

1 Cyanobacterial blooms mitigation using proteins with high isoelectric point and  
2 chitosan modified soil

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## 6 Abstract

7 A new environmental friendly method was developed for cyanobacterial blooms  
8 mitigation using local lake shore soil modified by protein with high isoelectric point  
9 (pI) and chitosan jointly. Results suggested that 5 mg/L lysozyme (pI  $\approx$  11) and 100  
10 mg/L bromelain (pI  $\approx$  9.5) modified 10 mg/L soil can both reduce the surface charge  
11 of *microcystis aeruginosa*, the dominant species forming cyanobacterial blooms, from  
12 -26 mv to -10 mv and remove 73% and 60% of algal cells in 30 min, respectively. The  
13 limited improvement of removal efficiency was due to the small flocs (< 60  $\mu$ m)  
14 formed by charge neutralization, which need more than 90 min to settle in static  
15 condition. However, when the small flocs were linked and bridged by the other  
16 modifier, chitosan with long polymer chain, large flocs of about 800  $\mu$ m and 300  $\mu$ m  
17 were formed and more than 80% of algal cells were removed in 5 min and 30 min by  
18 lysozyme-chitosan modified soil and bromelain-chitosan modified soil, respectively.  
19 The lower removal ability of bromelain-modified soil was due to the lower charge  
20 density leading to less powerful in destabilization of algal cells. Depending on the

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21 bi-component modification mechanism including charge neutralization of proteins  
22 with high pI and netting and bridging function of chitosan with long polymer chain, it  
23 is possible to flocculate cyanobacterial blooms in natural waters effectively using  
24 locally available materials.

25 Key words: Cyanobacterial blooms, algae flocculation, modified soil, protein  
26 coagulant, chitosan.

## 27 1. Introduction

28 Over the last two decades, the growth of cyanobacterial as harmful algal blooms  
29 (HABs) occurred more frequently in China, well in line with global trend (Anderson,  
30 1997). Among the technologies combating HABs in natural waters including  
31 mechanical, biological, chemical, genetic and environmental control (Anderson,  
32 2009), significant attention has been focused on the use of coagulants or flocculants  
33 modified clay/soil/sand to flocculate and sediment HAB cells (Li & Pan, 2013; Pan et  
34 al., 2006a; Pierce et al., 2004; Sengco et al., 2001; Sun et al., 2004). Coagulants such  
35 as polyaluminium chloride (PAC) with high charge density can neutralize the negative  
36 surface charge of algal cells and then destabilize the cell suspension to promote their  
37 aggregation (Sengco et al., 2001), but small, fluffy and light flocs are often formed by  
38 the electrostatic interactions (Renault et al., 2009), which are not easy to settle or  
39 re-suspended with only modest currents (Beaulieu et al., 2005) in natural waters.  
40 Furthermore, the chemical coagulants may also cause adverse ecological effects, such  
41 as killing the zooplankton, *Daphnia magna* (Tomasik et al., 1995). Flocculants are  
42 often vital for the growth of floc size because of the long polymer chain with netting

43 and bridging function. Chitosan, a natural and biodegradable flocculants, modified  
44 soil has been proved to flocculate Cyano-HABs effectively in Lake Taihu (Pan et al.,  
45 2006b). However, the charge density of flocculants is often not as high as coagulants,  
46 the removal efficiency was significantly affected by surface properties of particles  
47 (Huang & Chen, 1996). Some stable algal cells may not be able to be destabilized and  
48 then captured and flocculated by using flocculants alone (Li & Pan, 2013).

49         Responding to the shortcomings of coagulants or flocculants as modifiers for the  
50 HABs flocculation in natural waters, a universal, environmental friendly modification  
51 method using moringa oleifera seed extract (MO), a natural coagulant, and chitosan  
52 jointly to turn sand/soil into effective flocculants for mitigating HABs under broad  
53 water conditions was proposed (Li & Pan, 2013). The coagulant, MO, firstly  
54 neutralize the surface charge of algal cells, destabilize them to form small flocs, this  
55 process not only create the optimized condition for chitosan to play its netting and  
56 bridging function to link the small flocs into large ones, which significantly speed up  
57 the sedimentation process, but also increase the removal efficiency since chitosan  
58 works better for the unstabilized, less negatively charged small flocs (Li & Pan, 2013).  
59 Using this mechanism, the flocculation efficiency, floc size and sedimentation process  
60 were all optimized and hence HABs can be removed effectively in short time.

