Removal of *Microcystis aeruginosa* cells using the dead cells of a marine filamentous bacterium, *Aureispira* sp. CCB-QB1

Go Furusawa[1] and Koji Iwamoto[2]

[1] Centre for Chemical Biology, Universiti Sains Malaysia, Bayan Lepas, Penang, Malaysia
[2] Malaysia-Japan International Institute of Technology, Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur, Malaysia

ABSTRACT

Inorganic and synthetic flocculants are widely investigated for removing harmful microalgae, such as *Microcystis aeruginosa*. However, their toxicity and non-biodegradability are shortcomings. Bioflocculants based on extracellular polysaccharides have attracted much attention as alternative flocculants. However, its high production cost is a limiting factor for applying bioflocculants. Here, we investigate the potential of the dead cells of a marine filamentous bacterium, *Aureispira* sp. CCB-QB1, as a novel flocculant on *M. aeruginosa* cells. The removal efficiency of *M. aeruginosa* cells by the dead cells was measured by mixing and shaking both components in a buffer with 5 mM $CaCl_2$ in different incubation times and concentrations of the dead cells. After that, the minimum effective concentration of $CaCl_2$ was determined. The combination effect of $FeCl_3$ and the dead cells on the removal efficiency was tested. The structure of cell aggregates consisted of the dead cells and *M. aeruginosa* cells were also observed using a scanning electron microscope. The maximum removal efficiency (75.39%) was reached within 3 min in the presence of $CaCl_2$ when 5 mg/ml of the dead cells (wet cells) were added. The optimal concentration of $CaCl_2$ was 5 mM. The combination of the dead cells and a low concentration of $FeCl_3$ (10 mg/L) with 5 mM of $CaCl_2$ significantly improved the removal efficiency by about 1.2 times ($P < 0.05$). This result indicates that the combination usage of the dead cells can reduce the use of $FeCl_3$. These results indicated that the dead cells could potentially be a novel biolfocculant to remove *M. aeruginosa* cells.

INTRODUCTION

Harmful blooms of cyanobacteria (cyanoHABs) by toxic cyanobacterial species have been recognized in many countries (*Briand et al., 2003*) and generate direct economic damages because of the pollution of freshwater environments, as sources of drinking water, agriculture, fishing, and industry water (*Hamilton, Salmaso & Paerl, 2016*). CyanoHABs also seriously affect health problems in humans and animals because of the production of toxins such as microcystin and anatoxin (*Azevedo et al., 2002*; *Katırcıoğlu, Akin & Atici,*

2004). For instance, microcystin and its derivatives disrupt the structure and function of the liver and cause deaths by liver hemorrhage (*Azevedo et al., 2002*; *Katırcıoğlu, Akin & Atici, 2004*). CyanoHABs occur in many countries in tropical and temperate regions. In addition, it is known that climate change, such as rising CO_2 and global warming, is likely to stimulate the development of harmful cyanobacterial blooms in eutrophic waters (*Visser et al., 2016*).

To remove microalgae, including cyanobacteria cells, from the water column, several types of flocculants are used. The flocculants are mainly classified into three groups, such as inorganic flocculants (*e.g.*, polyaluminum chloride (PAC) and ferric chloride ($FeCl_3$)), organic synthetic flocculants (*e.g.*, polyacrylamide derivatives), and some kinds of clay or bioflocculants mainly consisting of polysaccharides produced by microorganisms (*Salehizadeh & Shojaosadati, 2001*). Inorganic flocculants, such as PAC and $FeCl_3$, were low-cost materials and exhibited a high removal efficiency on *M. aeruginosa* cells (*Li et al., 2015*; *Sun et al., 2012*; *Zhang et al., 2011*). However, a high dosage of the flocculants is required for its high removal efficiency. In addition, the sludge containing inorganic flocculants consisted of heavy metals is toxic to the environment (*Fast, Kokabian & Gude, 2014*). Especially, aluminum may be one of the risk factors for Alzheimer's disease (*Flaten, 2001*; *Fast, Kokabian & Gude, 2014*).

Polyacrylamide derivatives are extensively used to treat natural water and sewage because of their high removal efficiency on pollutants (*Kurenkov, Hartan & Lobanov, 2002*). Polyacrylamide derivatives are also used in removing microalgae, including *M. aeruginosa* (*Pugazhendhi et al., 2019*; *Cai et al., 2021*). However, acrylamide is considered a probable human carcinogen and mutagen *in vitro* and *in vivo* experiments using animals (*Dearfield et al., 1988*; *Dearfield et al., 1995*). Thus, there are concerns about their toxicity and less biodegradability.

To date, bioflocculants, which are non-toxic and biodegradable compounds, have attracted attention as an alternative to chemical flocculants. Bioflocculants are biological polymers, mainly extracellular polysaccharides (EPS) or proteins, produced by living organisms, particularly plants and microbes. As the plant-based natural coagulants, some seeds and fruit/ peels, such as *Cicer arietinum*, *Moringa oleifera*, and cactus, are reported (*Kumar, Othman & Asharuddin, 2017*). Bioflocculants composed of EPS produced by bacteria belonging to genera *Bacillus* (*Deng et al., 2003*; *Zheng et al., 2008*), *Corynebacterium* (*He, Li & Chen, 2004*), *Klebsiella* (*Wang et al., 2007*), *Paenibacillus* (*Li et al., 2013*), *Proteus* (*Xia et al., 2008*) and *Pseudomonas* (*Azzam & Tawfik, 2015*; *Liu et al., 2016*) were also found during past decades. These bioflocculants were exhibited high removal efficiency (80–99.6%) on negatively charged clay particles, kaolin. In addition, several investigations demonstrated that the bioflocculants were helpful for removing *M. aeruginosa*. For instance, Sun and colleagues reported that bioflocculant, EPS-1, produced by *Bacillus amyloliquefaciens* could remove approximately 88% of *M. aeruginosa* cells (*Sun et al., 2015a*). Bioflocculant produced by *Pseudomonas aeruginosa* ZJU1 combined with kaolin, $CaCl_2$, and $KAl(SO_4)_2$ also exhibited high removal efficiency on *M. aeruginosa* cells (*Sun et al., 2015b*). The investigation conducted by *Zhang et al. (2019)* demonstrated that the bioflocculant produced by *Halobacillus* sp. strain H9 also successfully removed

M. aeruginosa cells. Besides plants and microbes, another type of polysaccharide, chitosan, produced from shells of crustaceans has also attracted much attention as a bioflocculant to remove *M. aeruginosa*. The study conducted by *Park et al. (2020)* demonstrated that chitosan fiber removed almost 89% of *M. aeruginosa* cells. *Ma et al. (2016)* also reported that chitosan-aluminum chloride combined coagulants exhibited high removal efficiency (>90%) on *M. aeruginosa* cells. Thus, bioflocculants consisted of non-toxic and biodegradable biopolymers might be useful for removing *M. aeruginosa* cells. However, the high cost of the natural macromolecules, especially EPS from microorganisms, limits their application.

