RESEARCH ARTICLE

Novel fibromonas and fibrous microbial flocculant preparation and practical application

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Sewage treatment is of great significance for environmental protection and sustainable development and is related to the quality of human life. Developing new and efficient sewage treatment methods has important research value and social significance. A new type of fiber monocyte and fiber microbial flocculant was developed in this study for wastewater treatment. During the process, a treatment method for microbial flocculant-producing bacteria was designed from the perspective of bacterial selection and cultivation. The safety of microbial flocculants was analyzed from the perspective of flocculation mechanism and toxicity, and a novel method for the wastewater treatment performance of flocculants was designed. The results showed that the optimal inoculation amount of bacterial strains was 1%, and the optimal concentration for glucose addition was 12 g/L. In Zeta potential analysis, the developed method showed that the lowest Zeta potential decreased to -55 mV after flocculation of phenolic substances at a pH value of 12. In the fractal dimension testing of flocs, the developed method maintained a two-dimensional fractal dimension of over 1.182 after flocculation. In the analysis of sewage purification efficiency, the developed method maintained a total nitrogen removal rate of over 46.89% and a chemical oxygen demand removal rate of over 43.52% in 7 samples. The microbial flocculant developed by this research could effectively treat wastewater with good treatment performance.

Keywords: sewage treatment; microorganisms; flocculant; single celled bacteria; toxicity analysis.

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Introduction

Wastewater treatment has always been an important issue in the field of environmental protection. With the advancement of industrialization and urbanization, the issue of sewage discharge is becoming increasingly prominent [1, 2]. With the development of industry and agriculture, there are many emerging pollutants in sewage, such as drug residues, hormones, personal care products, *etc*., and the treatment technology of these pollutants is not fully matured. Traditional wastewater treatment methods are often costly and have limited treatment efficiency. The development of low-cost and high-efficiency processing technology is an urgent need. Existing wastewater treatment technologies, especially the use of chemical flocculants, have problems such as high cost, large amounts of sludge generated after treatment, and the possible introduction of toxic substances.

As an alternative solution, microbial flocculants have the advantages of low cost, environmental friendliness, and no secondary pollution. Flocculation technology is widely used as an effective water treatment method in the sewage treatment process. Mohamed *et al*. designed a

magnetic nano flocculant for wastewater treatment, using trivalent and divalent iron ions as magnetic sources. After completing wastewater flocculation, the flocculant was recycled and reused using an external magnetic field [3]. However, the use of flocculants with chemical components such as aluminates and iron salts can easily generate a large amount of waste residue during application, which requires treatment and disposal and increases additional treatment costs. Excessive use of chemicals may lead to residual chemicals in wastewater, which can harm the environment. In addition, biological treatment is an environmentally friendly wastewater treatment method, but when dealing with high-concentration industrial wastewater, it usually requires a long treatment time and a large amount of biological accumulation, which can lead to low treatment efficiency [4]. Microbial flocculant is a low-cost, high-efficiency, and environmentally friendly sewage treatment tool that can quickly colonize and adsorb fiber substances in high-concentration fiber wastewater, forming larger flocculants. Liu *et al*. successfully analyzed microbial flocculants and found that the production and application costs of microbial flocculants were mainly influenced by strain screening and cultivation methods. Scientific cultivation methods were needed for large-scale applications [5]. Monga *et al*. proposed a remediation method based on indigenous bacterial flocculants for heavy metal pollution in rivers and measured that the proposed method had high flocculation activity in high chromium content environments [6]. Yang *et al*. designed a flocculant based on microalgae to treat food processing wastewater. The proposed method effectively treated protein wastewater and humic organic wastewater during wastewater treatment [7]. Microbial flocculants can coagulate and precipitate non-degradable solid suspended particles in liquids, thereby achieving the goal of purifying water quality [8, 9]. There are particles in raw water when microbial flocculants are flocculated. After adding microbial flocculants, the colloid and flocculant adsorb each other. The aggregation between particles and flocculants is caused by electrostatic and van der Waals forces, forming bridging adsorption [10-12]. The particles and flocculants are interwoven into a grid shape and gradually settle down. When optimizing microbial cultivation conditions, there are two aspects including medium optimization and cultivation condition optimization. When optimizing the culture medium, the carbon-nitrogen ratio of carbon and nitrogen sources is selected. When optimizing the culture conditions, different culture temperatures, shaking table revolutions, and culture times are selected for culture testing, and the optimal culture conditions are determined [13, 14]. When conducting flocculant research and development, the treatment of flocculant-producing bacteria, toxicity analysis of flocculants, and flocculation mechanism analysis are important links that play a decisive role in the effectiveness and safety of flocculants [15, 16]. Many countries and regions are promoting the development of green technology. Microbial flocculants, as a green technology, in line with policy orientation, help to obtain policy support and financial investment. Microbial flocculants can significantly reduce the use of chemical flocculants, reduce wastewater treatment costs, and reduce the generation of waste residue [17, 18].