61         However, MO grows only at low-altitude areas, including arid zones (Morton,  
62 1991), they are not immediately available at some locations that have HAB problems,  
63 and transportation costs may render this method uneconomical. A possible solution of  
64 this problem might be the development of new coagulants, preferably from natural

65 and renewable sources. According to the function of MO in the bi-component  
66 modification method (Li & Pan, 2013), the point for screening new materials should  
67 focus on the positive charge density, which destabilize negatively charged algal cells  
68 and improve the removal efficiency. In this aspect, proteins with high isoelectric point  
69 (pI) show promise because of the abundance of  $\text{NH}_2$  groups, which turn into the  
70 positive charged  $\text{NH}_3^+$  after the protonated process. Although some kind of proteins  
71 extracted from common bean (Antov et al., 2010), grape seed (Chang et al., 2009),  
72 chestnut and acorn (Sciban et al., 2009) as coagulants for water turbidity removal  
73 have been reported, Ghebremichael et al. (2005) have also found that cecropin A, a  
74 small peptide showed similar coagulation activity to both MO and alum, the  
75 mechanism of protein as coagulants to modify clay/soil/sand for HABs control in  
76 natural waters is still far from comprehensive and systematic. There are at least three  
77 questions needed to be answer: (1) what common characteristics these proteins have,  
78 (2) what effects of these coagulants have when used for HABs flocculation and how  
79 to take advantage of these effects, and (3) how to screen materials which possess  
80 these characteristics.

81 In this paper, we selected two proteins with high pI, lysozyme (pI  $\approx$  11) and  
82 bromelain (pI  $\approx$  9.5) and chitosan as modifiers to soil collected from the beach of  
83 Lake Taihu, China to remove *microcystis aeruginosa* (M.A.), the main species  
84 forming cyanobacterial blooms in fresh waters. Through the examination of removal  
85 efficiency, floc size growth process, floc structure and zeta potential, the interactions  
86 between proteins and algal cells were discovered. The effects of the two modifiers for

87 the algae removal were also studied by comparing the flocculation properties of  
88 protein, chitosan and protein-chitosan modified soil. Finally, the principle for  
89 screening materials which possess similar effects was also discussed. The main  
90 objective of this study was to propose an effective modification method for  
91 Cyano-HABs mitigation using materials which are environmentally benign and  
92 locally available.

## 93 2. Material and methods

### 94 2.1 Algae culture

95 M.A. cells were obtained from Freshwater Algae Culture Collection at the  
96 Institute of Hydrobiology, Chinese Academy of Sciences, and cultured in BG11  
97 medium, which was adjusted to pH = 8.0 before autoclaving by adding either 0.1  
98 mol/L HCl solutions or 0.1 mol/L NaOH solutions. The sterilized 500 mL glass flasks  
99 containing 300 mL aqueous *M. aeruginosa* medium were maintained at  $25 \pm 1$  °C  
100 under cool white fluorescent light of 2000–3000 lx on a 12 h light and 12 h darkness  
101 regimen in the illuminating incubator (LRH-250-G, Guangdong Medical Apparatus  
102 Co.Ltd., China).

### 103 2.2 Soil and modifiers

104 The soil was collected from the bank of Meiliang Bay, Lake Taihu in China,  
105 washed with deionized water, dried at 100 °C for 10 h, and then grounded and sieved  
106 through 180 mesh (< 90 μm) before use.

