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

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Abstract

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

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

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

1. Introduction

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

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

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

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

and renewable sources. According to the function of MO in the bi-component modification method (Li & Pan, 2013), the point for screening new materials should focus on the positive charge density, which destabilize negatively charged algal cells and improve the removal efficiency. In this aspect, proteins with high isoelectric point (pI) show promise because of the abundance of NH_2 groups, which turn into the positive charged NH_3^+ after the protonated process. Although some kind of proteins extracted from common bean (Antov et al., 2010), grape seed (Chang et al., 2009), chestnut and acorn (Sciban et al., 2009) as coagulants for water turbidity removal have been reported, Ghebremichael et al. (2005) have also found that cecropin A, a small peptide showed similar coagulation activity to both MO and alum, the mechanism of protein as coagulants to modify clay/soil/sand for HABs control in natural waters is still far from comprehensive and systematic. There are at least three questions needed to be answer: (1) what common characteristics these proteins have, (2) what effects of these coagulants have when used for HABs flocculation and how to take advantage of these effects, and (3) how to screen materials which possess these characteristics.

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

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

2. Material and methods

2.1 Algae culture

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

2.2 Soil and modifiers

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

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

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

2.3 Algae flocculation

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

the flocculation experiments were conducted in triplicate and the results were presented as the mean values. The removal efficiency of cells was calculated as (initial cell concentration−sample cell concentration)/initial cell concentration ×100%. The surface charge of M.A. cells after affected by lysozyme modified soil or bromelain modified soil was quantified by zeta potential (Zetasizer 2000, Malvern Co. United Kingdom).

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

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

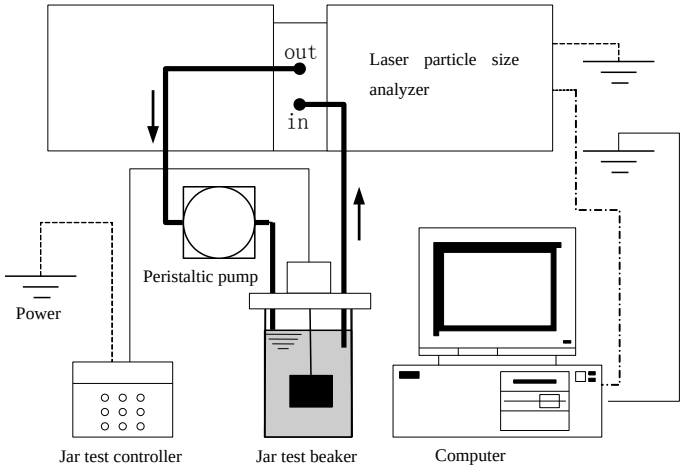


Figure 1. Instrument for floc size growth process online monitoring.

2.4 Influence of pH on the flocculation of protein modified soil

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

3. Results and discussion

3.1 Dosage-effect of modified soil

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

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

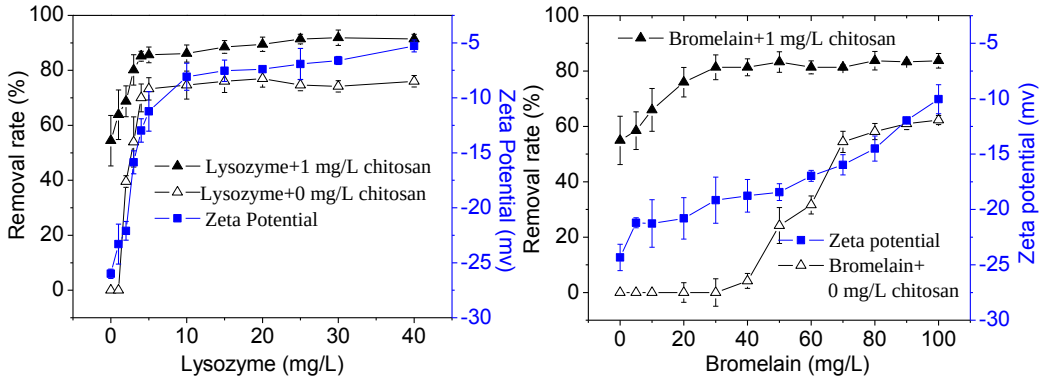


Figure 2. The increase of zeta potential and removal efficiency of M.A. cells by different dosage of lysozyme, bromelain, lysozyme-chitosan and bromelain-chitosan modified 10 mg/L soil.