This research aimed to provide a new and more effective flocculation technology for wastewater treatment by developing and optimizing microbial flocculants, while reducing the degree of dependence on chemical components to reduce environmental secondary pollution, promoting the development of wastewater treatment technology in a greener and more sustainable direction. A new type of fiber monocyte and fiber microbial flocculant was proposed. This study linked microbial technology with wastewater treatment technology to help foster interdisciplinary collaboration and drive innovation in wastewater treatment.

Materials and Methods

Treatments for microbial flocculant producing bacteria

The strains of flocculant-producing bacteria were obtained from New Hope Livestock Farm (Shenyang, Liaoning, China). The strains of gel fibromonas, Denver fibromonas, and Finn fibromicrobacteria were obtained from the strain bank of Dalian University of Technology (Dalian, Liaoning, China). 10 (5 males and 5 females) of 6 to 8 weeks old SPF grade BALB/c mice (Changsheng Biotechnology Co., Ltd., Shenyang, Liaoning, China) with the body weight from 18 to 22 g were used for flocculant preparation.

The bacterial strains were enriched, isolated, and purified before initial screening. The samples of bacterial strains were cultured in a liquid medium for 24 to 48 hours. Then the bacterial solution was inoculated into a solid culture medium for single colony separation and purification followed by after separation cultivation. For the initial screening, purified colonies were seeded in an agar medium with 4-5 colonies per plate. Colonies were incubated at 37°C for 48 hours and then were stained for 1 minute. Strains with a larger ratio of hydrolytic zone to colony diameter were selected.

The flocculation activity measurement was performed thereafter for re-screening. The selected colonies were incubated at 37°C, 155 rpm for 48 hours before centrifugation for 5 minutes. The flocculating activity of bacterial cells was measured at 550 nm wavelength using UV5200 spectrophotometer (Shanghai Yuanxi Instrument Co., Ltd., Shanghai, China) and was calculated as follows:

$$P_x = (A - B) / A \times 100\% \quad (1)$$

where P_x was the flocculation rate. A was the absorbance of the control group. B was the absorbance of the experimental group.

Morphological identification, scanning electron microscopy identification, bacterial identification, biochemical characteristic identification, gene sequence analysis, and whole genome sequencing on the bacterial strains obtained from the re-screening process were performed to complete bacterial identification. The morphological identification was done by observing the single stained colonies' morphology under XS-213-201 microscope (Nanjing Jiangnan Yongxin Optics Co., Ltd, Nanjing, Jiangsu, China). The morphological characteristics of the colony were recorded. SU5000 Scanning Electron Microscopy (Hitachi, Tokyo, Japan) was employed to identify the colony following the manufacturer's instructions for sample preparation. Further, 10 colonies were selected for bacterial identification. The bacterial proteins were extracted and processed for mass spectrometry experiment using mass spectrometry sample processing kit (Brooke Technology Co., Ltd., Beijing, China). The strain genus was determined by comparing detected map with the online reference map database. The biochemical characterization was determined using bacterial biochemical identification cards and kits (Biomerieux China Limited, Shanghai, China) following the manufacturer's instructions. The gene sequence analysis and whole genome sequencing were done by Shanghai Shenggong Biotechnology Co., Ltd. (Shanghai, China).

Toxicity and flocculation mechanism analysis of microbial flocculants

Oral acute toxicity and blood cell chromosomal tests of experimental mice were performed. Briefly, the mice in the experimental group were given a bacterial suspension by gavage in 10 consecutive days and observed and collected vital sign data. The half-lethal dose was determined by:

$$\lg LD_{50} = \log^{-1}\left[X_m - i\left(\sum P - 0.5\right)\right] \quad (2)$$

where $\lg LD_{50}$ was the half-lethal dose. i was the logarithmic difference in dose. P was mouse mortality rate. The chromosomal tests on blood cells were performed by feeding experimental mice with bacterial suspension for seven

consecutive days before blood sample collection. About 300 to 600 mL of blood were collected from each mouse and then were incubated at 37°C for 48 to 52 hours. Following cultivation, colchicine was added to the blood culture and centrifugated to remove the upper layer of clear liquid. A low osmotic solution was added to the samples and thoroughly mixed. The low permeability operation was conducted followed by the addition of the fixed solution. The supernatant was removed again through centrifugation, completing the pre-fixation stage. Two additional centrifugations were done to obtain the cell suspension. The cell suspension was deposited onto a clean glass slide for air drying and Giemsa staining before applied to scanning imaging.

