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**Surface-retained organic matter of *Microcystis aeruginosa*
inhibiting coagulation with polyaluminum chloride
in drinking water treatment**

By

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ABSTRACT

Algogenic organic matter produced by the excess growth of cyanobacteria in semi-closed water areas causes coagulation inhibition in drinking water production. In this study, hydrophilic substances of *Microcystis aeruginosa*, which were mainly composed of lipopolysaccharide (LPS) and RNA, were prepared, and the involvement of these cyanobacterial hydrophilic substances in coagulation inhibition was investigated. As a result, it was found that the negatively charged hydrophilic substances with a molecular weight higher than 10kDa have a significant role in coagulation inhibition. Further fractionation of cyanobacterial hydrophilic substances revealed that surface-retained organic matter (SOM), including LPS, could exhibit a potent inhibitory effect on the coagulation using polyaluminum chloride (PACl), presumably because of the direct interaction of hydrophilic SOM with cations originated from PACl, which could impede the hydrolysis of the coagulant.

Keywords: *Microcystis aeruginosa*, surface-retained organic matter (SOM), coagulation inhibition, polyaluminum chloride (PACl), lipopolysaccharide (LPS)

1. Introduction

Semi-closed water areas such as reservoirs are the main sources of drinking water in urbanized areas. The eutrophication of the semi-closed water areas has been one of the critical issues in drinking water production, because it brings about the excess growth of cyanobacteria, thereby causing various disorders in drinking water treatment processes (Widrig et al., 1996; Hitzfeld et al., 2000; Ma et al., 2002; Westerhoff et al., 2005; Huang et al., 2009). In particular, coagulation efficiency at the destabilization step is fairly susceptible to the presence of algogenic organic matter (AOM) in source water (Bernhardt et al., 1991; Cheng and Chi, 2003; Pivokonsky et al., 2006). The reduction of coagulation efficiency caused by AOM could be temporarily overcome by increasing coagulant dose, which creates subsidiary problems, including an increased cost for the coagulant and sludge treatment. It is important to elucidate the inhibitory mechanism caused by AOM, which could contribute to the establishment of effective countermeasures to coagulation inhibition.

One of the proposed inhibitory mechanisms is that AOM can form complexes with cations originated from coagulant, which seriously deteriorates the coagulation capability (Bernhardt et al., 1987; Bernhardt et al., 1991). Our previous studies showed that proteins from *Microcystis aeruginosa* consume polyaluminum chloride (PACl) in the coagulation process due to the formation of chelate complexes with the coagulant

(Takaara et al., 2005; Takaara et al., 2007). Since cyanobacterial proteins are mainly located inside the cell, it was speculated that these inhibitory proteins released by cell lysis or prechlorination significantly affect coagulation efficiency. However, the presence of these cyanobacterial inhibitory proteins does not fully explain the involvement of AOM in coagulation inhibition, because the reduction of coagulation efficiency without accompanying cell destruction also has been reported (Ma et al., 2007). The presence of inhibitory organic matter located on the outside of cyanobacterial cells would be one of the plausible explanations for the nonproteinaceous substance-induced coagulation inhibition (Hoyer et al., 1985; Bernhardt et al., 1987). The exterior-located organic matter can be categorized into surface-retained organic matter (SOM) and extracellular organic matter (EOM), and EOM has been extensively studied as an inhibitory substance (Liu and Bernhardt, 1991). On the other hand, the involvement of SOM in coagulation inhibition has not been investigated well because it has been technically difficult to analyze the contribution of SOM to coagulation inhibition separately from EOM and intracellular organic matter (IOM). The evaluation of cyanobacterial SOM as an inhibitory substance could be very informative to address the problems of the reduction of coagulation efficiency in water treatment processes.

In this study, we analyzed the inhibitory potential of SOM produced by *M. aeruginosa* against the coagulation with PACl. Firstly, in order to confirm the presence of inhibitory substances on the cell surface of a laboratory strain of *M. aeruginosa*, the

removal rates of *M. aeruginosa* cells and kaolin under various dosage of PACI was evaluated. Then, hydrophilic organic substances, including SOM, were extracted from *M. aeruginosa* cells by a phenol-water extraction, and fractionated by ethanol precipitation, ultrafiltration and ion exchange chromatography. RNase A treatment was also employed to eliminate RNA co-extracted with SOM in the hydrophilic organic substances from *M. aeruginosa* cells. The involvement of the extracted SOM in the coagulation inhibition was evaluated by the coagulation test using a kaolin suspension in the presence of the extracted SOM.

