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ENVIRONMENTAL BIOTECHNOLOGY

Characterization and application of bioflocculant prepared by *Rhodococcus erythropolis* using sludge and livestock wastewater as cheap culture media

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Abstract A new bioflocculant was produced by culturing *Rhodococcus erythropolis* in a cheap medium. When culture pH was 7.0, inoculum size was 2 % (*v*/*v*), Na_2HPO_4 concentration was 0.5 g L^{-1}, and the ratio of sludge/livestock wastewater was 7:1 (*v*/*v*), a maximum flocculating rate of 87.6 % could be achieved. Among 13 different kinds of pretreatments for sludge, the optimal one was the thermal-alkaline pretreatment. Different from a bioflocculant produced in a standard medium, this bioflocculant was effective over a wide pH range from 2 to 12 with flocculating rates higher than 98 %. Approximately, 1.6 g L^{-1} of crude bioflocculant could be harvested using cold ethanol for extraction. This bioflocculant showed color removal rates up to 80 % when applied to direct and disperse dye solutions, but only 23.0 % for reactive dye solutions. Infrared spectrum showed that the bioflocculant contained functional groups such as –OH, $–NH_2$, and $–CONH_2$. Components in the bioflocculant consisted of 91.2 % of polysaccharides, 7.6 % of proteins, and 1.2 % of DNA. When the bioflocculant and copper sulfate ($CuSO_4$) were used together for decolorization in actual dye wastewater, the optimum decolorization conditions were specified by the response surface methodology as pH 11, bioflocculant dosage of 40 mg/L, and $CuSO_4$ 80 mg/L, under which a decolorization rate of 93.9 % could be reached.

Keywords Pretreatment · Bioflocculant · Polysaccharides · Excess sludge · *Rhodococcus erythropolis* · Response surface methodology

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Introduction

Flocculants are usually effective in aggregating colloids and have been widely used in industrial fields such as tap-water preparation, wastewater treatment, downstream techniques, fermentation process, and food industries (Salehizadeh and Shojaosadati 2001). Compared with traditional inorganic and organic synthetic polymer flocculants such as polyacrylamides, natural flocculants, or bioflocculants are more readily biodegradable and less harmful to human and ecosystems (Simphiwe et al. 2010). Microbes has been investigated in different kinds of wastewater treatment as well, such as Pant and Adholeya (2007, 2009, 2010) who used bacterial-, fungal-, algal-, and plant-based systems in the color removal of distillery effluent. Zhou et al. (1990) investigated the treatment of phenol-containing wastewater by an immobilized microorganism (*Candida tropicalis*). Moletta (2005) studied the winery and distillery wastewater treatment by anaerobic digestion, which was the transformation of organic matter by a consortium of anaerobic microorganisms.

In bioflocculant preparation, different kinds of microbial species have been studied and reported, such as *Enterobacter* sp. (Haruhiko et al. 1997), *Virgibacillus* sp. (Sekelwa et al.

2011), *Halomonas* sp. (Jin et al. 2010), *Citrobacter* sp. (Fujita et al. 2000), *Bacillus* sp., and *Rhodococcus erythropolis* (Daolun and Shinhong 2008; Kurane et al. 1994; De Carvalho and Da Fonseca 2005).

High cost of bioflocculant preparation has prevented bioflocculants from being more widely used. To solve this problem, several investigators have tried to prepare bioflocculants using food wastewater as a cheap culture medium for fermentation. For instance, alcohol was used by Kurane et al. (1994) to produce bioflocculant with *R. erythropolis*. Fujita et al. (2000) studied the characterization of a bioflocculant produced by a bacterial strain isolated from a biofilm formed inside a kitchen drain. Wang et al. (2007) produced a novel bioflocculant by the culture of *Klebsiella mobilis* using dairy wastewater. Most of those cheap media could be easily reutilized in other industries; meanwhile, the reuse of excess sludge from municipal wastewater treatment plants is a tough issue. The major outlets for sludge are agriculture and landfill, with only a relatively small amount incinerated. About 60 % of excess sludge from a wastewater treatment plant is disposed in landfill (Suresh et al. 2004). Hence, the study of its reutilization is needed.

To some extent, excess sewage sludge is also a resource, which could be reutilized in manufacturing building materials, producing biohydrogen, and feeding fuel cells for electricity generation (Yang 2011; Sevda et al. 2013). So far, Suresh et al. (2004) have studied the preparation of biodegradable plastics with excess sludge. In the field of bioflocculant preparation, Sun et al. (2012) have investigated directly extracting flocculating activity ingredients from biological sludge. But, these ingredients only showed a high flocculating activity under alkaline conditions.

Excess sludge contains plenty of high corruptible organic substances and microorganisms, and 60 % of its components are proteins and carbohydrates (Tang et al. 2010). Certain chemical and physical conditions such as alkaline, acid, ultrasound, and so forth could disrupt excess sludge cells (Zhiqiang et al. 2013; Sun et al. 2012). After being disrupted, excess sludge can provide microbes with a certain amount of carbon source, nitrogen source, inorganic salts, and microelements (Neyens and Baeyens 2003). In addition, livestock wastewater rich in nitrogen (N) and phosphorus (P) can lead to aquatic eutrophication if discharged untreated, whereas these nutrients could also be used by bacteria. As reported by Obaja et al. (2003), nutrients in piggery wastewater could be biologically removed in sequencing batch reactors (SBR). However, the report of fermentation using excess sludge and livestock wastewater is really documented. Hence, in this paper, excess sludge and livestock wastewater were selected as a cheap medium in bioflocculant preparation.

In addition, copper sulfate ($CuSO_4$) possesses flocculating and coagulating properties, and a few studies on $CuSO_4$ as a coagulant have been documented, namely Jun and Wei (2001) used copper sulfate to remove algae by coagulation; Chaudhari et al. (2010) studied the treatment of paper and pulp mill effluent by coagulation using poly aluminum chloride (PAC) and $CuSO_4$. So it is probably feasible to treat dye wastewater using *R. erythropolis* sludge bioflocculant (RSF) and $CuSO_4$ as composite flocculant.

In this research, strain *R. erythropolis* (ACCC 10543) was used to produce bioflocculant with the supernatant of pretreated excess sludge and livestock wastewater. Furthermore, since dewatered sludge is much easier to be transported and in order to exclude the impact of excess sludge's activity, dewatered sludge was studied as well in the current study. Different pretreatments were employed to disrupt sludge cells, releasing nutrients into the liquid phase. The characteristics, performances, and components of this bioflocculant were also investigated. At last, the application conditions of the composite flocculant RSF-$CuSO_4$ in actual dye wastewater treatment were optimized using the response surface methodology (RSM).