The sugar component, protein component, and structural composition were determined for the analysis of the flocculation mechanism. The sugar components were identified through a combination of Molish reaction and bioanalysis liquid chromatography-mass spectrometry. Protein composition was determined using the biuret method and ninhydrin method followed by protein quantification and gel electrophoresis analysis. The total protein was extracted, and the protein composition data were collected using a Bruker microTOF-Q-I mass spectrometer (Bruker, Billerica, Massachusetts, USA) following manufacturer's instructions. For structural analysis of the flocculant, a 90Plus Zeta potential analyzer (Brookhaven Instruments, Holtsville, NY, USA) combined with infrared spectroscopy was employed following the manufacturer's instructions.

Microbial flocculants in wastewater treatment

The process of microbial flocculants treatment of wastewater mainly includes treatment of aquaculture wastewater, treatment of processing plant wastewater, and treatment of domestic wastewater. The wastewater samples used for the analysis of flocculant application in this study were all obtained from Panjin City, Liaoning Province, China. The wastewater samples of poultry and pig farming, food, chemical, and paper factories, pharmaceutical, and ship were obtained from Zhongwang White Feather Chicken Farm and Shengmu Fangzhou Animal Husbandry Co., LTD., Fusheng Food Co., LTD., and Haoye Chemical Co., LTD., paper mills, Chinese Medicine Branch of Panjin No. 2 Pharmaceutical Factory, and ocean-going ships in the Bohai Sea area, respectively. The aquaculture wastewater treatment included the treatments of experimental groups with either fixed dosage of calcium chloride or fixed dosage of flocculant. In the fixed calcium chloride dosage experiments, 100 mL of aquaculture wastewater was adjusted to neutral pH before 0.5 1.0, 1.5, and 2.0 mL of flocculants and 1 mL of 10% calcium chloride were added to different groups of sewage, respectively. The reaction mixtures were placed at room temperature for 1 to 2 hours to complete the settlement. The flocculation rate, total nitrogen, and ammonia nitrogen values of the wastewater before and after treatment were measured to calculate the removal rate. For the fixed flocculant dosage experiments, 100 mL of aquaculture wastewater was mixed with different types of flocculants at the optimal dosage followed by adding different amounts of 10% calcium chloride. The mixture was mixed evenly and left to settle at room temperature for 1-2 hours to complete sedimentation. The flocculation rate, total nitrogen, and ammonia nitrogen values of the wastewater before and after treatment were measured to calculate the removal rate. For processing plant wastewater and domestic wastewater treatment, 100 mL of wastewater from different types of processing plants was first adjusted to neutral pH. Then, 1 mL of 10% calcium chloride and 2 mL of flocculant were added to the wastewater before being allowed to settle for at least one hour. Various indicators of the settled wastewater were measured to evaluate the removal rate.

The effects of wastewater treatment were evaluated through scanning electron microscopy observation, third-party testing, and shelf-life evaluation. For scanning electron microscopy observation, both the original wastewater and solid flocculants after purification were collected

