Characteristics and Culture Conditions of a Bioflocculant Produced by *Penicillium sp.*

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Objective To study the characteristics of a bioflocculant named MBF7 produced by *Penicillum* strain HHE-P7 and the effects of cultivation conditions on bioflocculant production. **Methods** The chemical group in the bioflocculant molecules was shown by Fourier transform infrared (FTIR) spectra, and the average molecular weight of MBF7 was estimated by gel permeation chromatography. The effects of medium components on bioflocculant production and flocculating activity were studied. **Results** Phospho-, amino-, hydroxyl, and carboxyl groups were the major fractions of MBF7, and the molecule weight was about 3.0×10^5 Da. In addition, the carbon and nitrogen sources favorable for the bioflocculant production were glucose and yeast extract respectively. When the initial pH of the medium was adjusted to 5.0, high flocculant efficiency could be achieved. **Conclusion** The bioflocculant MBF7 is a new macromolecule with high flocculating efficiency for Kaolin suspension, and could be produced under appropriate culture conditions.

Key words: Bioflocculant; *Penicillium sp*.; Flocculating activity; Carbon and nitrogen sources

INTRODUCTION

Compared with conventional chemical flocculants, bioflocculants are biodegradable and nontoxic, and produce no secondary pollution. Microorganisms, such as *Bacillus mucilaginosus*[1], *Gordonia polyisoprenivorans* CCT 7137[2], and *Enterobacter aerogenes*[3], have been found to produce flocculating substances, most of which are extracellular biopolymers of the microorganisms[4]. The molecular weight and composition of bioflocculants are important factors affecting the flocculating efficiency and flocculating mechanisms. The weight is found to range from 1×10^5 to 2×10^6 Da[5]. The compositions of most bioflocculants reported are polysaccharides and proteins[6], and amino- and carboxyl groups are effective for flocculation.

HHE-P7 was another bioflocculant-producing microorganism which could excrete some flocculating material during growth. Cheng Wen[7] indicated that HHE-P7 could grow on the culture medium containing glucose and urea, etc. The flocculating activity of MBF7 for Kaolin suspension was influenced by pH and dosage[8]. In this study, attention was paid to the characteristics of MBF7 and the cultivation conditions. The molecular weight and compositon of MBF7 were investigated by gel permeation chromatography (GPC) and Fourier transform infrared (FTIR) spectrophotometer respectively. The cultivation conditions such as carbon source, nitrogen source, and the optimal initial pH of the culture broth were also determined herein.

MATERIALS AND METHODS

Strain and Materials

The strain HHE-P7 identified as *Penicillium purpurogenum* was taken in our laboratory.

The average diameter of Kaolin particles was 4 μm, and the specific gravity was 2.58 g/cm^3.

Culture Conditions

The medium used in this study contained 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 L deionized water. A colony of the strain HHE-P7 was inoculated into a 250 mL flask containing 100 mL such medium. The culture was incubated in a rotary shaker at 150 rpm at 30 ℃ for

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120 h without control of pH during the operation. Then the medium samples were retrieved for the subsequent determination of the flocculation properties.

Distribution of Flocculating Activity in Culture

The culture broth (80 mL) after 5d cultivation was centrifuged in a centrifuge (LDZ5-2, China) at 3 000 r/min for 15 min. The precipitated cells were washed twice with distilled water, and resuspended in 80 mL distilled water for later use on the one hand. On the other hand, the cell-free supernatant was poured into four volumes of cold anhydrous alcohol to precipitate the biopolymer flocculant. The resultant precipitate was collected by centrifugation at 6 000 r/min for 15 min and re-dissolved in 80 mL distilled water. After three such ethanol precipitation steps, the crude bioflocculant MBF7 was obtained. Thereafter, the flocculating activity of culture broth, supernatant, washed cells and MBF7 was tested, respectively.

The biomass, which was the amount of the cell weight after precipitation, was dried at 80 ℃ to constant weight for subsequent analysis.

