

Study on the flocculability of the *Arthrobacter* sp., an actinomycete resuscitated from the VBNC state

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Abstract A bioflocculant with high flocculating activity, LC13-SF, produced by strain LC13^T which was in a viable but nonculturable (VBNC) state, and which was woken up by Rpf (resuscitation promoting factor), was systematically investigated with regard to its fermentation conditions and flocculating activity. The key parameters influencing the bioflocculant LC13-SF were investigated through measuring the optical density at 660 (OD₆₆₀) of the fermentation liquid and the optical density at 550 (OD₅₅₀) of the centrifugal supernatant. The flocculating efficiency and the Zeta potentials were chosen as the response variables for the study of the flocculating activity. The results showed that the optimal conditions for bioflocculant LC13-SF production were a fermentation time of 72 h, an initial pH of 7.0, a fermentation temperature of 30°C and a shaking

speed of 150 r/min. The optimized flocculating process was as follows: a final volume percentage of bioflocculant LC13-SF and 0.5% (w/w) CaCl₂ were 1.5 and 5%, respectively in a 4 g/L Kaolin suspension, and the system pH was adjusted to 8.0. Under these conditions, the flocculating efficiency and the absolute value of the Zeta potential reached 94.83% and 4.37, respectively.

Keywords VBNC · Rpf · LC13-SF · Fermentation condition · Flocculating activity

Introduction

Soils may be the most challenging of natural environments to characterize microorganism. Through comparing the dilution plate counts of culturable microorganisms with direct microscopic counts of total microorganisms, it has been established that only 0.01–4% of microorganisms in soils can be cultured on organic growth media (Faegri et al. 1997; Olsen and Bakken 1987). Most soil bacteria are in a viable but nonculturable (VBNC) state, in which microbial cells are metabolically active but cannot be cultured by current techniques. Therefore, an important topic of research is how to wake up bacteria from such a non-growth state. A major breakthrough in the understanding of VBNC states has arisen from the study of the high G + C gram-positive bacterium, *Micrococcus luteus*, which secretes a small protein called Rpf (resuscitation-promoting factor). Rpf plays a very important role in studying unknown microorganisms, because it can resuscitate the large number of “non-culturable” bacterial groups to become cultivable (Mukamolova et al. 1998).

In our previous study, using MPN (most-probable-number) method (repeat the experiment three times, according to

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MPN table, can determine the total bacterial count of cultivable), Rpf was added as a component of culture system to investigate the VBNC state bacteria in response to Rpf. Through comparing the treatment group (with Rpf) with control group (without Rpf), many bacteria were confirmed in VBNC state, then were isolated by plate isolation method (Ding 2004; Ding et al. 2007, 2009; Li et al. 2007; Ding and Yokota 2010). Nowadays, it is highly anticipated to develop efficient microbial flocculants (MBFs) (Zhang et al. 2007; Huang and Chen 2004) that partly or even completely substitute for the traditional flocculants (Christopher et al. 2006; Tareke et al. 2002). So we selected some of strains which isolated from activated sludge or sewage treatment system to investigate their function of flocculation. Adopting Kaolin suspension as the active evaluation system, a flocculant producing strain LC13^T which the flocculation efficiency was up to 80% was selected. The strain LC13^T (CCTCC 206018^T) was isolated using NY medium which was consisted of nutrient broth 1.6 g, yeast extract 0.5 g, cycloheximide 0.05 g, kabicidin 0.1 g and agar 15 g dissolved in 1 L distilled water with pH adjusted to 7.0, and was identified as a novel species of the genus *Arthrobacter* by its morphological, physiological and genotypic characteristics (Ding et al. 2009). In this study, attention was focused on its flocculating properties. We investigated its fermentation conditions and flocculating activity. Based on the experimental results, we optimized the flocculation characteristics of LC13^T.

Materials and methods

Materials

Kaolin was of chemically pure grade (Sinopharm Chemical China), and its suspension was prepared at the concentration of 4 g/L. The analytically pure grade CaCl₂ (Sinopharm Chemical China) was prepared at the concentration of 0.5% (w/w). The components of the culture and fermentation medium which were purchased from Sanpu Chemical Co. in China were of analytically pure grade.