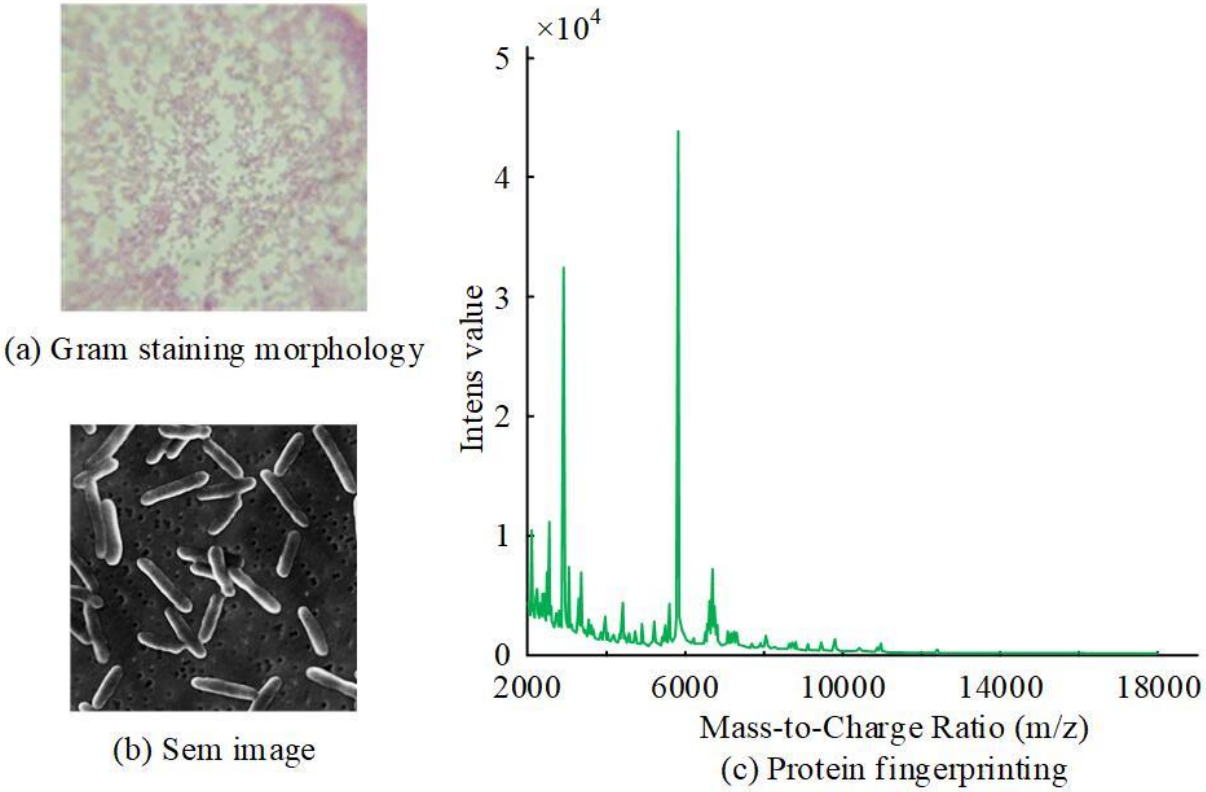

Figure 1. Bacterial identification image.

and dried in GRX-9241B constant temperature drying oven (Fucode Laboratory Equipment Co., LTD., Shanghai, China) at 75°C for 3-4 days. The flocculants were fixed with conductive adhesive and gold-plated to improve conductivity. The treated flocculants were then scanned with an electron beam to obtain information about their appearance. Third-party testing was carried out by analyzing the purification indicators of the flocculant according to national standards and evaluating the purification effects. Shelf-life evaluation involved storing the flocculant at 4 and -20°C and conducting performance tests once a month for 12 months. The change in the performance of the flocculant over time was analyzed to determine its shelf life.

Results and discussion

Identification of microbial flocculant strains

Three strains with the highest flocculation activity were selected as the experimental subjects and labeled as Alpha, Bravo, and Charlie. The strain with the highest flocculation activity during the experimental process for bacterial identification was selected. Gram staining morphology, electron microscopy images, and protein fingerprint images were combined to determine the bacterial strain (Figure 1). The Gram staining results showed a blue-purple positive form, indicating that the strain absorbed crystal violet dye and reacted with potassium iodide to form a complex. The strain appeared cylindrical in the scanning electron microscope image demonstrate curved edges and blunt circles at both ends. The two-dimensional width of the strain ranged from 0.3 microns to 0.6 microns, and the length ranged from 1.0 microns to 2.0 microns. The strain was single or paired, without spore or capsule structure. The signal intensity of the protein fingerprint reached 3.27 × 10^4 and 4.34 × 10^4 at a mass-to-charge ratio of