Flocculation Test

Kaolin suspension was used to measure the flocculating activity of the bioflocculants. 0.5 g of Kaolin clay was suspended in 100 mL deionized water, and then 2 mL of the bioflocculant and 5 mL $CaCl_2$ [1% (w/v)] were added to the Kaolin suspension. The pH was adjusted to 8 with 0.1 mol/L HCl or 0.1 mol/L NaOH. The mixture was stirred at 60 rpm for 30 s with a mixer and held still for 5 min at room temperature. The absorbance of the supernatant (A) and the blank control (without addition of the bioflocculant-A_0,) was measured at 550 nm with a spectrophotometer (Unico UV-2 000 spectrophotometer, China). The above method was also used to measure the flocculating activity of the culture broth, the supernatant, the washed cells, and the crude product. The flocculating efficiency (%) was defined and calculated as follows:

$$\text{Flocculating efficiency (\%)} \quad \eta = \frac{A_0 - A}{A_0} \times 100$$

Molecular Weight Determination

The number-average molecular weight of the flocculant was measured by gel permeation chromatography (GPC) with Sephadex G-200 series columns with a refractive index detector (410). The eluant containing 0.1 mol/L NaCl was brought to a pH of 7.2 with 0.2 mol/L phosphorous acid, and the flow rate was set at 0.6 mL/min.

FTIR Analysis

Bioflocculant MBF7 was analyzed with a Fourier transform infrared (FTIR) spectrophotometer (Perkin Elmer model 1 730). The sample was blended with KB_r and passed into a disc for FTIR analysis. The spectrum of the sample was recorded on the spectrophotometer over a wave-number range of 4 000-400 cm^{-1} under ambient conditions.

Optimized Cultivation Conditions for Bioflocculant Production

To identify a suitable carbon source for bioflocculant production, HHE-P7 was cultivated with various carbon sources (glucose, sucrose, maltose, starch, lactose, molasses). The effects of various nitrogen sources (beef extract, yeast extract, peptone, urea, NH_4Cl, $(NH_4)_2SO_4$, casein) were also investigated. The initial pH of the culture was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 with HCl or NaOH. The cultivation temperature was maintained at 30 ℃ and the rotary speed of the shaker was set at 150 r/min.

The flocculating activities of the bioflocculant in this study were the mean value of at least two replicates.

RESULTS

The Number-average Molecular Weight of Bioflocculant MBF7

As shown in Fig. 1, the elution operation lasted from 45 min to 440 min, and four peaks appeared at 65 min, 230 min, 310 min, and 340 min, respectively, and the corresponding molecular weight was 14.79×10^5, 4.17×10^5, 3.10×10^5, and 2.72×10^5, Da, respectively. The results showed that the bioflocculant MBF7 was composed of such four parts and the average molecular was over 3×10^5 Da, indicating that it was a high-molecular-weight biopolymer and could be considered potential for good flocculating efficiency in practical wastewater treatment.

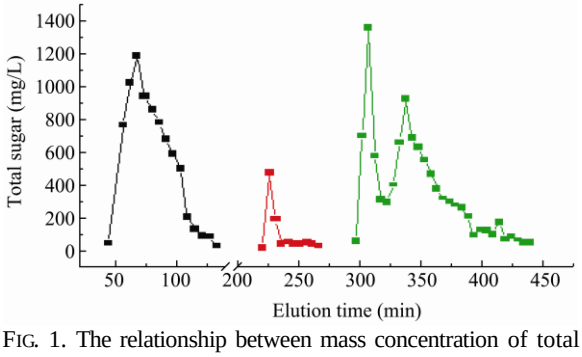

FIG. 1. The relationship between mass concentration of total saccharides and elution time in MBF7.

Composition of Bioflocculant MBF7

From the infrared spectrum of MBF7, the characteristics of chemical groups were analyzed (Fig. 2). The spectrum displayed clear absorption peaks at 3416.50, 2924.00, 1594.94, 1299.10, 1112.86, 535.29 cm^{-1} wavelength. The broad O-H and N-H stretching absorption band could be observed at 3416.50 cm^{-1} and a weak C-H stretching vibration band at 2924.00 cm^{-1}. The peak at 1594.94 cm^{-1} was characteristics of C=O. The peaks at 1299.10 cm^{-1} and 1112.86 cm^{-1} were P=O and C-O absorption bands, respectively. The strong absorption peak observed at 535.29 cm^{-1} was known to be typical characteristics of sugar derivatives. The infrared spectrum of the bioflocculant thus indicated the presence of phospho-, carboxyl, hydroxyl, and amino-groups in bioflocculant MBF7.