In a previous investigation, we reported that a marine filamentous bacterium, *Aureispira* sp. CCB-QB1, formed cell aggregates in the presence of CaCl$_2$ (*Furusawa, Hartzell & Navaratnam, 2015*). In addition, the dead cells of *Aureispira* sp. CCB-QB1, which has a negatively charged cell surface, could absorb negatively charged clay particles, kaolin, and form aggregates with kaolin particles in the presence of CaCl$_2$ (*Hasyimah, Furusawa & Amirul, 2021*). Our investigation demonstrated that the precipitation might be occurred by neutralizing the negative charge of both kaolin and the dead cells by adding a divalent cation, CaCl$_2$. Based on the result, we predicted that *M. aeruginosa* cells might also be precipitated by the dead cells in the presence of CaCl$_2$ (*Hasyimah, Furusawa & Amirul, 2021*). Furthermore, the preparation of the dead cells was more straightforward than that of known bioflocculants consisted of polysaccharides because the precipitation and purification steps required in known bioflocculants were not necessary to prepare the dead cells. This information indicates that the dead cells have the potential for an inexpensive flocculant compared with the other bioflocculants based on extracellular polymers. In this study, to confirm our prediction, we performed to remove *M. aeruginosa* cells by using the dead cells from aqueous samples under various conditions. To the best of our knowledge, this is the first report to remove the *M.aeruginosa* using the dead cells of a filamentous bacterium.

MATERIAL AND METHODS

Microorganisms and culture conditions

Aureispira sp. CCB-QB1 (referred to hereafter as CCB-QB1) deposited in CCB Microbial Biodiversity Library (CCB-MBL) (No. CCB-MBL0192) was the cells used as a bioflocculant in this study. The cells were cultured in a modified high nutrient artificial seawater medium (H-ASWM) (0.91% tryptones, 3.39% artificial sea salt, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6) described by *Hasyimah, Furusawa & Amirul (2020)* at 30 °C for 20 h with shaking (200 rpm). *Microcystis aeruginosa* NISE 102 strain (referred to hereafter as NISE 102) (*Gao et al. (2012)*) was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES Collection, Tsukuba, JAPAN). The NISE 102 cells were cultured in liquid BG-11 medium at 26 °C and under a fluorescent lump at 3000 Lux, with a light: dark period of 12:12 h for 14 days.

Preparation of the dead cells of CCB-QB1

The dead cells of CCB-QB1 were prepared based on the method described by *Hasyimah, Furusawa & Amirul (2020)*. To obtain exponential phase cells, the CCB-QB1 cells were cultured in 100 ml of modified H-ASWM at 30 °C for 20 h with shaking. An optical density at 600 nm (OD_{600}) of cell suspension was measured using a spectrophotometer (UV-1800, Shimadzu, Japan) and adjusted $OD_{600} = 1.0$ with modified H-ASWM broth. The cell suspension was exposed to 3 W/m^2 of UV-A in a laminar flow cabinet (AVC-4D, Esco Technologies Inc., USA) for 10 min, and then the suspension was centrifuged at $3,200 \times g$ for 5 min to harvest cells.

Observation of aggregation formation of the dead cells on NISE 102 cells

NISE 102 cells in the exponential phase were centrifuged at $1,000 \times g$ for 10 min. The cell pellet was suspended into 7 mM $CaCl_2$ solution with 10 mM HEPES buffer (pH 7.6) and adjusted to $OD_{680} = 0.6$ ($4.15 \times 10^6 \pm 5 \times 10^4$ cells/ml). The HEPES buffer was used as a basal buffer for preparing the cell suspension of NISE 102. The wavelength was referred from Sun's report (*Sun et al., 2015a*; *Sun et al., 2015b*). Fifty mg of the dead cells (wet cells) was added to 10 ml of NISE 102 cell suspension. The suspensions were shaken for 3 min at 30 °C with shaking (100 rpm). The aggregates were observed under a light microscope (Olympus BX51, Olympus, Japan).

Effect of incubation time and initial concentration of the dead cells on the removal efficiency

One hundred mg, 50 mg, and 25 mg of the wet dead cells were added to 10 ml aliquots of NISE 102 cells suspension ($OD_{680} = 0.6$). The final concentration of the wet dead cells is 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1 mg/ml in 10 ml of NISE 102 cells suspension, respectively. The suspensions were shaken at 100 rpm for 3, 10, and 30 min at 30 °C in a shaker incubator. The aggregates were settled down at the bottom of the flask for 5 min. After that, 1 ml of the supernatant was carefully removed and transferred to a 1 ml cuvette. The absorbance was measured by a UV spectrophotometer at a wavelength of 680 nm. The removal efficiency was calculated as follows:

Removal efficiency (%) $= (1 - A/0.6) \times 100$.

Where A is OD_{680} of samples. 0.6 indicates the initial optical density of NISE 102 cells.

Effect of CaCl$_2$ on the removal efficiency

Five mg/ml of the dead cells (wet cells) was added to NISE 102 cell suspensions ($OD_{680} = 0.6$) containing 50 mM, 20 mM, 5 mM, and 1 mM of $CaCl_2$. The suspensions were shaken at 100 rpm for 3 min at 30 °C in a shaker incubator, and the aggregates were settled down at the bottom of the flask for 5 min. The removal efficiency was calculated by the same method described above.

Effect of FeCl$_3$ on the removal efficiency

One hundred mg/L, 50 mg/L, 25 mg/L, and 10 mg/L of $FeCl_3$ were prepared in NISE 102 cell suspensions adjusted at $OD_{680} = 0.6$ with/without 5 mM $CaCl_2$. The aggregate formation

and the removal efficiency were calculated using the procedure described above. Next, to measure the effectiveness of the combination of the dead cells and $FeCl_3$ on the removal efficiency, 10 mg/L of $FeCl_3$ with 5 mg/ml of the dead cells (wet cells) was prepared in NISE 102 cell suspensions ($OD_{680} = 0.6$) containing 5 mM $CaCl_2$. The aggregate formation and calculation of the removal efficiency were conducted using the same procedure described above

Scanning electron microscope (SEM) observation of aggregates

Fifty mg of the dead cells were added into 10 ml of the suspension of NISE 102 cells ($OD_{680} = 0.6$) with $CaCl_2$ and mixed at 100 rpm for 3 min. In a sample with $FeCl_3$, 10 mg/L of $FeCl_3$ was prepared in the suspension after mixing both cells for 3 min. The suspension was also mixed at 100 rpm for 3 min to make samples for SEM observation. The freeze-drying method was used for preparing the SEM samples. The aggregates were transferred to aluminum plates, and the extra water from the samples was carefully removed. The plates were placed on wet filter paper on a petri dish. For fixing the samples, a few drops of 2% Osmium tetroxide (OsO_4) were dropped on the filter paper, and the petri dish was immediately closed and left in the fume hood for about 1 h. After that, the samples were frozen in liquid nitrogen for a few seconds and dried using a freeze-drying machine (Emitech K750X, Emitech, UK). Finally, the samples were coated with gold and observed by a Leo Supra 50 VP field emission scanning electron microscope (Carl Zeiss, Germany).