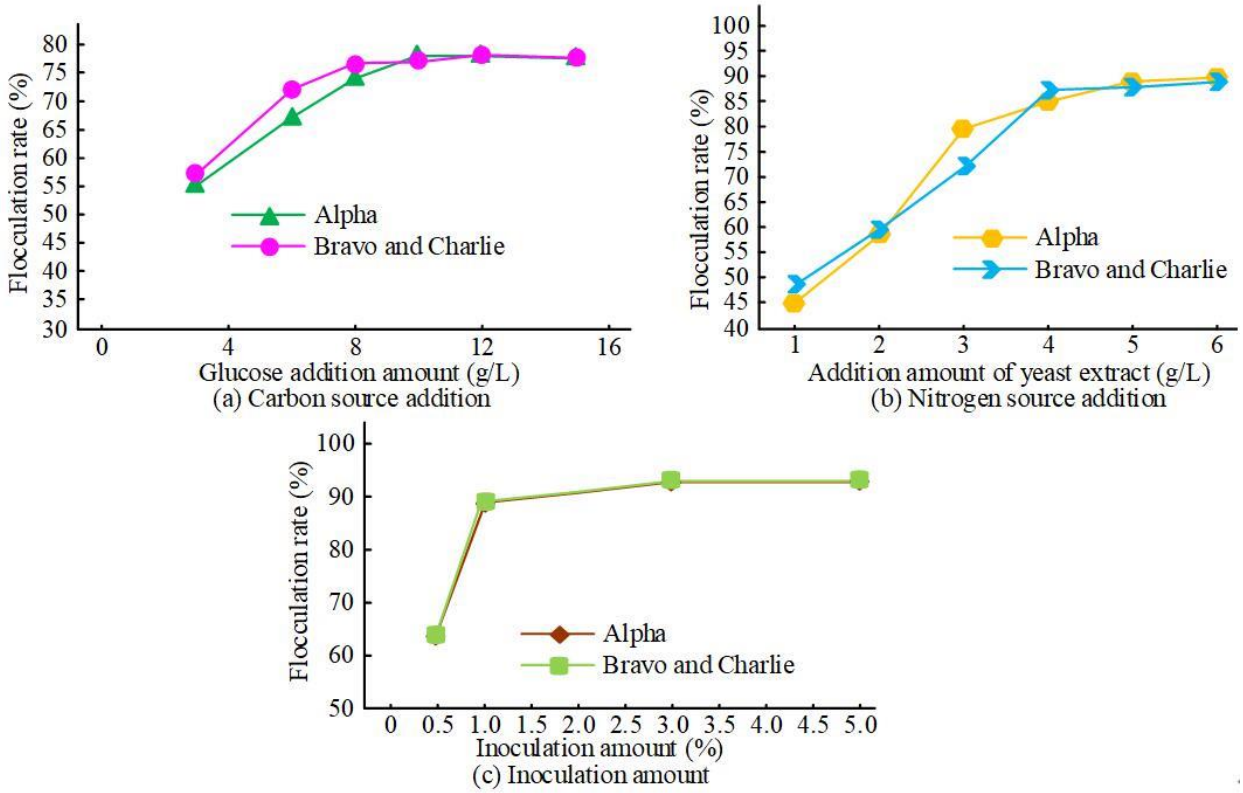

Figure 2. Testing of influencing factors.

2,876 m/z and 5,821 m/z, respectively. The identified strain was then determined to belong to the fiber monocellular strain.

Factors influencing the flocculation rate of microbial flocculants

When testing the impact of flocculation rate, different amounts of carbon source addition, nitrogen source addition, and inoculation were selected. Among them, Bravo and Charlie strains belonged to the same strain and had the same performance (Figure 2). In the carbon source addition test, the flocculation rate of the Alpha strain increased with the increase of glucose concentration before the glucose concentration reached 10 g/L. After the glucose concentration reached 10 g/L, the flocculation rate tended to stabilize and remained at about 78%. After glucose reached 12 g/L, the flocculation rate of Bravo and Charlie strains remained stable at about 78%, which indicated that 12 g/L was the optimal concentration for glucose addition. In the nitrogen source addition test, the flocculation rate of the Alpha strain tended to stabilize after the yeast extract reached 5 g/L. After the addition of 4 g/L yeast extract, the flocculation rate of Bravo and Charlie strains stabilized, indicating that 4 g/L was the optimal concentration for adding yeast extract. In the inoculation volume test, all three strains showed similar performance, and the flocculation degree began to stabilize when the inoculation volume was 1%, which indicated that the optimal vaccination dose was 1%.

Zeta potential and infrared spectral analysis of microbial flocculants

The Zeta potential of the microbial flocculant prepared in the study was analyzed using different specific components during flocculation. The Zeta potential of different components reached its highest value at a pH value of 2. The potential of oil without flocculation reached 21 mV, and the potential

