Research Article

Isolation, Identification, and Optimization of Culture Conditions of a Bioflocculant-Producing Bacterium Bacillus megaterium SP1 and Its Application in Aquaculture Wastewater Treatment

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A bioflocculant-producing bacterium, Bacillus megaterium SP1, was isolated from biofloc in pond water and identified by using both 16S rDNA sequencing analysis and a Biolog GEN III MicroStation System. The optimal carbon and nitrogen sources for Bacillus megaterium SP1 were 20 g L\(^{-1}\) of glucose and 0.5 g L\(^{-1}\) of beef extract at 30\(^\circ\)C and pH 7. The bioflocculant produced by strain SP1 under optimal culture conditions was applied into aquaculture wastewater treatment. The removal rates of chemical oxygen demand (COD), total ammonia nitrogen (TAN), and suspended solids (SS) in aquaculture wastewater reached 64, 63.61, and 83.8%, respectively. The volume of biofloc (FV) increased from 4.93 to 25.97 mL L\(^{-1}\). The addition of Bacillus megaterium SP1 in aquaculture wastewater could effectively improve aquaculture water quality, promote the formation of biofloc, and then form an efficient and healthy aquaculture model based on biofloc technology.

1. Introduction

Bioflocculant is an active substance produced by growing microorganisms and is composed of macromolecular polymers, such as glycoprotein, polysaccharide, protein, cellulose, and nucleic acid [1–3]. Bioflocculant offers many advantages for suspended solids (SS) removal, such as high security and efficiency, low cost, being nontoxic, and producing no secondary pollution for the environment [4–10]. The use of bioflocculant for SS removal has been widely used in industrial, domestic, and building material and livestock wastewater treatment as a new water treatment agent [11–14]. Although there were some general reports of bioflocculant in wastewater treatment, relevant research and application of bioflocculant in aquaculture wastewater treatment have rarely been reported.

In recent years, the aquaculture industry had developed rapidly with a worldwide presence, especially in China. However, low feeding utilization rates caused approximately 75% of the aquaculture feed to remain as nitrogen and phosphorous in the wastewater [15]. Aquaculture wastewater was discharged arbitrarily into rivers, lakes, and ocean, resulting in eutrophication and even red tide disasters. Many efforts have sought to reduce and regulate the generation and emission of aquaculture wastewater, such as upscaling aquaculture wastewater treatment by microalgal bacterial flocs [16], application of probiotics in carp aquacultures [17], removal of organic matter from polluted coastal waters by floating bed phytoremediation [18], and the application of artificial wetlands in multistage aquaculture wastewater purification [19]. Biofloc technology as one of the most advanced aquaculture technology models has been widely applied in shrimp, tilapia, and carp pond cultures. Using biofloc technology produced more aquaculture products without significantly increasing the usage of the basic natural resources of water and land, minimized damage to the environment, and provided an equitable cost/benefit ratio to support economic and social sustainability [20–24].
Compared with industrial and domestic wastewater, aquaculture wastewater is mainly composed of carbon, nitrogen, phosphorous, and other nutrients. Aquaculture wastewater also has its own characteristics, such as fewer poisonous metal materials and lower concentrations of nitrogen, phosphorous, SS, and chemical oxygen demand (COD). Therefore, bioflocculant-producing bacteria could feasibly be added to ponds and then used to effectively treat aquaculture wastewater. The aim of the present study was basically developed by two sections. The first section was to isolate and identify a bioflocculant-producing bacterium from fish pond and to optimize its culture conditions. In the second section, the bioflocculant produced by Bacillus megaterium SPI was applied in the aquaculture wastewater treatment to reduce the COD and inorganic nitrogen, promote the formation of biofloc, improve the utilization rate of nitrogen, and ultimately form a highly efficient and healthy aquaculture model suitable for China's pond aquaculture.

2. Materials and Methods

2.1. Biofloc Samples and Isolation of Bioflocculant-Producing Bacterium. Biofloc samples were collected by Imhoff cones from the carp biofloc technology pond at Hulan experimental station of Heilongjiang River Fisheries Research Institute in Heilongjiang Province, China (45.97°N, 126.63°E). The samples were stored at 4°C in sterile containers. First, each biofloc sample was homogenized and serially diluted in sterile water. Second, each dilution was spread on enrichment medium and incubated at 30°C for 72 h. Strains with different colony morphology were taken and repeatedly cultivated for purification, and the single pure colony was saved for later use. Third, each pure colony was spread on fermentation medium and cultured at 30°C in a rotary shaker at 160 r min⁻¹ for 72 h. The culture broth was used to determine for flocculating efficiency. The strain with the highest flocculating efficiency and good several subcultures was selected as the bioflocculant-producing bacterium for further study. Kaolin suspensions at a concentration of 5 g L⁻¹ were then used to evaluate the flocculating capability of a series of the culture. Among them, the enrichment medium included beef extract (3 g L⁻¹), peptone (10 g L⁻¹), and NaCl (5 g L⁻¹) and was amended with 1.8% agar. The fermentation medium included glucose (20 g L⁻¹), KH₂PO₄ (2 g L⁻¹), K₂HPO₄ (5 g L⁻¹), MgSO₄·7H₂O (0.5 g L⁻¹), (NH₄)₂SO₄ (0.2 g L⁻¹), NaCl (0.1 g L⁻¹), urea (0.5 g L⁻¹), and yeast extract (0.5 g L⁻¹).

