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Production and characterization of high efficiency bioflocculant isolated from *Klebsiella* sp. ZZ-3

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HIGHLIGHTS

- The high efficient bioflocculant was produced by isolated *Klebsiella* sp. ZZ-3.
- The bioflocculant is pH tolerant and thermal stable.
- The bioflocculant is a β-type heteropolysaccharide containing some protein.
- High molecular weight and multi-functional groups contributed to the flocculation.
- Bridging is the main flocculation mechanism of ZZ-3.

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ABSTRACT

In this study, a new bioflocculant (ZZ-3) is isolated and evaluated. This novel flocculant was derived *Klebsiella*, which was identified by 16S rDNA sequencing as well as biochemical and physiological analyses. The composition of ZZ-3 was found to be 84.6% polysaccharides and 6.1% protein. More specifically, the amount (moles) of the polysaccharides rhamnose, mannose, and galactose were found to be 6.48, 2.47, and 1.74 greater than glucose, respectively. Results show ZZ-3 has a relatively high molecular weight (603–1820 kDa) and contains many functional groups (hydroxyl, amide, carboxyl, and methoxyl) that likely contribute to flocculation. The results of microscopic observation, zeta potential measurements, and ZZ-3 bioflocculant structure suggested that bridging was the main mechanism for flocculation with kaolin. Based on a high flocculation efficiency, thermal stability, pH tolerance and the ability to flocculate without additional cations, ZZ-3 shows potential for industrial application.

1. Introduction

Chemical flocculants (e.g., inorganic aluminum, ferric salts and synthetic organic polymers) are widely used for wastewater and drinking water treatment as well as in the food industry (Salehizadeh and Shojaosadati, 2001). Due to their biochemical stability, residues and derivatives of chemical flocculants are found in the environment (Rudén, 2004). New evidence suggests some traditional flocculants may be toxic to humans. For example, aluminum salts (e.g. alum) are suspected of inducing Alzheimer's disease (Crapper et al., 1973). Polyacrylamides, a common type of anionic flocculant, are found to be both neurotoxic and strong human carcinogens (Salehizadeh and Shojaosadati, 2001).

In contrast, so called bioflocculants – flocculants produced by microorganisms – are typically biodegradable and considered relatively safe for the environment and humans (Salehizadeh and Shojaosadati, 2001; Salehizadeh et al., 2000). Due to their benign characteristics, bioflocculants show great potential to replace the traditional chemical flocculants. In recent years, the identification and characterization of bioflocculants has increased. Strains of highly efficient bioflocculants isolated from an array of environments include *Proteus mirabilis* TJ-1 (Xia et al., 2008) and *Bacillus licheniformis* X14 (Li et al., 2009) from water treatment process, *Bacillus* sp. (Zheng et al., 2008) and *Bacillus mojavensis* strain 32A (Elkady et al., 2011) from typical soils, and *Nannocystis* sp. NU-2

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(Zhang et al., 2002) and *Sorangium cellulosum* NUST06 (Zhang et al., 2002) from saline soils. However, high yield costs and lower flocculation efficiency, relative to synthetic organic flocculants, currently limit the application of bioflocculants. Hence, identifying new bioflocculants with high flocculation efficiency and low yield costs is urgently needed. After this initial identification, further characterize is also necessary to better understand the mechanisms responsible for flocculation so that their efficiency can be optimized.

In the present study, six strains of bioflocculant producing organisms were isolated from paper mill wastewater. Flocculation activity was then tested using a kaolin suspension without the aid of additional cations. The strain with highest flocculation efficiency was identified as *Klebsiella* sp. ZZ-3 by 16S rDNA sequences and subjected to further biochemical and physiological characterization.

2. Methods

2.1. Isolation bioflocculant producing microorganism

An activated sludge sample was obtained from the primary settling tank of a paper mill wastewater treatment system in Chongqing, China. One gram of the active sludge was dispersed in 10 ml of sterile distilled water. Serial dilutions (1:10) of this sample were then made until a maximum dilution of 10^{-6} was achieved. From the last 3 dilution aliquots, 500 μl was planted on Luria–Bertani (LB) agar plates (tryptone, 10 g; yeast extract, 5 g; and NaCl, 10 g; agar, 15 g; per liter) and cultured at 30 °C overnight. Strains with unique colony morphologies were picked and inoculated into 250 ml flasks containing 100 ml flocculant selecting medium (glucose, 10 g; KH_2PO_4, 2 g; K_2HPO_4, 5 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.1 g; urea, 0.5 g; yeast extract, 0.5 g; per liter) for 3 days at 30 °C on a rotary shaker at 180 rpm. From this culture, 1 ml was used for testing flocculation activity.