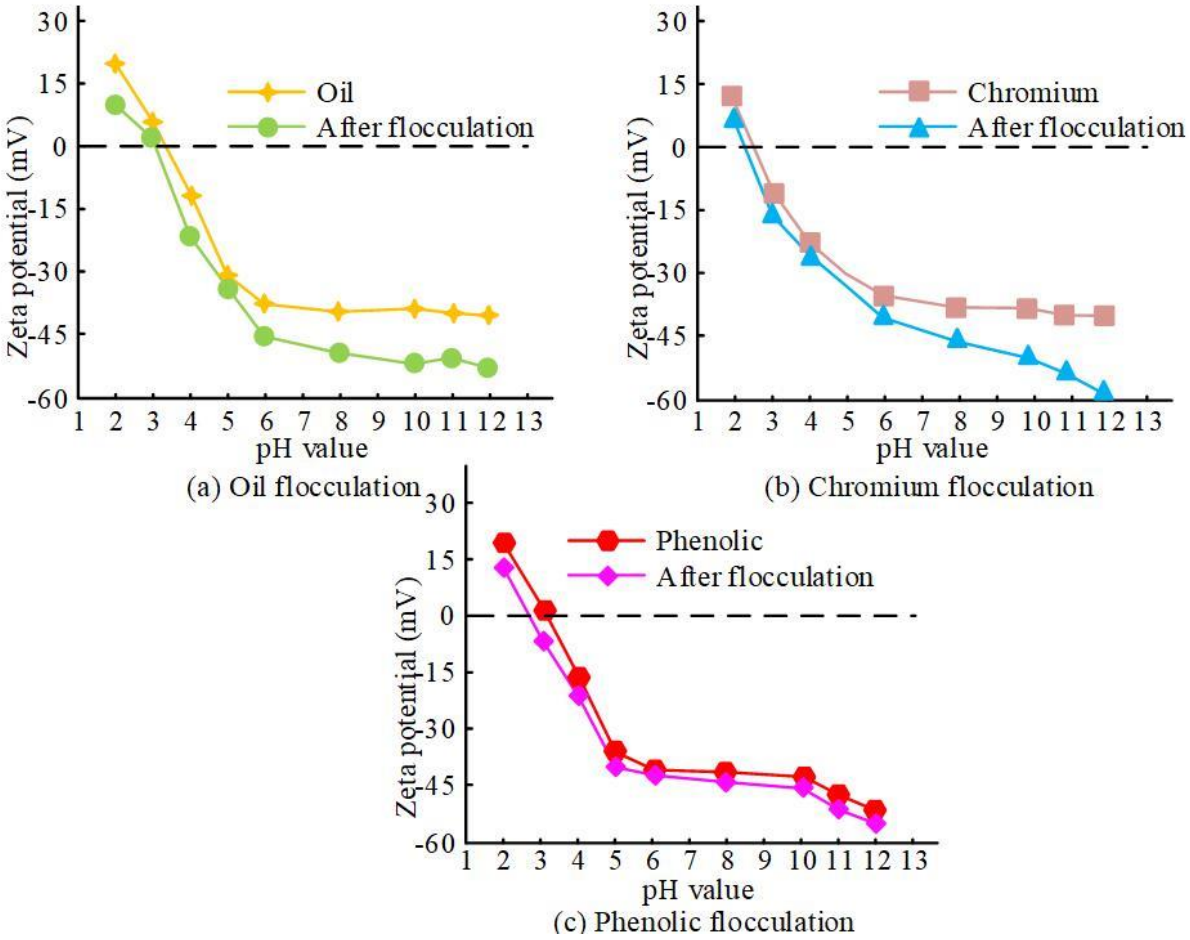

Figure 3. Zeta potential analysis.

after flocculation was 10 mV. The potential of chromium without flocculation reached 13 mV, and the potential after flocculation was 6 mV. The potential of phenolic substances without flocculation reached 19 mV, and after flocculation, the potential was 13 mV. At a pH of 12, the Zeta potential reached its lowest value, and the potential of the oil without flocculation reached -40 mV. The potential after flocculation was -52 mV. The potential of chromium without flocculation reached -40 mV, while the potential after flocculation was -59 mV. The potential of phenolic substances without flocculation reached -50 mV, and the potential after flocculation was -55 mV (Figure 3). The results indicated that the flocculant had a smooth electric neutralization effect with substances in the water, reducing the electrostatic repulsion between particles and accelerating particle flocculation. The infrared spectra of three strains were shown in Figure 4. There were obvious polysaccharide characteristic peaks in the infrared spectra of the three strains. The infrared spectrum of the Alpha strain had a broad and strong absorption peak at the wavelength of 3,313/cm, while the stretching peak at the wavelength of 2,927/cm was weak. The absorption peak at the wavelength of 1,659/cm came from the asymmetric stretching vibration of the secondary acylamide bond. The infrared spectra of Bravo and Charlie strains exhibited a broad and strong absorption peak at the wavelength of 3,313/cm, while the stretching peak at the wavelength of 2,928/cm was weak. The absorption peak at the wavelength of 1,241/cm was caused by the symmetric

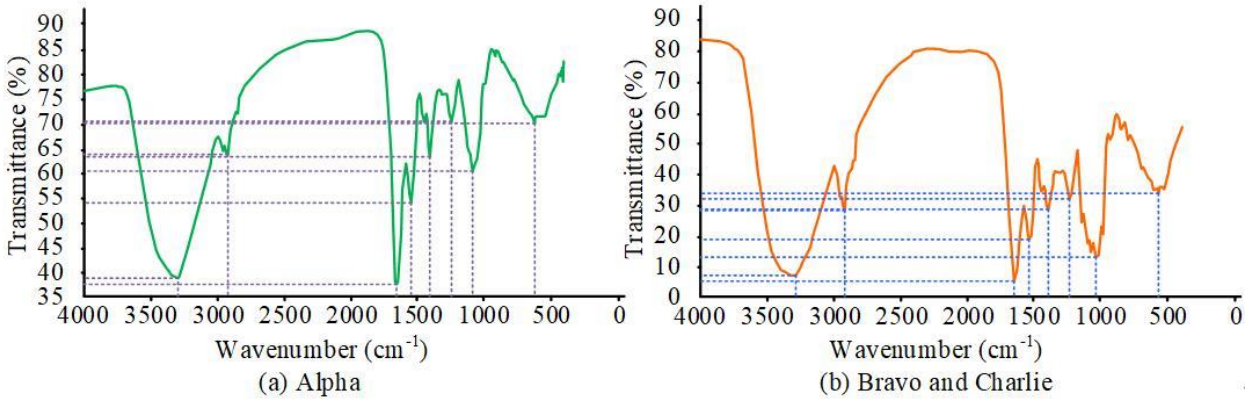

Figure 4. Infrared spectrum measurement.