Materials and methods

Materials

Bacterial strain

Strain *R. erythropolis* (ACCC 10543) was purchased from Agriculture Culture Collection Center of China (ACCC).

Sludge samples

The excess sludge samples in this project was taken from the Guozhen Municipal Wastewater Treatment Co., Ltd., Changsha, Hunan, China, and stored at 4 °C prior to use.

The characterization of excess sludge is presented in Table S1 (in ESM-electronic supplementary material).

Livestock wastewater

The livestock wastewater was obtained from an anaerobic digestion reactor in a livestock wastewater treatment plant of Tianxin Pig Farm, Changsha, Hunan, China. The pH and VSS/TSS of the wastewater were 7.3–8.0 and 88.14 %, respectively. Its concentrations of chemical oxygen demand (COD), turbidity, total phosphorus, and ammonium nitrogen were 1,400–1,600 mg L^{-1}, 170–220 NTU, 30–50 mg L^{-1}, and 910–1,420 mg L^{-1}, respectively.

Standard medium

The NOC-1 refers to the bioflocculant yielded by *R. erythropolis* in a standard medium. The standard medium consisted of (in grams per liter) the following: sucrose, 20; urea, 4; yeast powder, 1; NaCl, 1; K_2HPO_4, 5; KH_2PO_4, 2; and $MgSO_4$, 0.2. The initial pH of media was adjusted to 7.0±0.1 using 1.0 M of sodium hydroxide and 1.0 M of hydrochloric acid.

Methods

Pretreatments of excess sludge

According to the literatures (Neyens and Baeyens 2003; Penaud et al. 1999; Skiadas et al. 2005; Sun et al. 2012; Tanaka et al. 1997; Tang et al. 2010; Zhiqiang et al. 2013), different pretreatments applied to excess sludge were as follows: (1) 120 °C thermal pretreatment (120 °C); (2) 70 °C thermal pretreatment (70 °C); (3) alkaline pretreatment (Al); (4) acid pretreatment (Ac); (5) ultrasonic pretreatment (U); (6) microwave pretreatment (M); (7) 70 °C thermal-alkaline pretreatment (70 °C-Al); (8) 70 °C heating-acid pretreatment (70 °C-Ac); (9) 70 °C heating-ultrasonic pretreatment (70 °C-U); (10) 120 °C heating-alkaline pretreatment (120 °C-Al); (11) 120 °C heating-acid pretreatment (120 °C-Ac); (12) 120 °C heating-ultrasonic pretreatment (120 °C-U); (13) 120 °C heating-microwave pretreatment (120 °C-M). The corresponding conditions are presented in Table S2 (in ESM).

Bioflocculant production

Sludge livestock wastewater medium (SLWM) was prepared by mixing the supernatant of pretreated excess sludge and sterilized livestock wastewater at a specific broth ratio (*v*/*v*), then the pH of each SLWM was adjusted to a certain value. The SLWM was inoculated with strain *R. erythropolis* in a 250-mL flask, precultured in a reciprocal shaker at 30 °C, and stirred at 130 r min^{-1} for 20 h. Batch fermentation was carried out by cultivating the precultured inoculum in a 150-mL SLWM with an inoculum size of 2.0 % (*v*/*v*) in a 250-mL flask, then the mixture was cultivated for 72 h at the same condition as those for precultivation samples. Ultimately, RSF was obtained as the cell-free supernatant after a 6,000 r min^{-1} centrifugation for 10 min applied on the SLWM broth. The control sample was conducted with the same procedure but uninoculated.

When a pretreated dewatered sludge was used as a medium to replace the supernatant of pretreated excess sludge, SLWM was prepared by mixing the different masses of dewatered sludge with 130 mL of distilled water and 20 mL of livestock wastewater in a 250-mL Erlenmeyer flask. Subsequently, the pretreatment of 120 °C, 120 °C-Al, and 120 °C-U were carried out in parallel. pH was adjusted to 7.0 before fermentations.

Determination of flocculating rate

To measure the flocculating rates of bioflocculants in this study, the flocculating test was done as follows using kaolin clay suspension (Kurane et al. 1986a, b).

A 0.40-g amount of kaolin clay was suspended in 100 mL of deionized water, and 2.0 mL of the bioflocculant RSF and 5.0 mL of $CaCl_2$ (1 %) were added into the kaolin suspension. The pH of was adjusted to 7.0±0.1 using 1.0-M hydrochloric acid and sodium hydroxide solution if not specified. The mixture was firstly stirred at 180 r min^{-1} for 1 min then 130 r min^{-1} for 4 min using a six-breaker jar tester (ZR4-6, SY.36-ZR4-6, Shenzhen Zhongrun Company, China) and held still for 5 min. The absorbance of the supernatant and the blank control (i. e., without the addition of the bioflocculant) was measured at 550 nm (denoted as A and A_0, respectively) using a spectrophotometer. The flocculating rate (%) was defined and calculated by Eq. (1):

$$\text{Flocculating rate}\,(\%) = {}^{(A_0-A)}\Big/_{A_0} \times 100 \qquad (1)$$

Analyses of Fourier transform infrared spectrometry (FTIR)

FTIR spectrum of RSF was recorded using FTIR spectrophotometer (VARIAN 3100 FTIR) with the frequency range of 400–4,000 cm^{-1}. Solid samples were milled with potassium bromide (KBr) to form a very fine powder. Spectra were obtained at 4 cm^{-1} resolution and at room temperature.

RSM experimental design

In this study, RSM was used to determine the influence of three independent variables and the optimum conditions of the decolorization of dye wastewater. The dosages of RSF (x_1), $CuSO_4$ (x_2), and pH (x_3) were chosen as three independent variables. Each variable was coded at three levels: −1, 0, and 1 according to the following equation:

$$X_i = (x_i - x_0)/\Delta x_i \qquad (2)$$

where x_i is the uncoded value of the *i*th independent variable, X_i is the real value of the *i*th independent variable, x_0 is the real value of the *i*th independent variable at the center point, and Δx_i is the step change of the *i*th independent variable.