Bacteria strain and culture media

A. albidus LC13^T (CCTCC 206018^T) was a flocculant-producing strain that was isolated from a filtration substrate made from volcanic rock from Niigata, Japan, using NY medium (Ding et al. 2009). Through comparing the treatment group (with Rpf) with control group (without Rpf), the strain LC13^T was confirmed in VBNC state. The stock culture preserved in 20% (v/v) glycerol solution at -80°C was routinely used as a preinoculum to inoculate a 150-mL flask containing 50 mL of fermentation broth.

Assay of fermentation conditions

The fermentation medium consisted of peptone 5 g, yeast extract 0.5 g, glucose 5 g and NaCl 2.5 g dissolved in 1 L distilled water with the pH adjusted to 7.0 with NaOH (0.1 M) and HCl (0.1 M). All media solutions were sterilized at 121°C for 15 min. This fermentation cultivation process was divided into two stages. In the first stage, a 150-mL flask containing 50 mL fermentation medium was inoculated with 1.0 mL preculture of strain LC13^T and incubated at 30°C in a rotary shaker at 160 r/min for 12 h. In the following stage, the pre-fermentation medium was inoculated into a 150-mL flask according to the same volume ratio used in the fermentation liquid in the first stage. Various conditions of fermentation time (24–120 h), the initial pH (5–9) of the fermentation medium, fermentation temperature (24–34°C) and shaking speed (130–180 r/min) were tested for bioflocculant LC13-SF production. In the meantime, the cell growth was monitored by measuring the optical density (OD) of the fermentation broth at 660 nm (OD₆₆₀) with a spectrophotometer (UV-7504, Shanghai, China). At the end of the cultivation, the obtained fermentation broth was centrifuged (12,000×g, 10 min) to separate the cells. The centrifugal supernatant was the liquid bioflocculant LC13-SF, which was used for the analysis of flocculating efficiency. All experiments were performed in triplicate and the mean average was calculated.

Flocculation experiments

The flocculating efficiency (FE) was evaluated using a previous method with a slight modification, in which a Kaolin suspension was chosen (Zhang et al. 2007; Yokoi et al. 1995). In a 100-mL test tube, 0.5% (w/w) CaCl₂ and bioflocculant LC13-SF were added to 100 mL of Kaolin suspension (4 g/L). The pH of the mixture was adjusted with NaOH (0.1 M) and HCl (0.1 M). After each addition, the reaction was allowed to proceed for 1 min with rapid mixing, then, gravity settlement was allowed to occur via letting the mixture stand for 5 min. The optical density (OD) of the upper phase was measured at 550 nm (OD₅₅₀) with a spectrophotometer (UV-7504, Shanghai, China). A control experiment was prepared by the same procedure except that the bioflocculant LC13-SF was replaced by fresh fermentation medium. Flocculating activity was evaluated by the flocculating efficiency according to the Eq. (1) below. All experiments were performed in triplicate and the mean average was calculated.

$$FE = [(b - a)/a] \times 100\% \quad (1)$$

where *a*, and *b* are the optical densities (OD) of the sample and control experiments, respectively, at 550 nm. The key parameters influencing the flocculation behavior, such as

pH, the dose of bioflocculant LC13-SF and CaCl_2 were systematically investigated by analyzing the Zeta potentials and FE of the flocculation systems.

Results and discussion

Effects of fermentation conditions on bioflocculant LC13-SF production

Bioflocculant production is affected by several factors, such as the constituents of the culture medium and culture conditions (Zhou et al. 2003). The key parameters of the fermentation conditions, such as fermentation time, initial pH, fermentation temperature and shaking speed were investigated with the aim of obtaining the maximum bioflocculant LC13-SF production as well as the optimal cost of cultivation.

Determination of the fermentation time

The initial pH of the fermentation medium, fermentation temperature and shaking speed were respectively fixed at 7.0, 30°C and 160 r/min, respectively. Then, the effect of fermentation time on the bioflocculant LC13-SF production was investigated by varying the time from 24 to 120 h. As can be seen from Fig. 1, the FE increased concomitantly with the increase of the fermentation time, and it went from 26.36% using a fermentation time of 24 h, to 83.16% when 72 h of fermentation time were tested. The fermentation time longer than 72 h did not result in a larger increase in the FE but conversely, it decreased slightly to 71.05% with a fermentation time of 120 h. The maximum production of bioflocculant LC13-SF was achieved at 72 h, but the cell growth reached its peak at 48 h. However, after 96 h, both the flocculating efficiency and the cell growth decreased. Therefore, 72 h was chosen as the optimal fermentation time.