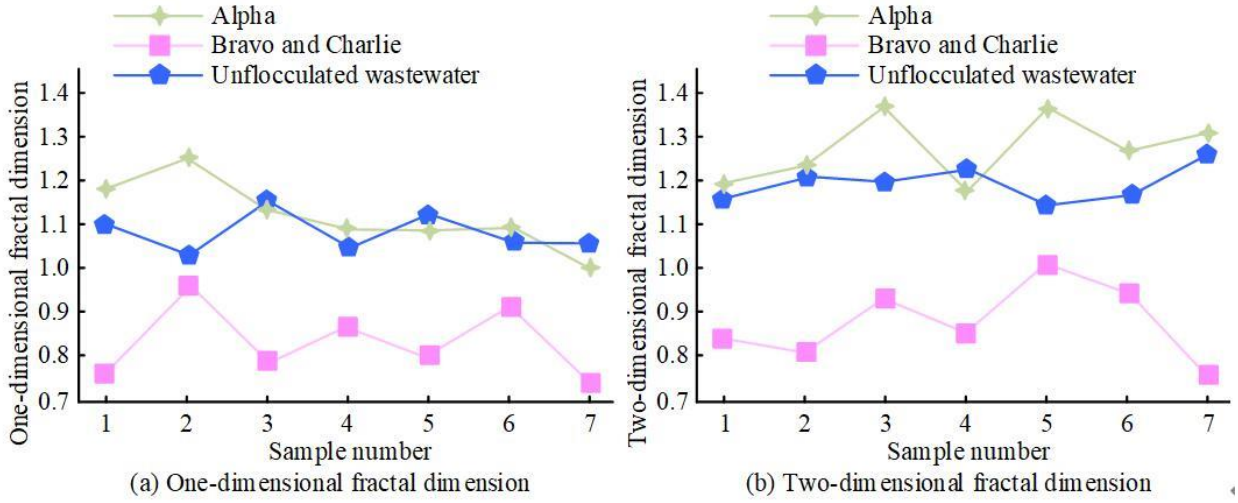

Figure 5. Fractal dimension of flocs.

stretching vibration of nitrogen dioxide groups. The results proved that polysaccharides were the main component of flocculants, and the sugar molecules contained hydroxyl carboxyl and amino groups.

Application effect and shelf-life testing of microbial flocculants

To analyze the application effects and the development of flocculants in sewage treatment, the fractal dimension of the flocculant and the purification effect were conducted. The one-dimensional fractal dimension of particles in wastewater without flocculation was in the range of 0.734 to 0.966, while the two-dimensional fractal dimension was in the range of 0.755 to 1.062. The one-dimensional fractal dimension of the flocs treated with the Alpha strain was within the range of 1.009 to 1.258, and the two-dimensional fractal dimension was within the range of 1.182 to 1.375. The one-dimensional fractal dimension of the flocs treated with Bravo and Charlie strains was within the range of 1.027 to 1.169, and the two-dimensional fractal dimension was within the range of 1.143 to 1.262 (Figure 5). The results indicated that the use of the developed flocculants for sewage treatment resulted in lower porosity of the flocs and the overall compactness of the flocs, effectively flocculating particles in the sewage. The

Table 1. Sewage purification effect.

Flocculants	Index (%)	1	2	3	4	5	6	7
Alpha	Ammonia nitrogen removal rate	67.59	87.53	75.34	65.98	78.19	75.62	84.81
Bravo & Charlie		68.32	69.75	72.31	69.34	76.15	68.39	77.31
Alpha	Total nitrogen removal rate	87.31	46.89	75.38	62.39	81.34	76.96	79.87
Bravo & Charlie		76.31	65.34	74.56	70.25	77.84	71.20	67.20
Alpha	Chemical oxygen demand	45.67	56.58	59.76	86.42	75.35	65.38	58.90
Bravo & Charlie	removal rate	76.38	68.96	43.52	75.20	53.89	58.97	63.15
Alpha	Flocculation rate	86.34	79.35	84.36	85.26	79.31	82.67	78.66
Bravo & Charlie		84.20	74.69	78.32	75.26	82.19	75.98	78.30

