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Screening and Identification of Flocculant Producing Bacteria and Its Application in Wastewater

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Abstract: Background: with the increasingly serious global water pollution, industrial and agricultural wastewater treatment is becoming more and more important. Flocculant precipitation is the first process in wastewater treatment. One of the keys to achieve efficient water purification is to add microbial flocculant with excellent performance. Objective: the research of high-efficiency and non-toxic microbial flocculant for wastewater treatment has increasingly become important in environmental protection. Method: CMC-Na medium and dilute iodine staining was used to separate and screen flocculant producing bacteria. The distribution of flocculant activity and the effect of different types of wastewater treatment were studied. Results: the flocculant producing strain 71-1 separate from ruminant feces was identified as *Cellulosimicrobiums sp*. The flocculating rate of MBF-P71 was 97.83%, which had obvious degradation effect on COD value of pig farm manure water and ship port sewage. The removal rates of cod were 66.95% and 46.68% respectively. Conclusion: MBF-P71 has the advantages of good flocculation effect, low production cost, high efficiency and low dosage. It has a good development and application prospect for wastewater treatment.

Keywords: Microbial Flocculant, *Cellulosimicrobiums*, Screening and Identification, Sewage Purification, Flocculant-producing Strain, Wastewater

1. Introduction

Water is the source of human life. It is estimated that by 2025, two thirds of the world population will probably live in lack of water resources. With the continuous growth of population and the development of urbanization in China, the demand for water resources keeps increasing, and the problem of water pollution is becoming increasingly prominent.

Microbial flocculant is a novel water treatment agent extracted from micro-organisms or their secretions by biotechnology and purified, which may be degraded naturally. It is also the key and core of flocculation technology for water treatment, and can be widely used in sewage or waste water treatment and sludge decontamination [1, 2].

In the natural environment, many microorganisms can be used as flocculant producing bacteria.

Microbial flocculant-producing bacteria are widely distributed in air, soil, ore, sewage, sludge and animals. They include fungi, bacteria and actinomycetes. The research on bacterial flocculant is relatively few, and the research on fungal flocculant is more than others. However, bacteria grow fast and have high practical significance in industrial production.

Microbial flocculant is high efficient, safe, non-toxic, and without secondary pollution. So it is promising for wide application. At present, a large amount of sewage is produced in China every year, and it is urgent to have an economical and simple treatment technology for industrial wastewater and domestic sewage treatment. For this, the study aims to explore the research and development concerning screening and identification of the strains for flocculant production, as well as application of microbial flocculants in degradation for different kinds of sewage or wastewater, so that the microbial flocculants could be used for kinds of sewage or wastewater

efficiently and specifically, and the large-scale industrial production of flocculants become economic and feasible.

2. Materials and Methods

2.1. Experimental Materials

The strains were from the feces samples of ruminant collected from large-scale farms and slaughtering plants in three provinces of northeast China; the piggery liquid manure samples were from Wufengwang pig professional cooperative in Heishan, Jinzhou City, Liaoning Province.

China; domestic sewage samples were from Panjin City First Sewage Treatment Plant; food factory wastewater samples were from a Food Co. Ltd. of Panjin City; and marine ship sewage samples were from the ship that near the oceangoing shipsmoored at the port of Liaodong Bay, Panjin.

2.2. Main Reagents

Peptone, yeast extract and AGAR were purchased from OXOID, UK; sodium carboxymethyl cellulose from Beijing Solarbio Biotechnology co., LTD; Kaolin, potassium iodide, iodine, calcium chloride and other reagents from the Chemical Reagent Co., Ltd. of Sinopharm Group; API 20E, API 20NE and API 50CH kits for biochemical identification of bacteria and GP gram-positive bacteria identification card from BioMérieux; MALDI-TOF mass spectrum matrix and buffer from Brucker, Germany; bacterial genomic DNA extraction kit from Beijing Tiangen Biochemical Technology Co., Ltd.; 2×M5 HiPer plus Taq HiFi PCR mix from Mei5 Biotechnology Co. Ltd, Beijing; DL2000 DNA Marker from TaKaRa Biotechnology (Dalian) Co. Ltd., and agarose from Amresco Company.

