Research article

The mechanism of kaolin clay flocculation by a cation-independent bioflocculant produced by *Chryseobacterium daeguense* W6

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Abstract: In recent years, several novel cation-independent bioflocculants have been reported, which can avoid the secondary contamination caused by addition of cations. However, compared with cation-dependent bioflocculants, the flocculating mechanism of cation-independent bioflocculants is largely unknown. In this study, a cation-independent bioflocculant MBF-W6 produced by *Chryseobacterium daeguense* W6 was used as a model to investigate the flocculating mechanism. The results showed that the major flocculating component of MBF-W6 is a complex of proteins and polysaccharides. The zeta potential results indicated that kaolin clay particles were not precipitated due to charge neutralization and the bridging mediated by cations did not play a major role in the flocculating process. These results are consistent with the fact that MBF-W6 is a cation-independent bioflocculant. Further scanning electron microscopic observation showed that MBF-W6 induced flocs formed tight packed structure, suggesting that the kaolin clay particles maybe directly attached and bridged by bioflocculant MBF-W6. In addition, we also found out that Fe^{3+} ions inhibit the flocculating activity of MBF-W6 by affecting $-COO^-$ and $-NH$ groups. Therefore this study can improve our understanding on flocculating mechanism of cation-independent bioflocculants.

Keywords: bioflocculant; cation-independent; flocculating mechanism; polysaccharide; *Chryseobacterium daeguense*

1. Introduction

Flocculants are widely used in various industrial processes, such as wastewater treatment, drinking water purification and downstream fermentation processes [1,2,3]. Chemically synthetic flocculants are predominantly used because of their effectiveness and low cost, but most of them are harmful to human health, such as acrylamide monomer from one of the most popular synthetic flocculants poly-acrylamide that is categorized as both neurotoxin and highly carcinogenic to humans [4,5,6]. Bioflocculants are mainly composed of extracellular polymeric substances, such as glycoprotein, polysaccharide, protein and nucleic acid produced by microorganisms during their growth [7-10]. Compared with chemically synthetic flocculants, bioflocculants are not toxic, harmless and biodegradable [11,12,13]. However, major bottlenecks for the commercialization of bioflocculants include the higher production cost and uncertain flocculating mechanism. The study on flocculating mechanism could help us to elucidate the role of bioflocculant in process applications and hence improve the overall treatment efficiency [6]. In recent years, many extracellular bioflocculant have been reported, and it has also been reported that certain cations such as Ca^{2+} can improve their flocculation [3,14,15,16]. Generally, bridging mediated by cations and charge neutralization are the two main mechanisms for these cation-dependent bioflocculants [17,18]. For example, the treatment of kaolin suspension by a bioflocculant secreted by *Bacillus mucilaginosus* GY03 served as a model for studying flocculating mechanism characterized by bridging mediated by cations and charge neutralization [17]. Bioflocculant HBF-3 produced by *Halomonas* sp. V3a' are mainly composed of extracellular polysaccharides. Flocculation of kaolin suspension with bioflocculant HBF-3 served as a model to investigate the flocculating mechanism in which bridging mediated by Ca^{2+} was proposed [6]. However, the addition of cations can cause the secondary contamination. In recent years, several novel cation-independent bioflocculants have been reported. For instance, *Bacillus* sp. F19 produces a cation-independent bioflocculant, whose flocculating activity is inhibited by the presence of Fe^{3+} ions [3]. The bioflocculants produced by *Klebsiella pneumoniae* and *Aspergillus flavus* show a good flocculating activity in kaolin clay suspension without cation addition [19,20]. However, the flocculating mechanism of these cation-independent bioflocculants is largely unexplained.

Our previous study discovered a bioflocculant producing strain *Chryseobacterium daeguense* W6 which produces a cation-independent bioflocculant MBF-W6 and Fe^{3+} ions inhibit its flocculating activity [21]. Therefore in this study, the MBF-W6 was used as a model to elucidate the flocculating mechanism of cation-independent bioflocculants. Specifically, we found that (i) the major flocculating component of MBF-W6 is a complex mixture of proteins and polysaccharides; (ii) the charge neutralization and bridging mediated by cations are not the underlying flocculating mechanism of MBF-W6, which is consistent with the fact that the flocculant of MBF-W6 is cation-independent; (iii) kaolin clay particles may get attached and bridged by MBF-W6 directly and form a tight packed structure; (iv) Fe^{3+} ions can inhibit the flocculating activity of MBF-W6 by influencing $-COO^-$ and $-NH$ groups of these polymers. Therefore this study can improve our understanding to flocculating mechanism of cation-independent bioflocculants.