2. Materials and methods

The preparation procedure of SOM samples for the evaluation of inhibitory potential on the coagulation with PACI is summarized in Fig. 1. Details of the sample preparation and the coagulation test are described below.

Cultivation of *M. aeruginosa*

M. aeruginosa NIES-91 strain was provided by the National Institute for Environmental Studies, Japan. *M. aeruginosa* was cultured in a 500 mL conical flask containing 250 mL MA medium at pH 8.6 (Ichimura, 1976) under illumination of a fluorescent lamp (4000 Lux) with a cycle of 12 hrs light and 12 hrs dark at 30 °C. *M.*

aeruginosa was harvested in a steady growth phase when its optical density at 660 nm (OD_{660}) reached 0.90 ± 0.05 . The excess production of a slime layer and floating colony formation of *M. aeruginosa* were not observed.

Coagulation test of *M. aeruginosa* cells

Harvested *M. aeruginosa* cells in a steady growth phase were concentrated by being centrifuged at $1500 \times g$ for 10 min. The pellet was suspended in 50 mL of MA medium, and washed twice by centrifugation ($1500 \times g$, 10 min), decantation and resuspension into MA medium in order to remove EOM components. The cell pellet after the third centrifugation of *M. aeruginosa* was used for the following experiment.

M. aeruginosa cells were suspended in 300 mL autoclaved tap water at OD_{660} of 0.0450 ± 0.0005 (corresponding to OD_{660} of a kaolin suspension at 20 mg/L), and the alkalinity of the suspension was adjusted to 50 mg/L with 100 mg/L of $NaHCO_3$. Then, the pH value of each sample was adjusted to 7.00 ± 0.05 , which is the optimal pH condition for the coagulation with PACl, with 1N HCl or 1N NaOH. The concentration of dissolved organic carbon (DOC) of the filtered sample water (cyanobacterial cells are removed by the filtration) was 5.54 ± 1.51 mg/L, which was measured with TOC-5000 (SHIMADZU, Kyoto, Japan). The DOC constituents in the filtrate is likely to be the residual EOM produced by *M. aeruginosa*, and this concentration of EOM has been proved not to be involved in the coagulation inhibition (Takaara et al., 2007). PACl

(Al₂O₃: 10.0 to 11.0 %) was added at the concentration between 10 and 130 mg/L, and the suspension was mixed at 80 rpm for 2 min (G value = 267 s⁻¹) and 30 rpm for 15 min (G value = 61 s⁻¹). Then, the suspension was left for 30 min. The supernatant of each sample (50 mL) was collected by using a U-shaped pipette in order to avoid the suction of precipitated solids. The OD₆₆₀ of the collected supernatant was measured by UV1600 (SHIMADZU, Kyoto, Japan).

Preparation of hydrophilic substances from *M. aeruginosa*

Cell-associated hydrophilic organic matter was extracted from *M. aeruginosa* by the phenol-water extraction method (Westphal and Jann, 1965) with minor modifications. Firstly, the cell pellet of washed *M. aeruginosa* cells was lyophilized by a vacuum freeze dryer (Yamato, Japan). Lyophilized cells of *M. aeruginosa* were suspended in 4 mL of autoclaved distilled water at 65 °C, and then 4 mL of 90 % phenol (65 °C) were added. After vigorous stirring with vortex, samples were stirred for 15 min at 65 °C and left for 15 min on ice. Cooled samples were centrifuged at 4,000 x g for 30 min at 2 °C. Components of cell-associated hydrophilic substances were fractionated in the aqueous phase. The aqueous phase (4 mL) was then mixed with 4 mL of 90 % phenol at 65 °C. Samples were cooled on ice and centrifuged at 4000 x g for 30 min at 2 °C. The aqueous phase was collected, donated as cyanobacterial hydrophilic compound (CHC), and stored at -80 °C until further analysis.

CHC was further fractionated by the ethanol precipitation. Four milliliters of 99 % ethanol (chilled at -20 °C) were added to 4 mL of CHC and mixed by tumbling. After centrifugation at 15,000 x g for 20 min at 2 °C, the supernatant was decanted. Then, 2 mL of 80 % ethanol chilled at -20 °C was added to the collected pellet and centrifuged at 15,000 x g for 20 min at 2 °C. The supernatant was decanted, and the pellet (hereinafter denoted as CHCpellet) was stored at -80 °C until further analysis.