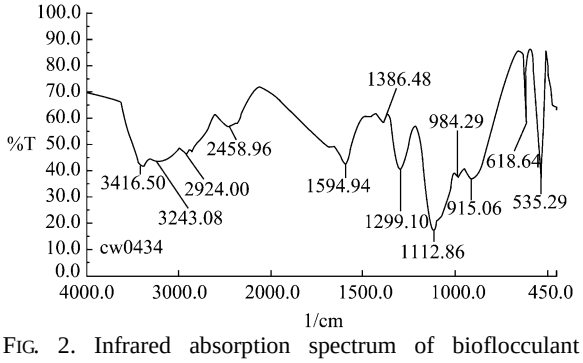

FIG. 2. Infrared absorption spectrum of bioflocculant MBF7.

Distribution of Flocculating Activity Components

The distribution of flocculating efficiency of the culture broth was examined, as shown in Fig. 3. The highest flocculating activity (96%) was present in the culture broth. The flocculating efficiency of the cell was only 1%. Most of the flocculating efficiency could be found in the cell-free supernatant, and more than 93% was recovered from ethanol precipitation. It was evident that the bioflocculant was the material excreted by HHE-P7 and could be used in the form of culture broth, supernatant or crude bioflocculant MBF7.

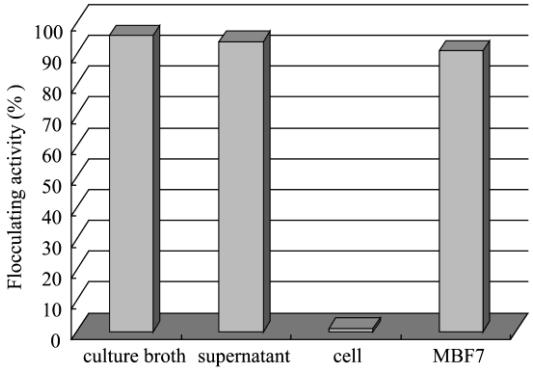

FIG. 3. Distribution of flocculating efficiency com- ponents in culture broth.

Effect of Carbon Source on Flocculation Efficiency

Flocculation efficiency of the substance produced by HHE-P7 was investigated with glucose, sucrose, maltose, lactose, starch, and molasses as the sole carbon source at the same concentration of 20 g/L. As shown in Fig. 4, HHE-P7 could utilize all the materials provided in this experiment, and after 5 day's cultivation, the flocculating efficiency in excess of 70% was achieved. When glucose, sucrose, maltose, and lactose were used as C source, the flocculating efficiency was above 80% only after 1 day's cultivation. Starch and molasses were not easily utilized by HHE-P7 at the beginning, but after a 2-day incubation, the flocculating efficiency increased gradually and reached about 90% and 75%, respectively. The optimal C source to cultivate HHE-P7 was glucose because the highest flocculating efficiency was achieved with the shortest incubation time compared to other carbon sources.

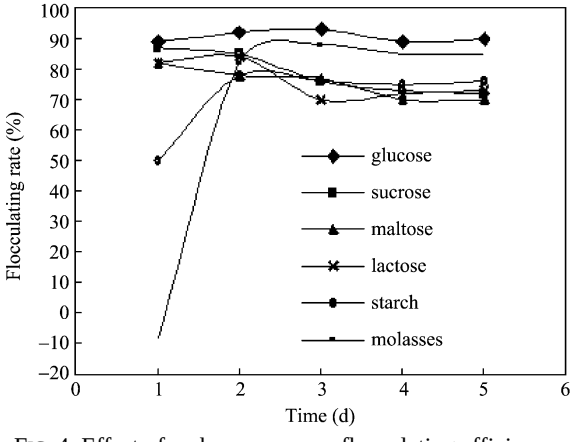

FIG. 4. Effect of carbon source on flocculating efficiency.