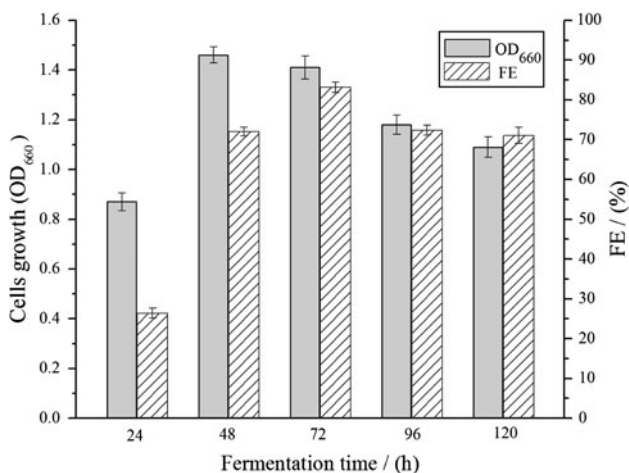


Fig. 1 Effect of fermentation time on bioflocculant LC13-SF production

These phenomena suggest that some LC13-SF bioflocculants were released in the post-fermentation period. In a previous study, bioflocculants produced by *Bacillus licheniformis* were also collected in the latter logarithmic growth phase and the early stationary phase (Shin et al. 2001). However, the results are discrepant with those of some other reports: for example, the bioflocculant production of *Enterobacter aerogenes* occurred in parallel with cell growth, concomitant with its maximum flocculating activity (Lu et al. 2005).

Determination of the initial pH of the fermentation medium

With other parameters fixed at the central level, the variation of the initial pH of the fermentation medium among 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0 was designed to measure the influence of initial pH on bioflocculant LC13-SF production. The terminal pH was also investigated. As shown in Table 1, the results showed that the FE of bioflocculant LC13-SF reached a peak when pH was 7.0. At higher pH levels, the FE in the system decreased, but the cell growth increased slightly until pH 7.5. The terminal pH increased when the initial pH of the fermentation medium was increased. Thus, when the initial fermentation pH was 9.0, the terminal pH reached 5.21.

The initial pH of the fermentation medium determines both the electric charge of the cells and the oxidation–reduction potential which, in turn, can affect nutrient absorption and enzymatic reaction. For the LC13^T strain, bioflocculant LC13-SF was possibly produced at maximum levels under neutral conditions. Additionally, within a certain range, an initial alkaline condition can stimulate cell growth, and the terminal pH has a proportionally direct relationship with initial pH of the fermentation medium.

Determination of the fermentation temperature

The effect of the fermentation temperature on the FE and cell growth was investigated by fixing other parameters at

Table 1 Effect of initial pH of fermentation medium on bioflocculant LC13-SF production (mean ± SE)

Initial pH	Terminal pH	Cells growth (OD ₆₆₀)	FE (%)
5.0	3.27 ± 0.03	0.32 ± 0.03	52.04 ± 1.02
6.0	3.89 ± 0.04	0.63 ± 0.04	78.93 ± 1.47
6.5	3.95 ± 0.02	0.91 ± 0.03	80.06 ± 1.06
7.0	3.98 ± 0.04	1.25 ± 0.03	82.47 ± 1.28
7.5	4.12 ± 0.05	1.28 ± 0.04	77.52 ± 1.73
8.0	4.87 ± 0.02	1.21 ± 0.05	70.13 ± 1.74
9.0	5.21 ± 0.02	1.17 ± 0.04	43.19 ± 1.49

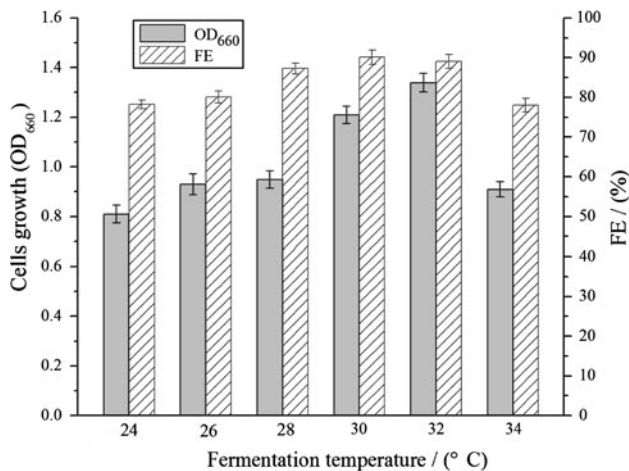


Fig. 2 Effect of fermentation temperature on bioflocculant LC13-SF production

the central level. As can be seen from Fig. 2, when the fermentation temperature was 30°C, the FE of bioflocculant LC13-SF rose to 90.1%, which was the optimal level. However, the cell growth continued to increase when the FE was already starting to decrease, at the temperature of 32°C, OD₆₆₀ reached a maximum of 1.34. For the best FE, 30°C was used in the following studies.