107 The protein modifiers, lysozyme and bromelain were purchased from ACROS  
108 Organics, bio-pure. According to the solubility, 200 mg lysozyme and 500 mg

109 bromelain was dissolved in 100 mL deionized water, respectively. The biopolymer  
110 modifier, chitosan was obtained from Qingdao Yunzhou Bioengineering Co. Ltd in  
111 China. 500 mg chitosan was added into 100 mL of 0.5% HAc and stirred until all the  
112 chitosan was dissolved. This solution was then diluted with deionized water to obtain  
113 a final concentration of 100 mg/mL before use. The lysozyme, bromelain and chitosan  
114 solutions were prepared freshly for each experiment.

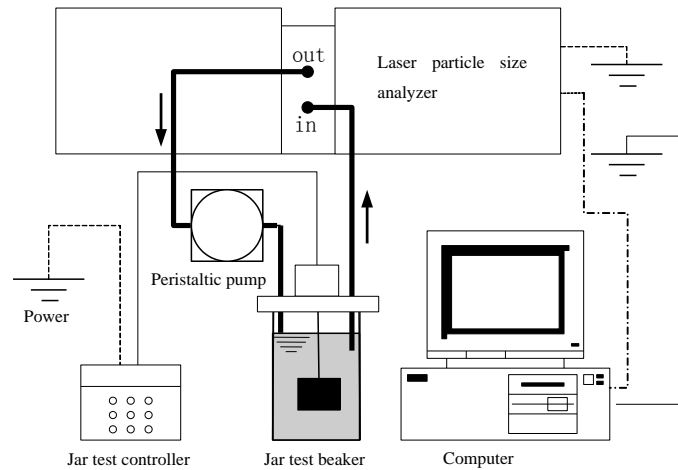
### 115 2.3 Algae flocculation

116 Flocculation experiments were conducted with a jar-test apparatus using M.A.  
117 cells in the mid- to late-exponential growth phase. The initial cell concentration was  
118 diluted to  $7.29\text{--}7.69 \times 10^9$  cells/L, or optical density of 0.150 at the wavelength of  
119 680 nm ( $OD_{680\text{ nm}}$ ) (Pan et al., 2006a) with BG11 culture medium. A volume of 200  
120 mL algae culture was transferred into a 300 mL beaker for all the flocculation  
121 experiments and the pH was adjusted to 8.0 by adding either 0.1 mol/L NaOH or 0.1  
122 mol/L HCl solutions. Different dosage of lysozyme, bromelain, chitosan  
123 lysozyme-chitosan and bromelain-chitosan modified 10 mg/L soil were added to the  
124 culture (Zou et al., 2006). The control culture was run without adding any soil or  
125 modifiers. The solutions were stirred in a jar test apparatus (ZR3–6, Zhongrun Water  
126 Industry Technology Development Co. Ltd., China) with 300 rpm for 1 min, then 120  
127 rpm for 2 min, followed by 40 rpm for another 10 min, the solutions were then kept  
128 standing for 30 min. Samples from 2 cm below the water surface were collected. The  
129 cells were enumerated in a counting chamber of an electromotive microscope  
130 (Axioskop 2 mot plus, Carl ZEISS, Germany) after being fixed by Lugol solution. All

131 the flocculation experiments were conducted in triplicate and the results were  
132 presented as the mean values. The removal efficiency of cells was calculated as  
133  $(\text{initial cell concentration} - \text{sample cell concentration}) / \text{initial cell concentration} \times 100\%$ .  
134 The surface charge of M.A. cells after affected by lysozyme modified soil or  
135 bromelain modified soil was quantified by zeta potential (Zetasizer 2000, Malvern Co.  
136 United Kingdom).

137 According to the dosage-effect experiment, studies of flocculation kinetics and  
138 floc size growth process of lysozyme, bromelain, chitosan, lysozyme-chitosan and  
139 bromelain-chitosan modified soil were conducted. The optimal dosage of each  
140 modified soil was added to the prepared algae culture, the solutions were then  
141 flocculated as the same procedure as described above. After sedimentation for 0, 2, 5,  
142 10, 15, 20, 30, 60, 90, 120, 180, 240, 300, 360 and 420 min, the samples were  
143 collected and the removal efficiencies were calculated.

