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A novel bioflocculant produced by *Klebsiella sp*. and its application to sludge dewatering

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Keywords
bioflocculant; combination; flocculation rate; *Klebsiella sp*; sludge dewatering.

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Abstract

A bioflocculant-producing bacterium, named N-10, was isolated from activated sludge and was identified as *Klebsiella sp*. The bioflocculant (named MBF10) produced by *Klebsiella sp*. had a good flocculating capability and could achieve a flocculating rate of 86.5% to kaolin suspension at the dosage of 0.034 g/L. Compared with chemical flocculants, such as $Al_2(SO_4)_3$, polyaluminum chloride (PAC) and cationic polyacrylamide (PAM) at their optimal dosages, MBF10 showed a similar performance for sludge dewatering. After preconditioning, dry solids (DS) and specific resistance to filtration (SRF) of the sludge were 17.5% and 3.36×10^{12} m/kg, respectively. The combined use of MBF10 and $Al_2(SO_4)_3$ resulted in optimum sludge dewatering properties, SRF reduced from 10.87×10^{12} m/kg to 1.72×10^{12} m/kg, and DS increased from 13.1% to 21.3%. Charge neutralisation and interparticle bridging were proposed as the reasons for the enhanced performance based upon the experimental observations.

Introduction

Bioflocculants (microbial flocculants) are metabolic products of microorganisms produced during their growth (Lu *et al*. 2005). In recent years, the study of bioflocculants has attracted wide attention (Liu *et al*. 2009). Many microorganisms, such as *Citrobacter* (Ike *et al*. 2000), *Bacillus mucilaginosus* (Deng *et al*. 2003), *Serratia ficaria* (Gong *et al*. 2008) and *Bacillus licheniformis* (Li *et al*. 2009), have been found to produce bioflocculants. Bioflocculants are widely used in the treatment of water and wastewater, in the processing of downstream, and in disposing of food and chemicals (Salehizadeh & Shojaosadati 2001; You *et al*. 2008).

Sludge produced at various stages of wastewater cleaning may contain only 0.25% of dry solids (DS). It has to be further thickened so that the amount of water is lowered, which results in lower economic demands (Thapa *et al*. 2009). Flocculation is the most conventional method to increase sludge dewatering efficiency (Vaxelaire & Cezac 2004; Nguyen *et al*. 2008), which is useful especially in sludge separation based on different particle weights. Many chemical flocculants, including aluminum sulfate [$Al_2(SO_4)_3$], ferric chloride ($FeCl_3$), polyaluminum chloride (PAC) and polyacrylamide (PAM), have been widely used because of their high flocculating performance and low cost. However, there is evidence that the neurotoxic and carcinogenic acrylamide monomers caused by chemical flocculants are harmful to human and environment; such considerations restrict the use of these flocculants (Ruden 2004). Bioflocculants are attractive alternatives to substitute existing chemical flocculants because of their nontoxic and harmless characters. But so far, there is hardly report on the application of bioflocculants in sludge dewatering.

In this study, a bioflocculant-producing bacterium (named N-10) from activated sludge was screened, which was identified as *Klebsiella sp*. A series of experiments were conducted to investigate the bioflocculant production and flocculation performance. The comparison and combination of the bioflocculant (MBF10) with other chemical flocculants in sludge dewatering were also studied. Moreover, based on the experimental results, the enhanced flocculation mechanism of combined MBF10 with other chemical flocculants was further proposed.

Materials and methods

Screening and culturing of bioflocculant-producing bacteria

Many types of bacteria that were previously isolated from soils and activated sludge were screened. The composition of the medium for screening were as follows: 20 g glucose, 2 g KH_2PO_4, 5 g K_2HPO_4, 0.2 g $(NH_4)_2SO_4$, 0.1 g NaCl, 0.5 g urea and 0.5 g yeast extract dissolved in 1.0 L distilled water with the initial pH adjusted to 8.0. After sterilisation and inoculation of the medium, the bacterium was cultured at 30°C on a rotary shaker at 150 rpm for 72 h. Kaolin suspensions as a test material was used to evaluate the flocculating capability of the culture broths, and a bacterium with flocculating efficiency was selected as the preliminary bioflocculant-producing bacterium. Thereafter, it was cultivated in a 250-mL flask containing 50 mL screening medium and incubated for 60 h on a rotary shaker at 30°C and 150 rpm. Kaolin suspensions were used to evaluate the flocculating capability, and then the strain with high flocculating efficiency was selected as the bioflocculant-producing bacterium for further investigation. The morphological character of the best strain was observed with a microscope (CX40RF200, Olympus Optical Co. Ltd, Japan), and it was named *Klebsiella sp.* N10.