Statistical analysis

All results were represented by the mean $\pm$ standard deviation (SD). All data were subjected to one-way analysis of variance (ANOVA) with Tukey tests to examine significant differences between individual mean values. All statistical tests were carried out using SPSS Version 27.0 (IBM). Differences were considered statistically significant at P values <0.05. Independent t-test was used to compare the differences between samples with and without $CaCl_2$ and samples with and without $FeCl_3$. The analysis was also conducted by using SPSS Version 27.0.

RESULTS

Aggregate formation of the dead cells with NISE 102 cells

To test the absorption ability of the dead cells of CCB-QB1 against NISE 102 cells, the dead cells of CCB-QB1 and NISE 102 cells were mixed in 10 mM HEPES buffer (pH 7.6) with and without 7 mM $CaCl_2$. Figure 1A showed that both cells formed small aggregates within 3 min incubation period only in the presence of $CaCl_2$. In contrast, the cell aggregates were not observed in the absence of $CaCl_2$ (Fig. 1A). In light microscopic observation, isolated cells of NISE 102 and QB1 were observed in the absence of $CaCl_2$ (Fig. 1B, top). On the other hand, NISE 102 cells were surrounded and trapped by many CCB-QB1 cells in the presence of $CaCl_2$ (Fig. 1B, bottom), indicating that the dead cells of CCB-QB1 are capable of removing NISE 102 cells.
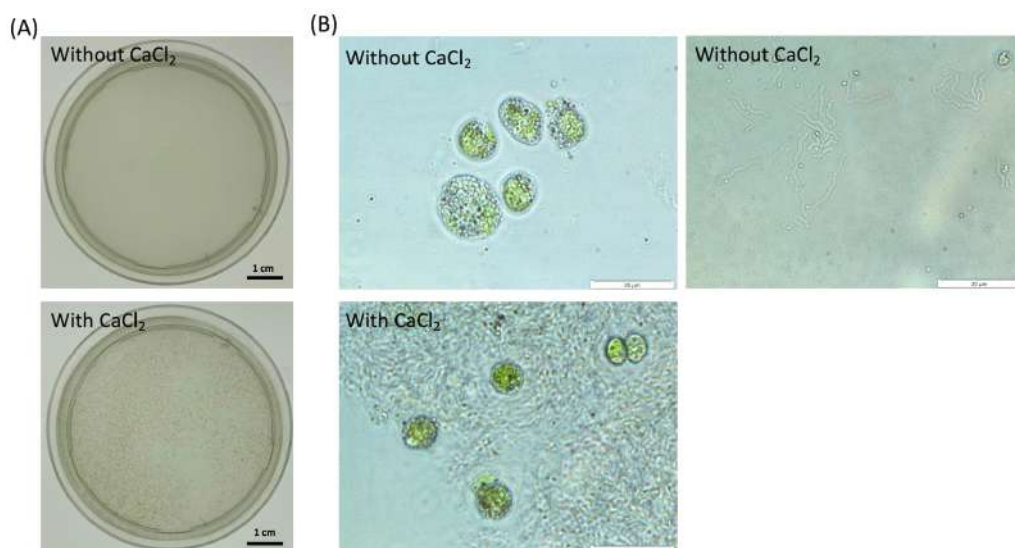
**Figure 1 Observation of cell aggregates consisted of the dead cells and NISE 102 cells in the presence of 7 mM CaCl$_2$.** (A) Aggregation formation by the dead cells with NISE 102 cells. Top is the sample without CaCl$_2$. Bottom is the sample with CaCl$_2$. (B) Light microscopic observation of both the dead cells and NISE 102 cells (Top) and the inside of the aggregate (Bottom) (×1,000). Top is the sample without CaCl$_2$. Bottom is the sample with CaCl$_2$. The scale bar (A) one cm, (B) 20 μm.

Full-size 🖼 DOI: 10.7717/peerj.12867/fig-1

Effect of incubation time and dosage of the dead cells on removal efficiency

Generally, incubation time and initial concentration of flocculant are critical factors for forming flocs and removing the target. Hence, the removal rate of NISE 102 cells was measured using 10.0, 5.0, 2.5, and 1.0 mg/ml of the dead cells for each incubation period (3, 10, and 30 min). For 3 min incubation, the removal efficiency of 10.0 and 5.0 mg/ml of the dead cells was 76.77 and 75.39%, respectively (Fig. 2). The removal rate decreased with decreasing the dosage of the dead cells, such as 71.89 (2.5 mg/ml) and 68.06% (1.0 mg/ml) (Fig. 2). Especially, the removal efficiency of the sample with 1.0 mg/ml was significantly lower than that of the sample with 10.0 and 5.0 mg/ml ($P < 0.05$). As shown in Fig. 2, samples incubated for 10 and 30 min also exhibited similar tendencies. The samples added of 1.0 mg/ml of the dead cell exhibited the lowest removal efficiency (68.06 and 66.83%, respectively) in both samples. In addition, the removal efficiency of the samples with 10.0 and 5.0 mg/ml of the dead cells was similar to among the three different incubation periods. These results indicated that 3 min incubation period was enough to remove NISE 102 cells from the aqueous sample. In addition, the removal efficiency between 10.0 and 5.0 mg/m samples after 3 min incubation period was not significantly different ($P > 0.05$). Therefore, the following experiments were conducted with 5.0 mg/ml of the dead cells for 3 min incubation period.
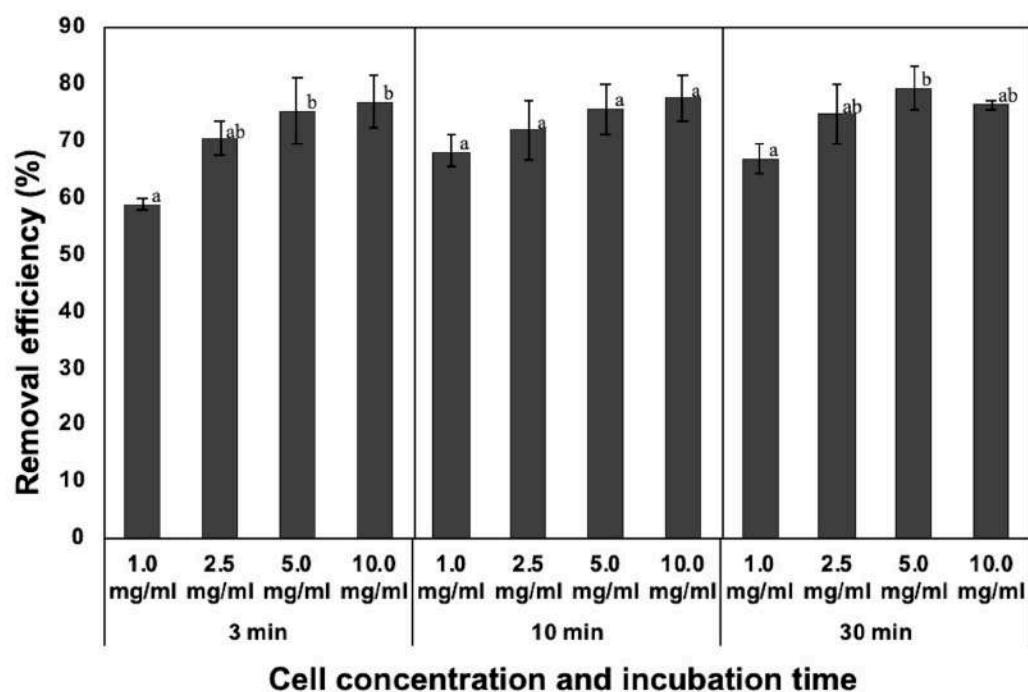
Figure 2 **Effect of incubation time and dosage of the dead cells on removal efficiency of NISE 102 cells.** **1.0, 2.5, 5.0, and 10.0 mg/ml of the dead cells (wet cell) with 7 mM $CaCl_2$ were mixed with NISE 102 cells for 3, 10, and 30 min with shaking (100 rpm).** Different letter indicates a significant difference (One-way ANOVA and Tukey's analysis, $P < 0.05$).