The metabolism of microorganisms has a direct relationship with the culture temperature (Zhang et al. 2007; Salehizadeh and Shojaosadati 2002). A lower fermentation temperature might make the LC13^T strain hibernate partially, and its enzyme system for bioflocculant LC13-SF production may not be activated completely. On the other hand, a higher fermentation temperature might have an adverse effect on the nucleic acid and the enzyme system of the LC13^T strain, leading to the low flocculating efficiency.

Determination of the shaking speed

Various shaking speeds of 130, 140, 150, 160, 170 and 180 r/min were investigated by fixing the fermentation time, initial pH and fermentation temperature as 72 h, 7.0 and 30°C, respectively. The FE and cell growth change at the different shaking speeds were shown in Fig. 3. The maximum FE was obtained when a shaking speed of 150 r/min was used, but the cell growth continued to increase with the increase of the shaking speed until a shaking speed of 160 r/min was reached.

The shaking speed determines the concentration of dissolved oxygen, which can also affect the nutrient absorption and enzymatic reaction of microorganisms (Salehizadeh and Shojaosadati 2001). The results suggest that a shaking speed either higher or lower than the optimal speed caused a decrease of flocculating efficiency in the flocculation system.

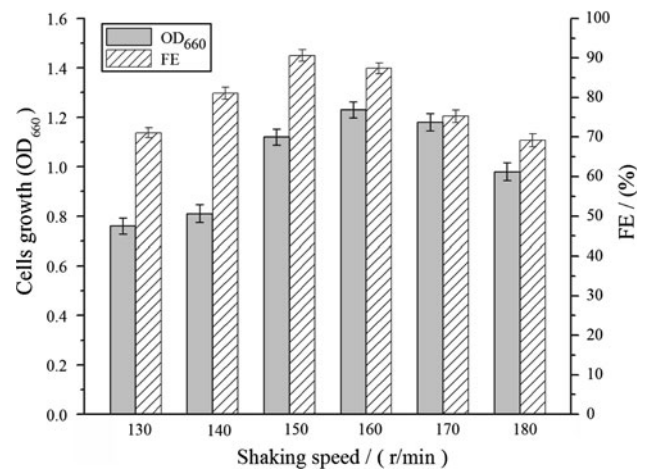


Fig. 3 Effect of shaking speed on bioflocculant LC13-SF production

On the basis of the above experimental results, the optimal conditions for bioflocculant LC13-SF production were: (a) a fermentation time of 72 h; (b) an initial pH of 7.0; (c) a fermentation temperature was 30°C; and (d) a shaking speed was 150 r/min. Under these conditions, the flocculating efficiency reached 90.60%.

Experimental results of flocculating activity

The effect of pH on the flocculation behavior

Volume percentage of bioflocculant LC13-SF and 0.5% (w/w) CaCl₂ were 2 and 5%, respectively in a 4 g/L Kaolin suspension. Then, the system pH was adjusted to 5.0, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0, and the flocculating activity was determined by estimating the FE and Zeta potential. As shown in Fig. 4, when the pH was 8.0, the FE reached a peak (92.5%). Under the weak alkaline condition, the