144 The floc size growth process during flocculation was monitored with a laser  
145 particle size analyzer (Mastersizer 2000, Malvern Co. United Kingdom) (Figure 1).  
146 After the addition of each modified soil and flocculation started, samples were drawn  
147 into the analyzer and back to the jar by a peristaltic pump (BT00-300M, Baoding  
148 Longer Precision Pump Co. Ltd., China) at a flow rate of 35 mL/min. The floc size of  
149 the samples was determined first before going through the pump head to avoid floc  
150 breakage. The flow rate should not be too fast to avoid breakage of flocs or too slow  
151 to avoid sedimentation in the pipe (Jarvis et al., 2005). The size was denoted by the  
152 measured mean diameter ( $D_{0.5}$ ).



153

154 **Figure 1.** Instrument for floc size growth process online monitoring.

155 2.4 Influence of pH on the flocculation of protein modified soil

156 Different pH between 5.0 - 10.0 with an increment of pH 1.0 was controlled using  
 157 either 0.1 mol/L NaOH or 0.1 mol/L HCl solutions before flocculation experiment.  
 158 Optimal dosage (determined by dosage-effect experiment) of lysozyme modified soil  
 159 and bromelain modified soil was added into the culture. After flocculation and  
 160 sedimentation for 30 min, samples were collected and the removal efficiencies were  
 161 calculated. The initial concentration of M.A. cells, flocculation procedure and  
 162 calculation equation were the same as described in 2.3.

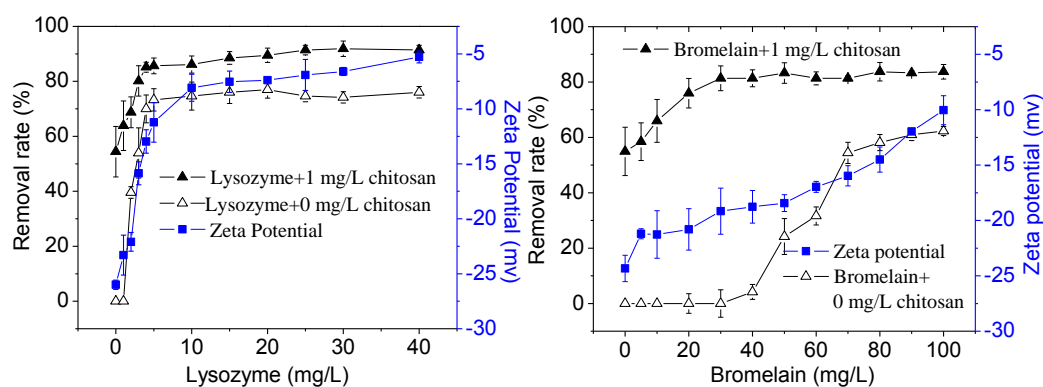
163 3. Results and discussion

164 3.1 Dosage-effect of modified soil

165 The positive charged protein, lysozyme and bromelain can both neutralize the  
 166 negative surface charge of M.A. cells (Figure 2), the zeta potential was increased from  
 167 -26 mv to -10 mv at the dosage of 5 mg/L lysozyme and 100 mg/L bromelain  
 168 modified 10 mg/L soil, respectively. The charge neutralization process destabilized  
 169 the algae suspension and created flocculation potential since algal cells are normally



170 stable negatively charged bio-particles in natural waters (Tenney et al., 1969). The  
 171 removal efficiency changed as the same trend with zeta potential, a maximum of 73%  
 172 and 60% algal cells were removed at the optimal dosage of 5 mg/L lysozyme and 70  
 173 mg/L bromelain modified 10 mg/L soil, respectively. The increasing slope of zeta  
 174 potential and algae removal rate when using lysozyme (pI  $\approx$  11) or bromelain (pI  $\approx$  9.5)  
 175 modified soil suggested that higher positive charge density leads to more effective in  
 176 destabilization of algae suspension and hence achieved higher flocculation efficiency.