Production of bioflocculant

The *Klebsiella sp.* N10 was inoculated into a 150-mL flask containing 50 mL screening medium and incubated for 48 h on a rotary shaker at 30°C and 150 rpm. This preculture procedure was then used as the standard inocula preparation for all experiments. The fermentation medium (pH 8.0) consisted of (per 1.0 L) 20 g glucose, 0.5 g yeast extract, 0.5 g urea, 2 g KH_2PO_4, 5 g K_2HPO_4 and 0.1 g NaCl. A 150-mL flask containing 50 mL fermentation medium was incubated at 30°C on a rotary shaker at 150 rpm for 96 h. The fermentation broth obtained at appropriate time intervals was centrifuged (4000 × g, 30 min) to separate the cells. The cell-free culture supernatant was the liquid bioflocculant, which was used for the analysis of flocculating activity. All experiments were performed in triplicates for the mean calculation.

Determination of the flocculating activity

Flocculating rate was used as a measurement of flocculating activity of the bioflocculant. A 0.5 g kaolin clay (average diameter 4 μm) was suspended in 100 mL distilled water, and then 5 mL 1% (wt%) $CaCl_2$ and 2 mL of bioflocculant (culture broth) were added to the kaolin suspension. The mixture was stirred with rapid mixing at 200 rpm, followed by slow mixing at 50 rpm and then kept still for 10 min. The absorbance of the supernatant and the blank without bioflocculant was measured at 550 nm (OD_{550} and OD_{blank}, respectively) with a spectrophotometer. The flocculating rate was defined and calculated as follows:

$$\text{Flocculating rate } (\%) = \frac{A-B}{A} \times 100\% \quad (1)$$

where A and B are optical density (OD) of the control and sample, respectively, at 550 nm.

Bioflocculant purification

To purify the bioflocculant, the viscous culture broth was diluted with distilled water and then centrifuged to remove cell pellets at 8000 × g for 15 min to concentrate the culture. The supernatant was poured into four volumes of cold ethanol to precipitate the biopolymer flocculants. The resultant precipitate was collected by centrifugation at 8000 × g for 15 min and redissolved in distilled water. After three such ethanol precipitation steps, the crude biopolymer obtained was dialysed at 4°C overnight against distilled water, and the resultant precipitate was vacuum-dried for 24 h, and then MBF10 was obtained.

Bioflocculant composition analysis

The total sugar content of the bioflocculant was determined by the phenol-sulfuric acid method using glucose as standard solution (Chaplin & Kennedy 1994). The protein content was measured by the Lowry–Folin method using a UV spectrophotometer (Shimadzu, Japan). GC-MS analyses were conducted to analyse the content of pure bioflocculant on a Trace GC, Palaris Q GC-MS spectrometer (Thermo-Finnigan, Austin, TX, USA) using carbon capillary column, DB-1(film thickness, 0.25 mm; column dimensions, 30 m × 0.25 mm), with helium as carrier gas. The column temperature of GC used in this study was programmed from 60 to 230°C, with an increasing rate of 5°C/min. The temperature of the injection chamber was 250°C, and the temperature of transfer line was 230°C.

Sludge dewatering by flocculants

Sludge used in this study was collected from the secondary settling tank of Jin-Xia municipal wastewater treatment plant (Changsha, China). The collected sludge had undergone gravity thickening and was ready to be dewatered by a belt press filter after adding P(AM-DMC). After it was transported to the laboratory, it was stored at 4°C and analysed within 2 days.

The dewaterability of the sludge was expressed in terms of specific resistance to filtration (SRF) and DS. Various conditioners, including MBF10, $Al_2(SO_4)_3$, PAC and cationic poly-

acrylamide [CPAM, molecular weight (MW) = 1.2×10^8] were separately added into a 200-mL mixing chamber with 100 mL of sludge. After stirring for 1 min, the mixture was left still for 5 min, and then it was poured into the funnel fitted with a filter paper. After 2 min of gravitational drainage, a vacuum of 0.04 MPa was applied. The volume of the filtrate collected every 15 s was recorded.