Effect of Nitrogen Source on Flocculation Activity

The effects of nitrogen sources on the flocculating activity and biomass weight (with glucose as the carbon source) were also studied (Fig. 5). Among the various nitrogen sources examined, yeast extract was the most suitable nitrogen source for HHE-P7 because the greatest biomass (8.8 g/L) and the largest production of MBF7 (6.4 g/L) were obtained. It was also found that the culture media with different types of nitrogen showed no obvious difference in flocculating activity, which was all more than 80%. Organic nitrogen sources like yeast extract, beef extract and casein were favorable for the biomass production. In the case of inorganic materials, $(NH_4)_2SO_4$ and NH_4Cl were disadvantageous to biomass production. From the fact of HHE-P7 cultivation, it can be learnt that higher biomass was uncorrelated with higher flocculating activity.

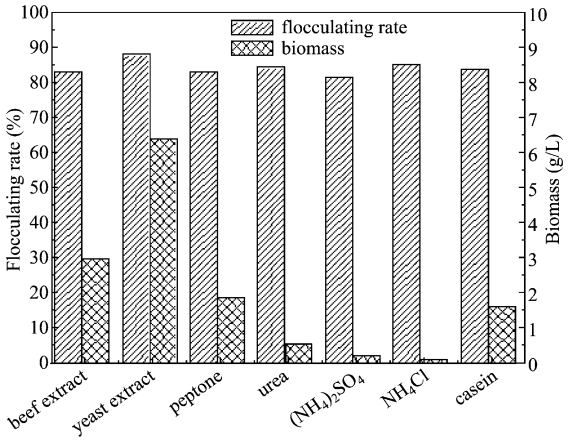

FIG. 5. Effect of nitrogen source on flocculating efficiency and biomass production.

Effect of Initial pH on Flocculation Activity

To optimize the culture conditions for bioflocculant production, the fluctuation of pH during the incubation time of HHE-P7 and the effect of the initial medium pH on flocculating efficiency were investigated (Fig. 6 and Fig. 7). As shown in Fig. 6, when the initial pH was set at 5.0-8.0, the pH changed to 5.5 after 1 day of cultivation, and kept steady till the 4th day, which indicated that HHE-P7 had the ability to produce some substances to regulate pH, and that 5.5 was the optimal pH for the strain to grow. In Fig. 7, when the initial pH of the medium was set at 4.0 or 5.0, the flocculating activity was about 85%; otherwise, the flocculating efficiency fluctuated between 50% and 80%. The results suggested that the optimal initial pH for both HHE-P7 growth and bioflocculant production was 5.0.

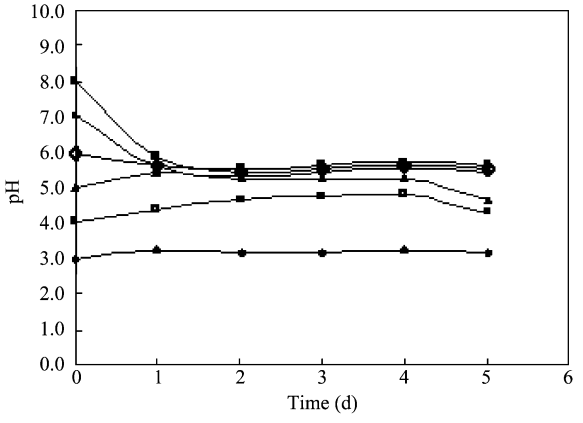

FIG. 6. pH of the medium during the growth of HHE-P7. ■, pH 8.0; ○, pH 7.0; ◇, pH 6.0; ▲, pH 5.0; □, pH 4.0; ●, pH 3.0.

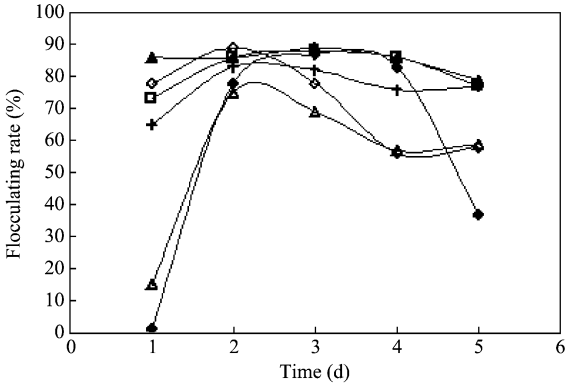

FIG. 7. Effect of initial pH value of the medium on flocculating efficiency. ◆, pH 3.0; □, pH 4.0; ▲, pH 5.0; +, pH 6.0; ◇, pH 7.0; △, pH 8.0.