2. Materials and Method

2.1. MBF-W6 production

Chryseobacterium daeguense W6 was cultured in 500 mL flasks containing 100 mL fermentation medium with 180 rpm agitation at 30 °C. The composition of the fermentation medium were as follows: glucose 1 g/L, Tryptone 2 g/L, $Mg(NO_3)_2$ 0.2 g/L at pH 6.0. The fermentation broth corresponding to 72 h incubation was used to extract the bioflocculant MBF-W6. The fermentation broth was centrifuged at 8000 rpm at 4 °C for 20 min to remove residual cells, and then the supernatant was collected, dialysed at 4 °C for 24 h in distilled water, and lyophilized to obtain the purified bioflocculant MBF-W6.

2.2. Flocculating activity assay

The flocculating activity of bioflocculant MBF-W6 at different conditions was monitored by calculating the flocculating activity as previously described with slight modification [21]. Briefly, 4 g/L kaolin clay suspension (pH 7.0) was used as the solid phase to which the storage bioflocculant solution (1 g/L) was added, and stirred for 2 min. After settling for 1 min, the absorbance (OD) of the sample was recorded by using a spectrophotometer (Unic-7200) at 550 nm. A control, without the addition of any agent, was measured in the same manner. The flocculating activity was calculated according to the following equation: flocculating activity = $(A_0 − A_1)/A_0 × 100\%$, where A_1 is the absorbance of the supernatant sample at 550 nm and A_0 is the absorbance of the control at 550 nm.

2.3. Properties of MBF-W6

Thermal stability of MBF-W6 was analyzed as follows: the purified MBF-W6 was dissolved in water to a concentration of 1 g/L and heated at 100 °C for 0, 10, 20, 30 and 60 min in the boiling water bath. After cooling the MBF-W6 solution to the room temperature (24 °C), different volume of bioflocculant solution was added into kaolin clay suspension, and the flocculating activity was determined.

To test whether the charge neutralization occurs during the flocculation, the zeta potentials of the flocculating systems with different concentration of MBF-W6 were recorded. MBF-W6 solution was added into 4 g/L kaolin clay suspension. The zeta potentials of kaolin suspension containing different concentrations of MBF-W6 were measured respectively using a Zeta Potential Sizer (Zetasizer Nano ZS England). To analyze the effect of Ca^{2+} on flocculating activity, the flocculating activity of MBF-W6 was compared with that of MBF-W6 plus $CaCl_2$ at different concentrations.

2.4. Bonding type assay and scanning electron microscopic (SEM) observation

The bioflocculant solution was introduced into the kaolin clay suspension to reach a concentration of 1.2 mg/L and stirred for 2 min. After settling for 1 min, the supernatant was removed and the flocs were collected for bonding type assay. Three kinds of chemical treatments were performed to test the bonding type in MBF-W6 induced flocs. The flocs were treated by 2 mol/L Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) (pH 7.8), 2 mol/L HCl and 5.0 mol/L urea, respectively. The individual mixture was gently stirred, and then the flocculating activity was

determined. One drop of kaolin suspension or MBF-W6-induced flocs was added onto a slide and it was fixed by air drying. The fixed specimen was observed with a Scanning Electron Microscope (SEM) (Hitachi S-570, Japan).

2.5. Mechanism of inactivation of bioflocculant by Fe^{3+} ions

1 mL 10 g/L $FeCl_3$ was mixed to 9 mL 1 g/L purified MBF-W6 solution, and incubated at 4 °C for 1 h. The mixture was lyophilized directly or lyophilized after dialyzing at 4 °C for 12 h in distilled water (change fresh distilled water every 2 h) to remove Fe^{3+}. The flocculating activity of MBF-W6 before and after removing Fe^{3+} was determined. The functional groups in the MBF-W6 before and after removing Fe^{3+} were compared using the Fourier Transform Infrared (FTIR) spectrophotometer (Bio-Rad FTIR Model FTS135). The spectrum of the sample was recorded on the spectrophotometer over a wave-number range of 650–4000 cm^{-1} under ambient conditions.