Full-size ⬜ DOI: 10.7717/peerj.12867/fig-2

Effect of $CaCl_2$ on the removal efficiency

To confirm the effect of $CaCl_2$ against the removal of NISE 102 cells, 50, 20, 5, and 1 mM of $CaCl_2$ were added to the suspension of NISE 102 cells. Although we expected that high $CaCl_2$ concentration conditions, 50 mM and 20 mM, stimulated robust removal of the cells compared to 5 mM samples, the removal rate of the three samples was quite similar (75. 11, 76.44, and 75.44%, respectively) ($P > 0.05$) (Fig. 3). On the other hand, the sample added of 1 mM of $CaCl_2$ exhibited significantly lower removal efficiency (30.05%) ($P < 0.05$) than those of samples containing a higher concentration of $CaCl_2$ (Fig. 3). The results showed that the minimum effective concentration of $CaCl_2$ for removing NISE 102 cells was 5 mM, and the concentration was used in the following experiments.

Effect of $CaCl_2$ and $FeCl_3$ on the removal efficiency

First, four different concentrations of $FeCl_3$, 100, 50, 25, and 10 mg/L, were added to the suspension of NISE 102 cells without dead cells of QB1. The removal efficiency of samples with 100 and 50 mg/L of $FeCl_3$ exhibited 100 and around 90% in the absence and presence of $CaCl_2$, respectively (Fig. 4A). On the other hand, as shown in Fig. 4A, the removal efficiency decreased with decreasing the concentration of $FeCl_3$. The samples with 25 and 10 mg/L of $FeCl_3$ removed 61.67 and 40.23% of the cells without $CaCl_2$ (Fig. 4A). Interestingly, the removal efficiency increased with adding the 5 mM $CaCl_2$ in the samples
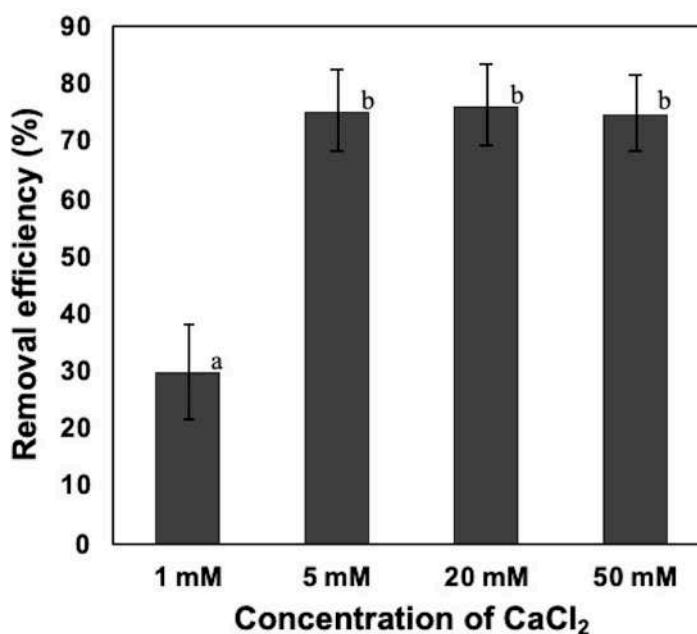
Figure 3 **Effect of calcium ion on the removal efficiency of NISE 102 cells.** 1, 5, 20, 50 mM of $CaCl_2$ were added into NISE 102 cell suspension with 5.0 mg/ml of the dead cell. The suspension was mixed for 3 min. Different letter indicates a significant difference (One-way ANOVA and Tukey's analysis, $P <$ 0.05).

Full-size ◩ DOI: 10.7717/peerj.12867/fig-3

with 25 and 10 mg/L of $FeCl_3$ as 76.44 and 57.61%, respectively (Fig. 4A). Especially, the removal rate with 10 mg/L of $FeCl_3$ with $CaCl_2$ was 1.43 times higher than that without $CaCl_2$ ($P < 0.05$). This result showed that the removal efficiency with 10 mg/L of $FeCl_3$ was improved by adding 5 mM $CaCl_2$.

In Fig. 4B, the removal efficiency of 10 mg/L $FeCl_3$ with the dead cell on NISE 102 cells was tested. The removal efficiency of $FeCl_3$ with the dead cell was 70.28%. Expectedly, the removal efficiency was significantly improved from 70.28% to 84.17% by adding 5 mM $CaCl_2$ ($P < 0.05$) (Fig. 4B).

