Short Communication

Characterization and flocculation mechanism of an alkali-activated polysaccharide flocculant from *Arthrobacter* sp. B4

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**highlights**

- An alkali-activated polysaccharide flocculant from *Arthrobacter* sp. B4 was studied.
- It could spontaneously flocculate and decrease the system’s pH at a high pH.
- The self-flocculation was mediated by ionization and charge neutralization mechanism.
- It had great potential for the strong alkaline wastewater treatment.

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**abstract**

The characterization and flocculation mechanism of a bioflocculant produced by *Arthrobacter* sp. B4 were investigated. The bioflocculant’s active ingredient was a polysaccharide (B4-PS) that consisted of three main fractions corresponding to the molecular weights of approximately $3.97 \times 10^4$ Da, $6.84 \times 10^3$ Da and $5.9 \times 10^6$ Da, respectively. These fractions were composed of galactose, glucose, mannose and glucuronic acid. Flocculation experiments showed that B4-PS could spontaneously flocculate in the presence of Ca$^{2+}$ ions at a high pH (>12.0), followed by the pH reduction to ~6.0. The self-flocculation of B4-PS may be mediated by ionization and charge neutralization mechanism. Furthermore, B4-PS exhibited excellent capabilities for pollutant removal and pH reduction in alkaline wastewater. These data suggest B4-PS may be a promising tool for use in industrial alkaline wastewater pretreatment.

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1. Introduction

Flocculation is a process in which flocculants absorb freely-suspended particles which leads to aggregation forming larger particles and sedimentation. This process plays an important role in removing pollutants from wastewater containing various toxic suspended particles. Flocculants are classified into three types: inorganic flocculants, organic synthetic flocculants and naturally occurring bioflocculants. Many inorganic and organic flocculants are known to increase environmental risk of secondary pollution which can cause human health problems (Lofrano et al., 2013). In contrast, the bioflocculants are eco-friendly, biodegradable and biocompatible.

Over the past decades, bioflocculants have attracted great attention in the field of environmental engineering. They are mainly produced by microorganisms, such as algae, yeast and bacteria, whose flocculation behavior was proposed to result from the microbial cell surface (Cui et al., 2014; Kregiel et al., 2012), or from biomacromolecules synthesized during cell growth process. These include polysaccharides (Li et al., 2013a), proteins (Poorni and Natarajan, 2014), nucleic acids (Dominiak et al., 2011), etc. These bioflocculants were found to efficiently remove suspended pollutants of wastewater including dye pigments, heavy metal ions and other contaminants on the laboratory scale (Li et al., 2013a; Patil et al., 2011). However, to date, studies on bioflocculants have focused on their production and utilization. As a result, their active ingredients and flocculation mechanism are not entirely clear.

Determination of the active ingredients of bioflocculants is important to elucidate their flocculation mechanisms, which would be beneficial for optimizing the flocculating parameters thus improving their efficiency in practical application. The literature is ripe with hypotheses about possible flocculation mechanisms, including charge neutralization, Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory and divergent cation bridging (DCB) theory (Sobeck and Higgins, 2002). Charge neutralization occurs when the flocculant carries an ionic charge that is opposite
to that of the target particles. In this case, the particle surface charge density is reduced by adsorption of the flocculant and the particles draw sufficiently close together to promote flocculation. The DLVO theory is a classical colloidal theory that describes charged particles as having a double layer of counterions surrounding the particle (Guo et al., 2014). The addition of cations will aid aggregation of the charged particles in a suspension by decreasing the size of the double layer and the repulsive forces between particles. DCB theory describes the process where divalent cations bridge the negatively charged functional groups within the biocompount mole from Brunauer-Emmett-Teller (BET) biocompount amorphous filter paper in favor of flocculation. The effectiveness of the bridging depends on the molecular weight and electric charge of bioflocculant (Zhang et al., 2010). Although these models have aided in the understanding of the flocculating process, a clear explanation of the process is still lacking.

In this paper, a novel bioflocculant (BFB4) was produced by *Arthrobacter* sp. B4. Its active ingredient, characterization, flocculation mechanism and actual application in wastewater treatment were investigated by a series of experiments.

### 2. Methods

#### 2.1. Flocculant production and preparation

*Arthrobacter* sp. B4 was grown in a medium consisted of 16 g/L glucose, 2.5 g/L yeast extract, and 5 g/L KH2PO4, then cultivated at 30 °C with shaking at 200 rpm for 72 h. The resulting fermentation broth was centrifuged at 10,000g for 20 min. The cell-free supernatant was obtained as the liquid BFB4, which was used for the flocculation experiments.