2.2. PCR Amplification and Phylogenetic Analysis. The bacterial genomic DNA of strain SPI was extracted using the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-Tek, Inc., USA). PCR amplification of the 16S rDNA was performed with universal primers (27F, 5′AGAGTTTGATCCTGGCTCAG3′, and 1492R, 5′GGTTACCTTGTTACGACTT3′). The amplification system was composed of a total volume of 50 μL containing 3 μL of total DNA, 1 μL of 27F, 1 μL of 1492R, 1 μL of dNTP, 5 μL of 10x buffer, 0.6 μL of Taq DNA polymerase, and 38.4 μL of ddH₂O [25]. The reaction conditions were as follows: 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1.5 min, annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min and a final extension at 72°C for 10 min [26]. PCR products were purified using PCR production purification kit, and the purified PCR products were sent to Suzhou GENEWIZ Biotechnologies Co. Ltd. (China) for sequencing. The sequence results were submitted (accession number: KU529280) to the GenBank database. Software MEGA 6.0 was used to construct a phylogenetic tree by the neighbor-joining method [27].

2.3. Identification with Biolog GEN III MicroStation System. The Biolog GEN III MicroStation System is an automated microbial identification system based on aerobic metabolic activities without the labor-intensive requirements of conventional strips or panels. The strain SPI was characterized using Biolog GEN III microplate (Biolog Inc., Hayward, CA, USA). GEN III plate contains 95 different carbon substrates, which is based on interpreting patterns of sole-carbon substrate utilization indicated by color development in a 96-well microtiter plate. By analyzing the similarity of the metabolic fingerprints between SPI and standard strains in the kinetic database by Biolog Retrospect 2.0 Data Management Software, the strain was identified. Among them, when SIM is > 0.5 and DIST is < 5.00, this is a more satisfactory result [28].

2.4. Analysis of Flocculating Efficiency. The flocculating efficiency of the bioflocculant produced by the bacterial culture was measured by kaolin suspension. In general, 2 mL of the bioflocculant (the culture broth of strain SPI), 5 mL of CaCl₂ (1%, w/v), and 93 mL of kaolin suspension were mixed in a 200-mL beaker. The mixture was stirred at 180 r min⁻¹ for 1.5 min and at 80 r min⁻¹ for 3 min with a vortex mixer (QL-86, Shanghai Jingmi Instrument Co., Ltd., China) and then kept still for 10 min. The supernatant portion was absorbed to determine its optical density (OD) at 550 nm by a 752 spectrophotometer [4]. The steps for the blank control were similar to the above steps except that the culture broth of strain SPI was replaced with distilled water. All assays were conducted in three duplicates. The flocculating efficiency was defined and calculated as follows:

\[
\text{Flocculating efficiency (\%) = } \left( \frac{A_0 - A}{A_0} \right) \times 100, \quad (1)
\]

where \(A_0\) and \(A\) were OD₅₅₀ of the blank control and of the supernatant, respectively.

2.5. Optimization of Culture Conditions. Experiments were designed in which carbon and nitrogen sources of fermentation medium were replaced by various carbon and nitrogen sources in fresh fermentation medium. The one-way medium was used to determine the flocculating efficiency for kaolin suspension and to select the optimum carbon and nitrogen source for strain SPI.

According to the one-way experiment results, specific carbon and nitrogen sources in the original fermentation medium were replaced by the optimum carbon and nitrogen sources for optimal performance. The four factors of carbon...
source, nitrogen source, initial pH, and temperature were the major factors influencing flocculation and were selected to design L_{16}(4^3) orthogonal experiment. The optimum culture conditions were obtained by the analysis of the orthogonal experiment results.

2.6. Preliminary Application in Wastewater Treatment of Aquaculture. The aquaria (90 × 55 × 45 cm) selected for the experimental tanks had 100 L of three types of water (aquaculture wastewater, Hulan river water, and urban domestic wastewater) with continuous aeration. Culture broth of strain SPI containing 1 × 10^7 CFU mL^{-1} was added to the aquaria water samples by an adding ratio of 1 × 10^4 CFU mL^{-1} to evaluate its effect on wastewater, especially by comparing how the data changed before and after inoculation. The experiment was divided into three groups, each group containing three duplicates. The indexes of chemical oxygen demand (COD), total ammonia nitrogen (TAN), suspended solids (SS), and volume of biofloc (FV) were determined.