CHCpellet was further fractionated by ultrafiltration. The ultrafiltration was performed by using a polysulphone membrane with a molecular weight cut off (MWCO) of 10 kDa (KURABO, Japan). Prior to the ultrafiltration, the membrane was washed by 20 mL of double distilled water. CHCpellet was dissolved in 2 mL of double distilled water, and filtered by the centrifugation at 3,000 x g for 30 min. The filtrate from CHCpellet (denoted as CHCpellet-filtrate) was sampled and stored at -80 °C until further analysis.

Anion exchange chromatography was employed to fractionate CHCpellet based on its net surface charge. Five milliliters of anion exchange resin (ORGANO, Japan) were stuffed into the column, and 15 mL of 20 mM Tris-HCl (pH 8.0) were added. CHCpellet dissolved in 3 mL of 20 mM Tris-HCl was applied to the column, and then 2 mL of 20 mM Tris-HCl (pH 8.0) were sequentially added to the column. Six milliliters of the permeate from the column, including positively- and non-charged CHCpellet (CHCpellet-anion exchanged), were collected at the column outlet and

stored at -80°C until further analysis.

CHCpellet was treated with RNase A to eliminate RNA components. CHCpellet dissolved in 2 mL of double distilled water was treated with RNase A of 100 µg/mL (SIGMA, Japan) for 6 hrs at 37 °C. After the RNase A treatment, remaining organic matter was purified by the ethanol precipitation as already described. The degradation of RNA molecules by the RNase A treatment was visibly confirmed by the agarose gel (1.5%) electrophoresis, ethidium bromide staining and UV light excitation. The collected pellet after the ethanol precipitation was donated as CHCpellet-RNase treated, and stored at -80 °C until further analysis.

Coagulation test of kaolin suspension in the presence of cell-associated hydrophilic substances from *M. aeruginosa*

Coagulation test of suspended kaolin using PACl was performed in order to investigate the contribution of CHC, CHCpellet, CHCpellet-filtrate and CHCpellet-anion exchanged to coagulation inhibition. The occurrence of the coagulation inhibition was assessed by the difference in OD₆₆₀ of the supernatant after the sedimentation of coagulated kaolin in the presence or absence of CHC as described previously (Takaara et al., 2007). Kaolin (23000-02, Kanto, Tokyo, Japan) was added to autoclaved tap water at a final concentration of 20 mg/L, and alkalinity was adjusted to 50 mg/L with 100 mg/L of NaHCO₃. Then, the pH value of each sample was adjusted to

7.00 ± 0.05 with 1N HCl or 1N NaOH. This suspension was used as the control in the coagulation test. The test samples were separately prepared by adding each fractionated CHC into the kaolin suspension as shown in Table 1. Then, the quantity of kaolin was adjusted to the final concentration of 20 mg/L, and the pH was adjusted to 7.00 ± 0.05 with 1N HCl or 1N NaOH. Two hundred milliliters of each test sample were poured into a 200-mL glass beaker and agitated with a shaking apparatus at 80 rpm for 1 min. Then, PACl was added to each sample at a concentration of 20 mg/L, and the sample was mixed at 80 rpm for 2 min (G value = 267 s⁻¹) and 30 rpm for 15 min (G value = 61 s⁻¹). Samples were left for 30 min, and then the supernatant of each sample (50 mL) was collected by using a U-shaped pipette in order to avoid the suction of precipitated solids. The OD₆₆₀ of the collected supernatant was measured by UV1600.

A coagulation test of the suspended kaolin in the presence of CHCpellet-RNase treated was separately performed to investigate the contribution of SOM from *M. aeruginosa* to coagulation inhibition. The amount of CHCpellet-RNase fractionated from cyanobacterial culture is far smaller than other fractions such as CHCpellet-filtrate and CHCpellet-anion exchanged. Therefore, it was necessary for us to conduct the coagulation test with CHCpellet-RNase in a smaller scale, and we adjusted the experimental conditions. SOM (CHCpellet-RNase treated) was added to autoclaved tap water, and test solutions at different concentrations of SOM (17 to 516 mg-C/L) were prepared. Kaolin was added to the solution at a final concentration of 20

mg/L, and alkalinity was adjusted to 50 mg/L with 100 mg/L of NaHCO₃. Then, the pH value of each sample was adjusted to 7.00 ± 0.05 with 1N HCl or 1N NaOH. Fifty milliliters of samples were used for the coagulation test. After test samples were agitated thoroughly, PACl was added to each sample at a final concentration of 10 mg/L. Then, the samples were mixed at 100 rpm for 2 min (G value = 162 s⁻¹) and 50 rpm for 15 min (G value = 57 s⁻¹). Samples were left for 10 min, and then the supernatant of each sample (25 mL) was collected. The OD₆₆₀ of collected supernatant was measured by UV1600.