#### 2.2. Flocculating activity assay

The flocculating activity was evaluated by calculating the flocculating efficiency of the BFB4 towards a kaolin suspension as a model system (Li et al., 2013a). This was accomplished using a 100 mL cylinder in which 90 mL of kaolin suspension (4 g/L) was mixed with 5 mL BFB4 and 5 mL CaCl2 (1%, w/v). The mixture was inverted once every 5 s for 10 times and then allowed to stand for 5 min. Absorption values of upper phase at the depth of 4 cm from the top surface were measured with a spectrophotometer (Thermo Scientific Evolution 600, USA) at 550 nm. A control experiment was performed in the same manner by substituting deionized water for the flocculant solution. The flocculating efficiency (FR) was calculated by the following equation:

$$\text{FR} = \left( \frac{B - A}{B} \right) \times 100\%$$  

where A and B are the absorbance values at 550 nm of the sample and control, respectively.

#### 2.3. Physical and chemical analysis

The chemical components of BFB4 were determined by a series of chemical reactions followed by spectrophotometric measurements. Total sugar, uronic acid, amino sugar and protein were measured by the methods described by our previous study (Li et al., 2013b). Nucleic acid content was detected using an ultraviolet spectrophotometer (Thermo Scientific Evolution 600, USA). The polysaccharide fraction (B4-PS) in BFB4 was prepared by removing the protein using pepsin and Sevag reagent (chloroform/m-butyl alcohol, 4:1, v/v) and digesting the nucleic acid fractions with DNase I and RNase A, respectively. Monosaccharide composition analysis was performed using High Performance Liquid Chromatography (HPLC, TSK gel GMPWXL column, 7.8 mm × 300 mm) after hydrolysis at 110 °C for 2 h with trifluoroacetic acid (2 M) and derivation at 70 °C for 1 h with 1-phenyl-3-methyl-5-pyrazole (0.5 M). The average molecular weight was determined by HPLC equipped with multi angle laser light scattering (Wyatt, DAWN HELOS) and refractive index detector (Agilent, G1362A). The infrared spectra were recorded in the frequency range of 4000–400 cm⁻¹ by a Fourier transform (FT-IR) spectrophotometer (Thermo Electron, Nicolet IR200). X-ray photoelectron spectroscopy (XPS) was conducted for major element composition analysis at a broad survey scan (20 eV pass energy). Scanning electron microscope (SEM, LEO 1530, Germany) analysis was performed using the coated sample with a thin layer of gold by means of ion sputter coating with an accelerating voltage of 15 kV. The Zeta potential were measured by Zetasizer 3000HS (Malver Instruments Ltd., England).

### 2.4. Purification of real wastewater

Five milliliters of B4-PS was added to 100 mL of propylene oxide saponified wastewater obtained from the wastewater treatment plant in Shandong, China. After 10 min of sedimentation, the supernatant was collected for the further analysis. The pH, chemical oxygen demand (COD), total carbon concentration (TOC), calcium ion concentration and total chlorine contents were determined using a pH meter, the dichromate method (Li et al., 2013a), ion chromatography and atomic absorption spectroscopy, respectively. Turbidity and color were measured using a spectrophotometer at 860 nm and 665 nm, respectively. The removal efficiency (RE) of the pollutants was calculated by the following equation:

$$\text{RE} = \left( \frac{C_0 - C}{C_0} \right) \times 100\%$$  

where \(C_0\) is the initial value and \(C\) is the value after treatment.

