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Production of a value added compound from the H-acid waste water—Bioflocculants by *Klebsiella pneumoniae*

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ABSTRACT

A novel strain (designated as ZCY-7) which could convert H-acid into bioflocculants was isolated from H-acid wastewater sludge. Conditions for bioflocculants production were optimized by response surface methodology (RSM) and determined to be inoculum size 9.65%, initial pH 7.0, and COD_{Cr} of the H-acid wastewater 520 mg/L. The highest flocculating efficiency achieved for kaolin suspension was 95.1%, after 60 h cultivation. The yielded bioflocculant was mainly composed of polysaccharide (82.4%) and protein (14.2%), and maintained its flocculating activity in 0.4% (w/w) kaolin suspensions over pH 2–8 and 20–80 °C. Fourier transform infrared (FTIR) spectra showed that amino, amide and hydroxyl groups were present in the bioflocculant molecules. A viable alternative treatment technology of H-acid wastewater using this novel strain is suggested, which could largely reduce bioflocculants costs. In addition, flocculating mechanism investigation reveals that the bioflocculant could cause kaolin suspension instability by means of charge neutralization firstly and then promoted the aggregation of suspension particles by adsorption and bridge. It is evident from the results that H-acid wastewater could be used as a source to manufacture bioflocculants.

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1. Introduction

In view of environmental concern, there are renewed interests on microbial flocculants in the recent past due to their low levels of toxicity, high biodegradable nature, high selectivity, and specificity even under extreme conditions like pH, temperature, and salinity, considering them as potential alternatives to synthetic flocculants [1,2]. These salient features make them potential candidates for a wide range of applications in diverse areas including water supply, wastewater treatment and downstream processing for fermentation industries [3,4].

The high-cost and low yield of production, however, is the major limitation of bioflocculants development for commercial use in wastewater treatment. Although several studies using synthetic and low-cost substrates for bioflocculant production have been reported [5,6], there has been no report on the production of bioflocculants using H-acid wastewater as a substrate.

H-acid (amino-8-naphthol-3,6-disulfonic acid) is a xenobiotic compound (PAH) used in the production of several direct, acid, reactive dyes and medicine [7–9]. The H-acid wastewater from the manufacturing processes is rich in various substituted derivatives of Naphthalene compound, which are not readily degradable compared with glucose or sucrose [7–9]. Additionally, wastewater from the manufacturing processes is released from the acidulation precipitation process and exhibits very high COD (30,000–50,000 mg/L), acidity (pH 1.5–3.5) and chroma, which can inhibit microbial activities. Strains that can effectively utilize H-acid wastewater to produce bioflocculants are of academic and practical interests. Therefore, specific objectives of this study were focused on the following factors: (1) isolation and identification of bioflocculant-producing strains from H-acid wastewater sludge; (2) production of bioflocculant using isolated strains from H-acid wastewater; (3) the performance of this bioflocculant and its flocculating mechanisms.

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2. Materials and methods

2.1. Wastewater sample collection and isolation of bioflocculant producing strains

Wastewater samples were collected from industrial effluent of JiHua Chemical Plant in Jiangsu Province, China. With serial dilution techniques, different strains of bacteria were isolated onto agar plates containing the following sterilized medium at pH 7.5 (per liter): 0.2 g H-acid, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.5 g urea, 0.5 g yeast extract and 20 g agar. Plates were incubated at 25 °C for 48–72 h. Isolated strains were named as ZCY1–ZCY25. Kaolin suspensions were then used to evaluate the flocculating capability of these microorganisms at the concentration of 4 g/L, and then the strains with high flocculating efficiency would be selected as the bioflocculant-producing strains for further investigation.

Morphological, physiological and biochemical characteristics of the bacteria were identified according to Bergey's Manual of Systematic Bacteriology. PCR amplification of 16 S rDNA was identified by Takara Biotechnology Co., Ltd [10].

2.2. Bioflocculant production and flocculating activity tests

H-acid wastewaters (COD_{Cr} = 41,000 mg/L, pH 3.0, BOD_5 = 90 mg/L, color = 3900, Na_2SO_4 = 250 g/L) were blending black liquor from the primary sedimentation tank of JiHua Chemical Plant in Jianggsu, China. The culture medium consisted of: diluted H-acid wastewater (COD_{Cr} = 500 mg/L^{-1}) 1 L, 5 g K_2HPO_4, 2 g KH_2PO_4, 0.2 g $MgSO_4$, 0.5 g urea and 0.5 g yeast extract. Prior to cultivation, H-acid wastewater was diluted to the desired COD_{Cr}. Initial pH of the H-acid wastewater medium was adjusted to the determined value. Batch fermentations were performed in a 5-L stirred tank fermenter (Model KF5L, KoBio-Tech Co., Inchon, Korea) with 3-L working volume at 37 °C for 96 h and agitation of 200 rpm. Samples were drawn at appropriate time intervals and monitored for pH, biomass, COD, BOD_5 and flocculation properties. The fermented medium was harvested (10,000 rpm; 4 °C, 20 min) and the cell-free supernatant was used as the source of bioflocculant. Purification of the bioflocculant was performed as described by the researchers [5]. The flocculating activity (FA) was measured using jar testers [11].

