitosan/g dry biomass). Likewise,
245 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.



257

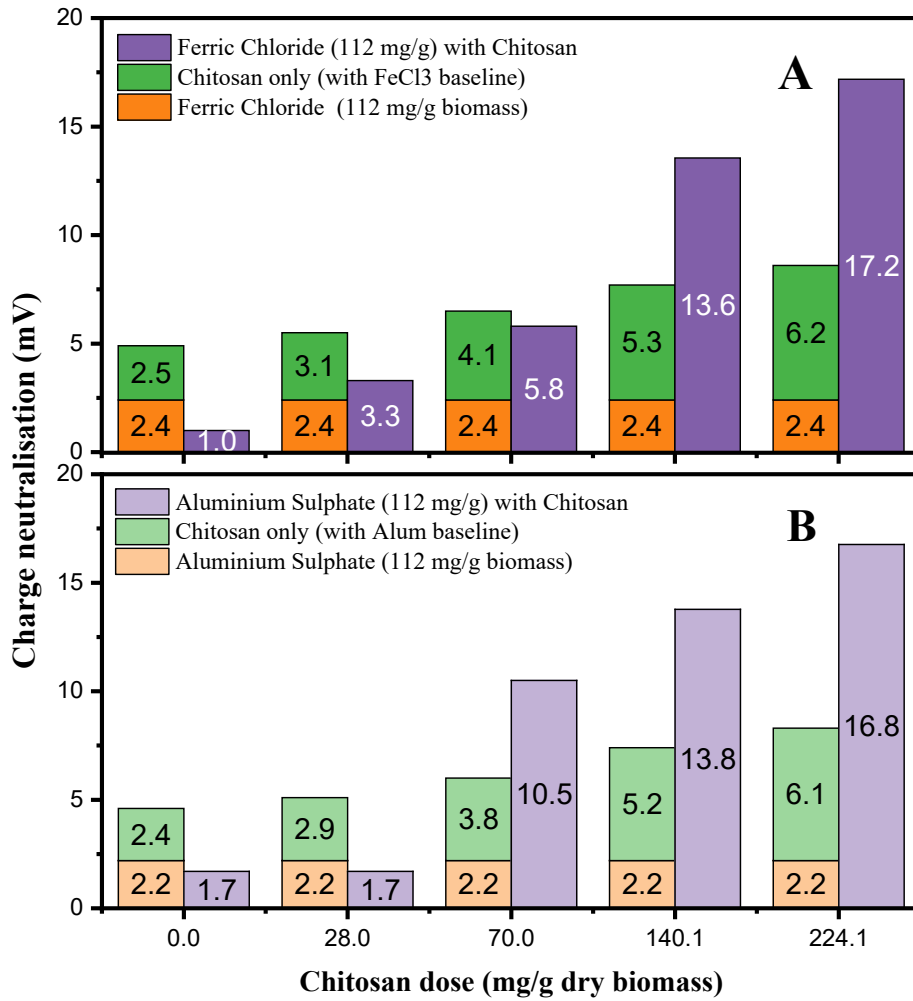
258 **Figure 4:** The synergistic effect of combining inorganic flocculant (a) ferric chloride and (b)
 259 aluminium sulphate with organic polymer Chitosan in flocculating *C. vulgaris*, indicated by the
 260 optical density removal efficiency at $\lambda = 680$ nm.

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.



284

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). Flopan™ 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 <i>C. vulgaris</i> 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

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™
 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.

318 **5 Acknowledgements**

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320 Information Technology, University of Technology Sydney under Tech lab BlueSky Project
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