### 3. Results and discussion

#### 3.1. Flocculating activity under various conditions

The flocculating activity of BFB4 towards kaolin suspension (4 g/L) was investigated under a variety of conditions. As shown in Fig. S1, BFB4 exhibited a strong flocculating capability in alkaline conditions (pH > 8.0) and its flocculating efficiency (FR) reached 99% at pH 12.0. This result was different from other similar studies, which claimed that microbial flocculants had a maximum flocculating efficiency in the range of pH 5.0–8.0 (Zhang et al., 2010; Patil et al., 2011). This may be due to variations in the BFB4 causing different structures and surface properties from other flocculants under a strong alkaline condition. A high pH may lead to the release of chains, swelling and low density structure of biomolecules as a result of electrostatic repulsion (Wang et al., 2012), which may be propitious to the flocculation of BFB4 in the presence of Ca²⁺ ions. Therefore, the following experiments were performed under alkaline conditions (pH > 12.0). The flocculating activity of BFB4 was Ca²⁺-dependent and unaffected by other test metal ions. Its maximum FR (98%) was observed in the presence of 0.8% CaCl2 (w/v). No flocculation occurred in the absence of Ca²⁺ ions. This result agreed with the reports that many microbial flocculants were cation-dependent (Zulkiflee et al., 2012). Besides, BFB4 also appeared to mediate flocculation of other suspended particles, such as soil solid and Al₂O₃. Furthermore, BFB4 would spontaneously flocculate when suspended particles were absent. The maximum flocculation was achieved with an optimum BFB4 dosage of 5 mL in 100 mL of reactant. Under these conditions, the flocculation process was completed in 10 min.
3.2. Active ingredient and characterization

BFB4 was mainly composed of polysaccharide (63.1%) and protein (36.8%). The polysaccharide fraction consisted of neutral sugars (86.1%), uronic acid (12.8%), and no amino sugars were detected.

The active ingredient of BFB4 was determined to be a polysaccharide by assaying its flocculating activity distribution. The polysaccharide, namely B4-PS, was characterized by physical and chemical analysis. The monosaccharide composition analysis revealed that B4-PS comprised galactose, glucose, mannose and glucuronic acid at a ratio of 1.03:0.43:1:0.59, respectively. HPLC analysis of B4-PS showed one main peak (39%), one minor peak (4%) and a trace peak (0.4%) corresponding to the molecular weights of ~3.97 x 10^4 Da, 6.84 x 10^4 Da and 5.9 x 10^6 Da, respectively.

The infrared spectrum of B4-PS exhibited a broad stretched peak at around 3413 cm⁻¹, which was characteristic of hydroxyl groups (–OH). Peaks at around 2937 and 1384 cm⁻¹ can be assigned to C–H stretching. The band at 1614 cm⁻¹ was attributed to the stretching vibration of C=O in the carboxyl group. The broad peaks at 1000–1200 cm⁻¹ corresponded to C–O and the C–O–C stretching in the presence of carbohydrate and polysaccharide (Archana et al., 2013). The peak at 669 cm⁻¹ was associated with pyranose (Fig. S2A). XPS analysis showed that B4-PS contained carbon and oxygen, which was consistent with the results of monosaccharide composition analysis. SEM results indicated that the surface morphology of B4-PS to be a porous structure with randomly distributed small pores and interconnected channels, which provided more binding-sites for flocculation (Fig. S2B).

3.3. Flocculation mechanism

According to the flocculating activity study and active ingredient analysis, high pH, Ca²⁺ ions and the polysaccharide (B4-PS) should be key factors for self-flocculation of BFB4. The flocculating behavior of B4-PS was further investigated using alkaline deionized water (ddH₂O) prepared by adjusting the pH to 9.0–14.0 with 1 M NaOH in the presence of 0.5% CaCl₂ (w/v). The results showed that B4-PS could spontaneously flocculate when the pH was more than 12.0, accompanied by the formation of white particles and a decrease to pH 6.0 (Fig. S3A). Further experimentation revealed that the pH of the alkaline ddH₂O (pH 12.0) could decreased to ~7.0 after addition of B4-PS. Following this, the self-flocculation of B4-PS occurred after addition of CaCl₂, and the system pH was further reduced to 6.0 (Fig. S3B), suggesting that B4-PS was an alkali-activated polysaccharide flocculant and its self-flocculation of was induced by a high pH (>12.0). To explain this phenomenon, the variations of the Zeta potential and the pH in the flocculation process were measured in this manner: 0.2 mL of B4-PS solution (0.1 g/mL) was added to 10 mL of alkaline ddH₂O (pH 12.0); and 0.2 mL of B4-PS solution (0.1 g/mL) was added to 10 mL of alkaline ddH₂O (pH 12.0) and 0.5 mL of CaCl₂ solution (0.5%, w/v). As shown in Fig. 1, a stable Zeta potential occurred after the addition of each component. When B4-PS was added into alkaline ddH₂O, the Zeta potential of B4-PS quickly decreased from about −0.2 mV to about −4.5 mV and the system pH was reduced to 7.0. This occurred because the carboxyl groups (−COOH) in B4-PS may have ionized under the alkaline conditions to give carboxylate anions (COO⁻), and the hydroxyl ions (OH⁻) were converted into hydroxyl groups (−OH) or H₂O. After addition of B4-PS into alkaline ddH₂O and CaCl₂ solution, the self-flocculation of B4-PS occurred and the Zeta potential of the resulting flocs quickly increased to about 4.8 mV, and the system pH was reduced to about 6.0. This occurred because Ca²⁺ ions significantly decreased the static repulsive forces of the B4-PS molecules to promote its flocculation. These results suggested ionization and charge neutralization may be the main flocculation mechanism of B4-PS.