2.7. Analytical Methods. The COD and SS were determined using the methods given by the National Standard of China. TAN was determined by the YSI Professional Plus (YSI Incorporated, Yellow Springs, USA), FV was determined by sampling 1000 mL pond water into a series of Imhoff cones [29], and the volume of the floc plug accumulating on the bottom of the cone was determined 15 min following sampling [20].

2.8. Statistical Analysis. Data analysis was performed by one-way ANOVA using SPSS 17.0 software for Windows. Duncan’s multiple range tests were used to identify differences among experimental groups, and the level of statistical significance was accepted as \( P < 0.05 \).

3. Results and Discussion

3.1. Isolation of Bioflocculant-Producing Bacterium. Approximately 48 isolates were selected from the biofloc samples (Table 1). However, only six strains with flocculating efficiency exceeding 80% were able to actively flocculate kaolin suspension, as measured after five or more subcultures. Among them, the bacterium named SPI with the highest flocculating efficiency was selected as the bioflocculant-producing bacterium for further study.

3.2. Identification and Characterization of Bioflocculant-Producing Bacterium. Strain SPI was a circular, smooth, white, rod-shaped, Gram-positive bacterium with fermented liquid that was brown and turbid. Molecular analysis based on 16S rDNA confirmed the strain SPI to be a Bacillus sp.; therefore, it was named Bacillus sp. SPI. The nucleotide sequence obtained in the present study had been submitted to GenBank and assigned accession number KU529280. In the phylogenetic tree, strain SPI and the other closest Bacillus strains were grouped together (Figure 1). Strain SPI was further identified using the Biolog GEN III MicroStation System, which was Biolog’s latest generation product for the testing and microbial identification of aerobic Gram-negative and Gram-positive bacteria because they were in the same test panel; Gram stain and other pretests were no longer needed [30]. The results showed that strain SPI was Bacillus megaterium (probability 59.6%, SIM 0.596, and DIST 5.883) based on the carbon source metabolic characteristics (Table 2). Therefore, this strain was named Bacillus megaterium SPI.

Bioflocculants were produced by many microorganisms widely distributed in soils and waters [31]. More than 70 bioflocculant-producing microorganisms have been reported, such as Bacillus subtilis [5], Bacillus firmus [32], Bacillus licheniformis [33], Bacillus mucilaginosus [2], Proteus mirabilis [34], and Klebsiella sp. [35]. However, bioflocculant produced by Bacillus megaterium and its application in wastewater treatment have rarely been reported. It was found that the extracellular polymeric substances (EPS) from Bacillus megaterium TFB0 exhibit a high flocculation activity [36]. One report of a Bacillus megaterium strain producing a biodegradable flocculant was observed for turbidity and arsenic removal during growth [37]. Another bioflocculant produced by Bacillus megaterium YWO-5 was used for

| Number | Flocculating ratio (%) | Number | Flocculating ratio (%) | Number | Flocculating ratio (%) | Number | Flocculating ratio (%) |
|--------|------------------------|--------|------------------------|--------|------------------------|--------|------------------------|
| 1      | 58.2 ± 1.3             | 12     | 65.1 ± 3.2             | 25     | 68.5 ± 1.9             | 37     | 91.9 ± 2.2             |
| 2      | 62.1 ± 1.2             | 14     | 54.5 ± 2.1             | 26     | 75.9 ± 1.6             | 38     | 73.9 ± 1.5             |
| 3      | 66.9 ± 2.2             | 15     | 41.9 ± 1.8             | 27     | 64.2 ± 2.3             | 39     | 78.1 ± 4.2             |
| 4      | 65.1 ± 0.9             | 16     | 51.6 ± 1.1             | 28     | 52.5 ± 1.2             | 40     | 68.5 ± 3.1             |
| 5      | 89.2 ± 0.8             | 17     | 66.7 ± 0.8             | 29     | 58.1 ± 0.9             | 41     | 76.1 ± 2.1             |
| 6      | 65.1 ± 1.7             | 18     | 78.4 ± 2.1             | 30     | 62.9 ± 2.1             | 42     | 52.1 ± 3.6             |
| 7      | 75.4 ± 1.5             | 19     | 76.1 ± 3.2             | 31     | 57.2 ± 1.7             | 43     | 60.3 ± 1.5             |
| 8      | 68.1 ± 1.9             | 20     | 70.3 ± 1.5             | 32     | 56.6 ± 1.2             | 44     | 73.5 ± 0.8             |
| 9      | 62.1 ± 2.1             | 21     | 65.2 ± 3.5             | 33     | 75.2 ± 4.3             | 45     | 66.5 ± 1.3             |
| 10     | 70.3 ± 0.7             | 22     | 80.9 ± 2.1             | 34     | 88.7 ± 3.5             | 46     | 88.2 ± 2.7             |
| 11     | 42.1 ± 0.6             | 23     | 49.9 ± 1.4             | 35     | 75.2 ± 2.4             | 47     | 77.9 ± 1.6             |
| 12     | 72.5 ± 1.5             | 24     | 50.2 ± 2.3             | 36     | 43.9 ± 1.6             | 48     | 86.1 ± 2.1             |

Note. Each value represents a mean ± SE (\( n = 3 \)). Values in the line with different superscript letters are significantly different (\( P < 0.05 \)).
wastewater treatment [38]. In this work, the highly efficient bioflocculant-producing bacterium Bacillus megaterium SP1 was especially isolated from biofloc samples of aquaculture ponds for the purpose of accelerating biofloc formation and improving the water quality in aquaculture ponds.