The SRF was calculated by the following equation (Yin *et al*. 2004):

$$\frac{dt}{dV} = \frac{\mu}{A(\Delta p)}\left(\frac{\alpha c V}{A} + R_m\right) \quad (2)$$

where t is the time, V is the volume of filtrate, μ is the filtrate viscosity, A is the filter area, Δp is the pressure drop across filter, c is the slurry concentration, α is the SRF and R_m is the resistance of filter medium (neglected).

The DS of dewatered sludge was determined according to following equation:

$$DS = \frac{W_2}{W_1} \times 100\% \quad (3)$$

where W_1 is the weight of wet filter cake and W_2 is the weight of filter cake after drying at 105°C for 8 h.

Results and discussion

Screening and identifying of bioflocculant-producing bacteria

About 20 different types of strains, with a flocculating rate to kaolin suspension of over 50%, were isolated from soil and activated sludge samples. Among these bacteria, strain N-10, with the flocculating rate exceeding 86.5%, was the most effective bacterium. Colonies of N-10 were round, creamy coloured, smooth and mucoid. The bacterium was short rod-shaped, gram-negative, facultative aerobe, non-endospore-forming and nonmotile. The strain was identified as *Klebsiella sp.* using the Biolog system (Biolog, Hayward, CA, USA). The bioflocculant produced by strain N-10 was named MBF10.

Bioflocculant production

The growth curve of the N-10, the flocculating rate and pH variation of the culture broth is shown in Fig. 1. During the logarithm phase, the production of MBF10 was almost in parallel with cell growth and flocculating rates increased with increasing cultivation time, indicating bioflocculant was produced by N-10 during its growth, not by cell autolysis (Lu *et al*. 2005). This is supported by the fact that the flocculating rates increased rapidly during the first 60 h period. At the stationary phase, cell production reached its maximum (3.52 g/L) at 36 h, while MBF10 reached its maximum flocculating rates (86.5%) at 60 h. The production of biopolymer flocculants from *Bacillus licheniformis* reached maximum after 96 h of incubation (Shih *et al*. 2001), while N-10 produced bioflocculant in shorter time in this study. Both cell growth and MBF10 production decreased gradually in the death phase, which was possibly because of cell autolysis and enzyme activity decrease. The pH profile showed that the pH of the culture broth decreased from 8.04 to 5.02 within 48 h but rose later to 5.50 at about 72 h, and decreased slightly to 5.00 until the end, which was similar to the report of Dermlim *et al*. (1999), thus we presumed some organic acids were produced and released into the medium by strain N-10 during its growth period. The distribution of bioflocculant activity varies with the strains; Fig. 2 showed the distribution of N-10 activity. It was found that the fermentation broth (including biomass), the supernatant and the

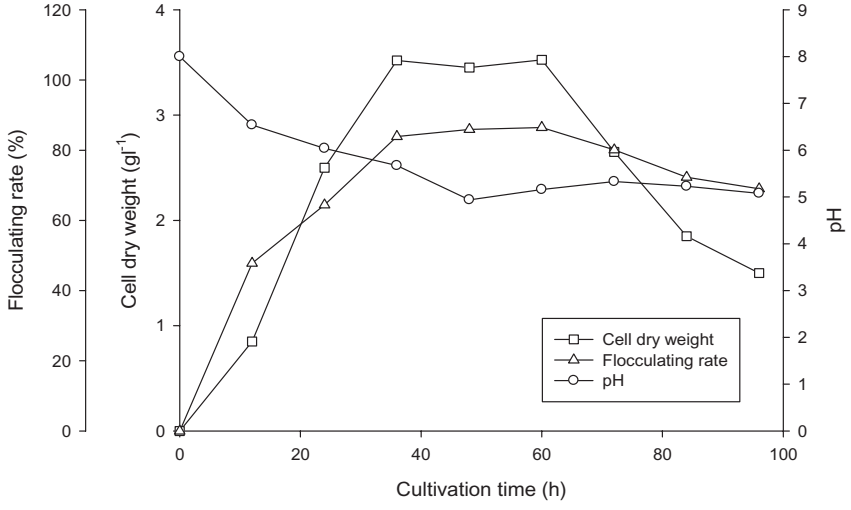

Fig. 1. Growth curve of *Klebsiella sp.*N10 on a rotary shaker at 150 rpm, 30°C for 96 h.