The response variable was fitted by a second-order model in the form of a quadratic polynomial equation:

$$y = \beta_0 + \sum_{i=1}^{m} \beta_i x_i + \sum_{i<j}^{m} \beta_{ij} x_i x_j + \sum_{i=1}^{m} \beta_{ii} x_i^2 \qquad (3)$$

where y is the response variable to be modeled, x_i and x_j are independent variables which determine y; β_0, β_i, and β_{ii} are the offset terms, the *i*th linear coefficient, the quadratic coefficient, and the *ij*th interaction coefficient, respectively. β_{ij} is the term that reflects the interaction between x_i and x_j. The actual design ran by the statistic software, Design-Expert 7.1.3 (Stat-Ease Inc., USA), is presented in Table S3 (in ESM).

Results

Bioflocculant preparation conditions

Optimization of initial culture pH and inoculum size

Initial culture pH could determine the charge of molecules at the surface of the cells (the zeta potential of the cells) which could reflect the net surface charge and the oxidation-reduction potential of the cells, thus affecting nutrient absorption and enzymatic reactions (Salehizadeh and Shojaosadati 2001). Bioflocculating efficiency of the bioflocculant yielded at pH 3.0–10.0 in SLWM conducted with thermal pretreatment was investigated. After fermentation for 3 days, flocculating rates were assayed and are shown in Fig. 1. The results indicate that initial pH 7.0 was the optimum for the production of bioflocculant, and this agreed with the finding of Lachhwani (2009) that neutral pH is more suitable for *R. erythropolis* fermentation.

Inoculum size (%) also played a significant role in bioflocculant efficiency (Fig. 1). The maximum flocculating rate of 89.9 % was achieved when the inoculum size was 2.0 % (*v*/*v*).

Effects of broth ratio on bioflocculating efficiency

The flocculating rates at different broth ratios of sludge/livestock wastewater in SLWM were investigated. The highest flocculating rate was observed when the broth ratio was 7.0 within the range of 0.125 to 10 (Table 1). At broth ratio 7.0, the carbon-nitrogen ratio (C/N) of SLWM was in the best condition for the production of bioflocculant by *R. erythropolis*.

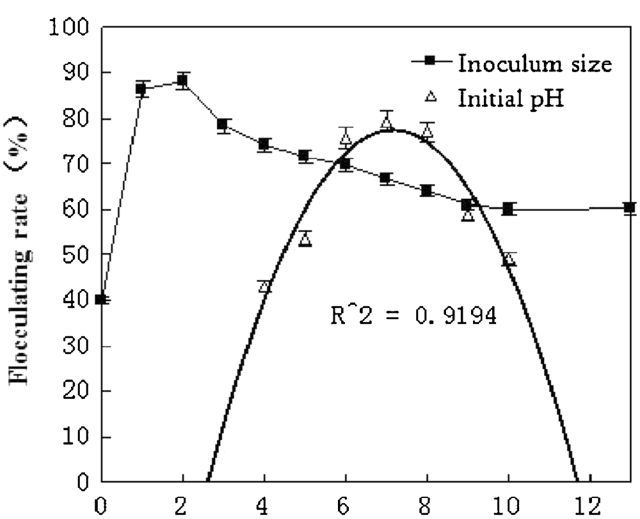

Fig. 1 Effects of culture pH and inoculum size (%) on flocculating efficiency

Optimization of sludge concentration in a cultural medium using dewatered sludge

The feasibility of bioflocculant preparation was investigated using dewatered sludge as a substitute for the supernatant of excess sludge. This experiment was carried out with three different pretreatments at various dewatered sludge concentrations. It can be seen from Fig. 2 that the optimal dewatered sludge concentration laid within 70–110 g L^{-1} for all the three pretreatment groups, and the bioflocculating efficiency dropped dramatically when sludge concentration was higher than 150 g L^{-1}.

Figure 2 also shows that the combined pretreatment of 120 °C-Al was more effective in producing bioflocculant than the single 120 °C pretreatment or combined pretreatment of 120 °C-U when dewatered sludge were used to replace excess sludge.

Effects of additional inorganic salts and nutrients on flocculating efficiency

In five groups of 70 °C-Al-treated SLWM (ratio 7.0), sucrose, NaH_2PO_4, NaCl, $CaCl_2$, and $MgSO_4$ of dosage 10, 0.5, 0.5, 0.5, and 0.2 g L^{-1} were added, respectively. Their flocculating rates at different incubation periods are shown in Fig. 3. It turned out that Na^+, Ca^{2+}, and carbon resource (sucrose) in the treated SLWM were enough for fermentation, whereas phosphorus resources were not. Furthermore, additional Na^+, Ca^{2+}, and carbon resource even led to decreases in flocculating rates.

In terms of the period of incubation, almost every group increased greatly in day 1 and reached its maximum flocculating rate on day 3.

Optimization of pretreatments with SLWM

Effects of different pretreatments on bioflocculating efficiency

In the experiment with ultrasound pretreatment, excess sludge was pretreated for 10 min with 80 W ultrasound (Weijie et al. 2009) at pH 7.0±0.1, since a low frequency and neutral pH condition can promote boifloccuulant extraction (Zhiqiang et al. 2013).

As expected, Table 2 indicates that most of these pretreatments were effective in sludge cell disruption. It was confirmed by More et al.'s (2012) finding that, to some extent, any kind of pretreatment could lead to bacterial cell lysis in

Table 1 Effect of the different ratios of SLWM on bioflocculating efficiency

Ratio (*v/v*)	No. of sludge	0.125	0.17	1	2	3	4
Flocculating rate (%)	10.1	36.3	51.3	63.1	65.2	67.2	69.9
Ratio (*v/v*)	5	6	7	8	9	10	No livestock wastewater
Flocculating rate (%)	75.2	80.3	84.9	72.4	72.2	72.1	68.8

sludge to release soluble organics and nitrogenous compounds into the liquid phase. As in Table 2, the maximum flocculating rate was observed in the thermal-alkaline pretreatments groups.

Moreover, after being applied with 120 °C thermal, microwave, and ultrasonic pretreatment individually, each SLWM sample showed a slight decrease of pH from 7.07 to 6.81, from 7.07 to 6.98, and from 7.03 to 7.00, respectively.

As for combined pretreatments, most of them were not as effective as single pretreatments. It was observed that the combined pretreatments of thermal treatment led to noticeable drops in flocculating rate. Among them, the group of 120 °C-M showed the most remarkable decrease, with a flocculating rate of 56.3 % compared with 77.2 % for the group of 120 °C. But combined pretreatment of thermal with alkaline was an exception, its flocculating rate even rose by 7 % when compared with the group of 120 °C.