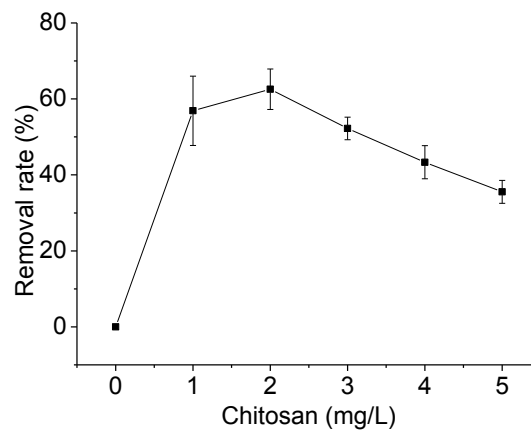


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178 **Figure 2.** The increase of zeta potential and removal efficiency of M.A. cells by  
 179 different dosage of lysozyme, bromelain, lysozyme-chitosan and bromelain-chitosan  
 180 modified 10 mg/L soil.

181 When chitosan modified soil was used, maximally 60% of algal cells were  
 182 removed at the dosage of 2 mg/L chitosan modified 10 mg/L soil (Figure 3). However,  
 183 in previous reports, 80% of the M.A. cells were removed by 1 mg/L chitosan modified  
 184 10 mg/L soil in 10 min in 0.5% NaCl solution (Zou et al., 2006). The difference  
 185 between previous and current results was due to the different flocculation systems. In  
 186 the previous study, algal cells were firstly harvested and re-dispersed in 0.5% NaCl  
 187 solution before flocculation, which reduced the stability of algae suspension and

188 create a better condition for the netting and bridging function of chitosan (Li & Pan,  
189 2013). In this study, the flocculation was directly conducted in the culture medium,  
190 the algal cells were more stable and harder to flocculate. However, when  
191 lysozyme-chitosan modified soil and bromelain-chitosan modified soil was used, the  
192 removal efficiency was increased and the dosage needed was significantly reduced  
193 (Figure 2), more than 80% of algal cells were removed at the optimal dosage of 5  
194 mg/L lysozyme + 1 mg/L chitosan + 10 mg/L soil, 30 mg/L bromelain + 1 mg/L  
195 chitosan + 10 mg/L soil, respectively.



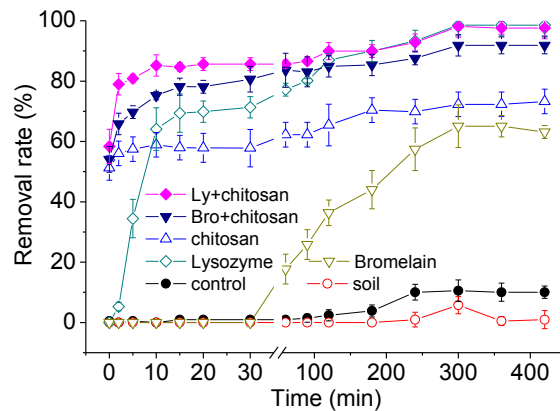
196

197 **Figure 3.** Removal of M.A. cells using chitosan modified 10 mg/L soil.

### 198 3.2 Flocculation kinetics and flocs growth process

199 Compared to control, soil alone was ineffective to flocculate M.A. cells (Figure  
200 4). When lysozyme-chitosan modified soil was used, more than 80% algal cells were  
201 removed in 5 min, while 90 min was needed for the lysozyme alone modified soil to  
202 achieve this removal rate. In the first 30 min, there was almost no effect of bromelain  
203 modified soil, after that, the removal efficiency gradually increased and reached 60%  
204 in 300 min, while 80% of algal cells were removed in 30 min when  
205 bromelain-chitosan modified soil was used. When chitosan modified soil was used, 60%