3. Results and discussion

Coagulation efficiency of *M. aeruginosa* cells with PACl

The coagulation test for *M. aeruginosa* cells using PACl was performed to determine the presence of inhibitory substances on the surface of *M. aeruginosa* NIES-91. EOM was washed away, and cells of *M. aeruginosa* suspended in autoclaved tap water at the turbidity corresponding to the kaolin suspension of 20 mg/L were used. The kaolin suspension at 20 mg/L was employed as a control for the coagulation test of *M. aeruginosa* cells. Fig. 2 shows the reduction rate of OD₆₆₀ in the coagulation test of *M. aeruginosa* cells with PACl. Suspended kaolin was well coagulated with 20 mg/L of

PACl (the reduction rate of OD₆₆₀ was 92.7 %), while strong coagulation inhibition was observed when the cell suspension of *M. aeruginosa* was destabilized by 50 mg/L or less concentration of PACl (the reduction rate was less than 5 %). Seventy milligrams per liter of PACl were required to obtain the OD₆₆₀ reduction rate of 93.8 % for the cell suspension of *M. aeruginosa*. The strong inhibition of coagulation with PACl could be attributed to the cell-associated substances of *M. aeruginosa*, because the concentration of DOC was too low (5.54 ± 1.51 mg/L) to cause the coagulation inhibition as proved by our previous study (Takaara et al., 2007). The contribution of cytoplasmic proteins to coagulation inhibition also can be ignored because cell destruction, represented by the change of color and the increase in organic matter in test water, did not occur during the destabilization. Since the flocculation of cyanobacterial cells was not observed at the PACl concentration of less than 50 mg/L, the aluminum speciation that is important for the coagulant to exert the coagulation capability (Zhao et al., 2009) seemed to be hampered by inhibitors possibly located on the surface of cyanobacterial cells. Since the majority of AOM located on the cyanobacterial cell surface could be hydrophilic carbohydrate substances, the involvement of cyanobacterial hydrophilic substances extracted from *M. aeruginosa* cells in the coagulation inhibition is analyzed in the following sections.

Involvement of CHC from *M. aeruginosa* in the coagulation inhibition

Fig. 3 shows the reduction rate of OD₆₆₀ on the coagulation of suspended kaolin in the presence or absence of CHC from *M. aeruginosa*. Suspended kaolin was well coagulated with PACl in the absence of CHC, in which the reduction rate of OD₆₆₀ reached 91.6 %. On the other hand, the reduction rate of OD₆₆₀ was dramatically reduced in the presence of CHC (13.3 %), which means that CHC from *M. aeruginosa* includes potent inhibitory substances for coagulation using PACl. The phenol-water extraction, which was employed to obtain CHC from *M. aeruginosa* in this study, is the method for isolating LPS of gram-negative bacteria, and the extract contains LPS and RNA at a ratio of 1:1 (Westophal and Jann, 1965). Components of the slime layer on the surface of *M. aeruginosa* cells could be also included in the extract because it is unlikely that the cell-associated slime layer was completely washed out during the cell collection step. However, the amount of slime layer constituents would be much less than that of LPS because excess production of the slime layer was not observed in the *M. aeruginosa* cell stock. This is why the low coagulation efficiency in the presence of CHC implies that RNA and/or LPS from *M. aeruginosa* caused coagulation inhibition.

Ethanol precipitation was employed as the first step to fractionate CHC. After ethanol precipitation, the precipitate was stored and denoted as CHC_{pellet}. CHC_{pellet} included 84 (±37) % of CHC on the carbon basis. Coagulation using PACl was considerably inhibited in the presence of CHC_{pellet} (the OD₆₆₀ reduction rate was 12.8 %), which means that the potent inhibitory substances in CHC were fractionated in

CHCpellet.