Optimization of single thermal pretreatments

Thermal pretreatment is preferred for the improvement of stabilization and could be realized at a relatively low cost and especially at low temperatures (Skiadas et al. 2005). Besides, a high temperature (130–180 °C) would lead to the formation of complex organic matters, which are hardly biodegradable (Tanaka et al. 1997). Hence, as in Fig. S1 (in ESM), in this study, single thermal pretreatments within 60–120 °C were investigated, with a processing time of 30 min. Results showed that the maximum flocculating rate of 82.5 % was observed using 70 °C thermal pretreatment. During 60–120 °C, flocculating rates above 65 % could be achieved. Flocculating rate beyond 75 % was not found when the temperature was below 70 °C.

Therefore, at 70 °C, different processing times were studied to specify the optimal conditions for single thermal pretreatment. As in Fig. S2 (in ESM), flocculating rate increased from 38 to 85 % as the processing time prolonged from 0 to 50 min, and the increasing rate slowed down gradually when it was beyond 30 min. Hence, 30–40 min was the relatively optimal treating time for sludge pretreatment, considering time and expense.

Characterizations and composition of RSF

Separation and purification of RSF

After a 72-h fermentation, the isolation of bioflocculant was carried out by centrifuging broth at 6,000 r min^{-1} at 4 °C for 10 min. The supernatant was collected. To remove insoluble substances, extraction was employed by adding two volumes of acetone or cold ethanol into the supernatant then centrifuged again (the sediments were collected), subsequently, washed with ether then vacuum-dried to obtain the white powdery purified bioflocculant (PRSF). It turned out that 1.6 g L^{-1} of the PRSF was obtained using ethanol for extraction, while using acetone, only 0.7 g L^{-1} was harvested. Along

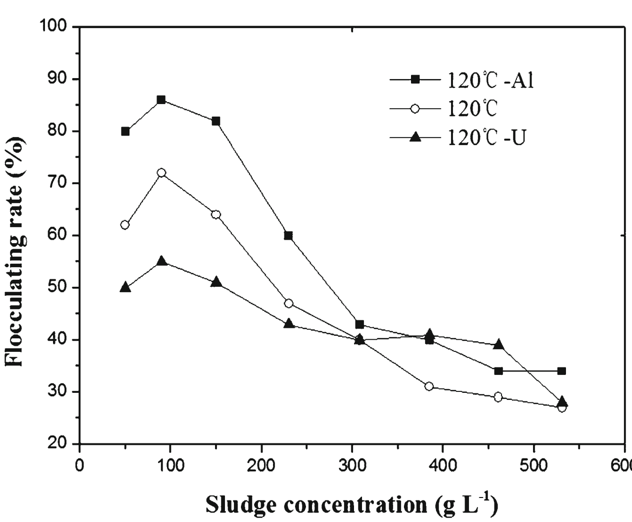

Fig. 2 Effects of sludge concentration and pretreatments on flocculating efficiency

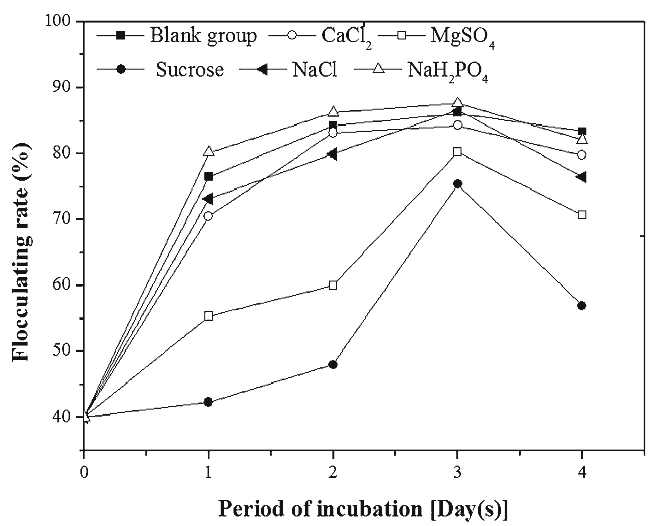

Fig. 3 Effects of additional inorganic salts and nutrition on flocculating rate

Table 2 Effects of different pretreatment of excess sludge on flocculating efficiency

Single pretreatment	70 °C	120 °C	Al	M	U	Ac	Blank
Flocculating rate (%)	81.1	77.2	64.8	53.5	18.4	15.0	3.0
Combined pretreatment	70 °C-Al	70 °C-Ac	70 °C-U	120 °C-Al	120 °C-Ac	120 °C-U	120 °C-M
Flocculating rate (%)	83.0	68.5	55.0	84.0	75.6	58.8	56.3

with an obviously bigger floc size, the flocculating rate of PRSF reached up to 94.5 % after the flocculating suspension was settled for only 1 min; however, it was 83.6 % using the unpurified RSF and settled for 5 min.

Flocculating efficiency of RSF at different flocculating pH

In comparison of the sludge supernatant group inoculated with the strain *R. erythropolis*, and the other one not, the former showed an improvement of more than 40 % in flocculating rate under neutral pH (Fig. 4). In addition, at pH 2.0, only a flocculating rate of about 30 % could be found using NOC-1, while it was higher than 95 % using RSF. It was interesting to find that the sediment of treated SLWM (STS) also showed high flocculating efficiency within a wide pH range from 2.0 to 12.0 when compared with untreated SLWM sediment.

To gain more insights into the characterizations and compositions of the RSF, its thermal stability and FITR spectrum were analyzed and recorded.

Thermal stability of RSF

With regard to the thermal stability of RSF, six crude RSF samples were placed at 4, 25, 40, 60, and 80 °C for 30 min, respectively, then their flocculating rates were determined using the flocculating test. After three replicates of these experiments, it turned out that flocculating rates of RSF only dropped slightly as the processing temperature increased from 4 to 80 °C, and a residual flocculating rate higher than 80 % could still be achieved using the RSF heated at 80 °C for 30 min.