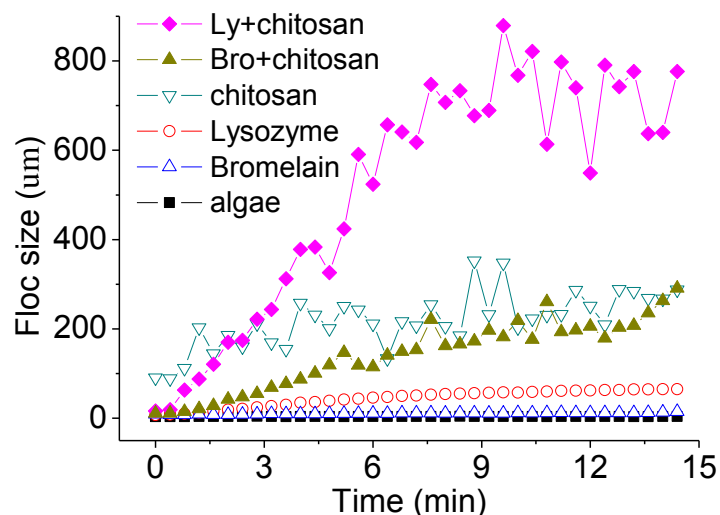
206 of algal cells were removed in 10 min and then increased slightly as time increased.



207

208 Figure 4. Flocculation kinetics of lysozyme, bromelain, chitosan, lysozyme-chitosan  
209 (LY+chitosan) and bromelain-chitosan (Bro+chitosan) modified 10 mg/L soil at the  
210 optimal dosage.

211 The flocculation kinetics of modified soil corresponded well to the algal floc  
212 formation and growth (Figure 5). The floc size of lysozyme and bromelain modified  
213 soil was about 12  $\mu\text{m}$  and 60  $\mu\text{m}$ , respectively. The limited improved floc size  
214 explained why more than 90 min was needed to achieve maximum removal efficiency  
215 when lysozyme or bromelain modified soil was used (Figure 4). In contrast, chitosan,  
216 lysozyme-chitosan and bromelain-chitosan modified soil can both increase the floc  
217 size to larger than 200  $\mu\text{m}$ , which greatly speed up the sedimentation process. The floc  
218 size formed by lysozyme and chitosan modified soil ( $\sim 800 \mu\text{m}$ ) was larger than  
219 bromelain and chitosan modified soil or chitosan alone modified soil ( $\sim 300 \mu\text{m}$ ),  
220 which suggested that higher positive charge density led to more powerful in  
221 destabilization of algal cells and created a better condition for chitosan to capture and  
222 bridge them into large flocs.



223

224 Figure 5. The formation and growth of algal flocs formed by lysozyme, bromelain,  
 225 chitosan, lysozyme-chitosan and bromelain-chitosan modified 10 mg/L soil during  
 226 flocculation process at the optimal dosage.

### 227 3.3 Effect of protein, chitosan and soil for algae flocculation

228 The lysozyme and bromelain can both neutralize the negative surface charge of  
 229 algal cells (Figure 2) and destabilize them to form small flocs (Figure 5), although the  
 230 former was more powerful than the latter. However, the small flocs were may be able  
 231 to settle gradually and increase the removal rate in static condition (Figure 4), but not  
 232 sufficient for particle application since the small and light flocs would not settle with  
 233 the disturbance of water flow and wind-induced waves in the field (Beaulieu et al.,  
 234 2005).

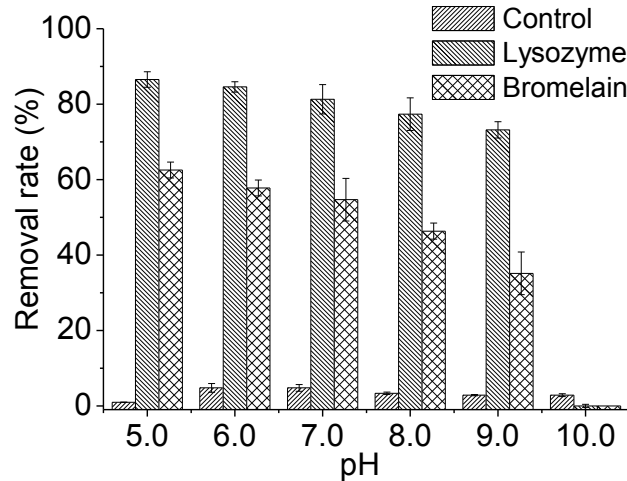
235 In contrast, chitosan was important for the floc size growth because of the long  
 236 polymer chain with netting and bridging function (Zou et al., 2006), chitosan alone or  
 237 used jointly with lysozyme or bromelain can both increase the floc size to more than  
 238 200  $\mu\text{m}$  (Figure 5). However, the removal efficiency of chitosan modified soil was  
 239 limited, the maximum removal rate was about 60% (Figure 3, Figure 4). This was