3. Results and Discussion

3.1. The identification of flocculating components of MBF-W6

Our previous report showed that the main components of the purified MBF-W6 are total proteins (32.4%), total sugar (13.1%) and nucleic acid (6.8%) [21]. In order to analyze the major flocculating components of MBF-W6, purified MBF-W6 was heated at 100 °C for different time, and the flocculating activity was determined. As shown in Figure 1, the flocculating activity of MBF-W6 decreased from 90% to 30% in the first 30 min, after which, the treated MBF-W6 maintained 30% flocculating activity, suggesting that both the proteins and polysaccharides are the major flocculating components. Previous study has reported that the bioflocculant produced by *Klebsiella pneumoniae* is mainly composed of extracellular polysaccharides that exhibited significant resistance to heat [22]. Bioflocculant produced by *Klebsiella mobilis* is a polysaccharide as no amino acids were detected [16]. Bioflocculant from *Proteus mirabilis* TJ-1 is a complex mixture of polysaccharides and proteins [15]. *Nocardia amarae* and *Rhodococcus erythropolis* secrete an extracellular protein bioflocculant [23,24]. Here we found out that the MBF-W6 is a complex mixture of polysaccharides and proteins, with the proteins showing more flocculating activity than the polysaccharides.
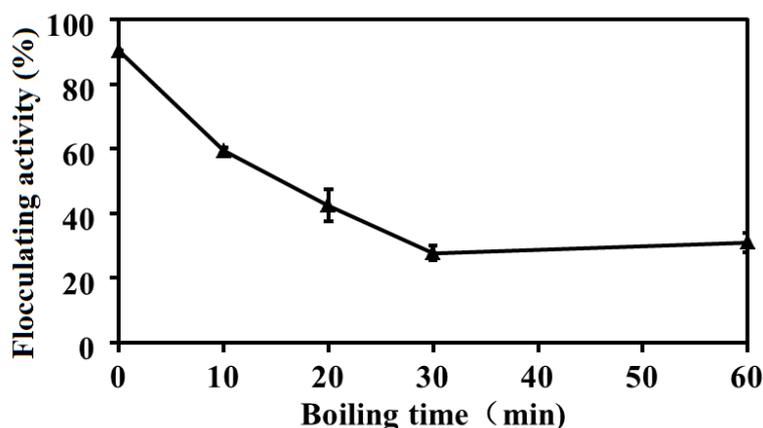

Figure 1. Effects of boiling time on the flocculating activity of MBF-W6.

3.2. The flocculating activity of MBF-W6 is cation-independent

The Ca^{2+} is regarded as a promoting factor for most extracellular polysaccharide bioflocculant. Bridging mediated by Ca^{2+} ions was considered to be one of the main flocculating mechanisms [6]. However, the addition of Ca^{2+} ions can cause secondary pollution and the cost increases. In this study, the flocculating activity of MBF-W6 was compared in the presence and absence of $CaCl_2$. As shown in Figure 2A, the addition of Ca^{2+} showed no positive effects on the flocculating activity of MBF-W6, on the contrary, the flocculating activity of MBF-W6 slightly decreased when the MBF-W6 concentration was lower than 1.0 mg/L. No significant effect was observed when the Ca^{2+} concentration was increased from 0 to 600 mg/L (MBF-W6 concentration is 1.2 mg/L) (Figure 2B). Our previous study also showed that other ions such as K^+, Na^+, Ba^{2+} Mg^{2+}, Al^{3+} cannot improve the flocculating activity, and certain cations such as Fe^{3+} can distinctly decrease the flocculating activity of MBF-W6 [21]. These results suggested that the flocculating activity is highly cation-independent, which avoids cost increase and secondary pollution. Several cation-independent bioflocculants have been reported in recent years. For example, the bioflocculant produced by *Bacillus* sp. F19 can achieve a flocculating activity without the addition of any cations [3]. Although the cations bound groups, such as carboxyl groups, present in these cation-independent bioflocculants, the flocculating activity is not enhanced by cations. This phenomenon could be explained by the carboxyl groups buried deep within protein structure, reducing the interaction between the carboxyl group and cations. In this case, the bridging mediated by cation is not the major flocculating mechanism, and some other novel flocculating mechanism might be driving these bioflocculants.