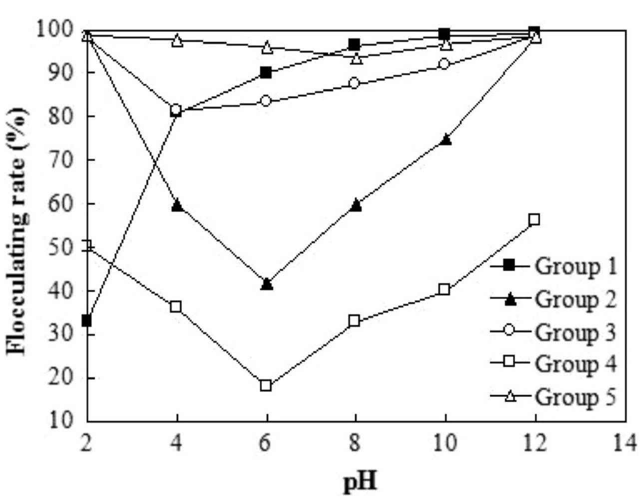

Fig. 4 Flocculating rate of different flocculants varied with pH. *1* NOC-1; *2* uninoculated SLWM supernatant; *3* inoculated SLWM supernatant; *4* untreated SLWM sediment; *5* treated SLWM sediment

Fourier transform infrared (FTIR) study and component analysis of RSF

The FTIR spectrum of the RSF produced with the thermal-alkaline pretreatment was recorded and shown in Fig. 5. As it can be seen in Fig. 5, the infrared spectrum of RSF was similar to that of Sun et al.'s (2012) research, in which they investigated the functional groups of a polysaccharide flocculant made from the sludge extracellular polymeric substance. Like in Fig. 5, their infrared spectrum displayed a broad stretching peak at around 3,436 cm^{-1} from 3,100 to 3,700 cm^{-1}, which can be assigned to the stretching vibration of –OH and –NH groups. Same bands within 1,000–1,200 cm^{-1} were also found in their results, indicating the characteristic absorption peaks of saccharides. Besides, the peak at 1,650 cm^{-1} in their infrared spectrum may be assigned to the –CO stretching in $–CONH_2$ group which is in accordance with Fig. 5 as well. Based on these, they concluded that polysaccharide were the main components of their purified bioflocculant. Furthermore, FTIR spectrum of the RSF in this study also exhibited several absorption peaks that were the same with that of chitosan (an alkaline polysaccharide polymer) (Cai et al. 2010).

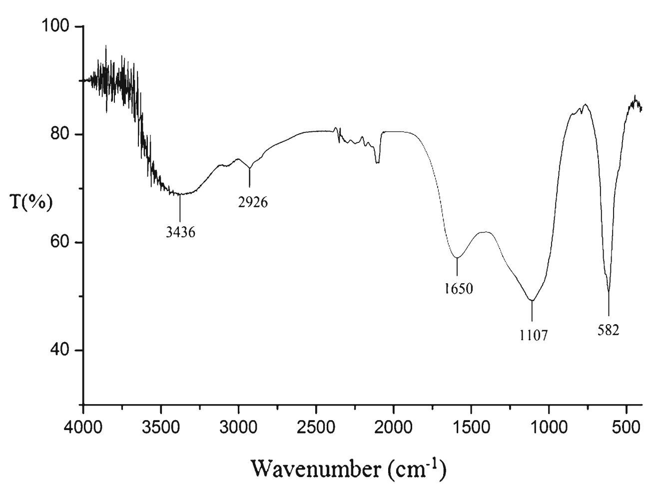

Fig. 5 Fourier transform infrared spectroscopy (FTIR) of RSF

Preliminary applications in simulated wastewater treatment

Simulated dyestuffs wastewater treatment

In order to analyze the specific flocculating properties of RSF in dye wastewater treatment, this experiment was conducted using simulated dyestuff wastewater containing three widely used dyes: monoazo-dye reactive brilliant red X-3B solution (Red X-3B), bis-azo direct sky blue 5B solution (Blue 5B), and disperse yellow E-3G solution (Yellow E-3G), respectively.

Simulated dye wastewater 1 g L^{-1} was prepared, and the decolorization rate was determined by the absorbance change at the maximum absorption wavelength of each dye solution, 526 nm for Red X-3B, 614 nm for Blue 5B, and 381 nm for Yellow E-3G.

After primary decolorization experiments under various pH, the optimal pH value for decolorization was specified as pH 2.5 for RSF and STS and pH 11.5 for NOC-1, respectively. Decolorization experimental results are presented in Table 3. As in Table 3, high decolorization rates were all observed for Blue 5B and Yellow E-3G using these three bioflocculants. When RSF was applied in these three simulated dye wastewaters, the highest decolorization (86.6 %) was found in direct Blue 5B group, which was similar to that of STS. On the contrary, it was in disperse Yellow E-3G simulated dye wastewater in terms of NOC-1.

Performances of RSF and STS on activated sludge settleability

In order to study the application of RSF (supernatant group) and STS (sediment group) on activated sludge settleability, to 100-mL activated sludge suspension, 4 % (m/m) RSF and STS were added. The result of sedimentation test is reported in Fig. S3 (in ESM). Data in Fig. S3 indicated that RSF and STS were all efficient in accelerating the precipitation process. The sludge volume fraction dropped from 100 to 40 % within 2.5 min, while it took over 4 min for the blank group.

Performance in flocculating activated carbon suspension

Two parallel flocculating tests were carried out with 1 g L^{-1} activated carbon suspension instead of kaolin clay suspension.

Table 3 Flocculating efficiency of NOC-1, RSF, and STS in simulated dye wastewater

Decoloration rate (%)	Red X-3B	Blue 5B	Yellow E-3G
NOC-1	16.7	62.4	85.7
RSF	23.0	86.6	73.8
STS	31.4	83.3	68.2

Compared with blank, the average flocculating rate of RSF and STS were 71.3 and 97.6 %, showing that these two bioflocculants could significantly accelerate the sedimentation velocity of activated carbon particles.