3.3. Optimization of Culture Conditions

3.3.1. The Selection of the Optimum Carbon Source. Carbon source is a carbonaceous material used in microbial cells to supply energy for microbial growth, reproduction, and movement. To investigate the effect of various carbon sources on flocculating rate (in a kaolin suspension) under optimal culture conditions, the carbon source glucose (carbon content 0.4%) was used as fermentation medium in the control group and was replaced by various carbon sources (sucrose, fructose, maltose, soluble starch, citric acid, glycerol, and ethanol at the same concentration; other components remain unchanged) (Figure 2(a)). It was evident that glucose, fructose, sucrose, and soluble starch were suitable for biofloculant production with the flocculating efficiency exceeding 80% after 72 h cultivation. The strain SP1 adapted well to a
Table 2: Carbon source metabolic characteristics of SP1 in GN III microplate.

| Carbonsource reactions | SPI |
|------------------------|-----|
| Polymers               |     |
| Dextrin                | /   |
| Glycogen               | –   |
| Tween 40               | /// |
| Sugars and sugar derivatives |     |
| N-Acetyl-d-galactosamine | –   |
| N-Acetyl-d-glucosamine | –   |
| N-Acetyl-β-d-mannosamine | –   |
| d-Arabinol             | –   |
| d-Cellobiose           | –   |
| d-Fructose             | /   |
| d-Fucose               | /   |
| d-Galactose            | /   |
| Gentiobiose            | –   |
| α-d-Glucose            | –   |
| 3-Methyl-glucose       | –   |
| Myoinositol            | –   |
| α-d-Lactose            | –   |
| d-Salicin              | –   |
| d-Maltose              | /   |
| d-Mannitol             | /   |
| d-Mannose              | –   |
| d-Melibiose            | –   |
| β-Methyl-glucomide     | /   |
| Stachyose              | –   |
| d-Raffinose            | /   |
| l-Rhamnose             | –   |
| d-Sorbitol             | –   |
| Sucrose                | +   |
| d-Trehalose            | /   |
| d-Turanose             | /   |
| Methyl esters          |     |
| Methyl pyruvate        | /// |
| d-Lactic acid methyl ester | –   |
| Carboxylic acids       |     |
| Acetic acid            | –   |
| Acetoacetic acid       | /   |
| Citric acid            | –   |
| Formic acid            | /   |
| l-Galactonic acid lactone | /   |
| d-Galacturonic acid    | /   |
| d-Gluconic acid        | /   |
| d-Malic acid           | +   |
| l-Malic acid           | +   |
| d-Gluturonic acid      | /   |
| α-Hydroxy-butyric acid | –   |
| β-Hydroxy-d,l butyric acid | /   |
| ρ-Hydroxy-phenylacetic acid | /   |
| d-Saccharic acid       | /   |
| Muic acid              | /   |
| Carboxylic acids       |     |
| α-Keto butyric acid    | –   |
| α-Keto glutaric acid   | –   |
| l-Lactic acid          | /   |
| Propionic acid         | –   |
| Quinic acid            | +   |
| Bromosuccinic acid     | /   |

Note. +: positive response; –: negative response; /: borderline; ///: mismatched positive; and ///: mismatched negative.

The specific flocculating rates of glucose and soluble starch were 87.9% and 86.8%, respectively. Therefore, glucose was chosen as the optimum carbon source of strain SP1 because it had the highest flocculating activity and it has the lowest cost.

3.3.2. The Selection of the Optimum Nitrogen Source. Nitrogen sources provide the raw material for microbial amino acid synthesis. The effect of various nitrogen sources on the flocculating efficiency (in a kaolin suspension) after 72 h cultivation was observed. Beef extract, peptone, urea, (NH₄)₂SO₄, and...
NH₄NO₃ replaced yeast extract (nitrogen content 0.03%) at the same concentration which was shown in Figure 2(b). The flocculating efficiency of six different nitrogen sources ranged from 66.88% to 89.37% and illustrated that certain nitrogen sources had a greater influence on the flocculating activity for the strain SPI. Specifically, beef extract and yeast extract produced bioflocculant with the flocculating efficiency exceeding 85% after 72 h cultivation. As a result, the beef extract was chosen as the best nitrogen source of strain SPI for further study because of its high flocculating efficiency, complicated composition, and abundant nutrition.