2.3. Response surface methodology experimental design

The Design Expert Software (version 8.0) was used for the statistical design of experiments and data analysis. The central composite design (CCD), a standard RSM, was selected for the optimization of the factors which made sense on the bioflocculant production. In this design, three factors were the COD_{Cr} of the H-acid wastewater, inoculum size, and initial pH respectively. Experiments were initiated as a preliminary study to determine a narrower range of H-acid wastewater COD_{Cr}, inoculum size, and initial pH before designing the experimental runs. Accordingly, COD_{Cr} of the H-acid wastewater from 100 mg/L was tried and the increments continued until appreciable reductions were observed during cell growth. Likewise, inoculum size range 1%–20% and initial pH range 2–11 were examined to search for a narrower and more effective range. As a result, it was chosen as follows: COD_{Cr} of H-acid wastewater 400–600 mg/L, inoculum size 5%–15% and initial pH 6–8. These three independent factors with five different levels (−1.682, −1, 0, +1, +1.682) of bioflocculant production were investigated and the experimental designs are shown in Table 1.

The response variable (y) that represented flocculating rate was fitted by a second-order model in the form of quadratic polynomial equation:

$$Y = \beta_0 + \sum \beta i X i + \sum \beta i X_i^2 + \sum \beta ij X i X j, \ldots i, \quad j = 1, 2, 3, \ldots, k \tag{1}$$

where Y is the predicted response, Xi and Xj are independent factors, β_0 is the intercept, βi is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

2.4. Characteristics of the bioflocculant

Purified bioflocculant was analyzed by a Fourier transform infrared (FTIR) spectrophotometer (Made in Germany Model EQUINOX55). Spectrum of the sample was recorded on the spectrophotometer over a wave number range 400–4000 cm^{-1} under ambient conditions. Total sugar content was determined by the phenol sulfuric acid method with glucose as a standard. Uronic acids were determined with carbazole sulfuric acid method [12] using galacturonic acid as a standard. Amino sugars were determined according to the Elson–Morgan method with glucosamine as a standard. Protein was determined by the Lowry-Folin method with bovine serum albumin as a standard[13,14]. Monosaccharide composition of the purified biopolymer flocculant was analyzed after hydrolysis with 3 M HCl at 100 °C for 4 h using cellulose TLC with ethyl acetate, pyridine, acetic acid and water (5:5:1:3, v/v) as solvent. Monosaccharides were detected by spraying with aniline phthalic acid reagent and heating at 110 °C for 5 min. The gel filtration chromatography (Sepharose gel column, Pharmacia) was packed in a glass Column (1.2 cm × 50 cm) and was performed to determine the molecular weight of bioflocculant. A sample solution (20 μL) was injected, and the column was eluted with 0.05 M NaCl solution at a flow rate of 0.6 mL/min. Molecular weight of the tested sample was based on the standard curve calibrated and established by standard-molecular-weight dextran [15]. Degradation temperature of the partially purified bioflocculant was studied using Thermogravimetric (STA 449/C Jupiter Netz, Germany; Perkin Elmer TGA7 Thermogravimetric Analyzer, USA) instrument. The bioflocculant measured was heated from 35 to 600 °C at a constant rate of 10 °C min^{-1} under constant flow of nitrogen gas. Characterization of charge (zeta potential) was implemented using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France) with the application of the Smoluckowski equation. *Zeta* potential values were obtained from the average of around 24 measurements, the average values are presented with its half-width confidence interval at 95% confidence level.

2.5. Flocculating properties of the purified bioflocculant

To obtain the optimal concentration of bioflocculant, 993 mL kaolin suspension (4.0 g/L) was added into a 1000 mL beaker supplemented with various amounts of bioflocculant solution (final concentration of 2–100 mg/L) and 9 mM $CaCl_2$ solution (5 mL) at pH7.0. Flocculating activity was then measured and calculated using the procedure described above. Effects of temperature and solution pH on flocculating activity were examined by measuring flocculating activity of the reaction mixture containing the optimal bioflocculant concentration at specified ranges pH 2–10 and at different temperatures 0–80 °C. PH stability of bioflocculant was determined by measuring the residual activity after 24 h of preincubation at various pH values (1–12) and compared with that of a normal bioflocculant solution at pH 7.0. Thermal stability of bioflocculant was determined by measuring the residual activity after 60 min of incubation at various temperatures (0–100 °C). Furthermore, effect of various cations and cation concentrations (0, 0.01, 0.1, 1, 10 and 100 mM) on flocculant activity was investigated

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