In order to obtain the information on the molecular weight of the inhibitory substances, CHCpellet was further fractionated by ultrafiltration. CHCpellet-filtrate was the permeate separated from CHCpellet by the ultrafiltration with MWCO of 10 kDa. Suspended kaolin was well coagulated with 20 mg/L PACl in the presence of CHCpellet-filtrate, in which the reduction rate of OD₆₆₀ reached 80.5 %. This coagulation efficiency in the presence of CHCpellet-filtrate was significantly higher than that of CHCpellet (12.8 %). The lower DOC concentration in CHCpellet-filtrate (Table 1) might contribute to the improvement of reduction efficiency of suspended solids. However, the DOC concentration in the test water including CHCpellet-filtrate was high enough to cause coagulation inhibition if coagulation inhibitors are the main components (Takaara et al., 2007). Therefore, it is considered that the coagulation inhibitors of interest are hardly included in the test water, and potent inhibitory substances are unlikely to be in the permeate by the ultrafiltration.

CHCpellet was also fractionated by anion exchange chromatography. CHCpellet-anion exchanged is the permeate from the anion exchange column when CHCpellet was introduced. Theoretically, CHCpellet-anion exchanged contains positively and noncharged substances in CHCpellet. The suspended kaolin was well coagulated with 20 mg/L PACl when CHCpellet-anion exchanged was included (the reduction rate of OD₆₆₀ was 76.1 %). These results lead to the speculation that the

coagulation inhibition is mainly caused by negatively-charged substances in CHCpellet. As a result, it is estimated that negatively charged hydrophilic substances with a molecular weight higher than 10 kDa could have a significant role in coagulation inhibition. Both LPS and RNA, the main components of CHCpellet, coincide with the criteria. In particular, LPS from *M. aeruginosa* could be suspected to be a plausible inhibitor, since LPS is located on the surface of the *M. aeruginosa* cells, and could be involved in coagulation inhibition without cell destruction as shown in Fig. 2. Although nucleotides can interact with aluminum (Kiss et al., 1996; Rubini et al., 2002), which raises the possibility of the interaction of RNA with components of the coagulant, RNA molecules existing in a cellular cytoplasm are difficult to contribute to the coagulation inhibition without the destruction of cyanobacterial cells. The vulnerability of RNA molecules in natural environment (Limsawat and Ohgaki, 1997) also should be taken into account to explain the factors of coagulation inhibition. Based on these discussions, we assayed in the following section the involvement of the CHCpellet-RNase treated, which are mainly composed of SOM, including LPS from *M. aeruginosa*, in the coagulation inhibition.

Involvement of CHCpellet-RNase treated in coagulation inhibition

In order to investigate the contribution of SOM from *M. aeruginosa* to the reduction of coagulation efficiency, the coagulation test using suspended kaolin with

PACl in the presence of CHCpellet-RNase treated was performed. Fig. 4 shows a reduction rate of OD₆₆₀ on the coagulation test in the presence of CHCpellet-RNase treated. The OD₆₆₀ reduction rate of more than 90 % was obtained when the concentration of CHCpellet-RNase treated was lower than 48 mg-carbon/L. However, the reduction of the coagulation efficiency was observed when the concentration of CHCpellet-RNase treated exceeded 55 mg- carbon/L. Then, coagulation with 10 mg/L PACl was completely inhibited (i.e., no flocculation was observed) when the concentration of CHCpellet-RNase treated reached 103 mg-carbon/L. These results indicate that CHCpellet-RNase treated significantly contributed to coagulation inhibition. It is suggested that the hydrolysis of PACl, which is required to exert a coagulation capability (Zhao et al., 2009), was strongly impeded by the presence of CHCpellet-RNase treated, since a strong inhibition against the formation of flocculation was observed.

If the majority of organic matter in CHCpellet-RNase treated consists of LPS, the concentration of CHCpellet-RNase treated that brought about the potent coagulation inhibition (103 mg-carbon/L) corresponds to about 10^{13} cells/L of *M. aeruginosa* according to the literature value of the LPS contents in a gram-negative bacterial cell (Raetz and Whitfield, 2002). This concentration of *M. aeruginosa* can be attained by the excess growth of cyanobacteria in semi-closed water areas (Zohary and Madeila, 1990), which supports the speculation that LPS from *M. aeruginosa* is a potent inhibitory

substance for coagulation. However, further evaluation is required to confirm that LPS from *M. aeruginosa* is the main inhibitory substances in the destabilization step with PACl as coagulant. For example, it should be noted that LPS molecules in CHC were used in a dispersed state in the coagulation test, but these molecules were originally located on the surface of a *M. aeruginosa* cells as components of its outer membrane. The interaction between LPS located on the cell surface of *M. aeruginosa* and coagulants needs to be analyzed in a future study. The components analysis of LPS from *M. aeruginosa* also contributes to the identification of the critical moieties in LPS causing coagulation inhibition. Outputs from these next challenges could create effective countermeasures against the problems of coagulation inhibition in drinking water production.