DISCUSSION

Flocculating agents are widely used in industrial processes including wastewater treatment, downstream processing, and food and fermentation processes[9]. Among the flocculants, aluminum salts represented by polyaluminum chloride and polyacrylamide derivatives have been commonly used because of their flocculating effectiveness and low cost. However, health problems caused by aluminum salts, including Alzheimer's disease, have been reported[10]. Poly-acrylamide derivatives are not easily biodegraded in natural environment and the monomers derived from them are both neurotoxic and carcinogenic[11]. Natural polymers are biodegradable and environment-friendly materials. Bacteria can utilize the nutrients in the culture medium to synthesize high molecular weight polymers within the cell under the action of specific enzymes, and these polymers can be excreted and survive in the medium or on the surface of the bacteria as capsule[12]. From the test, flocculating activity of the supernatant was 93%, indicating that the flocculant could be produced by HHE-P7 during its growth. As shown in Fig. 3, the culture broth after 5d cultivation and the crude product MBF7 both can be used as flocculant with high flocculating activity. An earlier report demonstrated that more than 99% of the flocculating activity in the culture broth of W7-1 was present in the supernatant with less than 1% bound to the cells[13].

The molecular weight and functional groups in the molecular chains are important factors to decide the flocculating activity of bioflocculants[14]. Flocculation with high molecular weight bioflocculants involves more adsorption points, stronger bridging, and higher flocculating activity than does flocculation with a low molecular weight

bioflocculant. In our research, MBF7 molecular weight was about 3×10^5, and was found to be an effective bioflocculant to flocculate kaolin suspension and suspended organic solids in starch wastewater[7]. The carboxyl groups present in the molecular chain make the chain stretched out because of electrostatic repulsion and the stretched molecular chains provide more effective sites for particle attachment. In addition, the high molecular weight makes bridging between bioflocculant and discrete particles effective, and as a result , MBF7 has high flocculating activity as some synthetic materials. Bioflocculant MBF7 contains carboxyl groups, which was also one of the reasons for the decrease in pH of the medium during cultivation as shown in Fig. 6.

Carbon and nitrogen sources have been reported to have an important impact on the production of bioflocculant[15]. As shown in Fig. 4, the best carbon source for the bioflocculant activity produced by HHE-P7 is glucose. However, the high cost of glucose, which has direct impact on production costs, limits the market potential of the biopolymers[16]. Starch is cheap, but is less suitable for HHE-P7. Molasses, a byproduct of many industrial processes, mainly from biodiesel production, is generated in large quantities, far beyond current consumption in traditional applications, thus making it a cheap material for HHE-P7 to produce bioflocculant. Mario Daniel Ferrari *et al*. have reported the use of molasses for the Baker's yeast production[17]. In the case of *Gordonia polyisoprenivorans* CCT 7 137, the growth of this strain on the molasses at various concentrations from 2% to 10% is found to produce exopolysaccharides[18]. Molasses is expected to be a substitute for glucose to be used in MBF7 production.

CONCLUSION

It is found that the optimal initial pH for the production of MBF7 was 5.0. HHE-P7 could produce flocculating materials using organic or inorganic N source. Yeast extract and glucose are the optimal N source and C source to enhance the production of MBF7, respectively. Other simple organics like sucrose, maltose and lactose are more easily used by HHE-P7 than starch and molasses. The high productivity and flocculating activity of MBF7 make it possible to be developed as a new biodegradable and harmless biopolymer flocculant. These properties make this polymer a good alternative to artificial synthesis such as aluminum salts, chemical and polyacrylamide in several applications in food, pharmaceutical, cosmetic, textile, paper and petroleum industries. However, high production cost and complicated production process have been the major obstacles to bioflocculant development in practical application. One measure to decrease the cost is to use some cheaper substrate to produce bioflocculant on industrial scale. Form this test, molasses is a potential carbon source to cultivate HHE-P7. Further studies on production of MBF7 with molasses and optimization of culture conditions are needed in order to enhance the bioflocculant production and realize its industrial utilization.

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