2.3. Instruments

BG-270 water-jacket incubator (Medical Equipment Plant, Shanghai Boxun Industry & Commerce Co. Ltd.), ZH-CHASL13 thermostatic oscillator (Jiangsu Photosynthesis Electromechanical Equipment Co. Ltd.), UV5200 spectrophotometer (Shanghai Yuanxi Instruments co., Ltd.), Allegra X-12R high-speed centrifuge (BECKMAN COULTER), GI 100DX autoclave (Xiamen Zhiwei Instruments Co., Ltd.), AC2-5S1 A2 model bio-safety cabinet (Esco Micro Pte Ltd, Singapore), NB-9 constant temperature magnetic stirrer (Suzhou JiulianTechnology Co., Ltd.), pH -100-a hand-held meter) (Zhejiang Lichen Technology Co., Ltd), matrix assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF)(Brucker, Germany), automatic microbial identification system (Biomerieux, French), ET99731 COD/TOC multi-parameter comprehensive water quality tester (Lovibond, Germany), AND etc.

2.4. Medium

CMC-Na agar medium: 10 g of CMC-Na, 1 g of K_2HPO_4, 0.5 g of Mg_2SO_4, 0.01 g of Fe_2SO_4, 3 g of NH_4SO_4, 15 g of agar, 1 L of water, pH 6.8-7.0, Enrichment medium: LB liquid medium.

2.5. Methods

2.5.1. Enrichment and Initial Screening of Strains

The samples of livestock feces were placed in 5 mL LB liquid medium and cultured at 37°C for 24-36 h. CMC-Na agar was separated by the streak plate method and cultured at 37°C for 36-48 h. A single colony of different morphology on the medium was selected for pure culture. The plate was dyed with a dye preparation of potassium iodide and dilute iodide solution for 1 min, and a Vernier caliper was used for measuring the hydrolytic circle diameter of colony. The strains with high ratio of hydrolytic circle diameter to colony diameter were selected for rescreening [3].

2.5.2. Strain Rescreening and Determination of Flocculation Activity

The strains selected through initial screening were inoculated in LB liquid medium, and then incubated at 37°C in a shaker of 155 rpm for 48 h. The culture broth was centrifuged at 8000 r/min for 5 min, the supernatant was discarded, and the precipitate was retained. Then the precipitate was resuspended in 0.1M PBS for washing twice, centrifuged at 8000 r/min for 5 min, resuspended with PBS solution and broken through ultrasonication. The liquid obtained after 30 min of ultrasonic treatment was the microbial flocculant.

The flocculation activity is expressed by flocculation rate. Add 100 mL of 4 g/L kaolin suspension, 1 mL of 10% $CaCl_2$ and 1 mL of culture medium in a 100 mL cylinder. Stir rapidly for 1min, slowly for 2 min, and then hold for 10 min. Then a spectrophotometer was used for measuring the absorbance of supernatant at 550 nm. Meanwhile, distilled water instead of culture medium and $CaCl_2$ solution was used as the control for experiment, and the following formula was used for calculating the flocculation rate:

$$\text{Flocculation rate}=(A-B)/A\times 100\%$$

where A is absorbance value of the control group at 550nm, and B is the absorbance value of the test group at 550 nm [4].

2.5.3. Identification of Strains

Strains with high flocculation rate were selected by colony morphology observation, and a single pure colony was picked out for gram staining. MALDI-TOF MS was used for the identification of bacterial species, API 20E and API 20NE kits as well as GP gram-positive bacteria identification card were used for the identification of biochemical characteristics. Gene sequence analysis on strain 16S rDNA: About 3 to 5 single colonies were picked out and emulsified in 50 μL of 0.25% sodium dodecyl sulfate -0.05N NaOH solution, and incubated in metal bath at 100°C for 15 min. Then 100 μL of H_2O was added to the mixture after incubation, and 2 μL was taken and used for DNA template for PCR. PCR amplification conditions: primers (5'-GAGTTTGATCCTGGCTCAG-3'), 1510R (5'-GGCTACCTTGTTACGA-3')

The 16SrDNA of the isolates was performed PCR

amplification with 25 μL of PCR reaction system, including 12.5 μL of 2×M5 HiPer plus Taq HiFi PCR mix, 1.0 μL of each primer, 2.0 μL of template DNA, and 8.5μL of ddH_2O. PCR amplification conditions: 95°C for 5 min, 94°C for 45 s, 52°C for 45s, 72°C for 2 min, and after 30 cycles, 72°C for 8 min. After 1.5% agarose gel electrophoresis, PCR products were sented to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. The obtained gene sequences were uploaded to the Genbank database, and compared with those of standard strains for the BLAST sequence alignmenthomology. Clustal X software was used for multiple sequence alignments, and a phylogenetic tree was constructed by adjacency method of MEGA10.0 software. The confidence was tested through 1000 times of bootstrap analysis [5].