4. Conclusions

The involvement of cyanobacterial hydrophilic compound (CHC) from *M. aeruginosa* in the coagulation inhibition was investigated. Negatively-charged CHC with a molecular weight higher than 10 kDa would have a significant role in coagulation inhibition. Lipopolysaccharide (LPS) and/or RNA from *M. aeruginosa* were suspected to be strong inhibitors for the coagulation. CHC treated by RNase A also exhibited strong inhibition in the coagulation using PACl, which leads to the speculation that LPS could have an important role in coagulation inhibition. The concentration of

CHC treated by RNase A that causes the coagulation inhibition was 103 mg-carbon/L. This concentration of CHC corresponds to 10^{13} *M. aeruginosa* cells/L, which can be observed when the excess growth of *M. aeruginosa* in freshwater occurs. The components analysis of LPS could provide important information on the mechanisms of coagulation inhibition caused by *M. aeruginosa*

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Fig. 1. Flow chart of the experiments in this study, including the preparation of cyanobacterial hydrophilic substances and the coagulation test using polyaluminum chloride.

Fig. 2. Coagulation test of *M. aeruginosa* cells using polyaluminum chloride. Kaolin suspension was used as a control. Error bars indicate standard deviations in triplicate.

Fig. 3. Reduction rate of optical density at 660 nm in the coagulation test of kaolin

suspension using polyaluminum chloride in the presence of cyanobacterial hydrophilic substances. Kaolin suspension was used as a control. Error bars indicate standard deviations in triplicate. Sample codes correspond to those in Table 1.

Fig. 4. Coagulation test of suspended kaolin using 10 mg/L polyaluminum chloride in the presence of RNase treated-cyanobacterial hydrophilic substances. Error bars indicate standard deviations in triplicate.

Table 1. Composition of samples used in the coagulation test with polyaluminum chloride

Sample code	Description	Volume (mL)		DOC±SD (mg/L)
		Cyanobacterial hydrophilic substances	Autoclaved tap water	
CHC	Extracts by phenol/water method	6	294	338±60
CHCpellet	Precipitation by ethanol precipitation	1	299	283±22
CHCpellet-filtrate	Permeate by ultrafiltration (MWCO is 10kDa)	1	299	63±22
CHCpellet-anion exchanged	Permeate from anion exchange chromatography	6	294	166±44