240 because chitosan was a weaker surface charge modifier (Huang & Chen, 1996), which  
241 was not sufficient to destabilize the highly negatively charged and stable algal cells.  
242 Therefore, the first step of charge neutralization not only created an optimized  
243 condition for chitosan to increase the floc size by netting and bridging function  
244 (Figure 5), but also improved the overall removal efficiency (Figure 4). The  
245 differences of removal rate and floc size between lysozyme-chitosan and  
246 bromelain-chitosan modified soil also suggested that higher charge density was more  
247 powerful in destabilization of algal cells and creating better condition for chitosan to  
248 play its netting and bridging function. To ensure the effectiveness, the pI should be no  
249 lower than 9.5 since the floc size had no increase when bromelain (pI  $\approx$  9.5) and  
250 chitosan modified soil was used compared to chitosan alone modified soil (Figure 5),  
251 although the overall removal efficiency was improved about 20% (Figure 4).

252 The soil, as we have discussed in our previous reports (Li & Pan, 2013; Pan et al.,  
253 2011), firstly provided the mass or ballast by bounding together with algal flocs  
254 tightly to carry them to bottom sediments. If there were just modifiers used, even  
255 when large flocs are formed, they may still float in the water surface. Sedimentation  
256 was regarded as a major challenge for coagulation and flocculation treatment of  
257 buoyant cyanobacterial cells (Gheraout et al., 2010). Second, soil particles are  
258 natural cheap carriers to hold and keep high concentration of the modifiers, which are  
259 otherwise easily diluted to below the working concentration in natural waters. In  
260 addition, the soil also enhances the collision frequencies between particles (Stumm &  
261 Morgan, 1996).

262 3.4 Influence of pH on the protein modified soil

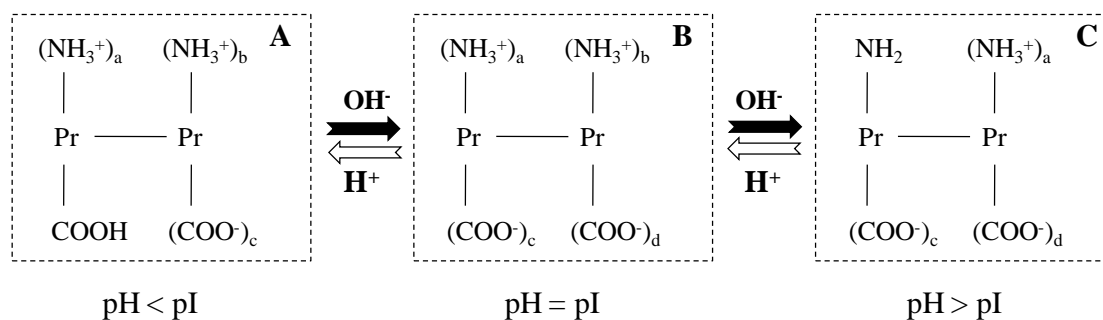
263 As pH increased, the removal efficiency decreased between pH 5.0 - 9.0, from 86%  
264 to 73% of lysozyme modified soil and 62% to 35% of bromelain modified soil,  
265 respectively (Figure 6). The reason was probably due to the increasing number of  
266 negatively charged groups, carboxyl-groups ( $-\text{COO}^-$ ) and consumption of positively  
267 charged groups, amino-group ( $-\text{NH}_3^+$ ) as pH increase (Figure 7). The positively  
268 charged groups was more, equal or less than negatively charged groups when  $\text{pH} < \text{pI}$ ,  
269  $\text{pH} = \text{pI}$  or  $\text{pH} > \text{pI}$ , respectively. The decrease of positive charge density led to  
270 less powerful in destabilization of algae suspension and thereby lowered the removal  
271 efficiency. When pH exceeded the pI, the protein was negatively charged and the  
272 destabilization effects were then disappeared.