### Table 3: The orthogonal experiment L₁₆(4⁵) of optimization of culture conditions.

| A (g L⁻¹) | B (g L⁻¹) | C (°C) | D | E | Flocculating efficiency |
|-----------|-----------|--------|---|---|-------------------------|
| 1 1 (10.0) | 1 (0.2) | 1 (20) | 1 (7.0) | 1 | 0.785 |
| 2 1 | 2 (0.5) | 2 (25) | 2 (6.5) | 2 | 0.813 |
| 3 1 | 3 (0.8) | 3 (30) | 3 (6.0) | 3 | 0.839 |
| 4 1 | 4 (1.0) | 4 (35) | 4 (5.5) | 4 | 0.765 |
| 5 2 (15.0) | 1 | 2 | 3 | 4 | 0.786 |
| 6 2 | 2 | 1 | 4 | 3 | 0.822 |
| 7 2 | 3 | 4 | 1 | 2 | 0.806 |
| 8 2 | 4 | 3 | 2 | 1 | 0.836 |
| 9 3 (20.0) | 1 | 3 | 4 | 2 | 0.840 |
| 10 3 | 2 | 4 | 3 | 1 | 0.866 |
| 11 3 | 3 | 1 | 2 | 4 | 0.858 |
| 12 3 | 4 | 2 | 1 | 3 | 0.856 |
| 13 4 (25.0) | 1 | 4 | 2 | 3 | 0.788 |
| 14 4 | 2 | 3 | 1 | 4 | 0.906 |
| 15 4 | 3 | 2 | 4 | 1 | 0.827 |
| 16 4 | 4 | 1 | 3 | 2 | 0.808 |

R 0.055 0.052 0.049 0.025 0.020

Note: A: glucose; B: beef extract; C: culture temperature; D: medium initial pH; and E: blank control.

NH₄NO₃ replaced yeast extract (nitrogen content 0.03%) at the same concentration which was shown in Figure 2(b). The flocculating efficiency of six different nitrogen sources ranged from 66.88% to 89.37% and illustrated that certain nitrogen sources had a greater influence on the flocculating activity for the strain SPI. Specifically, beef extract and yeast extract produced bioflocculant with the flocculating efficiency exceeding 85% after 72 h cultivation. As a result, the beef extract was chosen as the best nitrogen source of strain SPI for further study because of its high flocculating efficiency, complicated composition, and abundant nutrition.

#### 3.4. Optimization of Culture Medium and Culture Conditions by Using Orthogonal Experiments.

Orthogonal test factors and levels for flocculation of strains SPI, including glucose, beef extract, culture temperature, and culture medium initial pH values (with A, B, C, and D), were shown in Table 2. Orthogonal experiments were conducted to determine the optimal culture conditions. Orthogonal experimental results were shown in Table 3. The results of the range analysis suggested that the flocculating efficiency was influenced by the following factors in the descending order: glucose > beef extract > culture temperature > culture medium initial pH.

Microbial growth is influenced by culture medium composition and various survival factors. Lower concentration of carbon and nitrogen sources keeps strains such as SPI from getting enough nutrients, thus affecting its growth and flocculating efficiency of the bioflocculant. In contrast, higher concentrations of carbon and nitrogen sources can make higher concentrations of inhibitory substances that negatively affect microbial growth as well as the flocculating rate of bioflocculant [31, 39, 40]. Microbial activity and metabolism are related to temperature; the suitable temperature is beneficial to microbial growth and metabolic rate. It was generally believed that the optimum temperature for bioflocculant formation was between 25 and 35°C, with low temperatures slowing bacterial growth and high temperatures changing the structure of the protein or peptide chain included in the bioflocculant (leading to degeneration) [41]. Initial pH also can affect the growth of bioflocculant-producing bacteria; in general, the optimal pH value of bioflocculant-producing bacteria is from neutral to weak alkaline. For different microorganisms, the optimum pH value is not the same [42].

In this study, the optimal factor combination for flocculating efficiency from the result above was A₃B₁C₁D₁: 20 g of glucose, 0.5 g of beef extract, culture temperature of 30°C, and a medium initial pH of 7. Under these optimum culture conditions, the flocculating efficiency of bioflocculant produced by strain SPI for kaolin suspension was 94.32%.

#### 3.5. Preliminary Application in Wastewater Treatment of Aquaculture.

Based on the orthogonal experiment results, two types of wastewater and Hulan river water were treated under optimal conditions (A₃B₁C₁D₁), and the results were shown in Figure 3. The aquaculture wastewater quality after the treatment improved significantly. COD decreased from 35.6 to 12.8 mg L⁻¹ (P < 0.05), TAN decreased from 6.43 to 2.34 mg L⁻¹ (P < 0.05), SS decreased from 271 to 4.34 mg L⁻¹ (P < 0.05), and FV increased from 4.93 to 25.97 mL L⁻¹ (P < 0.05). Under optimal culture conditions, the strain SPI produced bioflocculant for aquaculture wastewater with a better purification effect: the removal rate of COD was from 44.19% to 64.04%, the removal rate of TAN was from 33.83% to 63.61%, and the removal rates of the SS were all over 70%. Interestingly, the FV ratio increased from 255.25% to 426.35%, which demonstrated that adding the culture broth of strain SPI to wastewater could effectively accelerate the formation of biofloc. Adding SPI could not only solve the problem of accumulation of harmful substances in aquaculture water but also promote the volume of biofloc which could be eaten by fish, improve the efficiency of protein generation in fish, reduce the feed demand for fish, and increase the income gained from aquaculture [20, 43].