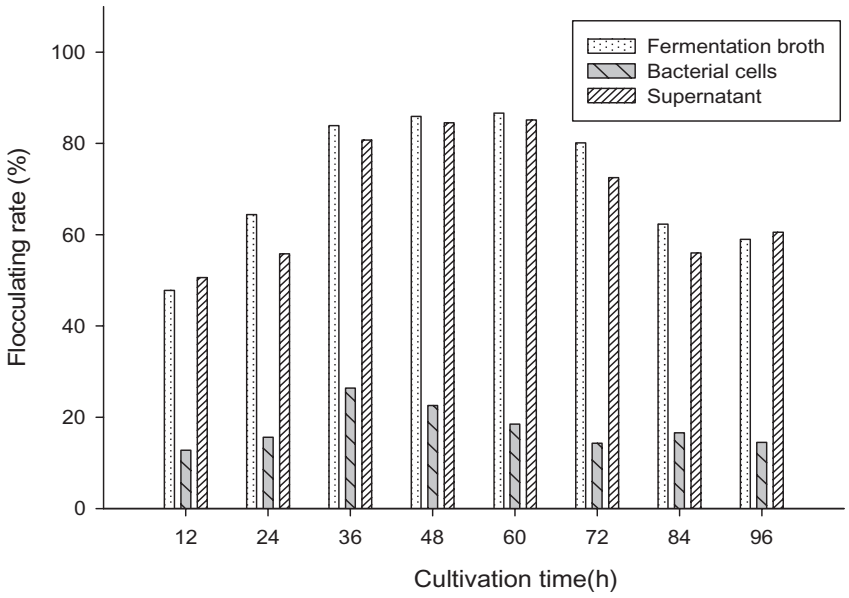

Fig. 2. The distribution of N-10 activity.

bacterial cells had flocculating activity throughout the process of fermentation. More than 75% of the flocculating activity was released into the culture medium, while the residual bioflocculant appeared to localise to the bacterial cell, which showed that the bioflocculant was an extracellular product. The distribution of flocculating activity was slightly different in various growth stages, and it might be because of the growth status and external environment of bacteria.

Composition analysis of the bioflocculant

The extracted dried flocculation looked like white powder. About 1.7–1.8 g of the purified MBF, whose MW was about 8.0×10^5 Daltons, could be recovered from 1.0 L of fermentation broth. It was similar to the previous report that about 1.58–2.19 g bioflocculant could be obtained per 1000 mL fermentation liquid by Lian *et al*. (2008). To investigate the content of bioflocculant, pure bioflocculant was analysed by chemical method, UV spectrophotometric method and GC-MC. The pH of fermentation medium decreased from 8.04 to 5.00 after the fermentation of MBF10 (Fig. 1) indicated that some organic acids produced and released into the medium. The phenol–sulfuric acid and Lowry–Folin methods showed that polysaccharide was the component of bioflocculant and no protein was detected. Furthermore, the UV scan chromatography (from 150 to 350 nm) displayed an absorption peak at around 200 nm characteristic for polysaccharide, and no characteristic absorption peak at around 250–280 nm, which indicated that there was no nucleic acid or protein in this bioflocculant. The results of GC-MC analysis further indicated that pure bioflocculant consisted of polysaccharide, including uronic acid, acetic acid, glucuronic acid and amino sugar as the principal constituents.

Sludge dewatering by flocculants

Comparison of the dewaterability of the sludge by various flocculants

As mentioned, it was confirmed that MBF10 had high flocculating activity in kaolin suspensions. Bioflocculant was applied to sludge dewatering in the following investigation to get its knowledge of flocculation characteristics. Table 1 presented the comparison of experimental results obtained using bioflocculant MBF10 with other conventional chemical flocculants, such as $Al_2(SO_4)_3$, PAC and CPAM at their optimal dosages on sludge dewatering. The results indicated that MBF10 performed much better than $Al_2(SO_4)_3$, but poorer than PAC and CPAM. After conditioning, DS and SRF of the sludge treated with MBF10 were 17.5% and 3.36×10^{12} m/kg, respectively. The comparison of flocculating ability between MBF10 and chemical flocculants revealed that MBF10 had similar flocculating activity.