B4-PS participates in the flocculation through available carboxyl and hydroxyl groups, which means that ion bond and hydrogen bonds will be main forces between Ca²⁺ ions and B4-PS molecules. Thus, excess EDTA, HCl and urea were added to the flocs suspension of B4-PS. As shown in Fig. S4, the flocs were destroyed after addition of excess EDTA (2%, w/v) due to the ionic bond rupture between Ca²⁺ ions and B4-PS molecules caused by EDTA chelation of Ca²⁺ ions and compression of the electrical double layer of the colloidal particles. When the excess HCl (1 M) was added, the flocs were destroyed. At the same time, the white particles dissolved, which suggested those particles could be CaCO₃ crystals. However, the flocs were stable after addition of the excess urea solution (2%, w/v) which could destroy hydrogen bonds between molecules. This indicated that there was no obvious hydrogen bonding interactions between B4-PS molecules. Based on these data, ionic bonds

![Fig. 1. Variations of the Zeta potential and the pH in the flocculation process. All the data are the mean of triplicates ± S.D.](image-url)
should be the critical crucial forces between Ca$^{2+}$ ions and B4-PS molecules, and self-flocculation of B4-PS may be mediated by the charge neutralization mechanism. This process was completed by two steps, ionization and flocculation. Initially, the –COOH of B4-PS was ionized to give COO$^-$ in the presence of OH$^-$, which decreased the Zeta potential of B4-PS. Subsequently, flocculation occurred owing to electrostatic interaction between Ca$^{2+}$ ion and B4-PS molecule after addition of Ca$^{2+}$ ions. In this process, Ca$^{2+}$ ions drew closer to the negatively charged B4-PS molecules, which shortened the distance between the negatively charged objects and reduced the thickness of the diffuse double layer of adjacent P-B4 molecules. This process may also have led to an attractive force between like-charged B4-PS molecules as they close on an oppositely charged area on a flat surface, which may contribute to the formation of ionic bonds between Ca$^{2+}$ ions and B4-PS molecules (Andrij et al., 2014). On the other hand, the formation of CaCO$_3$ in the flocculation system could further aid flocculation by the sweep coagulation mechanism (Leentvaar and Rebhun, 1982).

3.4. Purification of alkaline wastewater

This study confirmed that B4-PS could efficiently flocculate under strong alkaline conditions. Thus B4-PS was used to purify the strong alkaline propylene oxide saponified wastewater (pH > 12.0) that contained a large amount of Ca$^{2+}$ ions (17,758 mg/mL), Cl$^-$ ions and organic chloride (29,890 mg/mL) and other inorganic ions. The results showed that the pollutant removal efficiency for COD, TOC, Ca$^{2+}$, Cl$^-$ and turbidity were 51%, 61%, 89%, 20% and 33%, respectively. After treatment, the pH was reduced to ~6.5. In addition, B4-PS showed an excellent decolorization ability for the simulated alkaline print and dye wastewater (pH > 12.0) with a decolorization efficiency of ~63.4%, and the pH decreased to ~7.0. Overall, the satisfactory pollutant removal, pH reduction and Ca$^{2+}$ consumption demonstrated the feasibility of using B4-PS for the alkaline wastewater treatment, especially for the alkaline wastewater containing calcium ions.

4. Conclusions

An alkali-activated and calcium-dependent polysaccharide flocculant produced by Arthrobacter sp. B4 was investigated in our study. The flocculant exhibited excellent capabilities for self-flocculation and pH reduction under the strong alkaline conditions in the presence of Ca$^{2+}$ ions. Its self-flocculation was mediated by ionization and charge neutralization mechanism. This flocculant will be an attractive and specific candidate for purifying the strong alkaline wastewater.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.07.112.

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