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A novel bioflocculant produced by a salt-tolerant, alkaliphilic and biofilm-forming strain *Bacillus agaradhaerens* C9 and its application in harvesting *Chlorella minutissima* UTEX2341

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ABSTRACT

A novel bioflocculant MBF-C9 produced by a salt-tolerant, alkaliphilic *Bacillus agaradhaerens* C9 was investigated in this study. The effects of culture conditions such as initial pH, carbon source, nitrogen source, C/N ratio, and NaCl concentrations on MBF-C9 production were studied. The result showed that 4.65 g/L purified MBF-C9 was extracted with the following optimized conditions: 10 g/L glucose as carbon source, 10 g/L yeast extract as nitrogen source and initial pH 10.2. The MBF-C9 contained 65.42% polysaccharides, 4.70% proteins, and 1.65% nucleic acids. The highest flocculating rate of 95.29% for kaolin suspension was achieved at a dosage of 1.5 mg/L, pH 6.53 and 29 °C, and the highest flocculating rate of 80.63% for *Chlorella minutissima* UTEX2341 cells was observed at a dosage of 8 mg/L, which is much lower than that used in previous study. In addition, C9 could form biofilm under alkaline condition. Therefore, C9 has the potentials to be used in harvesting microalgae or as a bacterial agent to strengthen the treatment of alkaline wastewater.

1. Introduction

Bioflocculants are extracellular polymeric substances (EPS), which include glycoproteins, polysaccharides, proteins, and nucleic acids produced by microorganisms during their growth [1–3]. Bioflocculants are advantageous over inorganic flocculants and chemically synthetic flocculants in numerous applications including wastewater treatment and downstream processing for food and fermentation industries, due to their nontoxic, harmless, and biodegradable properties [4,5]. However, a major bottleneck for its commercial application is the high production cost compared with inorganic flocculants and synthetic flocculants [6]. To reduce the production cost, screening high production strains, using mutational methods to get more efficient mutants, seeking for low-cost substrates and optimizing fermentation conditions are the main research directions [7–9]. Expanding the application fields of bioflocculants is also a hot research point. For example, bioflocculants have been applied in collection of the microalgae. In recent years, microalgae have received increasing attention in the production of biofuels. However, economic production of microalgae biodiesel is limited by high cost in biomass harvest, which usually counts for 20–30% of the total production cost [10,11]. Flocculant is a low-cost way to harvest the microalgae. A bioflocculant produced by *Solibacillus silvestris* W01 has been applied to harvest of the marine microalgae *Nannochloropsis oceanica* DUT01 using high dosage of bioflocculant (the culture supernatant of W01 was mixed with the microalgae culture at a ratio of 3:1) [10]. Hence, reducing the dosage of bioflocculant can further decrease the cost for harvesting the microalgae.

Biofilm is a microbial derived sessile community characterized by cells that are attached on a solid surface, embedded in a self-produced matrix of extracellular polymeric substances [12], and has been widely used in continuous treatment of wastewater. The immobilized cells in biofilm secreted various enzymes which degrade the organic matters in wastewater [13,14]. Moreover, as shown in Fig. 1, in the biofilm reactor, some bioflocculant-producing strains immobilized in biofilm secrete extracellular polymeric substances which improve the sludge dewatering in

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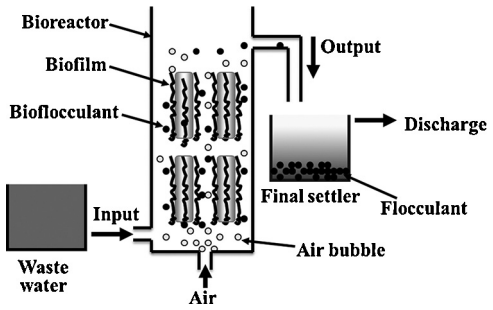

Fig. 1. A biofilm reactor modal used in wastewater treatment.

secondary settler [15]. However some alkaline wastewater, such as paper-making and dying industry wastewater could cause detachment of biofilm, leading to collapse of wastewater treatment systems. So it will be useful to obtain bioflocculant-producing strains which meanwhile can form biofilm at the alkaline condition.

In this study, a salt-tolerant, alkaliphilic strain *Bacillus agaradhaerens* C9 was isolated from alkaline lake. *B. agaradhaerens* is for the first time reported as bioflocculant-producing strain. The findings showed that (i) a high level production of 4.65 g/L bioflocculants MBF-C9 were achieved by strain C9 under the following conditions: 10 g/L glucose, 10 g/L yeast extract, initial pH 10.2, 37 °C culture; (ii) the highest flocculating rate of 80.63% for microalgae *Chlorella minutissima* cells was achieved using 8 mg/L purified MBF-C9, which was much lower than the dosage used for collecting *N. oceanica* DUT01 in a previous study [10]; (iii) the strain C9 could form biofilm in the alkaline condition. Therefore, strain C9 showed potential in low-cost microalgae harvesting or using as a bacterial agent to strengthen the treatment of alkaline wastewater.