2.5.4. Determination of Flocculation Distribution

A certain amount of fermentation medium was centrifuged at 8000 r/min for 5 min, dissolved in 0.1 mL PBS and resuspended for washing twice, and again centrifuged at 8000 r/min for 5 min. The precipitate was resuspended with PBS solution, broken through ultrasonication, and then diluted to volume of original fermentation broth with PBS. Then the flocculation rate of the fermentation supernatant, resuspended liquid and cell broken liquid were determined respectively.

2.5.5. Application of Flocculants in the Treatment of Various Sewage and Wastewater

For samples of piggery liquid manure, domestic sewage, food factory wastewater, and ship port sewage water, take 100 mL each, with 1.0 mL of 10% $CaCl_2$ solution, add in 1 mL of MBF-P71 cell wall broken liquid, shake well, and hold for 15 min for sedimentation. Then determine the flocculation rate, COD value and ammonia nitrogen content of samples.

3. Results

3.1. Preliminary Screening Results

Total 620 strains were isolated clinically, and preliminary screening by CMC-Na plate found 200 strains with relatively high cellulase activity. Table 1 showed 8 strains with the highest ratio of substrate hydrolytic circle diameter to colony diameter. As shown in Figure 1, dyeing with the dye preparation of potassium iodide and diluting iodide solution showed that strain 71-1 had a relative high hydrolysis capacity for cellulose, with obvious hydrolytic circle.

Table 1. *Comparison of 8 strains of CMC-Na substrate utilization and colony diameter.*

Strain	Substrate hydrolytic circle diameter to colony diameter (average)
P71-1	11.2±0.3
P85-1	8.6±0.2
P26-2	10.3±0.3
P40-2	27±0.3
P76-1	10.7±0.2
P99-1	11.1±0.1
P98-1	7.8±0.3
P72-3	8.1±0.2

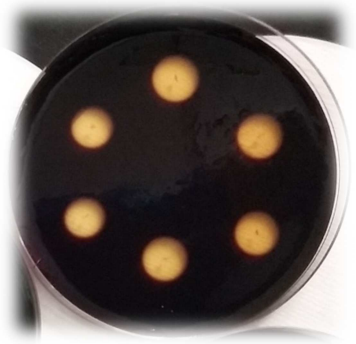

Figure 1. *Cellulase hydrolysis circle of strain 71-1.*

3.2. Rescreening Results

The 8 strains obtained through initial screening were respectively inoculated in LB liquid medium at 37°C for 48 h, and then the flocculation activity of the culture medium was measured with kaolin suspension. Figure 2 showed the flocculation effect of liquid resuspended after cell walls were broken for some strains.

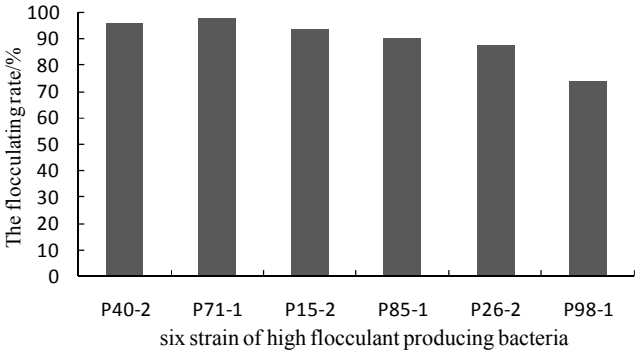

Figure 2. *Flocculation effect of strains P40-2, P71-1, P15-2, P85-1, P26-2, P98-1.*

3.3. Identification Of strain 71-1

3.3.1. Morphological Identification

Observed with naked eyes and under microscope, strain 71-1was yellow, round and slightly viscous on the LB plate, the surface was moist, smooth and opaque, with neat edge and a central bulge, diameter: 1-2mm. It was Gram positive, short rod-shaped, with obtuse ends, single or in pairs, without capsule and bud (Figure 3).