3.3. Charge neutralization assay

The charge neutralization is regarded as one of the underlying flocculating mechanisms for certain cation-dependent bioflocculants. For example, the charge neutralization plays an important role during the kaolin clay flocculation by a Ca^{2+}-dependent bioflocculant HBF-3, which is produced by a deep-sea bacterium mutant *Halomonas* sp. V3a' [6]. To test whether the charge neutralization occurs during the MBF-W6 induced flocculation, the zeta potentials and the flocculating activity of the flocculating systems with different concentrations of MBF-W6 were determined. As shown in Table 1. The flocculating activity over 90% was achieved for systems within the range of 0.6 to 11.6 mg/L MBF-W6 and the zeta potentials remained negative and decreased slightly with the increase of MBF-W6 concentration. These findings suggest that static repulsive forces are present among the particles. Kaolin clay particles bear a negative charge on the surface that enables it to be suspended well in solution due to the formation of electrical double layer (the Zeta potential of kaolin clay particles in the solution was − 14.8 mV). Polysaccharides and proteins contain the negative charged groups, such as carboxyl groups. The zata potential of bioflocculant solution (10 mg/L, pH 7) is − 33.8 ± 1.5 mV. The addition of MBF-W6 further decreased the Zeta potentials of the flocculant system. When the MBF-W6 dosage was enhanced from 1.2 mg/L to 11.6 mg/L, the zeta potential decreased from − 20.4 mV to − 25.2 mV, indicating that the increase of repulsive force between particles is an adverse factor for the flocculant of kaolin clay particles. As expected, the flocculating activity decreased from 96.27% to 92.14%. Therefore, the charge neutralization is not the principal mechanism behind the flocculating process of the negatively charged kaolin particles. This result is consistent with the cation independent theory as supplementation with positive charged cation is

required to neutralize the negatively charged bioflocculant and kaolin clay.
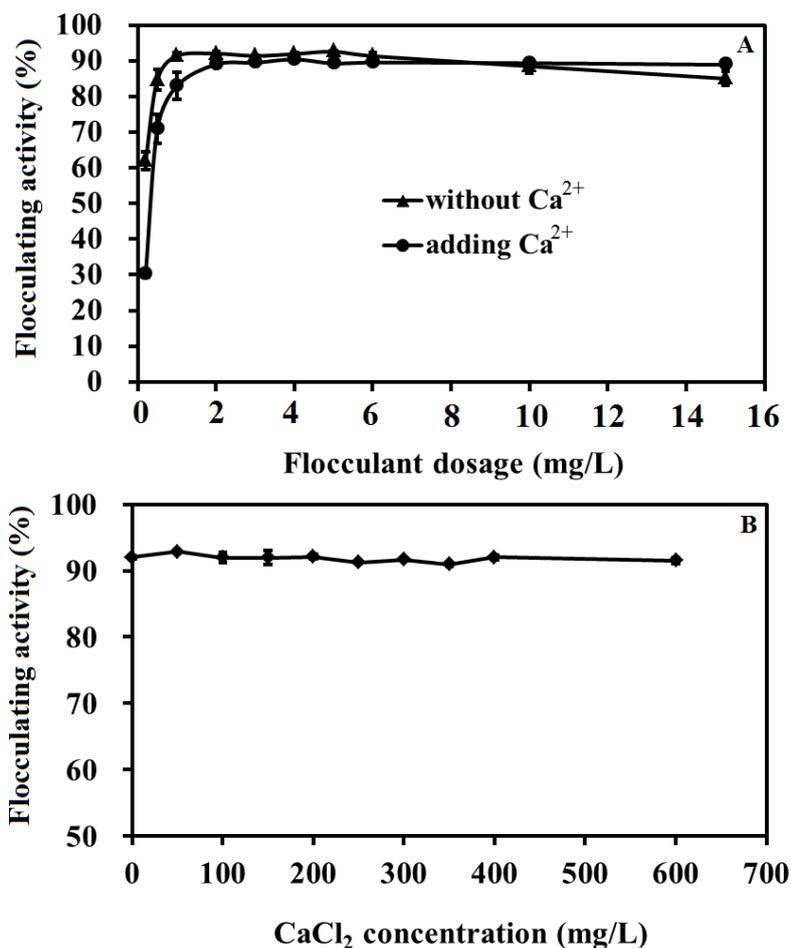