273

274 Figure 6 Effect of pH on M.A. cells removal using lysozyme (5 mg/L) or bromelain

275 (70 mg/L) modified 10 mg/L soil.



276

277 Figure 7. The changes of positively and negatively charged groups of proteins when  
 278 pH was lower, equal or higher than pI. In A, when  $pH < pI$ , the number of positively  
 279 charged groups was more than negatively charged groups, i.e.,  $a + b > c$ ; In B, when  
 280  $pH = pI$ , the number of positively charged groups was the same as negatively charged  
 281 groups, i.e.,  $a + b = c + d$ ; In C, when  $pH > pI$ , the number of positively charged  
 282 groups was less than negatively charged groups, i.e.,  $a < c + d$ .

### 283 3.5 Prospect of using protein-chitosan modified soil to control Cyano-HABs

284 Our results demonstrated that protein with high pI could neutralize the negative  
 285 charge of algal cells, destabilize them to form small flocs. Although like some other  
 286 natural or chemical coagulants, such as MO (Li & Pan, 2013), ferric salt (Ma et al.,  
 287 2012) and PAC (Beaulieu et al., 2005; Pan et al., 2011), the small flocs either settled  
 288 slowly or were too small to settle, the destabilization process was critical important  
 289 for the removal efficiency improving and floc size growth of chitosan. As the safe and  
 290 cheap carrier of these modifiers, soil provided mass or ballast to speed up the floc  
 291 sedimentation (Pan et al., 2011). Understanding the mechanism of this bi-component  
 292 modification method provided great opportunity for obtaining new modifiers from  
 293 locally available materials. The environmental concerns can also be greatly minimized  
 294 without using any chemical coagulants or flocculants.

295        There are maybe concerns about how to screen the proteins with the property of  
296 high pI, and if it is possible, the cost may be high to purify the proteins. Although the  
297 lysozyme and bromelain we used here were bio-pure products, the main purpose of  
298 this study was to select these two proteins as representatives to discover the effects of  
299 the positive charged proteins for algae flocculation and how to take advantage of these  
300 effects to improve the removal efficiency, by which a new bi-component modification  
301 method and guidelines for new modifiers screening was proposed. The lysozyme and  
302 bromelain, however, are still far from practical application since there are many other  
303 issues needed to be further studied, such as the efficiency in the field and most  
304 importantly, the ecological safety evaluation of these materials.

305        To screen the proteins with high pI, two methods show promise. One is protein  
306 precipitation in solutions with the pH equal to pI since the solubility of protein is  
307 minimum under this condition (Zhang et al., 2009). According to this principle,  
308 proteins with different pI could be separated roughly by adjusting the pH of solution.  
309 The other method is the ion exchange chromatography (Gassenschmidt et al., 1995;  
310 Ghebremichael et al., 2006). Proteins with different pI in one solution with a certain  
311 pH possess different charge properties (Figure 7), which means that the charge  
312 properties of proteins can be manipulated by adjusting the pH. If the pH increases, the  
313 proteins with pI lower than pH will possess negative charge and the others will  
314 possess positive charge, then the proteins with pI higher than the pH can be screened  
315 by cation exchange matrix absorption and elution. Once the high pI proteins are  
316 obtained, it is not important whether they are pure or not since the effect was



317 stimulated by charge neutralization between proteins and algal cells, it is not a  
318 material specific issue.

#### 319 4. Conculsion

320 M.A. cells could be effectively flocculated by positively charged protein and  
321 chitosan jointly modified soil. The protein with high pI firstly neutralized the negative  
322 charge of algal cells, destabilized them to form small flocs, chitosan with long  
323 polymer chain then linked and bridged the small flocs into large ones. Together with  
324 soil which helped to speed up the settle process, high removal efficiency was achieved  
325 in short time. This method greatly reduced the potential environmental impacts by  
326 using totally biodegradable modifiers in natural waters. With some additional research  
327 about protein screening, this approach shows promise to mitigate Cyano-HABs  
328 effectively using locally available materials.

#### 329 Acknowledgement

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