Fig. 1

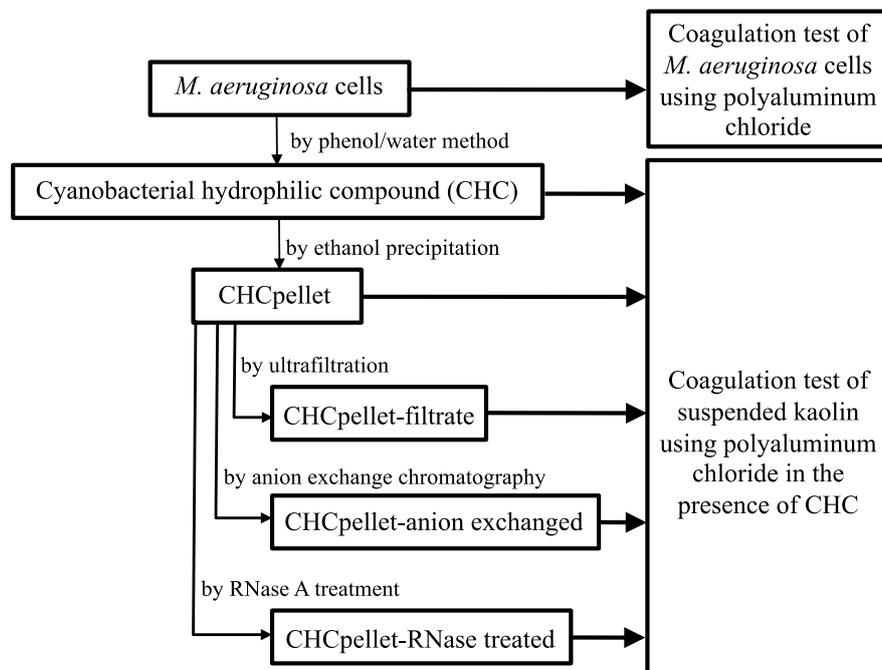


Fig. 2

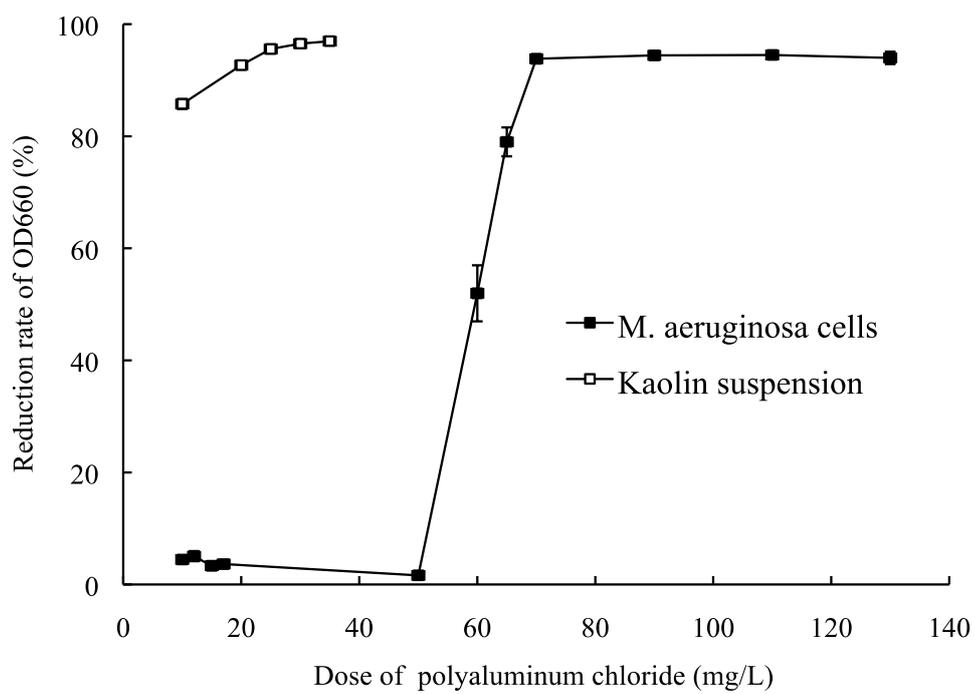


Fig. 3

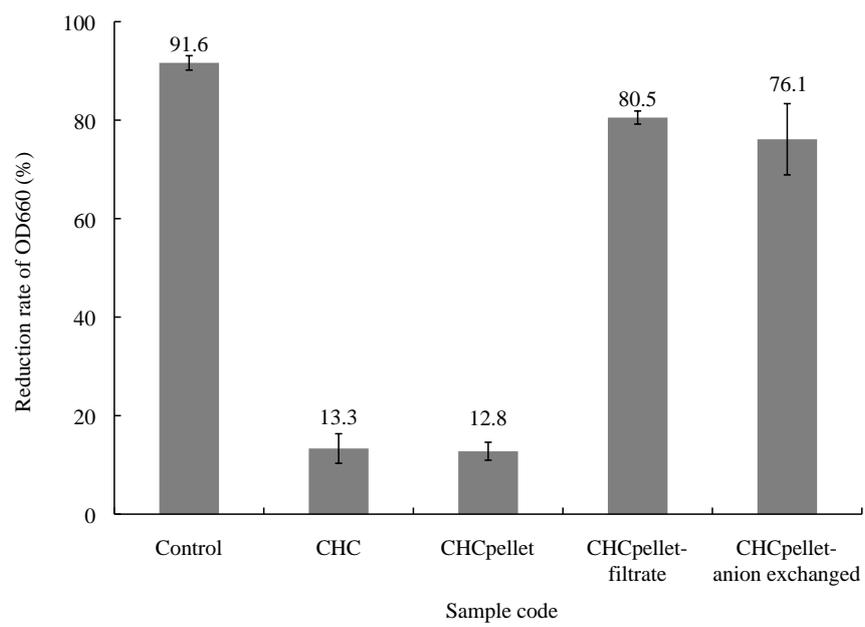


Fig. 4