SEM observation of cell aggregates

The aggregates were observed under SEM to investigate how dead cells formed the aggregates with NISW 102 cells. In the sample without $FeCl_3$, big aggregates were observed (Fig. 5A), and many NISE 102 cells were trapped in the aggregates (Fig. 5B). The dead cells that possessed filamentous structures got entangled in each filamentous cell. The dead cells tightly attached NISE 102 cells, and the entangled cells captured NISE 102 cells (Figs. 5C and 5F). Big aggregates were also observed in the sample with $FeCl_3$ (Fig. 5D). Many NISE 102 cells were also trapped in the aggregates as well as the sample without $FeCl_3$ (Fig. 5E). Compared to the sample without $FeCl_3$, the cells were packed more tightly in the aggregates (Fig. 5F).
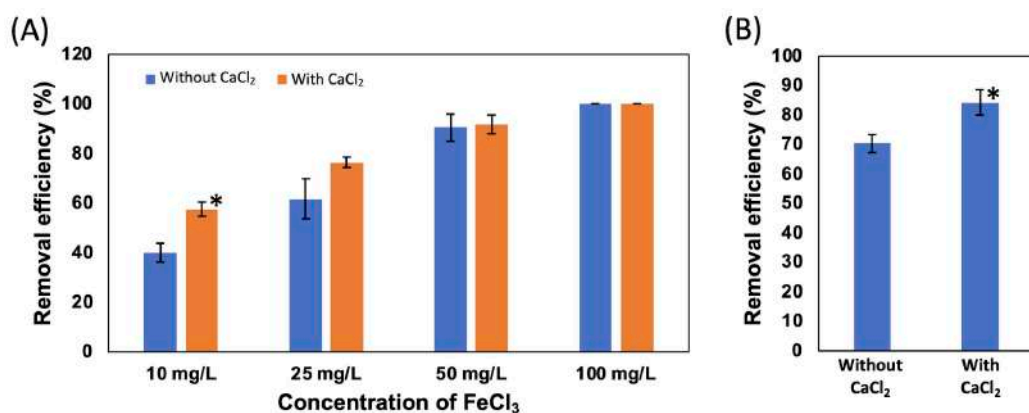
Figure 4 **Effect of CaCl$_2$ and FeCl$_3$ on the removal efficiency of NISE 102 cells.** (A) The removal efficiency of NISE 102 cells on different concentration of FeCl$_3$ (10, 25, 50, 100 mg/ml) without the dead cells. Blue bars indicate samples without CaCl$_2$, Orange bars indicate samples with CaCl$_2$. (B) The removal efficiency of NISE 102 cells with the dead cells in the presence of FeCl$_3$ (10 mg/ml) with and without 5 mM CaCl$_2$. Asterisks in (A) and (B) indicate the significant difference (Independent t-test, $P < 0.05$).

Full-size 🖼 DOI: 10.7717/peerj.12867/fig-4

DISCUSSION

In this study, we tried to apply the dead cells of CCB-QB1 for removing harmful algae, *M. aeruginosa*. Our previous investigations demonstrated that CCB-QB1 cells aggregated in the presence of 7 mM CaCl$_2$ (*Furusawa, Hartzell & Navaratnam, 2015*), and the dead cells of CCB-QB1 formed aggregates with kaolin clay particles in the same condition (*Hasyimah, Furusawa & Amirul, 2021*). In keeping with these data, we expected that the dead cells of CCB-QB1 could also form aggregates and precipitate on *M. aeruginosa* in the presence of CaCl$_2$. To confirm the hypothesis, the dead cells of CCB-QB1 and NISE 102 cells were mixed in the buffer with or without CaCl$_2$. As shown in Fig. 1A, although no cell aggregates were observed in the absence of CaCl$_2$, the dead cells could make aggregates with NISE 102 cells in the presence of CaCl$_2$. In addition, the removal efficiency of NISE 102 cells decreased under low CaCl$_2$ concentration (1 mM) (Fig. 3), indicating that CaCl$_2$ is crucial for removing NISE 102 cells. Several investigators described that the cell surface of *M. aeruginosa* is negatively charged in water (*Hadjoudja, Deluchat & Baudu, 2010*; *Shi et al., 2016*). Generally, the net charge of the bacterial cell surface is also negative (*Corpe, 1970*; *Li et al., 2019*; *Pajerski et al., 2019*). Our previous investigations also indicated that the cell surface of CCB-QB was negatively charged (*Furusawa, Hartzell & Navaratnam, 2015*). However, the dead cells of CCB-QB1 could adsorb negatively charged materials, such as kaolin clay particles, in the presence of CaCl$_2$ because Ca^{2+} might help neutralize the electrical forces (*Hasyimah, Furusawa & Amirul, 2021*). Sato and colleagues described that the addition of EPS produced by cyanobacteria and 1,000 mg/L of calcium (approximately 9 mM) was stimulated the colonization of *M. aeruginosa* cells (*Sato et al., 2017*). The addition of EPS and calcium decreased the zeta potential of *M. aeruginosa* cells and promoted its colonization. However, when only EPS was added, the zeta potential hardly decreased (*Sato et al., 2017*), resulting in inhibiting the colonization. These investigations revealed

that Ca^{2+} plays an important role in neutralizing the charge of *M. aeruginosa* cells. Thus, it is expected that the negative charge of NISE 102 and the dead cells might also be neutralized by Ca^{2+}, and the neutralization may eliminate repulsive electron forces between the dead cells and NISE 102 cells. As a result, Ca^{2+} might facilitate the biosorption of the dead cells against NISE 102 cells.

FeCl$_3$ was used as a flocculant for removing *M. aeruginosa* cells from aqueous samples (*Li et al., 2015*; *Hao et al., 2016*; *Gao et al., 2012*). In this study, we also performed to use FeCl$_3$ as a coagulant. Figure 4A showed that the removal efficiency of NISE 102 cells by a low concentration of FeCl$_3$ was enhanced by adding CaCl$_2$. Figure 5F also showed that the dead cells were more tightly aggregated in the presence of CaCl$_2$ and FeCl$_3$. The absorption of Fe(OH)$_3$ generated from FeCl$_3$ by organic matters could explain this result (*Amuda & Amoo, 2007*). Besides that, Liu et al. reported that Ca^{2+} increased the zeta potential of Fe(OH)$_3$ and facilitated Fe(OH)$_3$ floc aggregation (*Liu et al., 2007*). These investigations indicated that the increase of zeta potential and the promotion of floc aggregation by Fe(OH)$_3$ via the addition of Ca^{2+} enhance the removal of NISE 102 cells. Figure 4A demonstrated that a high removal efficiency (>90%) of NISE 102 cells was observed when the dosage of FeCl$_3$ was more than 50 mg/L. (*Li et al., 2015*) also described that the addition of 50 mg/L of FeCl$_3$ exhibited almost the maximum removal efficiency on *M. aeruginosa* cells. Hao and colleagues also showed that *M. aeruginosa* cells could aggregate when the dosage of FeCl$_3$ was 175 mg/L (*Hao et al., 2016*). Thus, the addition of a high dosage of FeCl$_3$ was required to remove *M. aeruginosa* cells. Figure 4B demonstrated that the addition of only 10 mg/l of FeCl$_3$ significantly improved the removal efficiency. These results also revealed that the combination of the dead cells and CaCl$_2$ could reduce the dosage of FeCl$_3$ for removing *M. aeruginosa* cells.