Figure 2. Effects of Ca^{2+} on the flocculating activity of MBF-W6. (A) The flocculant system with Ca^{2+} was compared with that without Ca^{2+} at different concentration of MBF-W6; (B) The flocculating activity of MBF-W6 was compared with that of MBF-W6 plus different concentrations of CaCl$_2$.

Table 1. Zeta potentials and flocculating activity of kaolin suspension with MBF-W6.

Bioflocculant dosage (mg/L)	Zeta potential (mV)	Flocculating activity (%)
0	− 14.8 ± 1.2	
0.6	− 21.9 ± 1.7	94.99 ± 0.13
1.2	− 20.4 ± 0.9	96.27 ± 1.22
2.3	− 22.3 ± 1.1	96.47 ± 0.35
4.1	− 22.1 ± 1.4	96.53 ± 0.87
5.8	− 22.6 ± 1.0	95.09 ± 0.98
11.6	− 25.2 ± 1.3	92.14 ± 1.84

3.4. Kaolin clay particles maybe attached and bridged by MBF-W6 directly

The bonding types in MBF-W6-induced flocs were tested by EDTA-2Na, HCl and urea treatment. Urea is known to disrupt hydrogen bonds, while EDTA-2Na and HCl destroy the ionic bonds [6,25]. It can be seen from Figure 3 that MBF-W6-induced flocs were not disintegrated in 5 mol/L urea, suggesting that hydrogen bonds does not exist predominantly in MBF-W6 induced flocs. When the MBF-W6-induced flocs were treated with 2 mol/L HCl, the flocculating activity decreased slightly from 92.43% to 75.09%, also indicating that the bonding in MBF-W6 induced flocs is not of ionic nature as well. This result is consistent with the fact that the flocculation of MBF-W6 is cation-independent. In previous studies, the bridging mediated by some ions has been reported as a major mechanism for cation-dependent bioflocculants [6,17,26]. In addition, we found out that MBF-W6 induced flocs were sensitive to 2 mol/L EDTA-2Na. This can be explained by the fact that high concentration of EDTA-2Na affect the stability of the protein components or disrupt the interaction between the proteins and polysaccharides. This confirmed that proteins were one of the major flocculating components in MBF-W6.
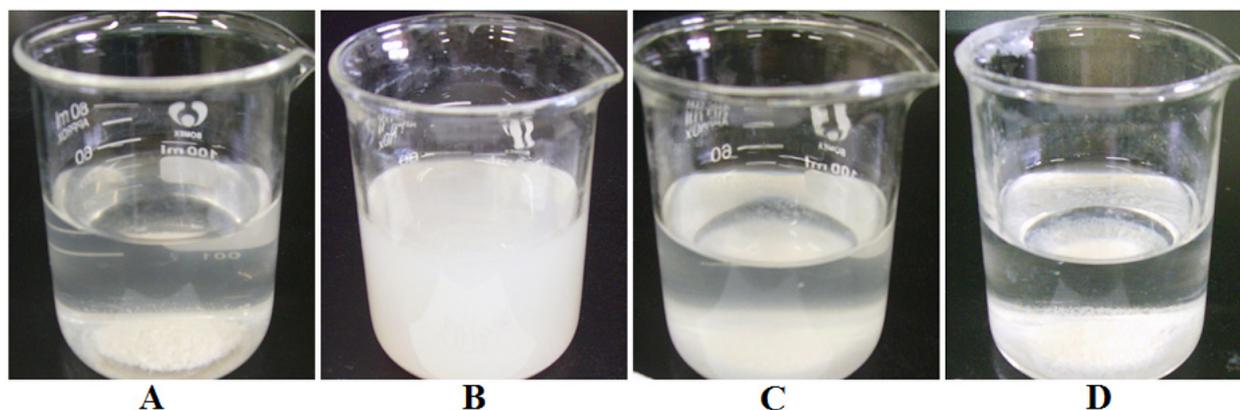