Optimization of dye wastewater decolorization with RSM

The application of RSM yields the following regression equation, an empirical relationship between decolorization rate (y_1) and the other factors (x_1–x_3), as given in the following equation:

$$\begin{aligned} Y_1 = {} & 88.66 + 9.00x_1 + 3.90x_2 + 6.50x_3 - 5.13x_1x_2 \\ & -11.58x_1x_3 + 3.58x_2x_3 - 16.39x_1^2 - 1.24x_2^2 - 7.54x_3^2 \end{aligned} \quad (4)$$

Statistical testing of the model was checked by Fisher's statistical test for the analysis of variance (ANOVA). The significance testing for the coefficient of the equation whose variables are in terms of coded factors was listed in Table 4. The results of ANOVA revealed that the second-order equation fitted well, since the Model F-Value (the ratio of mean square due to regression to mean square to real error) is 9.96 which implies that the model is significant. There is only a 0.31 % chance that a "Model F-Value" this large could occur due to noise. The value of the correlation coefficient (R^2= 0.9275) indicates that only 7.25 % of the total variation could not be explained by the empirical model (Ahamad et al. 2005). The value of "Prob>F"=0.0031 less than 0.0500 means that model terms are significant. The coefficient of variation (CV) as a measure of reproducibility of the models is the ratio of the standard error of estimate to the mean value of observed response expressed as a percentage. The CV of the model was calculated as 7.85 %. As a general rule, a model can be considered reasonably reproducible if its CV is less than 10 %.

With decolorization rate as the response variable, the three-dimensional surface plots (Fig. 6) were drawn to illustrate the

Table 4 Significance of the quadratic model coefficient of decolorization rate

Independent variables	Regression coefficients	Degrees of freedom	Standard error	Prob>F
x_1	9.00	1	2.13	0.0039
x_2	3.90	1	2.13	0.1100
x_3	6.50	1	2.13	0.0186
x_1x_2	−5.13	1	3.01	0.1329
x_1x_3	−11.58	1	3.01	0.0064
x_2x_3	3.58	1	3.01	0.2744
x_{11}	−16.39	1	2.94	0.0008
x_{22}	−1.24	1	2.94	0.6851
x_{33}	−7.54	1	2.94	0.0372

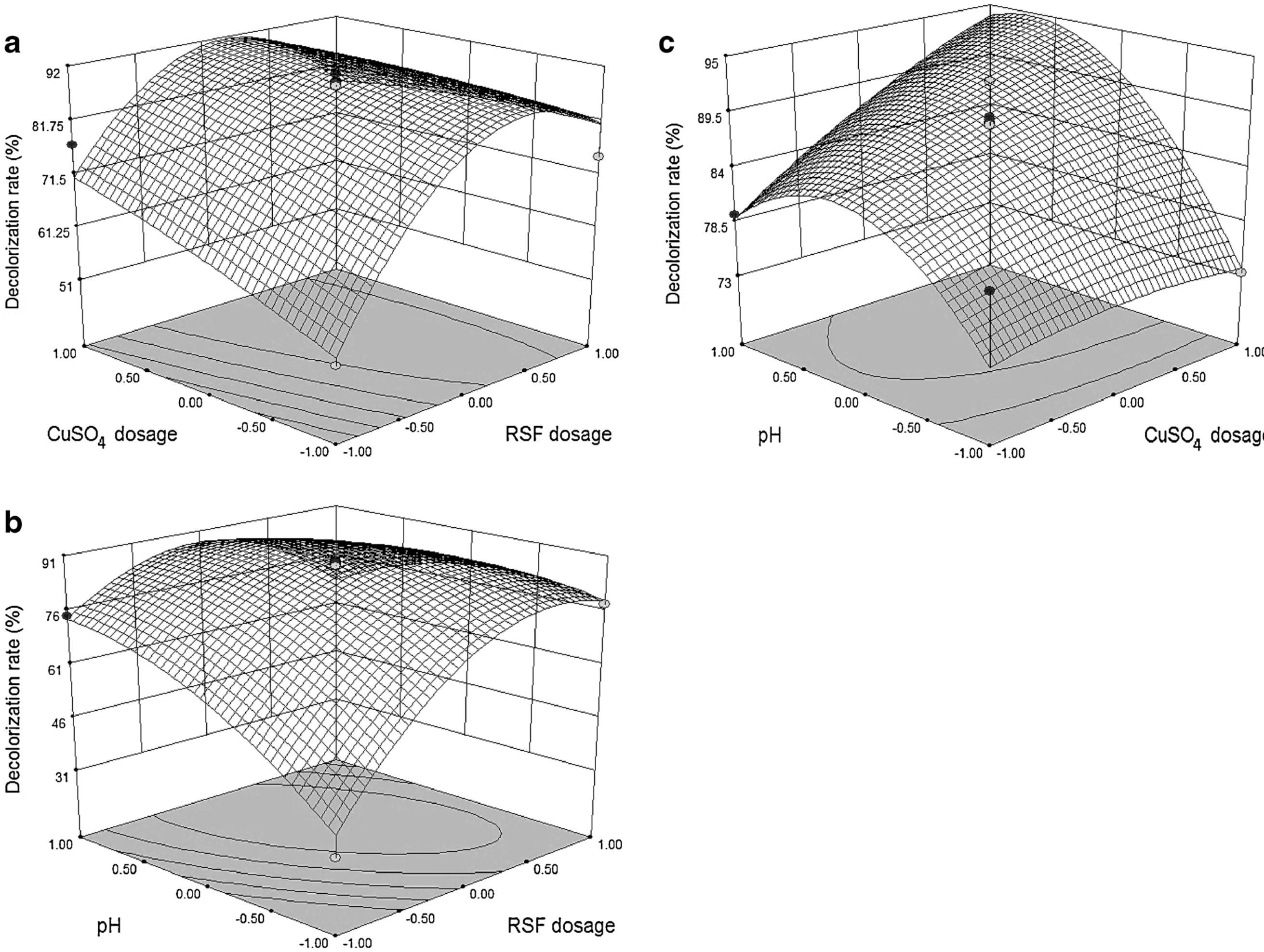

Fig. 6 Surface graphs of decolorization rate showing the effect of variables **a** $CuSO_4$ dosage-RSF dosage, **b** pH-RSF dosage, and **c** pH-$CuSO_4$ dosage

main and interactive effects of the independent variables on the dependent one. These graphs were obtained by varying the remaining two variables and predicting the decolorization rate.

The response surface reveals that the optimal conditions were exactly located inside the design boundary (Fig. 6). The corresponding two-dimensional contours indicate that there were considerable interactive effects on decolorization between RSF dosage and $CuSO_4$ dosage, pH, and RSF as well as pH and $CuSO_4$.

From Fig. 6a, in the region where pH was 7.0, with the RSF dosage of 30–65 mg/L and $CuSO_4$ dosage of 20–70 mg/L, the decolorization rate of 85.1 % could be reached. After the quadratic fitting empirical model optimization, when pH was 7.0, the optimal conditions for decolorization was SF dosage of 40 mg/L and $CuSO_4$ dosage of 40 mg/L, under which up to 88.5 % decolorization rate could be achieved.