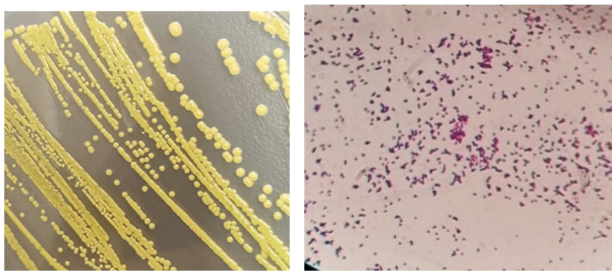

Figure 3. *Colony morphology (A) and Gram staining morphology of isolated strains (B) on LB solid medium.*

3.3.2. Mass Spectrometry and Physiological or Biochemical Identification

Strain 71-1 was identified by MALDI-TOF mass spectrometry (Figure 4). The biochemical identification results were shown in Table 2. It can be seen from Table 2 that strain 71-1 was partially different from the most approximate strain, *Cellulosimicrobium funkei* strain W6122 in biochemistry. Based on above morphological observation, mass spectrometry and biochemical identification results, the strain was preliminarily identified as *Cellulosimicrobiums* sp.

Table 2. *Physiological and biochemical characteristics of bovine Cellulosimicrobium sp.*

Test Item	Result		Test Item	Result	
	71-1	*C.funkei* W6122		71-1	*C.funkei* W6122
NO_3	+	−	TAD	+	−
GLU	+	+	SOR	−	−
ADH	−	−	RHA	−	−
URE	−	−	SAC	+	−
ESC	+	+	MEL	−	−
GEL	−	+	AMY	−	−
V-P	+	+	RIB	+	+
ARA	+	−	LXYL	+	+
MNE	+	+	OX	+	−
MAN	−	−	XYL	+	+
NAG	+	+	XLT	+	+
MAL	+	+	RAF	−	+
GNT	+	+	SAL	+	+
ADI	−	−	CEL	+	+
MLT	−	−	GNT	+	+
CIT	−	−	ARL	+	−
PAC	−	−	FUC	+	+
ONPG	−	+	LYX	+	+
GEN	+	+	LDC	−	+
ODC	−	−	IND	+	+

3.3.3. 16S rDNA Sequencing and Phylogenetic Analysis

With DNA of strain 71-1 as a template, a single strip with fragment of 1377 bp was obtained through PCR amplification (Figure 5), and 16 s rRNA gene sequence homology with *Cellulosimicrobium funkei strain* W6122 (NR_042937. 1) was 99.71%. The serial number MN 901961 was obtained through uploading 16SrRNA sequence of strain 71-1 to NCBI. Through construction of phylogenetic tree, strain 71-1 and *Cellulosimicrobium* constitute a stable group of the closest relationship, and belong to the same evolutionary branch as a known standard strain, *Cellulosimicrobium aquatile* strain 3 bp, with sequence similarity of 99% (Figure 6). In combination with colony morphology, protein fingerprinting, 16S rDNA identification results and physiological and biochemical characteristics, Strain 71-1 was identified as *Cellulosimicrobiums sp*. of effective species strain in *Cellulosimicrobium*.

