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Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1

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Abstract

A bioflocculant TJ-F1 with high flocculating activity, produced by strain TJ-1 from a mixed activated sludge, was investigated with regard to its production and characterization. By 16S rDNA sequence and biochemical and physiological characteristics, strain TJ-1 was identified as *Proteus mirabilis*. The most preferred carbon source, nitrogen source and C/N ratio (w/w) for strain TJ-1 to produce the bioflocculant were found to be glucose, peptone and 10, respectively. TJ-F1 production could be greatly stimulated by cations Ca^{2+}, Mg^{2+} and Fe^{3+}. The optimal conditions for TJ-F1 production were inoculum size 2‰ (v/v), initial pH 7.0, culture temperature 25 °C, and shaking speed 130 r/min, under which the flocculating activity of the bioflocculant reached 93.13%. About 1.33 g of the purified bioflocculant, whose molecular weight (MW) was 1.2×10^5 Da, could be recovered from 1.0 l of fermentation broth. Chemical analysis of bioflocculant TJ-F1 indicated that it contained protein (30.9%, w/w) and acid polysaccharide (63.1%, w/w), including neutral sugar, glucuronic acid and amino sugar as the principal constituents in the relative weight proportions of 8.2:5.3:1. Scanning electron microscopy (SEM) image of the purified solid-state TJ-F1 showed that it had a crystal-linear structure. Spectroscopic analysis of the bioflocculant by Fourier-transform infrared (FTIR) spectrometry indicated the presence of carboxyl, hydroxyl and amino groups preferred for the flocculation process.

Keywords: Bioflocculant; *Proteus mirabilis*; Culture condition; Protein; Acid polysaccharide

1. Introduction

Flocculating agents are generally classified into three groups: (1) inorganic flocculants, such as aluminum sulfate and polyaluminum chloride; (2) organic synthetic flocculants, such as polyacrylamide derivatives and polyethylene imine; (3) naturally occurring flocculants, such as chitosan, sodium alginate and bioflocculant (Salehizadeh and Shojaosadati, 2001; Zhang et al., 2007). Despite the effective flocculation performance and low cost of the synthetic chemical flocculants, their use has resulted in some health and environmental problems. For example, aluminum has been found to induce Alzheimer's disease (Arezoo, 2002). Furthermore, the acrylamide monomer is not only neurotoxic and carcinogenic, but also non-biodegradable in the nature (Rudén, 2004). On the contrary, bioflocculants produced by microorganisms during their growth are safe and biodegradable polymers (Deng et al., 2003). Because of the above alarming concerns, bioflocculants have been expected to increase in utilization (Salehizadeh and Shojaosadati, 2001).

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Over the past decades, some microorganisms, including algae, bacteria, actinomyces and fungi, have been reported to produce bioflocculant (Takagi and Kadowaki, 1985; Zhang et al., 1999; Huang et al., 2005). *Phormidium* J-1, a benthic filamentous cyanobacterium, isolated from a drainage channel, was found to produce a high molecular weight (MW) polymer which can flocculate bentonite particles from suspensions (Fattom and Shilo, 1984). *Rhodococcus erythropolis* produced a bioflocculant named NOC-1 with an efficient activity in causing flocculation of a wide range of suspended solids (Kurane et al., 1994). The bioflocculant purified from the culture broth of *Arthuadendron* sp. TS-49 could efficiently flocculate various microorganisms and organic/inorganic materials (Lee et al., 1995). *Enterobacter cloacae* WD7 was reported to generate a viscous culture broth exhibiting flocculating activity (Prasertsan et al., 2006). Significantly, many reported microorganisms which could secrete biopolymer flocculant belong to *Bacillus* sp. (Shih et al., 2001; Salehizadeh and Shojaosadati, 2002; Suh et al., 2002; Deng et al., 2003; Ganesh Kumar et al., 2004). He et al. investigated the production of a polygalacturonic acid bioflocculant REA-11 from a newly isolated strain, *Corynebacterium glutamicum* CCTCC M201005 (He et al., 2004). Kurane et al. reported that *Nocardia restricta*, *Nocardia calcarea* and *Nocardia rhodnii* could produce biopolymer flocculants (Kurane et al., 1986a). A strain *Saccharomycete* STSM-1 with flocculating activity was isolated from activated sludge (Cheng et al., 2004). Deng et al. found that *Aspergillus parasiticus* could produce a bioflocculant with a flocculating activity for Kaolin suspension and water-soluble dyes (Deng et al., 2005). Although so many studies about bioflocculants have been done, flocculating activity and culture cost of bioflocculants are still the major limiting factors with regard to their application (Li et al., 2003; Zhang et al., 2007). Consequently both the screening of new strains producing bioflocculants with high flocculating activity and reducing their culture cost are the primary aims in this field. To our best knowledge, no previous work describing the bioflocculant produced by *Proteus mirabilis* has been reported to date.