As shown in Fig. 6, when compared to each single use of RSF or $CuSO_4$, the RSF-$CuSO_4$ composite flocculant performed better in decolorization and adaptation to pH. $CuSO_4$, if used alone, showed a high flocculating ability under an alkaline condition rather than acid condition. The optimal decolorization conditions were calculated as the pH of 11, $CuSO_4$ dosage of 80 mg/L, and RSF dosage of 40 mg/L, and 93.9 % decolorization rate of RSF could be reached (Fig. 6b). While, when only 10 mg/L $CuSO_4$ were added, the decolorization rate was 72.5 %. In Fig. 6c, with RSF dosage increased from 10 to 80 mg/L added, the decolorization at pH 3.0 increased by 50 % or so. The RSM analyses also revealed that with RSF 25 mg/L and $CuSO_4$ 25 mg/L added, the same decolorization result of 86 % as that of $CuSO_4$ 80 mg/L could be obtained. Hence, RSF could reduce the dosage of $CuSO_4$ to a huge extent.

Discussion

This study obtained a new polysaccharide bioflocculant which possessed some characteristics of both bioflocculant NOC-1 (produced by *R. erythropolis* in a standard medium) and sludge flocculant. When the sludge were applied with thermal-alkaline pretreatment, a high flocculating rate above 80 % could be achieved within a wide pH range of 2–12. The method in this research could not only produce bioflocculant by fermentation but also make sludge particles into absorbent and flocculants at the same time. Therefore, both the reuse of the supernatant and sediments of excess sludge could be realized.

Excess sludge is rich in carbon source, and livestock wastewater is high in nitrogen source. The C/N in excess sludge might not meet the optimal condition for the strain (ACCC 10543) to yield bioflocculant. SLWM of various broth ratios (excess sludge: livestock wastewater (*v*/*v*)) could provide the fermentation with different C/N ratios; furthermore, carbon and nitrogen sources were well documented to have significant effects on bioflocculant production (Salehizadeh and Shojaosadati 2001; Zhang et al. 2002). High C/N ratio could stimulate cell growth but would lead to low flocculating rate, while low C/N ratio would result in a decrease of pH in culture medium. Hence, the flocculating rate reached its maximum at the broth ratio of 7.0 (Table 1).

The research on the effects of dewatered sludge concentration on the ESF production proved that it was feasible to prepare this bioflocculant by substituting dewatered sludge for excess sludge. When dewatered sludge was used, the optimal sludge concentration lied within 77–110 g L^{-1} (Fig. 2). On one hand, a high solid concentration can increase the viscosity of the broths having non-Newtonian, pseudoplastic flow behavior. High viscosity also exerts a negative impact on the mass transfer properties of the broth, especially the gas-liquid mass transfer rate. In addition, the mass transfer limitation and an inadequate supply of oxygen to microorganisms because of poor mixing at high sludge concentrations also attributed to a lower concentration of extracellular polymeric substance at the end of fermentation (Maria 2004). Therefore, oxygen was not sufficient in sludge broth for the strain to grow and yield bioflocculant. On the other hand, too much nutrition along with high sludge concentrations also contribute to low flocculating rates.

As for additional nutrients (Fig. 3), even though total-phosphorus (T-P) was rich in excess sludge, its available-phosphorus (A-P) was only of a small fraction. In addition, phosphorus is one of the most essential nutritional elements for bacterial growth. Hence, additional phosphorus stimulated bioflocculant production. But excess sludge consisted of too much Mg^{2+} which might inhibit bioflocculant production. Therefore, additional Mg^{2+} resulted in the decrease of flocculating rates in this experiment. Sucrose, added as a carbon resource, increased the ratio of C/N, and as stated earlier, a high ratio of C/N could stimulate cell growth but lead to a low flocculating rate. This also, to some extent, confirmed the results above that the optimal broth ratio was 7.0 considering the C/N ratio.

In terms of thermal pretreatments, flocculating rate beyond 70 % was not found at a processing temperature below 70 °C. If the processing temperature is too low, other bacteria substantially survived in SLWM might inhibit the breed of the fermentation strain ACCC 10543. Moreover, when the processing temperature was below 70 °C, cell lysis in sludge also was not enough to provide the strain with sufficient nutrients.

When thermal-alkaline combined pretreatment was applied, the flocculating rate rose was at least 9 % higher than other pretreatments (Table 2). It might be due to the fact that sludge solubilization increased considerably in alkaline pretreatment, however, to a lesser extent in acid pretreatment and ultrasonic pretreatment. Chen et al. (2007) reported that under acid condition, SCOD (soluble COD) and other water soluble carbohydrate with a low molecular weight released by cells in sludge was less than that under alkaline condition. In addition, it was also reported that, to some extent, the capacity of HCl in inducing cell lysis is lower than that of NaOH (Sun et al. 2012). Moreover, both COD solubilization and total solid elimination rates increased with NaOH added: the COD solubilization reached up to 63 %, and the observed COD solubilization was mainly due to protein hydrolysis which was directly linked to pH variations. Moreover, heating emphasized these effects of pH on COD solubilization (Penaud et al. 1999). In addition, Zhang et al. (2002) found that sludge disintegration degree (DDCOD) in combined treatment was higher than the sum of DDCOD in single-alkaline and single high-pressure homogenization treatments.

During pretreatments, slight declines of pH in SLWM might be the results of the pretreatments inducing further the hydrolysis of some macromolecular organic matter (protein and carbohydrate for example), producing acidoid such as fatty acid which would decrease pH.

At pH 2.0, only 30 % flocculating rates were found using the bioflocculant (NOC-1) yielded by the same strain which was cultured in a standard medium, while higher than 95 % flocculating rates could be reached using RSF. Among previous studies, almost no bioflocculants have been reported to have significant flocculating activities at a pH below 3 or above 8 (Salehizadeh and Shojaosadati 2001). Even though these two bioflocculants were produced by the same strain, their productions' characteristics would not be the same when the cultivation environments were different. This mechanism might be similar to the process of domestication or chemical mutagenesis.