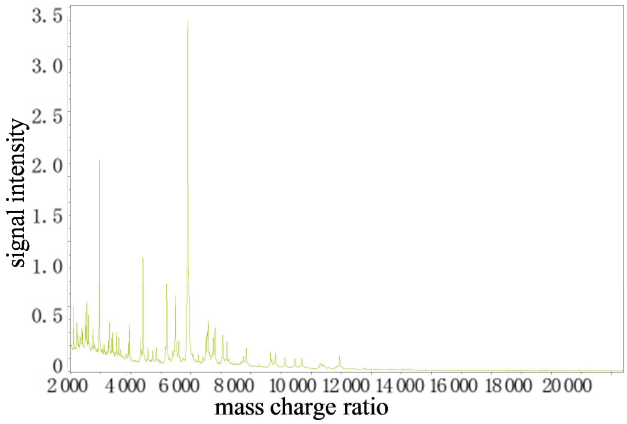

Figure 4. *Protein fingerprinting of strain 71-1.*

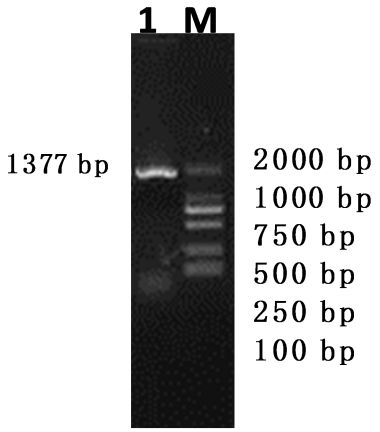

Figure 5. *PCR amplification results of strain 71-1 16SrRNA.*

3.4. Flocculation Distribution of Strain 71-1

The centrifuged fermentation broth, supernatant and bacterial suspension of strain 71-1 were taken for flocculation rate determination by kaolin test. The flocculation rate was 41.4%, 38.51% and 97.83%, respectively (Figure 7). The flocculant prepared from the bacterial suspension of strain 71-1was named as MBF-P71.

3.5. Application in Wastewater Treatment

3.5.1. MBF-P71 Treatment of Piggery Liquid Manure from Pig Farm

MBF-P71 treatment of piggery liquid manure from pig farm and determination of flocculation rate, COD value and ammonia nitrogen. The test results showed that the flocculation rate reached 96.63%. The COD value of piggery liquid manure from pig farm was 152 mg/L initially, and became 50.24 mg/L after MBF-P71 treatment. The COD removal rate was 66.95%. The ammonia nitrogen of piggery liquid manure from pig farm was 135.25 mg/L, and became110.90 mg/L after treatment. The ammonia nitrogen removal rate was 18.0%.

