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Production and characterization of a thermostable bioflocculant from *Bacillus subtilis* F9, isolated from wastewater sludge

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ABSTRACT

A bacterium isolated from wastewater sludge, identified as *Bacillus subtilis* F9, was confirmed to produce bioflocculant with excellent flocculation activity. The effects of culture conditions such as initial pH, temperature, carbon source, nitrogen source, and inoculum size on bioflocculant production were studied here. The results indicated that 2.32 g/L of purified bioflocculant could be extracted with the following optimized conditions: 20 g L^{-1} sucrose as the carbon source, 3.5 g L^{-1} peptone as the nitrogen source, an initial pH of 7.0, and a temperature of 40 °C. The purified bioflocculant consisted of 10.1% protein and 88.3% sugar, including 38.4% neutral sugar, 2.86% uronic acid, and 2.1% amino sugar. The neutral sugar consisted of sucrose, glucose, lactose, galactose, and mannose at a molar ratio of 2.7:4.7:3.2:9.1:0.8. Elemental analysis of the purified bioflocculant revealed that the weight fractions of carbon, hydrogen, oxygen, nitrogen, and sulfur were 30.8%, 5.3%, 54.7%, 6.4%, and 2.9%, respectively. Furthermore, the purified bioflocculant was pH tolerant within the range of 2–8 and thermotolerant from 10 °C to 100 °C, with optimal activity at pH 7.0 and at a temperature of 40 °C. The purified bioflocculant showed industrial potential for the treatment of drinking water. Considering these properties, especially its low molecular weight (5.3×10^4 Da), this bioflocculant with excellent solubility and favorable flocculation activity is particularly suited for flocculating small particles.

1. Introduction

Flocculating agents are widely used in various industrial fields such as wastewater treatment, textile manufacturing, cosmetology, pharmacology, and those involving fermentation processes (Li et al., 2009; Salehizadeh and Yan, 2014). Flocculants are usually classified into three groups: (i) organic polymer bioflocculants, including poly-acrylamide derivatives and poly-acrylic acid; (ii) inorganic flocculants such as aluminum sulfate and polyaluminum chloride; and (iii) bioflocculants consisting mainly of microbial polysaccharide, glycoprotein, protein, lipid, nucleic acid, and nucleoprotein (Aljuboori et al., 2013; Li et al., 2010). Synthetic organic or inorganic flocculants have been widely used in industrial fields due to low production costs and high efficiency; however, their use may cause environmental and health hazards. For example, acrylamide monomer is not only a strong carcinogen and neurotoxic to humans but is also non-biodegradable (Ruden, 2004). In addition, there is evidence that aluminum salts may induce Alzheimer's disease (Campbell, 2002). Use of synthetic organic polyelectrolyte bioflocculants has also been restricted in European countries because they are not readily biodegradable and even some of their degraded monomers cause health hazards (Li et al., 2010; Sam et al., 2011). Due to these concerns, bioflocculants produced by microorganisms have been attracting more attention in practical application.

"Bioflocculants are natural organic macromolecules that are derived from microorganisms; they can flocculate particles such as suspended solids, cells, and colloidal solids out of solution (Salehizadeh and Shojaosadati, 2001)." The degradation products of bioflocculants are harmless to the ecosystem (Buthelezi et al., 2010). Furthermore, bioflocculants can be produced economically on a large scale and be easily recovered from fermentation broth (Salehizadeh and Shojaosadati, 2001). However, high production

Abbreviations: BPM, bioflocculant-producing microorganisms; NCBI, national center for biotechnology information; FA, flocculating activity; HPLC, high-performance liquid chromatography; COD, chemical oxygen demand; NTU, nephelometric turbidity unit; COD, chemical oxygen demand

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costs and complicated production processes are major restraints of bioflocculant research (Salehizadeh and Yan, 2014). In addition, the application of bioflocculants in bioprocessing, wastewater treatment, and many other industrial operations mainly depends on further decreasing bioflocculant production costs using cheap substrates and novel fermentation and recovery strategies (Salehizadeh and Yan, 2014). Hence, research is needed to identify and select new bioflocculant-producing microorganisms (BPMs) that are capable of utilizing low-cost substrates, with high activity, high stability, and non-toxicity (Sharma et al., 2006; Wang et al., 2007). Thus far, many bacteria, and fungi have been reported to produce biopolymers such as polysaccharides, glycoproteins, and functional proteins with efficient bioflocculant activity (Zhao et al., 2012).

Activated sludge, soil and sediments, river and deep-sea water samples are the best sources for isolating extracellular biopolymer-producing microorganisms (Bala Subramanian et al., 2010; Salehizadeh and Shojaosadati, 2001). Bioflocculation is a dynamic process that usually occurs in activated sludge during the aerobic process (Salehizadeh and Yan, 2014). Organisms such as *Bacillus* sp., *Halomonas* sp., *Scenedesmus* sp., *Chryseobacterium* sp., *Aspergillus* sp., and *Pseudoalteromonas* sp. have been studied for bioflocculant production (Aljuboori et al., 2013; Salehizadeh and Yan, 2014; Wang et al., 2013). The composition and properties of bioflocculants depend on the type of BPMs, media composition, and environmental conditions. In most cases, glucose or sucrose has been used as organic substrates (Zhao et al., 2012); however, the mechanism of flocculation by bioflocculants is not yet clearly elucidated. Earlier experiments indicated that organic matter had substantial effects on flocculation time, floc size, density, and settling velocity (Elkady et al., 2011). The molecular weights of bioflocculants are generally over 10^5 Da. High molecular weight flocculants do not lead to the complete flocculation of small particles in suspension and hence, low molecular weight bioflocculants would be better in practical use (Liu et al., 2001). Furthermore, the data currently available on the environmental application of microbial flocculants (extracellular polymeric substances) are limited (More et al., 2014).