Sun and colleagues reported that maximum removal efficiency by bioflocculants from *B. amyloliquefaciens* strain DT and *P. aeruginosa* strain ZJU1 was reached within 10 min (*Sun et al., 2015a*; *Sun et al., 2015b*). The bioflocculant from *Klebsiella pneumonia* took 1.6 h for floc formation with *M. aeruginosa* cells (*Xu et al., 2016*). In contrast, in this study, the maximum removal efficiency by the dead cells was reached within 3 min (Fig. 2). This result revealed that the dead cells were able to quickly remove *M. aeruginosa* cells compared to other bioflocculants based on EPS from microorganisms. Another advantage of the usage of the dead cells is that the preparation process of the dead cells is more straightforward than that of other bioflocculants based on EPS from microorganisms. Generally, bioflocculant-producing bacteria were cultured for 2–3 days in a nutrient-rich broth. For instance, several types of saccharides (5.0 g/L sucrose, 2.0 g/L glucose, and 2.0 g/L maltose) were added to the culture broth to produce a bioflocculant from *P. aeruginosa* ZJU1 (*Sun et al., 2015b*). In addition, two volumes of cold ethanol were required to recover the bioflocculant released into the culture supernatant. Finally, the crude bioflocculant was centrifuged to harvest and dried by heat treatment or freeze-drying. In contrast, the CCB-QB1 cells were cultured in a seawater medium without adding any carbon sources. The incubation period to obtain the cells was 24 h (*Hasyimah, Furusawa & Amirul, 2021*). After 10 min UV treatment, the dead cells were able to use as a bioflocculant (*Hasyimah, Furusawa & Amirul, 2021*). Thus, the preparation procedure of the dead cells was quite
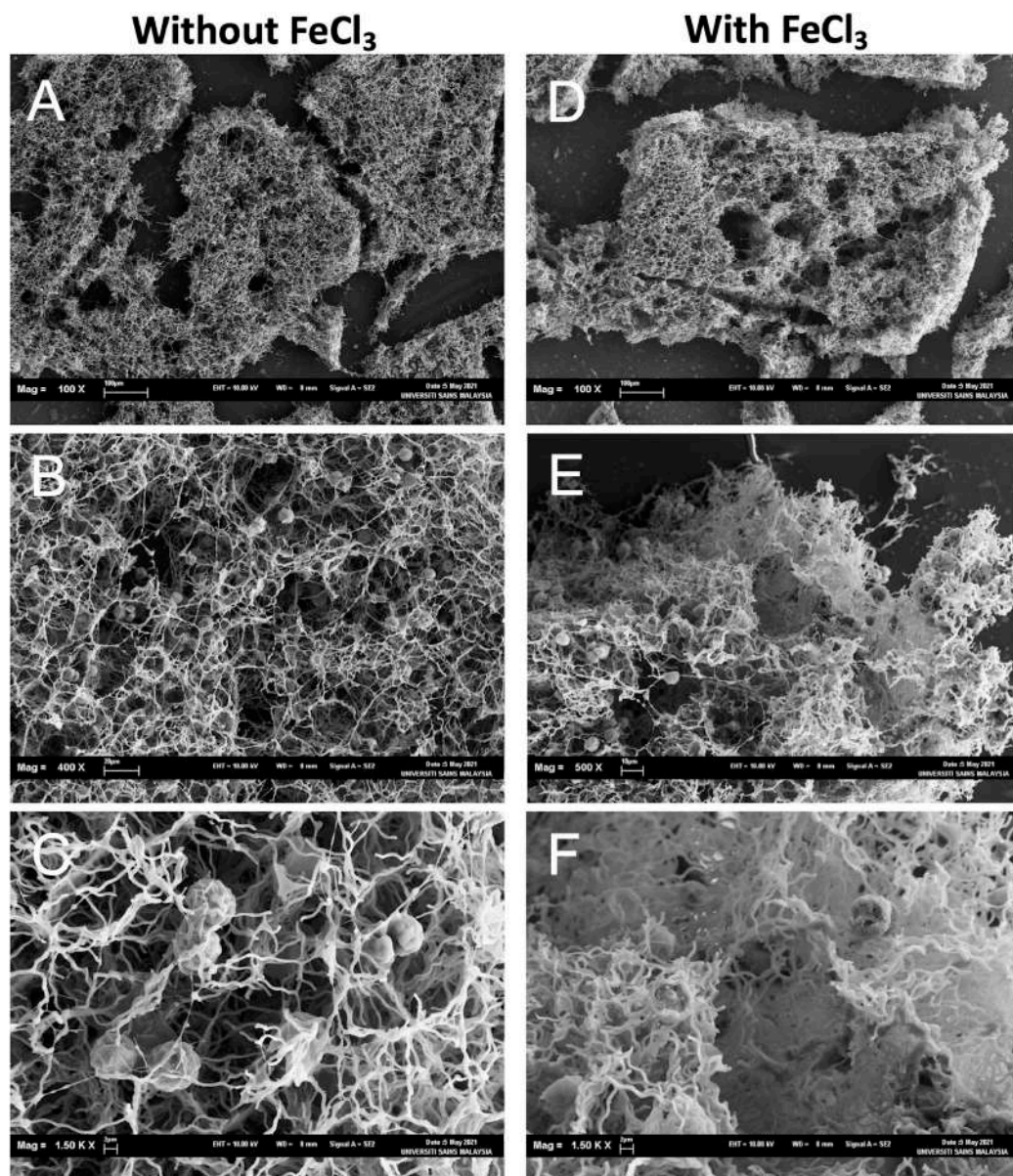
Figure 5   Scanning electron micrographs of the aggregates consisted of the dead cells and NISE 102 cells. (A, B, C): Samples without FeCl$_3$. (D, E, F): Samples with FeCl$_3$. The scale bars A: 100 μm (100×); B: 20 μm, (400×); C: 2 μm (1,500×); D, E: E; 2 μm (4,000×), 100 μm (100×); F: 10 μm (500×); G: 2 μm (1,500×).

Full-size ⊡ DOI: 10.7717/peerj.12867/fig-5

simple in comparison with the other bioflocculants, suggesting that the production cost of the dead cells might be cheaper than that of known bioflocculants consisted of EPS from microorganisms.