Figure 3. Bonding type analysis of MBF-W6. (A) MBF-W6-induced flocs used as control; (B) MBF-W6 induced flocs were treated by EDTA-2Na; (C) MBF-W6 induced flocs were treated by HCl; (D) MBF-W6 induced flocs were treated by urea.

In previous studies, some proteins or polysaccharides with high molecular weight have been reported as adhesins, which can promote the cells initial attachment on the solid surface to form biofilms [27,28]. So we speculated that the flocculation of cation-independent bioflocculants may be achieved by adhesion mechanism, in which the proteins or polysaccharides can attach on the surface directly and bridge the kaolin clay particles, and thus promoting their flocculation. SEM observation was performed to aid in interpreting the mechanism of kaolin suspension flocculation. Figure 4a shows the loose structure of kaolin clay before the addition of the bioflocculant MBF-W6. Compared with the kaolin clay without MBF-W6 treatment, the kaolin clay particles were bridged by the MBF-W6 directly and form tight packed structure (Figure 4b). This result confirmed our hypothesis that kaolin clay particles are attached and bridged by MBF-W6 directly.
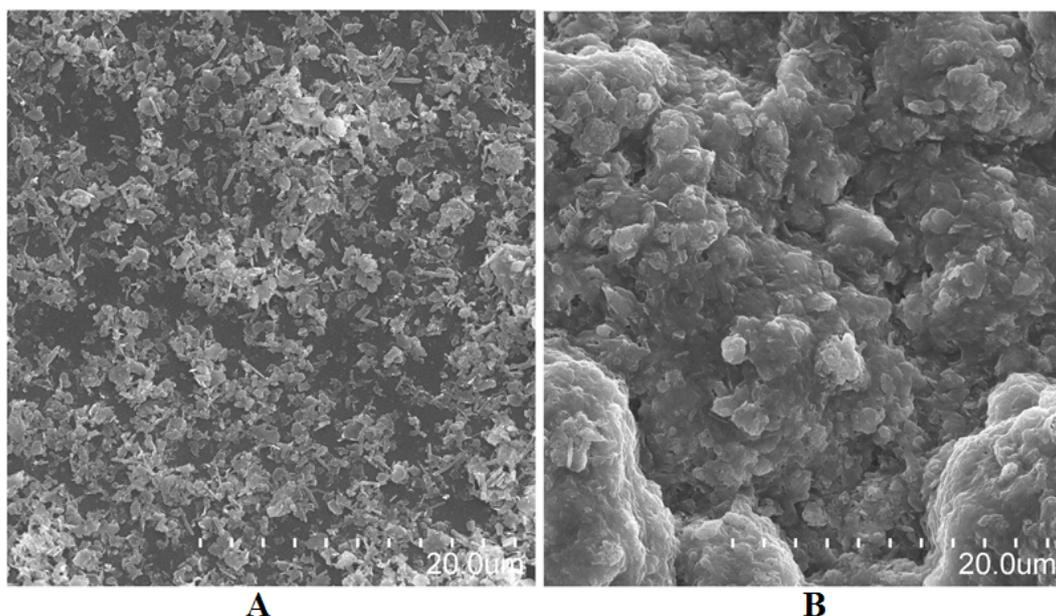
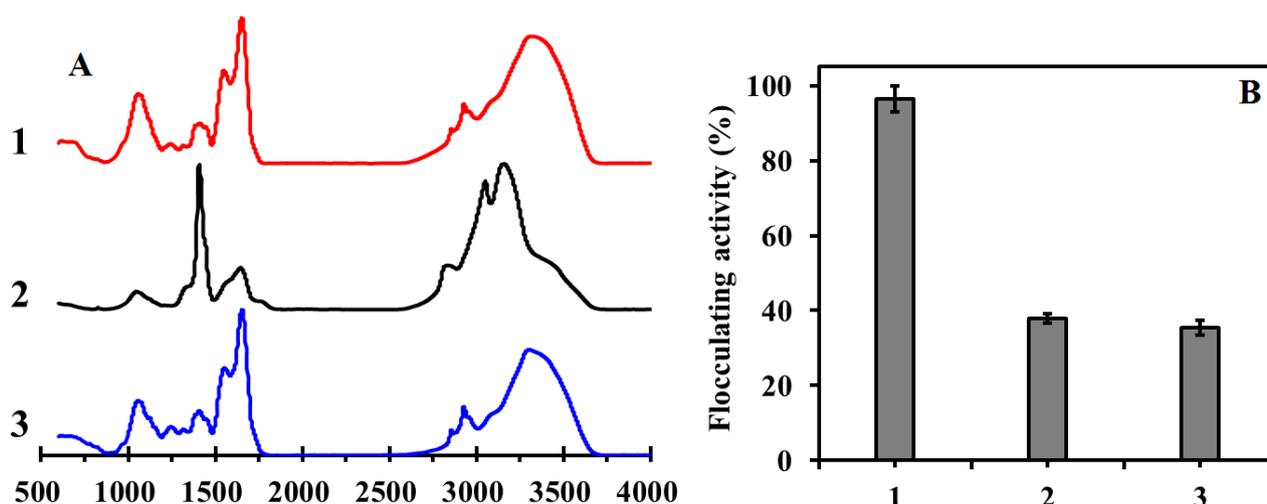**Figure 4. SEM images of kaolin clay (A) and MBF-W6-induced kaolin flocs (B).**