The price of $Al_2(SO_4)_3$, PAC, CPAM, MBF10 is approximately \$105, \$150, \$820, \$1320 per ton, respectively. According to the optimal dosage shown in Table 1, the cost for sludge dewatering with the four flocculants would be approximately \$15.8/t DS, \$15.0/t DS, \$1.0/t DS, \$7.9/t DS, respectively, which suggests that the application of bioflocculant in sludge dewatering is more worthwhile based on cost accounting. Moreover, the composition analysis of the bioflocculant indi-

Table 1 Experimental results of sludge dewatering with different flocculants

Flocculation	Optimal dosage [g/(kg ds)][a]	Optimum pH	DS (%)	SRF (10^{12} m/kg)
Blank	–	7	13.1	10.87
$Al_2(SO_4)_3$	150	8	15.0	3.03
PAC	100	8	20.8	2.54
CPAM	1	8	21.2	2.37
MBF10	6	8	17.5	3.36

[a]g/(kg ds) represents g/kg dry sludge.
DS, dry solids; SRF, specific resistance to filtration; PAC, polyaluminum chloride; CPAM, cationic polyacrylamide.

cates that polysaccharide is the mainly component of the bioflocculant, and it is nontoxic and harmless to environment. Therefore, it is potentially a promising flocculant used in sludge dewatering.

Sludge dewatering by combined use of bioflocculant and other flocculants

For the uniformity of material property, it is unfavourable for single flocculant to condition complex sludge. Lee & Liu (2000) had studied the application of two or more flocculants in sludge conditioning, which could exert the advantages of different flocculants and exhibit synergistic effect in sludge dewaterability.

The combined use of MBF10 with other flocculants on the dewaterability of sludge was investigated. When pH of sludge was adjusted to 8.0, 6 g (kg ds) $^{-1}$ of MBF10 was added into sludge with PAC (100 g(kg ds)$^{-1}$), $Al_2(SO_4)_3$ (150 g(kg ds)$^{-1}$) and CPAM (1 g(kg ds)$^{-1}$), respectively. It was found that there was an evident improvement of sludge dewaterability for the combined use of MBF10 with $Al_2(SO_4)_3$. Compared with single MBF10 and $Al_2(SO_4)_3$ treatment, SRF decreased by 48.8% and 43.3%, respectively. DS increased from 13.1% (raw sludge) to 21.3%, but it only increased to 17.5% and 15.0% for single MBF10 and $Al_2(SO_4)_3$ treatment, respectively. Meanwhile, the sludge dewaterability with combined MBF10- PAC or MBF10-CPAM decreased rather than improved (Fig. 3). $Al_2(SO_4)_3$, PAC and CPAM added positively charged sites to the micro flocs, but their dewatering performance was very different. According to Yu & Somasundaran (1996), the combined use of a low-MW cationic polymer followed by a high-MW anionic polymer might produce excellent flocculation, as having been demonstrated on alumina flocculation.

Enhanced flocculation mechanism of combined flocculants

Combining the results, the enhanced flocculation mechanisms of combined flocculants were elaborated. Many elements, such as particle size, extracellular polymeric substances (EPS), cationic salts and conditioning, were reported to influence sludge dewaterability (Chen *et al*. 2001; Vaxelaire

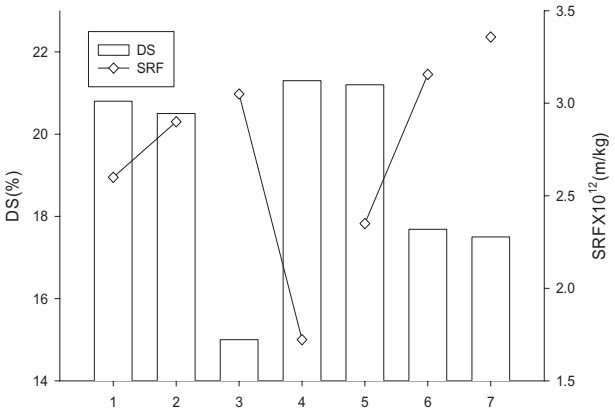

Fig. 3. DS and SRF of the sludge after various flocculants treatment (1. PAC; 2. MBF10-PAC; 3. $Al_2(SO_4)_3$; 4. MBF10-$Al_2(SO_4)_3$; 5. CPAM; 6. MBF10-CPAM; 7. MBF10). DS, dry solids; SRF, specific resistance to filtration; PAC, polyaluminum chloride; CPAM, cationic polyacrylamide.

& Cezac 2004). Based on the classic theory of double electrical layer, $Al_2(SO_4)_3$ compressed the double electrical layers of sludge particles by charge neutralisation. Therefore, relatively more compact primary flocs were accumulated to form small granules as a result of electrostatic attraction between cationic electrolyte and negatively charged sludge surfaces.