Besides, in this study, the STS also showed high flocculating efficiency within a wide pH range from 2.0 to 12.0. This may be because special environments such as high temperature and strong alkaline conditions could modify sludge particles. On one hand, they could induce the formation of high polymer matters of flocculating ability within cell debris and sludge particles. On the other hand, when sludge cells were cracked, the pore constructions in the sludge particles were also improved, which enhanced the flocculating ability of sludge particles. So, it can be concluded that the method of producing bioflocculant from strain *R. erythropolis* by using the nutrition in sludge and livestock suspension could also make sludge particles into flocculant as well. In that case, to a certain extent, the reuse of both of the supernatant and the sediments of the cultivation broth could be realized.

When RSF was applied in simulated dyestuff wastewater, as acid dye, Red X-3B's inert chemical structure and the attached phenyl, methyl, nitro, and sulfonate groups could account for its low decolorization results (Buthelezi et al. 2012). Moreover, bioflocculant was more efficient for treating suspended particles and colloids.

Discussion of components of RSF

It has been reported that Gao et al. (2009) purified polysaccharide flocculant with ethanol, while acetone was used for protein flocculant purification. So, more PRSF was obtained using ethanol than acetone for extraction, indicating that components of the bioflocculant were mainly polysaccharide rather than protein.

Performance of RSF in simulated dyestuff wastewater shown in Table 3 (the highest decolorization was found in direct Blue 5B simulated dye wastewater using RSF, which was similar to that of STS; however, it was disperse Yellow E-3G for NOC-1) also confirmed again that the bioflocculant prepared in SLWM were more different from NOC-1 in properties than from STS.

A residual flocculating rate of 80 % could still be achieved after heating the RSF at 80 °C for 30 min, and these thermostable characteristics suggested that active ingredients in RSF were mainly polysaccharide rather than protein. Moreover, it was reported that bioflocculant protein or peptide backbone in structure were usually sensitive to heat, while those consist of polysaccharide were thermostable (Gong et al. 2008).

Furthermore, on one hand, the typical functional groups of polysaccharide, namely, hydroxyl (–OH), amino ($-NH_2$), and acylamino ($-CONH_2$) groups were all observed in our FTIR spectrum of RSF (Fig. 5), revealing that RSF mainly contains polysaccharide. On the other hand, the point stated above that the first inoculation in sterilized SLWM could domesticate the strain was also confirmed; in other words, the bacteria *R. erythropolis* could adapt themselves to the SLWM's environment and ultimately yield bioflocculant RSF which shared some ingredients and properties with sludge.

But RSF in this study was somehow different from the flocculant produced by Sun et al. (2012) (who prepared flocculant directly from sludge cells). First of all, in terms of the pH adaptability, results in Fig. 4 showed that at least 80 % flocculating rate could be reached using RSF within the whole flocculating pH range, which is different from Sun's flocculant. Furthermore, a flocculating rate up to 90.1 % for RSF was observed at flocculating pH 3.0 compared to only 58.9 % for Sun's flocculant. Besides, even though the FTIR spectrum of RSF and that of Sun et al. (2012) did share some functional groups, the former exhibited an absorption at 582 cm^{-1}, corresponding to the existence of protein, which indicated that the RSF are protein-bound polysaccharides. Including the –OH groups at 3,436 cm^{-1}, the absorption peak at 2,926.40 cm^{-1} was also in accordance with that of NOC-1 (mainly glycoprotein) produced by *R. erythropolis* in a standard culture medium (Yang et al. 2013).

Hence, all in all, FTIR analysis has revealed that RSF possessed the same functional groups as both Sun's sludge flocculant and NOC-1. Generally speaking, RSF are proteoglycan complexes and among which polysaccharides are the majority.

Further chemical analysis was consistent with the results above, evidencing that purified RSF was composed of polysaccharides (91.2 %), protein (7.6 %), and DNA (1.2 %).

These results are different from our previous research (Junyuan et al. 2013) in which bioflocculant was prepared from sludge suspensions. And in that study, bioflocculant showed more properties of proteins than polysaccharides. This might be ascribed to the differences of sludge and livestock wastewater characters, since both the sludge and livestock used in these two researches were obtained from different sewage treatment plants and pig farms at different time, so the components and their concentrations in the fermentation media would not be the same as well. Besides, it was the activated sludge that was used in our former study; however, in the later one, the investigated sludge were excess sludge and dewatered sludge. In addition, in our previous research, both the supernatants and sediments of the sludge were used, while in our present study, only the supernatants of the pretreated sludge were investigated to exclude interference of the sludge particles on bioflocculant preparation and analyses. So the main nutrients attached on the sludge cells might differ from that in the sludge supernatants, and as discussed above, the concentration of sludge particles would exert influences on bacteria growth (Maria 2004). Therefore, different nutrition components and biophysical environments may account for the discrepancy in bioflocculant components.

RSM experiment

When compared to the single use of RSF or $CuSO_4$ in decolorization experiments, the composite flocculant RSF-$CuSO_4$ showed higher decolorization capacity and ability of dealing with different processing pH. In application to actual dye wastewater, RSF could reduce the dosage of $CuSO_4$ to a huge extent. The fact that solutes of RSF and $CuSO_4$ are of opposite charge could account for this phenomenon, and the existence of these two flocculants could stimulate the charge neutralization of each other in dye wastewater. Similarly, flocculant aids such as Ca^{2+} were not needed anymore using the RSF-$CuSO_4$ composite flocculant.

Cost estimate of NOC-1, PAC, $CuSO_4$, and RSF

The cost estimates of different flocculants were conducted when flocculants were applied in kaolin suspension with flocculating pH of 7.0 and flocculating rate of 85 %. Then, according to the media recipes in "Methods" section and the current price of each nutritional component (in dollars per kilogram), sucrose, 2.1; urea, 80.0; yeast powder, 47.7; NaCl, 16.5; K_2HPO_4, 8.8; KH_2PO_4, 5.3; and $MgSO_4$, 11.0, the treatment cost of NOC-1 was added up to 17.2 dollar/*t*. The treatment costs estimated for aluminum polychloride (2.2$/kg) and $CuSO_4$ (7.7$/kg) were 0.35 and 2.31 dollar/*t*, respectively. As for RSF, the material for its preparation (the excess sludge and livestock wastewater) are almost of no cost, and the majority part of its cost exists in the pretreatments of excess sludge and the fermentation process which is also inevitable in traditional bioflocculant preparation. Therefore, the method presented in this study could not only be environmentally friendly in comparison with aluminum polychloride and $CuSO_4$, but also cut the treatment cost to a huge extent compared to traditional bioflocculant NOC-1.

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