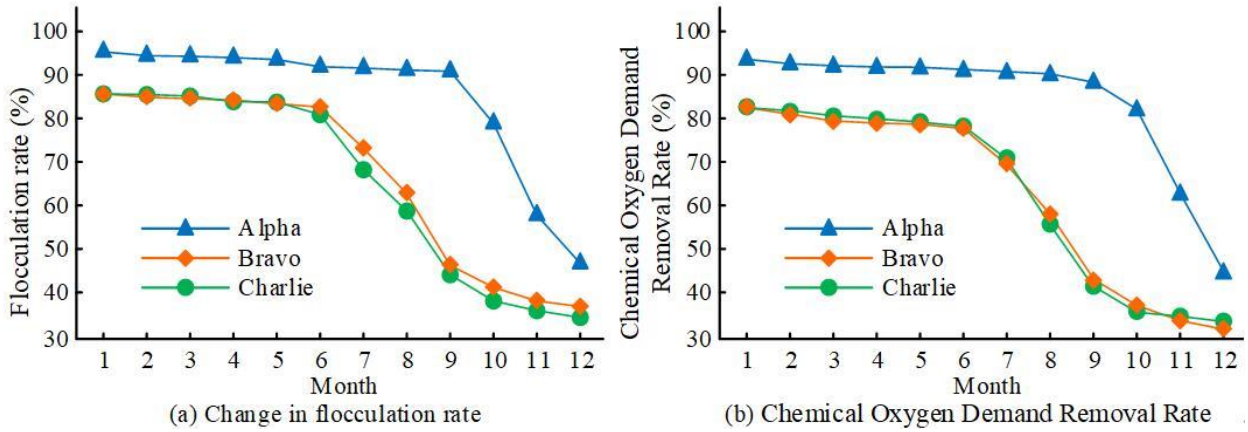

Figure 6. Flocculant shelf-life test.

purification effects were analyzed using different samples (Table 1). The results showed that the ammonia nitrogen removal rate of the Alpha strain in 7 types of samples remained above 65.98%. The total nitrogen removal rate remained above 46.89%. The removal rate of chemical oxygen demand remained above 45.67%. The flocculation rate remained above 78.66%. The ammonia nitrogen removal rates of Bravo and Charlie strains in 7 types of samples were above 68.39%. The total nitrogen removal rates were above 65.34%. The removal rates of chemical oxygen demand were above 43.52%. The flocculation rates remained above 74.69%. The results indicated that the development of flocculants effectively purified wastewater. The flocculant shelf-life tests showed that, in the test of the variation of flocculation rate with time, the flocculation rate of Alpha strain decreased slowly from January to September, reaching 90.8% by September, and then decreased sharply after September. The flocculation rate of the Bravo and Charlie strains decreased slowly from January to June. By June, the flocculation rate of the Bravo strain was 82.6%, while that of the Charlie strain was 80.9%. After June, the flocculation rate suddenly decreased (Figure 6(a)). In the test of the change of chemical oxygen demand removal rate with time, the chemical oxygen demand removal rate of Alpha strain decreased slowly from January to September, and by September, the chemical oxygen demand removal rate was 88.1%. After September, the chemical oxygen demand removal rate suddenly decreased. The removal rate of chemical oxygen demand of the Bravo and Charlie strains decreased slowly from January to June. By June, the removal rate of chemical oxygen demand of the Bravo strain was 77.8%, while that of the Charlie strain was 78.2%. After June, the removal

rate of chemical oxygen demand suddenly decreased (Figure 6(b)). The results suggested that the shelf life of the Alpha strain was 9 months, while that of the Bravo and Charlie strains was 6 months.

With the acceleration of industrialization and urbanization, the discharge of sewage has been increasing, causing serious pollution to the environment [19, 20]. Developing efficient and environmentally friendly wastewater treatment technologies is critical to protecting water resources and ecosystems. In the 7 wastewater samples, the total nitrogen removal rate was more than 46.89%, and the chemical oxygen demand removal rate was more than 43.52%, indicating that the flocculant could effectively treat sewage with high treatment efficiency. In the process of preparation of flocculant, the study determined that the best inoculation amount was 1% and the best concentration of glucose was 12 g/L. These optimization conditions were essential to improve the production efficiency and the quality of flocculants. In addition, through Zeta potential analysis, the lowest Zeta potential of phenolic substances after flocculation dropped to -55 mV, which indicated that the flocculant had good charge neutralization ability at high pH value, reducing electrostatic repulsion between particles and promoting flocculation. In the fractal dimension test of the flocculant, the flocculant maintained a two-dimensional fractal dimension of more than 1.143 after flocculation, which indicated that the flocculant treatment produced a more compact structure and lower porosity, which were conducive to improving the flocculation effect. The results further proved the effectiveness of the flocculant in wastewater treatment. The new fiber single-cell bacteria and fiber microbial flocculants developed in the research showed good potential in wastewater treatment. Future research could consider developing a wider range of application scenarios to adapt to the needs of different types of wastewater treatment.

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