The MW of subsequent additional MBF10 was 8.0×10^5 Daltons, which means ample binding sites and strong van der Waals forces with the sludge particles and the organic materials in the out layer. Scanning electron microscopy (SEM) image of the purified solid-state MBF10 showed that it had a crystal-linear structure, which assured the binding sites to be functional and bridge more sludge particles and out-layer organic materials to form compact sludge flocs (Salehizadeh & Shojaosadati 2001; Zhang *et al*. 2007). On the other side, MBF10 was negatively charged, the pretreatment with $Al_2(SO_4)_3$ reduced its difficulty of approaching to sludge particles (oppositely charged electrolytes reduced the particle surface charge density that particles may approach each other sufficiently close; therefore, the attractive forces became effective). If the extension of MBF from the particle surface is greater than the distance the interparticle repulsion acts, the MBF can be adsorbed onto another particle

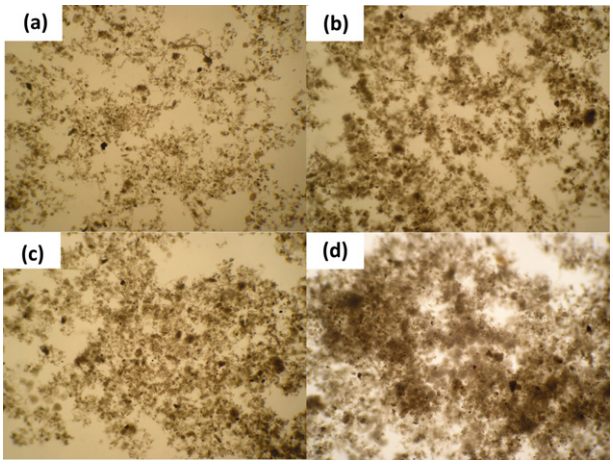

Fig. 4. Micrograph analysis of sludge conditioning (× 40) [(a) raw sludge; (b) sludge conditioned with $Al_2(SO_4)_3$; (c) sludge conditioned with MBF10; and (d) sludge conditioned with combined MBF10 and $Al_2(SO_4)_3$].

surface, thereby bridging the two, and then the aggregates entangled to form larger particles. Many particles could be adsorbed to form a long molecular chain, thus the particles adsorbed on the chain could be adsorbed simultaneously by other flocculant chains, leading to the formation of three-dimensional flocs (Deng *et al*. 2003), which further promoted the dewaterability of sludge by expelling the water in the exterior and the interior of the sludge flocs. Therefore, charge neutralisation and interparticle bridging contributed to the promotion of flocculation efficiency.

Micrograph analysis of sludge conditioning

The morphological differentiation between raw and conditioned sludge was shown in Fig. 4. It was clearly that the sludge particles were dispersed because of the repulsion of negative charge (Fig. 4a). Sludge components were cemented together by EPS (Liu & Fang 2002), which caused difficulty in sludge dewatering because EPS led to more interstitial bound water contained in the sludge and poor settleability by its large size (Chen *et al*. 2001). When $Al_2(SO_4)_3$ or MBF10 was separately added, the flocculation of sludge was observed and a part of interstitial water was excluded (Fig. 4b,c). Figure 4(d) revealed that cloud-like sludge was formed after conditioning with the combination of MBF10 and $Al_2(SO_4)_3$. Large numbers of sludge particles were accumulated into aggregated structure because of charge neutralisation and adsorption bridge building, therefore sludge dewaterability was greatly enhanced by the combination of MBF10 and $Al_2(SO_4)_3$.

Conclusions

(1) A bioflocculant-producing bacterium, N-10, was isolated from sludge and identified as *Klebsiella sp*. The bioflocculant (MBF10) produced by N-10 had 86.5% flocculating efficiency to Kaolin clay suspension of 5 g/L.

(2) The composition analysis showed that pure MBF10 mainly consisted of polysaccharide, including uronic acid, acetic acid, glucuronic acid and amino sugar as the principal constituents.

(3) MBF10 showed good flocculating performance on sludge dewatering, and DS and SRF of sludge treated by MBF10 were 17.5% and 3.36×10^{12} m/kg, respectively, which were similar to conventional chemical flocculants. However, the application of bioflocculant in sludge dewatering is worthwhile based on cost accounting.

(4) Sludge dewatering performance was greatly improved with the combination of MBF10 and $Al_2(SO_4)_3$. Charge neutralisation and interparticle bridging were proposed as the reasons for enhanced performance.

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