In the present study, we screened out a microorganism TJ-1 identified as *P. mirabilis* by 16S rDNA sequence and its biochemical and physiological characteristics. The bioflocculant produced by this strain, named TJ-F1, showed high flocculating activity to Kaolin suspension. Various factors affecting TJ-F1 production, such as culture time, initial pH of the culture medium, carbon source, nitrogen source, C/N ratio, metal ion, ionic strength, culture temperature, shaking speed and inoculum size, were investigated to identify the cost-optimal culture conditions for *P. mirabilis* TJ-1. The major components, structure and functional groups of TJ-F1 were obtained by chemical analysis, scanning electron microscopy (SEM) and Fourier-transform infrared (FTIR) spectrometry, respectively. The molecular weight of the bioflocculant was determined by gel permeation chromatography (GPC).

2. Methods

2.1. Microorganism

Strain TJ-1 is a flocculant-producing microorganism screened from the mixed activated sludge of four wastewater treatment plants (Quyang, Anting, Dongqu and Tongjixinchun) in Shanghai (China), by the State Key Laboratory of Pollution Control and Resource Reuse (SKL), Tongji University of China. Its stock culture was maintained at 4 °C on slant media and subcultured on a month basis.

2.2. Culture media

The medium for slant consisted of (per liter): 3 g beef extract, 10 g peptone, 5 g NaCl, and 20 g agar. The screening medium consisted of (per liter): 20 g glucose, 0.5 g urea, 0.5 g yeast extract, 0.2 g $(NH_4)_2SO_4$, 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 g K_2HPO_4, and 2 g KH_2PO_4 (Zhang et al., 2007). The production medium consisted of (per liter): 10 g glucose, 1 g peptone, 0.3 g $MgSO_4 \cdot 7\ H_2O$, 5 g K_2HPO_4, 2 g and KH_2PO_4. The initial pH of all media was adjusted to 7.0 by NaOH (0.1 M) and HCl (0.1 M). All media solutions were prepared with distilled water and sterilized at 115 °C for 30 min.

2.3. Bioflocculant production

The stock culture from the slant was inoculated into a 150-ml flask containing 50 ml screening medium and incubated for 48 h in a rotatory shaker at 30 °C, 160 r/min. This pre-culture procedure was then used as the standard inocula preparation for all experiments. A 150-ml flask containing 50 ml production medium was inoculated with 1.0 ml pre-culture of strain TJ-1 and incubated at 25 °C in a rotatory shaker at 130 r/min for 48 h. The fermentation broth obtained was centrifuged (4000*g*, 30 min) to separate the cells. The cell-free culture supernatant was the liquid bioflocculant, which was used for the analysis of flocculating activity. All experiments were performed in triplicates for the mean calculation.

2.4. Assay of flocculating activity

The flocculating activity was measured using a previous method, in which Kaolin clay was chosen as the suspended solid (Kurane et al., 1986b; Zhang et al., 2007). Three milliliter of 1% $CaCl_2$ and 2.0 ml of bioflocculant were added into 100 ml of Kaolin suspension (4.0 g/l) in 100-ml test tube in turn. The mixture was vigorously stirred and allowed to stand for 5 min. The optical density (OD) of the clarifying solution was measured with a spectrophotometer (Shimadzu UV-1700) at 550 nm. A control experiment was prepared using the same method but the bioflocculant was replaced by the fresh culture medium

(B). The flocculating activity was calculated according to Eq. (1)

$$\text{Flocculating activity} = \frac{B - A}{B} \times 100\% \quad (1)$$

where A is the optical density of the sample experiment at 550 nm; B is the optical density of control experiment at 550 nm.