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

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

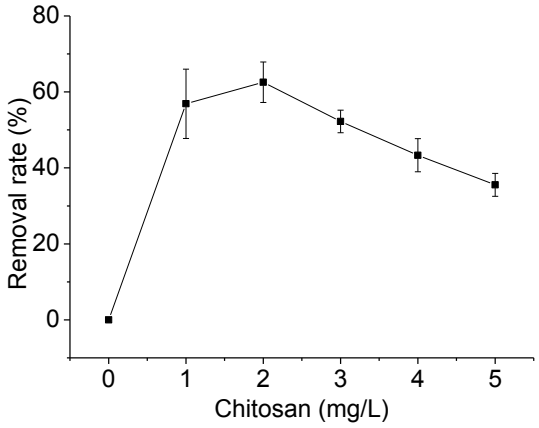
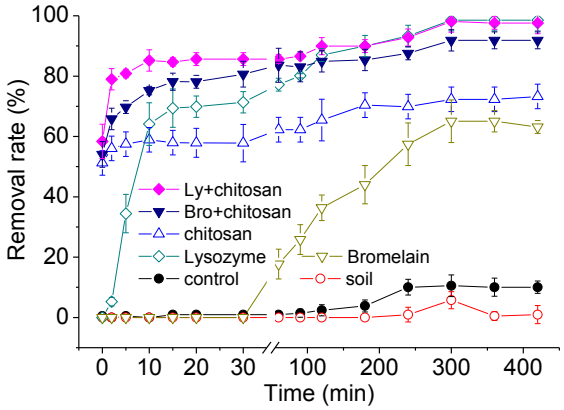


Figure 3. Removal of M.A. cells using chitosan modified 10 mg/L soil.

3.2 Flocculation kinetics and flocs growth process

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



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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 μm and 60 μm, 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 μm, which greatly speed up the sedimentation process. The floc
218 size formed by lysozyme and chitosan modified soil (～ 800 μm) was larger than
219 bromelain and chitosan modified soil or chitosan alone modified soil (～ 300 μm),
220 which suggested that higher positive charge density leaded 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.

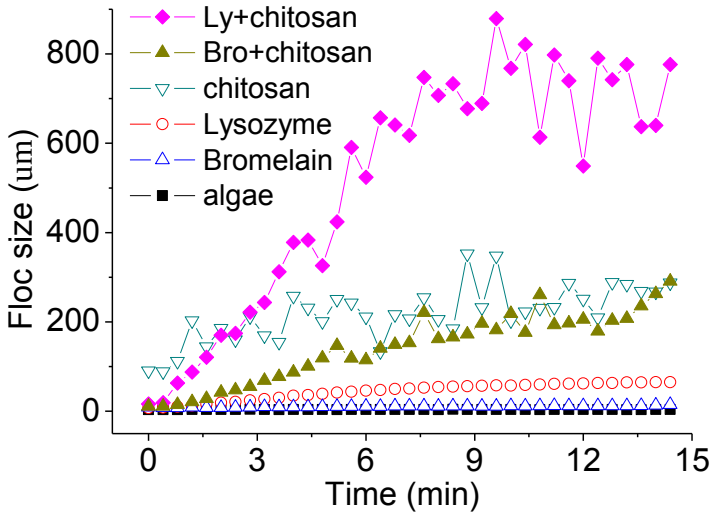


Figure 5. The formation and growth of algal flocs formed by lysozyme, bromelain, chitosan, lysozyme-chitosan and bromelain-chitosan modified 10 mg/L soil during flocculation process at the optimal dosage.