High levels of inorganic nitrogen such as ammonia nitrogen and nitrite nitrogen are harmful to fish and are regarded as a limiting factor to production in intensive aquaculture [44]. Compared with residential and industrial sewage, the aquaculture wastewater had its own characteristics with low pollutant concentration and large water flow. Nitrogen, phosphorus concentration, suspended solid content, and the COD of aquaculture wastewater are lower than those of other types of wastewater. Bioflocculant-producing bacteria can use these substances, which are harmful to the growth of fish, and produce bioflocculant with high flocculating
activity. These bioflocculant-producing bacteria were successfully used to flocculate particulate and organic matter, improve water transparency and dissolved oxygen, reduce oxygen consumption, and thus improve environment and water quality of aquaculture.

It was of great significance to generate a mutual fusion between the bioflocculant technology of industrial wastewater treatment and biofloc technology of aquaculture to enhance the quality and efficiency of aquaculture and to promote characteristics of Chinese aquaculture that are being friendly to the environment, being healthy, and being sustainable for development.

4. Conclusions

In this study, a bioflocculant-producing bacterium *Bacillus megaterium* SPI was isolated from biofloc in pond water. The optimal carbon and nitrogen sources for *Bacillus megaterium* SPI were 20 g L\(^{-1}\) of glucose and 0.5 g L\(^{-1}\) of beef extract at 30°C and pH 7. Under these optimum culture conditions, the flocculating efficiency of bioflocculant produced by strain SPI for kaolin suspension was 94.32%. It was demonstrated that adding strain SPI to aquaculture wastewater could effectively reduce the COD, TAN, and SS and accelerate biofloc formation.

**Abbreviations**

COD: Chemical oxygen demand  
TAN: Total ammonia nitrogen  
SS: Suspended solids  
FV: Volume of biofloc.

**Competing Interests**

There are no competing interests related to this paper.
Acknowledgments

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References

[1] J. Labille, F. Thomas, M. Milas, and C. Vanhaverbeke, “Floc- 
culation of colloidal clay by bacterial polysaccharides: effect of 
macromolecule charge and structure,” Journal of Colloid and 
Interface Science, vol. 284, no. 1, pp. 149–156, 2005.

[2] B. Lian, Y. Chen, J. Zhao, H. H. Teng, L. Zhu, and S. Yuan, “Mi-
crobial floculation by Bacillus mucilaginosus: applications and 
mechanisms,” Bioresource Technology, vol. 99, no. 11, pp. 4825–4831, 2008.

[3] J. Liu, J. Ma, Y. Liu, Y. Yang, D. Yue, and H. Wang, “Optimized 
production of a novel bioflocculant M-CII by Klebsiella sp. and 
its application in sludge dewatering,” Journal of Environmental 
Sciences, vol. 26, no. 10, pp. 2076–2083, 2014.

[4] R. Kurane, K. Hatamochi, T. Kakuno, M. Kiyohara, M. Hirano, 
and Y. Taniguchi, “Production of a bioflocculant by Rhodococ-
cus erythropolis S-1 grown on alcohols,” Bioscience Biotechnology 
and Biochemistry, vol. 58, no. 2, pp. 428–429, 1994.

[5] H. Yokoi, M. Shiraki, J. Hirose, S. Hayashi, and Y. Takasaki, “Flo-
culation properties of Xanthan produced by Xanthomonas 
campestris,” Biotechnology Techniques, vol. 10, no. 10, pp. 789–792, 1996.

[6] C. G. Kumar, H.-S. Joo, J.-W. Choi, Y.-M. Koo, and C.-S. Chang, 
“Purification and characterization of an extracellular polysac-
charide from haloalkalophilic Bacillus sp. I-450,” Enzyme and 
Microbial Technology, vol. 34, no. 7, pp. 673–681, 2004.

[7] S. Deng, G. Yu, and Y. P. Ting, “Production of a bioflocculant 
by Aspergillus parasiticus and its application in dye removal,” 
Colloids and Surfaces B: Biointerfaces, vol. 44, no. 4, pp. 179–186, 2005.

[8] R. Muñoz and B. Guieysse, “Algal-bacterial processes for 
the treatment of hazardous contaminants: a review,” Water 
Research, vol. 40, no. 15, pp. 2799–2815, 2006.