2.2. Identification of bioflocculant producing microorganisms

Extraction of genomic DNA was performed with TIA Namp Bacteria DNA Kit (TianGen, China) according to the vendor's protocol. The 16S rRNA gene was amplified using bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The conditions for PCR were as follows: 5 min of denaturation at 94 °C, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were visualized by 0.8% agarose gel (w/v) electrophoresis and purified by TIANgel Midi Purification Kit (TianGen, China). The purified DNA fragments were ligated into pMD19-T vectors (TaKaRa, China) according to the manufacturer's instructions. Approximately 200 clones were grown in a LB plate supplemented with 50 μg/ml ampicillin. Three clones were picked and cultured overnight in a LB broth with same concentration ampicilin. The plasmids were then extracted by TIANprep Mini Plasmid Kit (TianGen, China), purified by TIANpure Mini Plasmid Kit (TianGen, China), and sequenced by BGI Corp. (Beijing, China) using an Applied Biosystems 3730XL DNA analyzer based on the 27F primer. Resultant 16S rRNA gene sequences were assembled using Seqman II 5.0 at DNASTAR and analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment of related sequences was carried out via CLUSTAL_X (version 1.83). Gaps at the 5′ and 3′ ends and ambiguous bases were removed. Phylogenetic trees were constructed by the neighbor-joining and maximum-parsimony algorithms using MEGA (version 5.0). Evolutionary distances were calculated using Kimura's two-parameter model and bootstrap values were based on 1000 replications. The GenBank accession number of the 16S rRNA gene sequence is KJ681370.

Escherichia coli strains were used to clone the 16S rRNA gene and cultured in LB broth aerobically on a rotary shaker (200 rpm) or on LB plates at 37 °C with 100 μg/ml ampicillin.

Physiological and biochemical characteristics of the strain were identified according to the Manual of Systematic Bacteriology (Dong and Cai, 2001).

2.3. Determination of flocculating efficiency

The flocculating efficiency was determined based on the standard kaolin suspension method (Salehizadeh et al., 2000). Briefly, 1 ml of the flocculant was mixed into a 50 ml kaolin suspension (4 g/L) in a 50 ml graduated cylinder and covered. The test cylinder was then gently shaken and allowed to sit for 5 min at room temperature to allow settling. Three milliliters of supernatant was carefully removed from the upper layer of solution and the amount of absorbance at 550 nm was measured using a TU-1901 spectrophotometer (PERSEE, China). A control sample that did not receive a flocculation agent was also completed. Flocculation efficiency was calculated according to the following equation:

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

where A is the absorbance at 550 nm of a sample and B is the absorbance at 550 nm of the control.

2.4. Optimization of Klebsiella sp. ZZ-3 culture conditions

Culture time and the carbon and nitrogen sources were the most important factors influencing the yield cost of bioflocculant production. Culture times between 0 and 24 h were investigated using the flocculant selecting medium. To minimize the yield cost, the composition of the flocculant selecting medium was also optimized. The use of glucose as the primary carbon source was evaluated by assessing the impact of replacing it with sucrose, lactose, starch, maltose, mannitol, and citric acid. As for the source of nitrogen, peptone and urea were replaced with $NaNO_3$, NH_4Cl, yeast extract, and beef extract and results were compared.

2.5. Production, extraction, and purification of bioflocculant ZZ-3

Strain *Klebsiella* sp. ZZ-3 was inoculated into a 250 ml flask containing 100 ml optimized production mediums and incubated on a shaker (200 rpm) for 15 h at 30 °C. The flocculation efficiency of extracted precipitates (cells) and supernatant (bioflocculant) were compared using different extraction methods (centrifugation, 80 °C heating, and 32% sulfuric acid) (Sun et al., 2012). Extraction via centrifugation was performed by centrifuging broth that had been incubated over night at 12,000 rpm for 5 min and washed three times with 0.9% NaCl. Heated extraction consisted of dissolving centrifuged pellets into 0.9% NaCl and heating the sample to 80 °C for 15 min (Sun et al., 2012). After ambient cooling, the cell-free supernatant was obtained by centrifugation at 12,000 rpm for 10 min and filtered using a 0.22 μm membrane (JinTeng, China). Cold ethanol was then added to the supernatant and left overnight at 4 °C. The precipitate was collected by centrifugation at 12,000 rpm for 10 min and dissolved in ultrapure water. Sulfuric acid extraction was performed by suspending the centrifuged precipitate in 40 ml deionized water and adding 1.6 ml of 32% (w/v) sulfuric acid to the solution. After 15 min, the solution was centrifuged at 12,000 rpm for 5 min, and the supernatant was filtered using a 0.22 μm membrane (JinTeng, China). For both the heating and sulfuric acid extraction methods, the final supernatant was dialyzed overnight against deionized water with a molecular weight cutoff of 8–14.4 kDa (36 MM, Biosharp, USA). The purified bioflocculant (ZZ-3) was finally lyophilized by a freeze dryer (Virtis BT4KXL, USA).