3.3 Effect of protein, chitosan and soil for algae flocculation

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

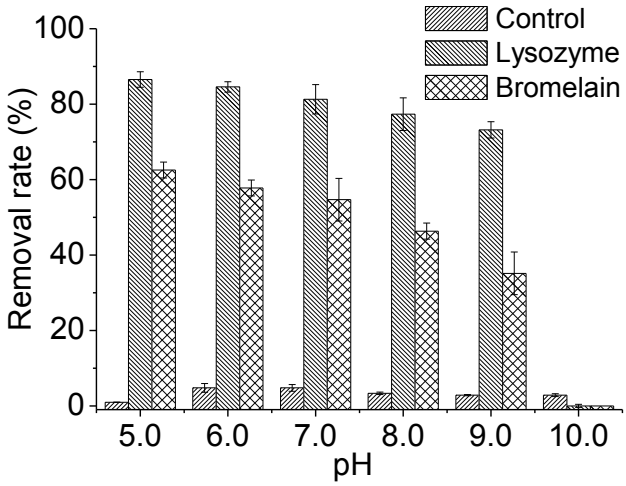
In contrast, chitosan was important for the floc size growth because of the long polymer chain with netting and bridging function (Zou et al., 2006), chitosan alone or used jointly with lysozyme or bromelain can both increase the floc size to more than 200 μm (Figure 5). However, the removal efficiency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

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

The soil, as we have discussed in our previous reports (Li & Pan, 2013; Pan et al., 2011), firstly provided the mass or ballast by bounding together with algal flocs tightly to carry them to bottom sediments. If there were just modifiers used, even when large flocs are formed, they may still float in the water surface. Sedimentation was regarded as a major challenge for coagulation and flocculation treatment of buoyant cyanobacterial cells (Ghernaout et al., 2010). Second, soil particles are natural cheap carriers to hold and keep high concentration of the modifiers, which are otherwise easily diluted to below the working concentration in natural waters. In addition, the soil also enhances the collision frequencies between particles (Stumm & 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 ($-COO^-$) and consumption of positively
267 charged groups, amino-group ($-NH_3^+$) as pH increase (Figure 7). The positively
268 charged groups was more, equal or less than negatively charged groups when pH < pI,
269 pH = pI or pH > pI, respectively. The decrease of positive charge density leaded 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.



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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.

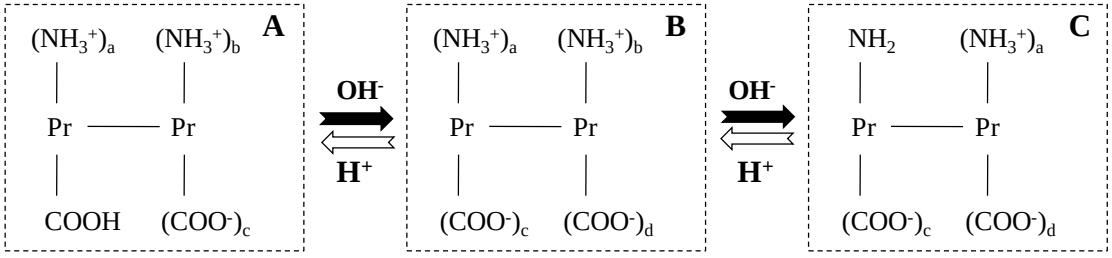

Figure 7. The changes of positively and negatively charged groups of proteins when pH was lower, equal or higher than pI. In A, when pH < pI, the number of positively charged groups was more than negatively charged groups, i.e., a + b > c; In B, when pH = pI, the number of positively charged groups was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

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

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

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

To screen the proteins with high pI, two methods show promise. One is protein precipitation in solutions with the pH equal to pI since the solubility of protein is minimum under this condition (Zhang et al., 2009). According to this principle, proteins with different pI could be separated roughly by adjusting the pH of solution. The other method is the ion exchange chromatography (Gassenschmidt et al., 1995; Ghebremichael et al., 2006). Proteins with different pI in one solution with a certain pH possess different charge properties (Figure 7), which means that the charge properties of proteins can be manipulated by adjusting the pH. If the pH increases, the proteins with pI lower than pH will possess negative charge and the others will possess positive charge, then the proteins with pI higher than the pH can be screened by cation exchange matrix absorption and elution. Once the high pI proteins are 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.

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