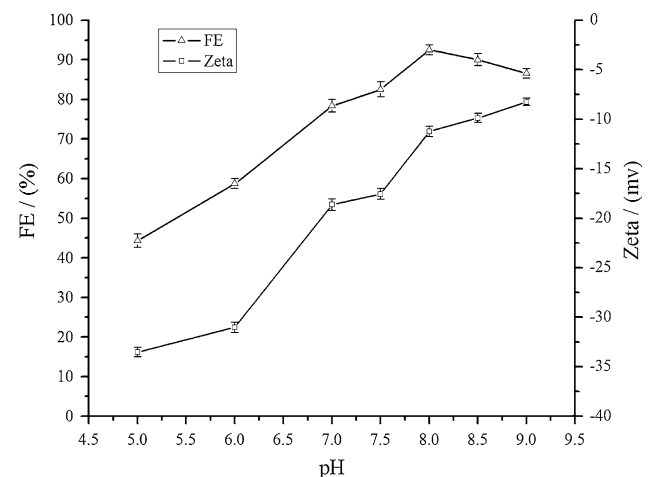


Fig. 4 Effect of pH on the flocculating efficiency of bioflocculant LC13-SF

absolute value of the Zeta potential sharply decreased, and with the increasing of the pH, the Zeta potential grew close to zero. However, the flocculation of the system was changed only slightly and even deteriorated. Furthermore, the weak alkaline condition can save large amounts of acid and alkali used to adjust pH. Therefore, the optimal pH value was selected as 8.0.

These results were consistent with one previous report, which indicated that microbial flocculant TJ-F1 exhibited excellent flocculating efficiency at pH 7.5 (Zhang et al. 2010), but were discrepant with another previous report, which indicated that the biopolymer produced by *Enterobacter cloacae* WD7 had optimum pH for flocculating efficiency at pH 6 (Prasertsan et al. 2006). As a natural organic macromolecular flocculant, LC13-SF can strengthen its bridging ability under an alkaline condition because $-\text{COOH}$ will be ionized into $-\text{COO}^-$, and $-\text{OH}$ will be increased, both of which can promote the flocculating activity of C13-SF, but under an acidic condition, this process will be blocked.

The effect of CaCl_2 dose on the flocculation behavior

Two milliliters LC13-SF and various volume percentage of 0.5% (w/w) CaCl_2 including 0, 2%, 4%, 5%, 6%, 8% and 10% were respectively added into a Kaolin suspension. Then the system pH was adjusted to 8.0 for the flocculating behavior test. The FE and Zeta potential changed at the different volume percentage of 0.5% (w/w) CaCl_2 as shown in Fig. 5. The maximum FE was obtained when volume percentage was 6%, but the value of FE 93.3% was only slightly different from 92.94% when volume percentage was 5%. Therefore, 5% was fixed as the optimal volume percentage of 0.5% (w/w) CaCl_2 . The absolute value of Zeta decrease concomitantly with the increase of the volume percentage of 0.5% (w/w) CaCl_2 , after the

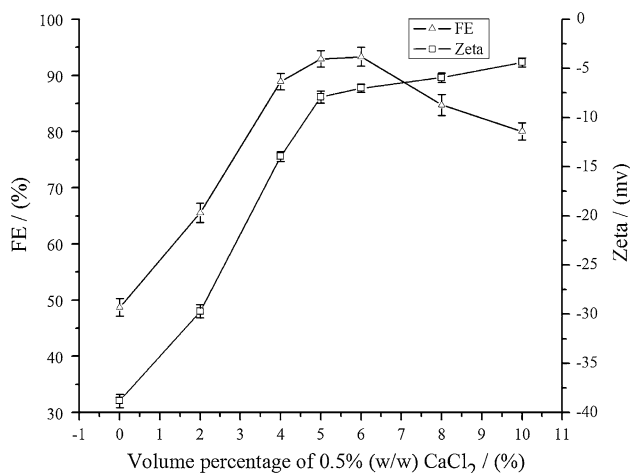


Fig. 5 Effect of CaCl_2 on the flocculating efficiency of bioflocculant LC13-SF

volume percentage increased to 5%, the value of the Zeta potential decreased slightly, and when the volume percentage was 10%, the absolute value of Zeta decreased to 4.39.

The main role of CaCl_2 in the flocculating process of bioflocculant LC13-SF to the Kaolin suspension was that Ca^{2+} efficiently neutralized the negative charges of the particles and decreased the absolute value of the Zeta potential in the flocculating process. With a deficient dose of CaCl_2 , not all of the suspended particles and the organic materials will be flocculated. On the other hand, an excessive concentration of CaCl_2 makes the suspended particles and the organic materials stable again in their original states because of superfluous positive charges (Xia et al. 2008). In addition, excessive CaCl_2 debased the floc size, because excessive Ca^{2+} might occupy the absorption sites with negative charge on MBFGA1 molecules (Yang et al. 2009).