2. Materials and methods

2.1. Isolation of bioflocculant-producing strain

A bioflocculant-producing strain, named C9, was isolated from an alkaline lake sample. The composition of the screening medium consisted of the following: glucose 10 g/L, yeast extract 5 g/L, peptone 5 g/L, K_2HPO_4 1.3 g/L and $MgSO_4{\cdot}7H_2O$ 0.2 g/L and Na_2CO_3 10 g/L. 1 mL of alkaline lake sample was inoculated in a 150 mL flask containing 50 mL screening medium and cultured in a shaker at 200 rpm at 37 °C. After 24 h cultivation, 0.25 g kaolin clay was added directly into the culture broth, and stirred for 2 min. After 1 min settlement, the supernatant was removed carefully. The bioflocculant-producing strains will settle down together with kaolin clay particles for their attachment on the particle surface. Then the sediment was washed and resuspended using 0.9% NaCl solution and vortexed 10 s. After brief centrifugation and dilution, the solution was streaked on screening medium agar plates. All the colonies presented on the plates were purified. Each isolated strain was first inoculated in 150 mL flasks containing 50 mL screening medium and then incubated at 200 rpm, 37 °C. Propitious culture broth was explored for flocculating activity. Strains with high flocculating ability were selected for further studies.

2.2. Measurement of flocculating activity

The flocculating activities were measured by calculating the flocculating rate according to a previous study [6]. Briefly, kaolin clay was used as the solid phase. Bioflocculant solution was added into a 5 g/L kaolin suspension and stirred for 2 min. After settling for 1 min, the absorbance (OD_{550}) of the supernatant sample was measured by a spectrophotometer (Unic-7200). A control experiment, without addition of any agent, was measured in the same manner. The flocculating rate was calculated according to the following equation: Flocculating rate = $(A_0 - A_1)/A_0 \times 100\%$, where A_1 is the absorbance of the supernatant sample at 550 nm and A_0 is the absorbance of the control at 550 nm.

2.3. Identification of strain C9

The 16S rDNA sequence was analyzed to identify the strain C9. The cells were cultured overnight. The genomic DNA was extracted using Genomic DNA Mini Kit (Invitrogen). The 16S rDNA gene fragment of C9 was then amplified by PCR amplification using forward primer (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and reverse primer (5′-CTA CGG CTA CCT TGT TAC GA-3′). The PCR product was purified using a PureLink PCR Purification Kit (Invitrogen) and sequenced. The sequencing results were compared to the 16S rDNA sequences available in the GenBank from the National Center for Biotechnology Information (NCBI) database.

2.4. Production and extraction of the bioflocculant

Production of the bioflocculant was performed in 500 mL flasks containing 100 mL fermentation medium with 200 rpm shaking at 37 °C. The composition of the optimal fermentation medium was as follows: glucose 10 g/L, yeast extract 10 g/L, K_2HPO_4 1.3 g/L and $MgSO_4{\cdot}7H_2O$ 0.2 g/L and Na_2CO_3 15 g/L, pH 10.2. After 48 h incubation, the fermentation broth was centrifuged at 12,000 rpm at 4 °C for 30 min to remove the cells. The supernatant was collected for bioflocculant extraction. To extract the bioflocculant MBF-C9, 2 volumes of cold absolute ethanol were added to the broth to precipitate the bioflocculant. The resulting precipitate was collected by centrifugation at 10000 rpm, 4 °C for 5 min, washed by 75% ethanol and lyophilized to obtain purified MBF-C9.

2.5. Characteristics of purified MBF-C9

The total polysaccharides of MBF-C9 were measured using the phenol-sulfuric acid method with glucose as the standard sample [16]. The total protein content was determined using a Qubit protein assay kit (Invitrogen) according to the manufacturer's instructions. The total DNA content was quantified according a previous paper using dsDNA BR assay (Qubits assay kit, Invitrogen, USA) using a Qubit 2.0 fluorometer [17]. Then the purified bioflocculant was analyzed using an attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Bruker Tensor 27, Germany). The spectrum of the sample was recorded on the spectrophotometer over a wave-number range of 600–4000 cm^{-1} under ambient conditions.

2.6. Flocculating properties of MBF-C9

To analyze the flocculating properties of bioflocculant, the effects of bioflocculant dosage, pH and temperature of the solution on flocculating activity were determined. The dosage of bioflocculant was varied from 0.5 to 50 mg/L. To test the effect of Ca^{2+} on the flocculating rate, 1 mL 10 g/L $CaCl_2$ solution was introduced into the 60 mL flocculating system. The pH of the kaolin suspension was adjusted using HCl and NaOH to be in the range of 1.49 to 11.32. The temperature of kaolin suspension was adjusted from 3 to 63 °C.

2.7. Culture of Chlorella minutissima and its harvest by bioflocculant MBF-C9

C. minutissima was preserved in our laboratory and cultivated in the medium reported previously [18], containing glycerin 67.5 g/L, casein 26.1 g/L, yeast extract 0.1 g/L, KH_2PO_4 0.1 g/L, Na_2HPO_4 0.03 g/L, $CaCl_2{\cdot}2H_2O$ 0.013 g/L, FeNa EDTA 0.01 g/L, $MgSO_4{\cdot}7H_2O$