It was reported that some plant-based natural coagulants also successfully removed *M. aeruginosa* cells. For example, protein-based coagulant extracted using a salt solution (1 M NaCl or 1 M CaCl$_2$) from *Moringa oleifera* seeds exhibited a high removal rate (78.9%) on

M. aeruginosa cells (*Carvalho et al., 2016*). Moreti and colleagues also reported that a saline extract from *M. oleifera* seeds could remove *M. aeruginosa* cells effectively (*de Oliveira Ruiz Moreti et al., 2019*). Interestingly, the extract could adsorb and remove microcystin-LR (*de Oliveira Ruiz Moreti et al., 2019*). The preparation procedure of the extract using saline solution was more straightforward than that of bioflocculants consisted of EPS. However, many pretreatment processes, such as dehusking, powdering, and drying, were required. Of course, the large agricultural land and long cultivating period to obtain seeds are necessary for culturing *M. oleifera*. In contrast, the biomass production of CCB-QB1 is faster and easier than the plant.

CONCLUSION

In this study, the dead cells of a marine filamentous bacteria, *Aureispira* sp. CCB-QB1, was used as a bioflocculant for removing a harmful freshwater microalga, *M. aeruginosa*. The dead cells were removed about 75% of *M. aeruginosa* cells with 3 min incubation in the presence of $CaCl_2$. The optimization of $CaCl_2$ concentration for removing the *M. aeruginosa* cells showed that the optimal concentration of $CaCl_2$ was 5 mM. The removal efficiency by inorganic flocculant, $FeCl_3$, was improved by the addition of $CaCl_2$. Besides that, the combination of the dead cells and $CaCl_2$ could reduce the dosage of $FeCl_3$ for removing *M. aeruginosa* cells. These results indicated that the dead cells have the potential for a novel and alternative bioflocculant.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Go Furusawa conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Koji Iwamoto conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12867#supplemental-information.

REFERENCES

Amuda OS, Amoo IA. 2007. Coagulation/flocculation process and sludge conditioning in beverage industrial wastewater treatment. *Journal of Hazardous Materials* **141**:778–783 DOI 10.1016/j.jhazmat.2006.07.044.

Azevedo SMFO, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru—Brazil. *Toxicology* **181**:441–446.

Azzam AM, Tawfik A. 2015. Removal of heavy metals using bacterial bio-flocculants of *Bacillus* sp. and *Pseudomonas* sp. *Journal of Environmental Engineering and Landscape Management* **23(4)**:288–294 DOI 10.3846/16486897.2015.1068781.

Briand JF, Jacquet S, Bernard C, Humbert JF. 2003. Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. *Veterinary Research* **34**:361–377 DOI 10.1051/vetres:2003019.

Cai Q, Song K, Tian C, Wu X, Li Y, Huang Y, Wang C, Xiao B. 2021. Harvesting of *Microcystis* from waterbody by flocculation and filtration: the essential role of extracellular organic matters. *Journal of Water Process Engineering* **41**:102053 DOI 10.1016/j.jwpe.2021.102053.

Carvalho MS, Alves BRR, Silva MF, Bergamasco R, Coral LA, Bassetti FJ. 2016. $CaCl_2$ applied to the extraction of *Moringa oleifera* seeds and the ues for *Microcystis aeruginosa* removal. *Chemical Engineering & Technology* **304**:469–475 DOI 10.1016/j.cej.2016.06.101.

Corpe WA. 1970. Attachment of marine bacteria to solid surfaces. In: Manly R, ed. *Adhesion in biological systems.* New York: Academic Press, 73–87.

de Oliveira Ruiz Moreti L, Takaoka AB, Marquettotti Salcedo Vieira A, Vieira S, Mantovani D, Bergamasco R. 2019. The use of *Moringa oleifera* seeds and their fractionated proteins for *Microcystis aeruginosa* and microcystin-LR removal from water. *The Canadian Journal of Chemical Engineering* **97**:1307–1316 DOI 10.1002/cjce.23382.

Dearfield KL, Abernathy CO, Ottley MS, Brantner JH, Hayes PF. 1988. Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity, and carcinogenicity. *Mutation Research—Genetic Toxicology and Environmental Mutagenesis* **195**:45–77.

Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ. 1995. Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **330**:71–99 DOI 10.1016/0027-5107(95)00037-J.

Deng SB, Bai RB, Hu XM, Luo Q. 2003. Characteristics of a bioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. *Applied Microbiology and Biotechnology* **60**:588–593 DOI 10.1007/s00253-002-1159-5.

Fast SA, Kokabian B, Gude VG. 2014. Chitosan enhanced coagulation of algal turbid waters—comparison between rapid mix and ultrasound coagulation methods. *The Chemical Engineering Journal* **244**:403–410 DOI 10.1016/j.cej.2014.01.081.

Flaten TP. 2001. Aluminium as a risk factor in Alzheimer's disease, with emphasis on drinking water. *Brain Research Bulletin* **55**:187–196 DOI 10.1016/S0361-9230(01)00459-2.

Furusawa G, Hartzell PL, Navaratnam V. 2015. Calcium is required for ixotrophy of *Aureispira* sp. CCB-QB1. *Microbiology* **161**:1933–1941 DOI 10.1099/mic.0.000158.

Gao YU, Shimizu K, Xue Q, Feng D, Utsumi M. 2012. Inhibition of growth and microcystins production on *Microcystis aeruginosa* by electrolytic method. *Japanese Journal of Water Treatment Biology* **48**:125–132 DOI 10.2521/jswtb.48.125.

Hadjoudja S, Deluchat V, Baudu M. 2010. Cell surface characterisation of *Microcystis aeruginosa* and *Chlorella vulgaris*. *Journal of Colloid and Interface Science* **342(2)**:293–299 DOI 10.1016/j.jcis.2009.10.078.

Hamilton DP, Salmaso N, Paerl HW. 2016. Mitigating harmful cyanobacterial blooms: strategies for control of nitrogen and phosphorus loads. *Aquatic Ecology* **50**:351–366 DOI 10.1007/s10452-016-9594-z.

Hao J, Lian B, Zhou R, Liu H, Shi L, Liu C. 2016. Microbial flocculant combined ferric trichloride facilitates floating aggregation of *Microcystis aeruginosa* for efficient removal. *Desalination and Water Treatment* **57(43)**:20483–20493 DOI 10.1080/19443994.2015.1108236.

Hasyimah NAR, Furusawa G, Amirul AA. 2021. Biosorption of a dye and heavy metals using dead cells of filamentous bacterium, *Aureispira* sp. CCB-QB 1. *International Journal of Environmental Science and Technology* **18(6)**:1627–1636.

He N, Li Y, Chen J. 2004. Production of a novel polygalacturonic acid bioflocculant REA-11 by *Corynebacterium glutamicum*. *Bioresource Technology* **94**:99–105 DOI 10.1016/j.biortech.2003.11.013.

Katırcıoğlu H, Akin BS, Atici T. 2004. Microalgal toxin (s): characteristics and importance. *African Journal of Biotechnology* **3**:667–674.

Kumar V, Othman N, Asharuddin S. 2017. Applications of natural coagulants to treat wastewater—a review. *MATEC Web of Conferences* **103**:06016 DOI 10.1051/matecconf/201710306016.