[9] J. H. Yim, S. J. Kim, S. H. Ahn, and H. K. Lee, “Characteriza-
tion of a novel bioflocculant, p-KG03, from a marine dinoflagellate, 
Gyrodinium impudicum KG03,” Bioresource Technology, vol. 98, 
nos. 2 and 3, pp. 361–367, 2007.

[10] Y. Zheng, Z.-L. Ye, X.-L. Fang, Y.-H. Li, and W.-M. Cai, “Pro-
duction and characteristics of a bioflocculant produced by Bacillus 
subtilis F9,” Biotechnology, vol. 99, no. 16, pp. 7686–7691, 2008.

[11] S. B. Deng, R. B. Bai, X. M. Hu, and Q. Luo, “Characteris-
tics of a bioflocculant produced by Bacillus mucilaginosus and its 
use in starch wastewater treatment,” Applied Microbiology and 
Biotechnology, vol. 60, no. 5, pp. 588–593, 2003.

[12] S. Yan, N. Wang, Z. Chen et al., “Genes encoding the produc-
tion of extracellular polysaccharide bioflocculant are clustered on a 
30-kb DNA segment in Bacillus licheniformis,” Functional and 
Integrative Genomics, vol. 13, no. 4, pp. 425–434, 2013.

[13] L. Y. Peng, C. P. Yang, G. M. Zeng et al., “Characterization and 
application of bioflocculant prepared by Rhodococcus erythro-
polus using sludge and livestock wastewater as cheap culture 
media,” Applied Microbiology and Biotechnology, vol. 98, no. 15, pp. 6847–6858, 2014.

[14] S. S. Giri, M. Harshini, S. S. Sen, V. Sukumaran, and S. 
C. Park, “Production and characterization of a thermostable 
bioflocculant from Bacillus subtilis F9, isolated from wastewater 
sludge,” Ecotoxicology and Environmental Safety, vol. 121, pp. 45– 
50, 2015.

[15] R. Crab, Y. Avnimelech, T. Defoirdt, P. Bossier, and W. Ver-
straebe, “Nitrogen removal techniques in aquaculture for a 
sustainable production,” Aquaculture, vol. 270, no. 1–4, pp. 1–14, 2007.

[16] S. Van Den Hende, V. Beeen, G. Bore, N. Boon, and H. Vervaeren, “Up-scaling aquaculture wastewater treatment by 
microagal bacterial flocs: from lab reactors to an outdoor 
raceway pond,” Bioresource Technology, vol. 159, pp. 342–354, 
2014.

[17] M. A. O. Dawood and S. Koshio, “Recent advances in the 
role of probiotics and prebiotics in carp aquaculture: a review,” 
Aquaculture, vol. 454, pp. 243–251, 2016.

[18] L. Huang, J. Zhuo, W. Guo, R. G. M. Spencer, Z. Zhang, and J. 
Xu, “Tracing organic matter removal in polluted coastal waters 
via floating bed phytoremediation,” Marine Pollution Bulletin, 
vol. 71, no. 1–2, pp. 74–82, 2013.

[19] S. Zhang, G. Li, X. Li, and L. Tao, “Multiple linear modeling 
of outflow nitrogen dynamics in vertical-flow constructed 
wetlands under two different operating states,” Ecological Engi-
neering, vol. 81, pp. 53–61, 2015.

[20] Y. Avnimelech, “Feeding with microbial flocs by tilapia in 
minimal discharge bio-flocculation technology ponds,” Aquacul-
ture, vol. 264, no. 1–4, pp. 140–147, 2007.

[21] R. Crab, M. Kochwa, W. Verstraebe, and Y. Avnimelech, “Bio-
flocculation technology application in over-wintering of tilapia,” 
Aqua-cultural Engineering, vol. 40, no. 3, pp. 105–112, 2009.

[22] E. Stokstad, “Down on the shrimp farm,” Science, vol. 328, no. 
5985, pp. 1504–1505, 2010.

[23] R. Crab, T. Defoirdt, P. Bossier, and W. Verstraebe, “Biofloc tech-
nology in aquaculture: beneficial effects and future challenges,” 
Aquaculture, vol. 356, pp. 351–356, 2012.

[24] Z. Zhao, Q. Xu, L. Luo, C. Wang, J. Li, and L. Wang, “Effect of 
feed C/N ratio promoted bioflocs on water quality and produc-
tion performance of bottom and filter feeder carp in minimum-
water exchanged pond polyculture system,” Aquaculture, vol. 434, pp. 442–448, 2014.

[25] W. G. Weisburg, S. M. Barns, D. A. Pelletier, and D. J. Lane, “16S 
ribosomal DNA amplification for phylogenetic study,” Journal 
of Bacteriology, vol. 173, no. 2, pp. 697–703, 1991.

[26] X. Zhang, J. Gao, F. Zhao, Y. Zhao, and Z. Li, “Characterization of a salt-tolerant bacterium Bacillus sp. from a membrane biore-
actor for saline wastewater treatment,” Journal of Environmental 
Sciences, vol. 26, no. 6, pp. 1359–1374, 2014.