In the present study, we isolated a BPM from activated sludge. We investigated the optimal culture medium composition and various factors influencing flocculation efficiency, such as carbon source, nitrogen source, C/N ratio, pH, temperature, inoculum size, and time course. The properties and composition of the bioflocculant were determined in order to assess its potential application in various industrial processes and in drinking water purification.

2. Material and methods

2.1. Isolation and identification of bioflocculant bacteria

BPMs were isolated from the wastewater sludge of Periyar Maniammai University campus, using agar plate techniques on a basal medium. The composition of the medium was as follows: glucose, 20 g L^{-1}; yeast extract, 3.5 g L^{-1}; K_2HPO_4, 5 g L^{-1}; KH_2PO_4, 5 g L^{-1}; NaCl, 0.1 g L^{-1}; $MgSO_4$, 0.2 g L^{-1}; and agar–agar, 20 g L^{-1}. The cultures were incubated at 37 °C for 72 h. Colonies that were established were screened for their ability to produce bioflocculant based on colony morphology: large, viscous, and ropy. The selected isolate was cultivated in 250 mL of broth medium (pH 7.0) in a flask on a rotary shaker at 37 °C for 72 h at 160 rpm.

The selected isolate was identified based on 16S rDNA sequencing. The genomic DNA was isolated and subjected to amplification of 16S rDNA using universal primers. The amplified product was gel purified using a QIAGEN gel extraction kit (QIAGEN Inc., USA) and the purified products were sequenced as previously described by Kim et al. (2006). The 16S rDNA sequence (~1089 bp) was compared with 16S rDNA sequences that are currently available in the NCBI GenBank database.

2.2. Optimization of culture conditions for bioflocculant production

Various factors such as carbon source, nitrogen source, C/N ratio, pH, temperature, inoculum size, and culture time could affect bioflocculant production. To determine the effect of carbon and nitrogen sources on bioflocculant production, glucose was replaced with sucrose, fructose, lactose, and starch (20 g L^{-1}) as the carbon source, and yeast extract was replaced with $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, peptone, urea, and glutamic acid (3.5 g L^{-1} as the nitrogen source. For the C/N ratio, different concentrations of glucose were used in order to get different C/N ratios of 0:1–50:1 while yeast extract remained constant. The initial pH of the production media was adjusted from 2 to 10. The temperature of the production media was adjusted from 15 °C to 50 °C. Inoculum size was adjusted from 0.2 to 10% v/v, while the time course of bioflocculant production was adjusted between 0 h and 96 h. All of the experiments were conducted in triplicates.

2.3. Purification of bioflocculant

The bioflocculant from the broth culture was purified as previously described by Li et al. (2009), with slight modifications. Briefly, the culture broth was centrifuged to remove cell pellets (5000*g*, 30 min). Thereafter, the supernatant was concentrated and dialyzed overnight at 4 °C in deionized water. Three volumes of cold anhydrous ethanol (4 °C) were added to the dialyzed broth. The precipitate was then dissolved in deionized water followed by the addition of 10% cetylpyridinium chloride (Sigma-Aldrich, USA) with continuous stirring. The resulting precipitate was collected by centrifugation (5000*g*, 15 min) and dissolved in 0.5 M NaCl. Three volumes of cold anhydrous ethanol (4 °C) were again added to obtain the precipitate, which was then washed three times with 75% ethanol and lyophilized to obtain the crude bioflocculant. The partially purified bioflocculant solution (0.1%) was loaded onto a Sephacryl S-500 column (16 × 100 mm^2; GE Healthcare Life Sciences, India) and eluted with distilled water at a flow rate of 0.2 mL/min to collect the active fraction, which was lyophilized until further use.

2.4. Measurement of flocculating activity

A kaolin suspension was used to measure the flocculating rate of the bioflocculant in culture broth (Aljuboori et al., 2013). In brief, 2 g of Kaolin clay (Sigma-Aldrich, USA) was suspended in 1 L of deionized water. One milliliter of culture broth was added to 99 mL of the kaolin suspension in a 400-mL beaker, and the pH value was adjusted to 7.0 using 1 M NaOH or HCl. The mixture was stirred at 200 rpm for 1 min, slowly stirred at 100 rpm for 5 min, and allowed to stand for 5 min. The optical density (OD) of the supernatant was measured with a spectrophotometer (Perkin Elmer, USA) at 550 nm. As a control, the above procedures were repeated using fresh culture medium instead of the culture broth. The flocculating activity (FA) was calculated according to the following equation:

$$FA = (B - A/B) \times 100$$

where, '*A*' is the OD of the sample experiment at 550 nm and '*B*' is the OD of the control experiment at 550 nm. Complete removal of particles yields FA=100%. If no flocculation, FA=0%.