Figure 5. Fourier Transform Infrared spectrum (A) and flocculating activity (B) of different treated MBF-W6. 1, untreated MBF-W6; 2, MBF-W6 treated by Fe^{3+}; 3, MBF-W6 removed Fe^{3+} by dialysis.

3.5. Mechanism of inactivation of bioflocculant by Fe^{3+} ions

Our previous study showed that the Fe^{3+} ions can significantly inhibit flocculating activity of MBF-W6 [21]. Similar results were reported for other bioflocculants [3,24]. But the mechanism of inactivation of bioflocculant by Fe^{3+} was unexplained. The flocculating activities and FTIR spectrophotometer readings of MBF-W6 after addition and removal of Fe^{3+} by dialysis were compared. It can be seen from Figure 5, the readings showed that the absorbance of MBF-W6 treated by Fe^{3+} changed at 1660 and 3360 cm^{-1} in comparison to that of untreated MBF-W6, suggesting that

the –COO⁻ and –NH groups were influenced by the presence of Fe^{3+}. The –COO⁻ and –NH groups have been reported as major functional groups during the flocculating process [9,21,29-33]. The flocculating activity of MBF-W6 was reduced to 37.9% on exposure to Fe^{3+} and was further decreased to 35.4% after Fe^{3+} removal by dialysis, although the FTIR spectrophotometer reading of MBF-W6 after removal of Fe^{3+} ions showed similar absorption peaks as that of untreated MBF-W6, indicating that the effects of Fe^{3+} was irreversible, and maybe achieved by influencing the stability of proteins and polysaccharide.

4. Conclusion

This study demonstrated that cation-independent bioflocculant MBF-W6 produced by *Chryseobacterium daeguense* W6 showed a different flocculating behavior from most cation-dependent extracellular bioflocculant. The major flocculating component is a complex of proteins and polysaccharides. The negatively charged kaolin particles are not precipitated by charge neutralization. And the bridging mediated by ions is not the major flocculating mechanism, which is consistent with the fact that the flocculation of MBF-W6 is cation-independent. The flocculation of MBF-W6 may be achieved by a novel mechanism, in which the kaolin clay particles are attached and bridged directly by cation-independent bioflocculant and promote their flocculant. Furthermore, the inactivation of Fe^{3+} on MBF-W6 was achieved by influencing –COO⁻ and –NH groups. These results broaden understanding to flocculating mechanism of cation-independent bioflocculants. In future, the efforts, such as molecular methods, should be taken to further increase the yield of MBF-W6.

Conflict of Interest

All authors declare no conflicts of interest in this paper.

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