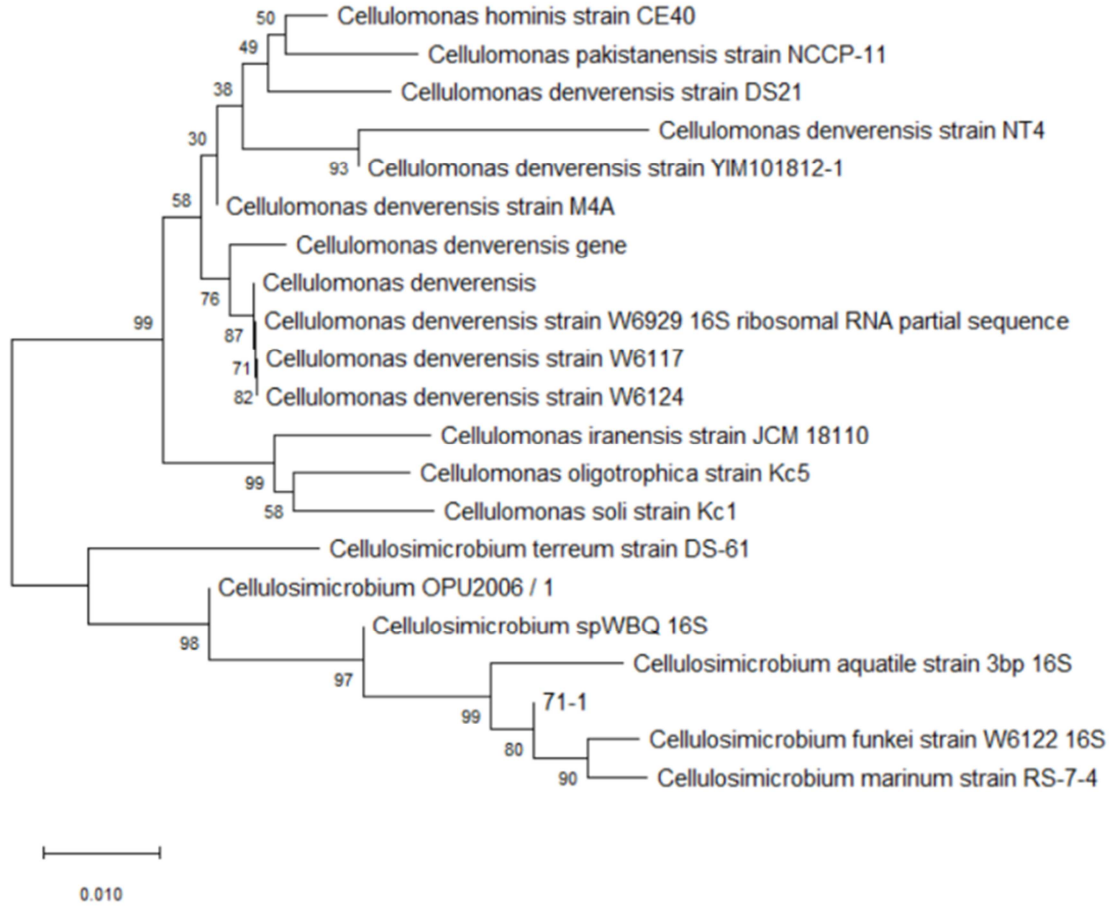

Figure 6. *Phylogenetic tree of 16SrDNA gene of strain 71-1.*

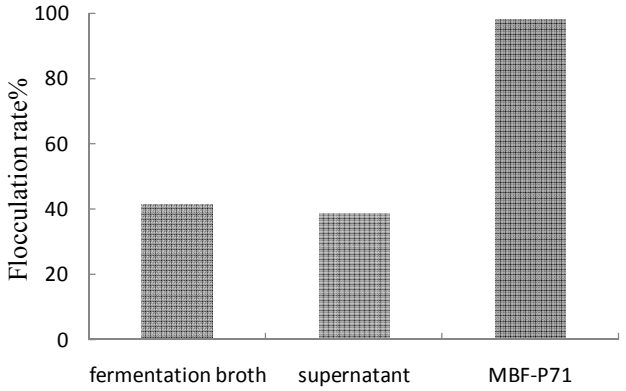

Figure 7. *Distribution of flocculating active components in fermentation broth.*

3.5.2. MBF-P71 Treatment of Wastewater from Food Factory

MBF-P71 treatment of wastewater discharged from bread factory and determination of flocculation rate, COD value and ammonia nitrogen. The test results showed that the flocculation rate reached 43.23%. The COD value of bread factory wastewater was 8.09 g/L initially, and became 7.41g/L after MBF-P71 treatment. The COD removal rate was 8.4%. The ammonia nitrogen of bread factory wastewater was 23.5 mg/L initially, and became 21.0 mg/L after MBF-P71 treatment, The ammonia nitrogen removal rate was 10.6%.

3.5.3. MBF-P71 Treatment of Domestic Sewage

MBF-P71 treatment of domestic sewage from Panjin City First Sewage Treatment Plant and determination of flocculation rate, COD value and ammonia nitrogen. The test results showed that the flocculation rate reached 64.28%. The COD value of untreated domestic sewage was 0.3 g/L, and after MBF-P71 treatment was 0.25 mg/L. The COD removal rate was 16.7%. The ammonia nitrogen of untreated domestic sewage was 1.3 mg /L, and after MBF-P71 treatment was 1.2 mg/L. The ammonia nitrogen removal rate was 7.7%.

3.5.4. MBF-P71 Treatment of Ship Sewage

For MBF-P71 treatment of sewage near shipsmoored at the port of Liaodong Bay, the flocculation rate was 56.73%. The COD value of untreated port sewage was 4.82 mg/L, and after MBF-P71 treatment was 2.57 mg/L. The COD removal rate was 46.68%. The ammonia nitrogen of untreated port sewage was 1.8mg/L, and after MBF-P71 treatment was 1.4 mg/L. The ammonia nitrogen removal rate was 22.2%.

4. Discussion

Since the 1980s, the research on microbial flocculants has

been initiated comprehensively. In 1986, Kurane produced a protein flocculant named NOC-1 with *Rhodococcus erythropolis* S-1 isolated from nature. NOC-1 has a high and extensive flocculation activity, which may be used broadly with low cost, and it is the best microbial flocculant so far [6]. In 1997,the flocculant DP-152 was isolated from rod-shaped bacteria by Suh et al. who discovered that rod-shaped bacteria could also produce flocculant for the first time [7]. In this study, *Cellulosimicrobium* strains were screened out from a large number of ruminant samples. *Cellulosimicrobium* was established in 2001 by Schumann, which belongs to order of actinomycetes, suborder of micrococci, *Cellulosimicrobium* [8]. There are 6 effective phenotypic strains for *Cellulosimicrobium*, i.e. *Cellulosimicrobium cellulans* [9], *Cellulosimicrobium aquatile sp*, *Cellulosimicrobium marinum sp*, *Cellulosimicrobium arenosum sp, Cellulosimicrobium funkei sp* and *Cellulosimicrobium terreum sp*. For the newly screened Strain 71-1, 16SrDNA sequencing and phylogenetic tree analysis were performed. The 16s rRNA gene sequence homology with *Cellulosimicrobium funkei* strain W6122 (NR_042937. 1) was 99.71%. It belongs to the same evolutionary branch as *Cellulosimicrobium aquatile* strain 3 bp, with sequence similarity of 99%, and the same group as *Cellulosimicrobium cellulans* and *Cellulosimicrobium marinum* sp. But analyses on hydrolysis capacity for cellulose and flocculation showed that it might be a new strain 71-1 of *Cellulosimicrobium aquatile strain* 3bp.