The effect of bioflocculant LC13-SF dose on the flocculation behavior

Volume percentage of bioflocculant LC13-SF varying from 0.5 to 3.5% were investigated by adding 5% volume percentage of 0.5% (w/w) CaCl_2 to a Kaolin suspension. Then the system pH was adjusted to 8.0 for the flocculating behavior test. As can be seen from Fig. 6, the dose of LC13-SF had an obvious effect on the FE as well as the Zeta potential. The FE initially increased with increasing LC13-SF dose, but then decreased. The optimal FE was obtained when the volume percentage of LC13-SF was 1.5%, and the absolute value of the Zeta potential reached its lowest value of 4.37. With a lower dose of LC13-SF, not all of the suspension particles were flocculated to flocs, but an excessive concentration may prevent the small flocs

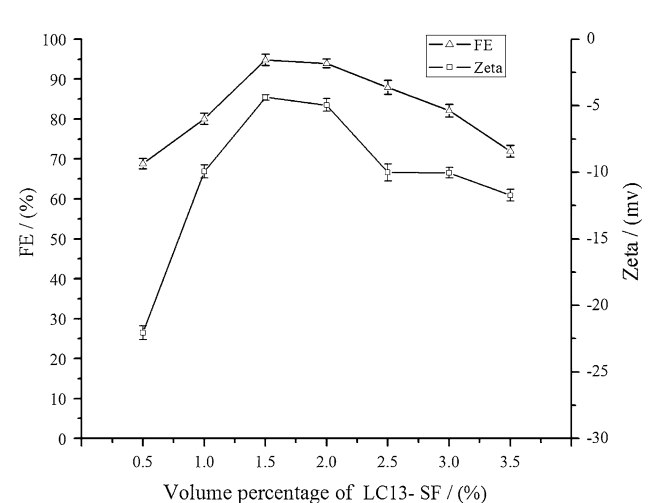


Fig. 6 Effect of LC13-SF dose on the flocculating efficiency

from growing into large flocs because the small flocs carry the same charges. Therefore, only the proper dose of LC13-SF resulted in excellent flocculating efficiency. These phenomena observed in Fig. 6 are consistent with most reports (Yu et al. 2008; Lian et al. 2003).

The bioflocculant LC13-SF was especially effective in terms of the flocculating behavior at a low concentration, because the adsorption of excessive flocculants restabilized the particles in the flocculation system. When excessive flocculants were used, only particles around them participated in the flocculating reaction, because of incomplete dispersion of the excesses. Therefore, other particles did not participate in the reaction, which may have led to a decrease of the FE (Suh et al. 1997). Thus, these results suggested that the bioflocculant LC13-SF held some advantages which were favorable for the flocculating effects.

Based on our results, the optimal conditions for flocculating efficiency were found to be as follows: a final volume percentage of bioflocculant LC13-SF and 0.5% (w/w) CaCl_2 were 1.5 and 5%, respectively in a 4 g/L Kaolin suspension. Then the system pH was adjusted to 8.0, and after each addition, the reaction occurred for 1 min with rapid mixing, then gravity settlement was allowed to occur for 5 min. Under these conditions, the flocculating efficiency and the absolute value of the Zeta potential reached 94.83% and 4.37, respectively.

Conclusions

LC13^T was demonstrated to be an effective flocculant-producing strain. The fermentation conditions and flocculating activity of bioflocculant LC13-SF were systematically investigated in this paper. The results showed that the optimal conditions for bioflocculant LC13-SF production were: (a) a fermentation time of 72 h; (b) an initial pH of 7.0; (c) a fermentation temperature was 30°C; and (d) a shaking speed was 150 r/min. Under these conditions, the flocculating efficiency reached 90.60%. The optimized flocculating process was as follows: a final volume percentage of bioflocculant LC13-SF and 0.5% (w/w) CaCl_2 were 1.5% and 5%, respectively in a 4 g/L Kaolin suspension. Then the system pH was adjusted to 8.0. Under these conditions, the flocculating efficiency and the absolute value of the Zeta potential reached 94.83% and 4.37, respectively.

The activity of LC13-SF under optimal conditions was determined and found that it was highly efficient for flocculating activity, which may find possible application in wastewater treatment as well as in the dewatering of waste sludge. Therefore, we believe that many of these VBNC-state bacteria which are, in fact, culturable using special

techniques, may be very useful in the application of microbial resources. Further studies should focus on cultivating bacteria that belong to these uncultured groups, as well as exploring their application value.

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