Kurenkov VF, Hartan H-G, Lobanov FI. 2002. Application of polyacrylamide flocculants for water treatment. *Chemistry and Computational Simulations. Butlerov Communications* **3**:31–40.

Li O, Lu C, Liu A, Zhu L, Wang PM, Qian CD, Jiang XH, Wu XC. 2013. Optimization and characterization of polysaccharide-based bioflocculant produced by *Paenibacillus elgii* B69 and its application in wastewater treatment. *Bioresource Technology* **134**:87–93 DOI 10.1016/j.biortech.2013.02.013.

Li X, Pei H, Hu W, Meng P, Sun F, Ma G, Xu X, Li Y. 2015. The fate of *Microcystis aeruginosa* cells during the ferric chloride coagulation and flocs storage processes. *Environmental Technology* **36**(7):920–928 DOI 10.1080/09593330.2014.966768.

Li Z, Ma J, Ruan J, Zhuang X. 2019. Using positively charged magnetic nanoparticles to capture bacteria at ultralow concentration. *Nanoscale Research Letters* **14**:195 DOI 10.1186/s11671-018-2843-4.

Liu R, Li X, Xia S, Yang Y, Wu R, Li G. 2007. Calcium-enhanced ferric hydroxide co-precipitation of arsenic in the presence of silicate. *Water Environment Research* **79**:2260–2264 DOI 10.2175/106143007X199324.

Liu W, Hao Y, Jiang J, Zhu A, Zhu J, Dong Z. 2016. Production of a bioflocculant from *Pseudomonas veronii* L918 using the hydrolyzate of peanut hull and its application in the treatment of ash-flushing wastewater generated from coal fired power plant. *Bioresource Technology* **218**:318–325 DOI 10.1016/j.biortech.2016.06.108.

Ma C, Hu W, Pei H, Xu H, Pei R. 2016. Enhancing integrated removal of *Microcystis aeruginosa* and adsorption of microcystins using chitosan-aluminum chloride combined coagulants: effect of chemical dosing orders and coagulation mechanisms. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **490**:258–267 DOI 10.1016/j.colsurfa.2015.11.056.

Pajerski W, Ochonska D, Brzychczy-Wloch M, Indyka P, Jarosz M, Golda-Cepa M, Sojka Z, Kotarba A. 2019. Attachment efficiency of gold nanoparticles by Gram-positive and Gram-negative bacterial strains governed by surface charges. *Journal of Nanoparticle Research* **21**:186 DOI 10.1007/s11051-018-4445-6.

Park YH, Kim S, Kim HS, Park C, Choi Y-E. 2020. Adsorption strategy for removal of harmful cyanobacterial species *Microcystis aeruginosa* using chitosan fiber. *Sustainability* **12**:4587 DOI 10.3390/su12114587.

Pugazhendhi A, Shobana S, Bakonyi P, Nemestóthy N, Xia A, Kumar G. 2019. A review on chemical mechanism of microalgae flocculation via polymers. *Biotechnology Reports* **21**:e00302 DOI 10.1016/j.btre.2018.e00302.

Salehizadeh H, Shojaosadati SA. 2001. Extracellular biopolymeric flocculants: recent trends and biotechnological importance. *Biotechnology Advances* **19**(5):371–385 DOI 10.1016/S0734-9750(01)00071-4.

Sato M, Amano Y, Machida M, Imazeki F. 2017. Colony formation of highly dispersed *Microcystis aeruginosa* by controlling extracellular polysaccharides and calcium ion concentrations in aquatic solution. *Limnology* **18**(1):111–119 DOI 10.1007/s10201-016-0494-7.

Shi W, Tan W, Wang L, Pan G. 2016. Removal of *Microcystis aeruginosa* using cationic starch modified soils. *Water Research* **97**:19–25 DOI 10.1016/j.watres.2015.06.029.

Sun F, Pei H-Y, Hu W-R, Ma C-X. 2012. The lysis of *Microcystis aeruginosa* in AlCl$_3$ coagulation and sedimentation processes. *Chemical Engineering Journal* **193**:196–202.

Sun P, Hui C, Bai N, Yang S, Wan L, Zhang Q, Zhao Y. 2015a. Revealing the characteristics of a novel bioflocculant and its flocculation performance in *Microcystis aeruginosa* removal. *Scientific Reports* **5**(1):17465 DOI 10.9734/JSRR/2015/14076.

Sun P-F, Lin H, Wang G, Lu L-L, Zhao Y-H. 2015b. Preparation of a new-style composite containing a key bioflocculant produced by *Pseudomonas aeruginosa* ZJU1 and its flocculating effect on harmful algal blooms. *Journal of Hazardous Materials* **284**:215–221 DOI 10.1016/j.jhazmat.2014.11.025.

Visser PM, Verspagen JMH, Sandrini G, Stal LJ, Matthijs HCP, Davis TW, Paerl HW, Huisman J. 2016. How rising CO_2 and global warming may stimulate harmful cyanobacterial blooms. *Harmful Algae* **54**:145–159 DOI 10.1016/j.hal.2015.12.006.

Wang SG, Gong WX, Liu XW, Tian L, Yue QY, Gao BY. 2007. Production of a novel bioflocculant by culture of *Klebsiella mobilis* using dairy wastewater. *Biochemical Engineering Journal* **36(2)**:81–86 DOI 10.1016/j.bej.2007.02.003.

Xia S, Zhang Z, Wang X, Yang A, Chen L, Zhao J, Leonard D, Jaffrezic-Renault N. 2008. Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1. *Bioresource Technology* **99(14)**:6520–6527 DOI 10.1016/j.biortech.2007.11.031.

Xu L, Huo M, Yang W, Zhou D, Huo H. 2016. *Klebsiella pneumoniae* metabolites enhance *Microcystis aeruginosa* biomass flocculation. *Chemistry and Ecology* **32(9)**:858–871 DOI 10.1080/02757540.2016.1201080.

Zhang D, Ye Q, Zhang F, Shao X, Fan Y, Zhu X, Li Y, Yao L, Tian Y, Zheng T, Xu H. 2019. Flocculating properties and potential of *Halobacillus* sp. strain H9 for the mitigation of *Microcystis aeruginosa* blooms. *Chemosphere* **218**:138–146 DOI 10.1016/j.chemosphere.2018.11.082.

Zhang Y, Tian J, Nan J, Gao S, Liang H, Wang M, Li G. 2011. Effect of PAC addition on immersed ultrafiltration for the treatment of algal-rich water. *Journal of Hazardous Materials* **186**:1415–1424 DOI 10.1016/j.jhazmat.2010.12.015.

Zheng Y, Ye ZL, Fang XL, Li YH, Cai WM. 2008. Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19. *Bioresource Technology* **99**:7686–7691 DOI 10.1016/j.biortech.2008.01.068.