This study established a simple and rapid method for screening strains used for flocculant production. The initial screening was based on ratio of hydrolytic circle diameter to colony diameter. Total 200 strains were rapidly picked out from more than 600 strains for determination of flocculation rate by kaolin test, and the strains of high flocculation rate were selected for further application studies. The screening efficiency was significantly improved and the duration for strain screening was significantly shortened compared with traditional methods. An investigation on flocculation distribution of strain71-1 found that the flocculating rate of ultrasonicated liquid resuspended after centrifugation (97.83%) was far higher than that of centrifuged fermentation supernatant (41.4%) and liquid resuspended after centrifugation (38.51%). This finding is quite different from the reported microbial flocculant prepared with centrifuged supernatant of fermentation liquid. Consulting a large number of domestic and foreign literatures found no report concerning microbial flocculant preparation with cell broken liquid. This finding provides reference for the studies on such microbial flocculant.

Chemical oxygen demand (COD) and ammonia nitrogen are important indicators for sewage treatment. A large amount of wastewater with high ammonia nitrogen and COD, if discharged into rivers, lakes or seas, will not only cause eutrophication or malodorous black of water, increase the difficulty and cost of water treatment, but even have toxic effects on people and organisms [10]. In 2010, Zhu Dan screened out Strain EH-5 from seabed mud, with which a microbial flocculantwas produced. The decrease rates of COD, chroma and turbidity of beer wastewater by the microbial flocculant were 64.15%, 69.57% and 95.91% respectively, and the decrease rates of COD, chroma and turbidity of dairy wastewater were 74.49%, 75.00% and 93.78% respectively [11]. In 2014, Ma Shuwen screened out from natural sediment of potato starch wastewater the mold and yeast with excellent flocculation performance, in which geotrichum candidum, after cultivation, made the removal rate of COD in the wastewater medium up to 90.32%, and the removal rate of BOD5 up to 86.0% [12]. Yang Jinfeng treated tap water with microbial flocculant M-127, which showed that M-127 was superior to conventional flocculant in treatment efficiency, with water turbidity decrease rate of 93.89% at maximum [13]. The flocculant MBF-P71 developed in this study had different degradation effects on piggery liquid manure from pig farm, bread factory wastewater, domestic sewage and ship sewage. But in contrast, MBF-P71 has a higher capability in COD removal for piggery liquid manure from pig farm and ship sewage, and the removal rate was 66.95% and 46.68% respectively. The reduction of COD indicates the degradation of a large number of reducing substances such as organic pollutants in the water.

For this study, the next step for the flocculant MBF-P71 was to analyze active ingredients of MBF-P71, expand the application of MBF-P71 in combination with other biological agents for treatment of different kinds of wastewater or sewage [14, 15]. The research, development and application of microbial flocculants are being oriented towards high efficiency, low cost and no pollution. With increasing awareness on environmental protection and ecological protection, the extensive application of microbial flocculants has brought practical benefits to production and life of humankind. In the long run, microbial flocculants are bound to become the hotspot for future development of flocculants in the aspect of wastewater or sewage treatment [16].

5. Conclusion

MBF-P71 produced by *Cellulosimicrobium sp*. separate from CMC-Na plate, the flocculation rate of MBF-P71 was 97.83%. MBF-P71 may degrade piggery liquid manure from pig farm, wastewater discharged from bread factory, domestic sewage and ship sewage to different degrees, especially wasterwater from pig farm and ship sewage, the COD removal rate was 66.95% and 46.68% respectively.

Author Contributions

The authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results. XJF and ZSS conceived the idea of the study; LM, WXD and WXF carried out the experiments and revised the manuscript.

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and testing for the collaborative study conducted together.

Data Availability

No data have been fabricated or manipulated (including images) to support my conclusions. The 16S rRNA gene sequence of strain 71-1 were deposited in GenBank under accession numbers MN 901961.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

I certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by SS Zhang. And the study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time. No data have been fabricated or manipulated to support our conclusions.

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