[27] N. Saitou and M. Nei, “The neighbor-joining method: a new 
technique for reconstructing phylogenetic trees,” Molecular 
Phylogenetics and Evolution, vol. 4, no. 4, pp. 406–425, 1993.

[28] X.-J. Hu, Z.-J. Li, Y.-C. Cao, J. Zhang, Y.-X. Gong, and Y.-F. 
Yang, “Isolation and identification of a phosphate-solubilizing 
bacterium Pantocea stewartii subsp. stewartii g6, and effects of 
temperature, salinity, and pH on its growth under indoor 
culture conditions,” Aquaculture International, vol. 18, no. 6, pp. 
1079–1091, 2010.

[29] A. D. Eaton, L. S. Cleeserci, and A. E. Greenberg, Eds., Standard 
Methods for the Examination of Water and Waste Water, American Public Health Association, Washington, DC, USA, 
10th edition, 1995.
[30] P. Wragg, L. Randall, and A. M. Whatmore, “Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest,” Journal of Microbiological Methods, vol. 105, pp. 16–21, 2014.

[31] T. Zhang, Z. Lin, and H.-L. Zhu, “Microbial flocculant and its application in environmental protection,” Journal of Environmental Sciences, vol. 11, no. 1, pp. 1–12, 1999.

[32] H. Salehzadeh and S. A. Shojaosadati, “Isolation and characterisation of a bioflocculant produced by Bacillus firmus,” Biotechnology Letters, vol. 24, no. 1, pp. 35–40, 2002.

[33] I. L. Shih, Y. T. Van, L. C. Yeh, H. G. Lin, and Y. N. Chang, “Production of a biopolymer flocculant from Bacillus licheniformis and its flocculation properties,” Bioresource Technology, vol. 78, no. 3, pp. 267–272, 2001.

[34] Z. Q. Zhang, S. Q. Xia, J. F. Zhao, and J. Zhang, “Characterization and flocculation mechanism of high efficiency microbial flocculant TJ-F1 from Proteus mirabilis,” Colloids and Surfaces B: Biointerfaces, vol. 75, no. 1, pp. 247–251, 2010.

[35] A. K. Mandal, K. K. Yadav, I. K. Sen et al., “Partial characterization and flocculating behavior of an exopolysaccharide produced in nutrient-poor medium by a facultative oligotroph Klebsiella sp. PB12,” Journal of Bioscience and Bioengineering, vol. 115, no. 1, pp. 76–81, 2013.

[36] S.-J. Yuan, M. Sun, G.-P. Shen et al., “Identification of key constituents and structure of the extracellular polymeric substances excreted by Bacillus megaterium TF10 for their flocculation capacity,” Environmental Science and Technology, vol. 45, no. 3, pp. 1152–1157, 2011.

[37] K. K. Devi and K. A. Natarajan, “Isolation and characterization of a bioflocculant from Bacillus megaterium for turbidity and arsenic removal,” Minerals and Metallurgical Processing, vol. 32, no. 4, pp. 222–229, 2015.

[38] H.-C. Seo, S.-J. Yeo, H.-Y. Cho, and H.-C. Yang, “Some cultural characteristics of Bacillus megaterium YWO-5 producing bioflocculant for wastewater treatment,” The Korean Journal of Applied Microbiology and Biotechnology, vol. 27, no. 1, pp. 80–85, 1999.

[39] Y. H. Wang, B. Dong, Y. B. Mao, and Y. S. Yan, “Bioflocculant-producing Pseudomonas alcaligines: optimal culture and application,” The Chinese Journal of Environmental Science and Technology, vol. 33, no. 3, pp. 68–71, 2010.

[40] R. Tao, Z. H. Yang, G. M. Zeng, E. J. Deng, and C. Li, “Screening and identification of bioflocculant-producing microorganism and optimal study on culture conditions,” The Chinese Journal of China Biotechnology, vol. 25, no. 8, pp. 76–81, 2005.

[41] J. Bao-jun and Y. Jiang-mei, “The research status and development trend of microbial flocculant,” Physics Procedia, vol. 24, pp. 425–428, 2012.

[42] W. Zhen, W. Kongxing, and X. Yumin, “Screening of flocculant-producing microorganisms and some characteristics of flocculants,” Biotechnology Techniques, vol. 8, no. 11, pp. 831–836, 1994.

[43] M. A. Burford, P. J. Thompson, R. P. McIntosh, R. H. Bauman, and D. C. Pearson, “The contribution of flocculated material to shrimp (Litopenaeus vannamei) nutrition in a high-intensity, zero-exchange system,” Aquaculture, vol. 232, no. 1–4, pp. 525–537, 2004.

[44] J. M. Ebeling, M. B. Timmons, and J. J. Bisogni, “Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems,” Aquaculture, vol. 257, no. 1–4, pp. 346–358, 2006.