2.5. Identification of strain TJ-1

The strain was cultivated in a stock culture for 24 h at 30 °C in a bio-incubation. The cell was lysed and DNA was extracted using the phenol/chloroform method (Rainey et al., 1996). The concentrated DNA was diluted and amplified using the PCR technique (Kim et al., 2006). The diluted DNA (2 μl) was used as a template, and forward primer (5′-GAGCGGATAACAATTTCACACAGG-3′) and reverse primer2 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) were used as PCR primers for amplification with TaKaRa 16S rDNA Bacterial Identification PCR Kit (Code No. D310). The conditions were 30 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (1.5 min). The amplification products were purified using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (Code No. DV805A). The sequencing of the purified products was finished by TaKaRa Biotechnology (Dalian) Co., Ltd.

The biochemical and physiological characteristics of strain TJ-1 were also identified with reference to Bergey's manual of systematic bacteriology (Buchanan and Gibbens, 1984).

2.6. Bioflocculant purification

The bioflocculant purification was obtained according to the method described previously (Salehizadeh et al., 2000). To purify the bioflocculant, the fermentation broth was centrifuged to remove cells by centrifugal separation (4000g, 30 min), and two volumes of cold ethanol were then added to the broth. The obtained precipitate was redissolved in distilled water followed by the addition of 2% cetylpyridinium chloride solution (CPC) with stirring. After 2 h, the precipitate collected by centrifugal separation of the bioflocculant and the CPC complex was dissolved in NaCl (0.5 M). Two volumes of cold ethanol were then added to obtain the precipitate, and then it was washed two times with ethanol and the precipitate was vacuum-dried.

2.7. Physical and chemical analysis of bioflocculant

The protein content was measured by the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976). The total sugar content was determined by the phenol–sulfuric acid method using glucose as the standard solution (Chaplin and Kennedy, 1994). The neutral sugar was determined by the anthrone reaction (Chaplin and Kennedy, 1994). The uronic acid was measured using the carbazole–sulfuric acid method (Chaplin and Kennedy, 1994). Amino sugars were determined according to the Elson–Morgan method with glucose amine as the standard solution (Chaplin and Kennedy, 1994). The SEM image of the bioflocculant was obtained using Philips XL 30 ESEM. The infrared spectrum of the dried bioflocculant sample (as KBr disks) was recorded at room temperature (25 °C) in the wavenumber range of 4000–400 cm^{-1} with a FTIR spectrophotometer Nicolet Nexus 670.

2.8. Molecular weight determination of bioflocculant

The molecular weight of the bioflocculant was determined by GPC. The purified bioflocculant was dissolved in distilled water and then filtered with a 0.45-μm membrane. The temperature and the pressure in the oven were 40 °C and 1.5 MPa. The filtrate was injected with distilled water at the rate of 0.5 ml/min. Different molecular weights exhibt different retention times on the GPC column (Tosoh TSK gel G4000PWXL, 7.8 mm × 300 mm). Blue Dextran ($MW > 2 \times 10^6$), standard Dextran T-70, Dextran T-40 and bioflocculant TJ-F1 were analyzed on this column, respectively. Based on these experiments, a standard curve between retention time and the logarithm of molecular weight was acquired, and a regressed equation was obtained as follows:

$$\text{Log}(\text{MW}) = K_1 T + K_2 \quad (2)$$

where MW (Dalton) and T (min) were the molecular weight and retention time, respectively. K_1 and K_2 were constants. The molecular weight of TJ-F1 was calculated from Eq. (2).

3. Results and discussion

3.1. Screening and identification of bioflocculant-producing strain

Hundred and twelve different strains were isolated from the mixed activated sludge. Among these strains, strain TJ-1 was screened out since the material extracted from its fermentation broth in the screening medium showed the strongest flocculating activity to Kaolin suspension (91.78%). Therefore, we chose strain TJ-1 as the aimed strain producing bioflocculant.

Strain TJ-1 was found to be rod-shaped with flagellums, and have a size of approximately (3.6–5.3) μm × (1.1–1.9) μm. Its colony was found to be circular, milk white, smooth and humid. Some of the biochemical and physiological characteristics of the strain are as follows: gram stain (−), indole test (−), methyl red reaction (+), voges-Prokauer test (−), glucose fermentation (gas and acid), lactose fermentation (−), phenylalanine test (+), sulfureted hydrogen (+) and motile (+). From these characteristics, strain TJ-1 resembled *P. mirabilis*.

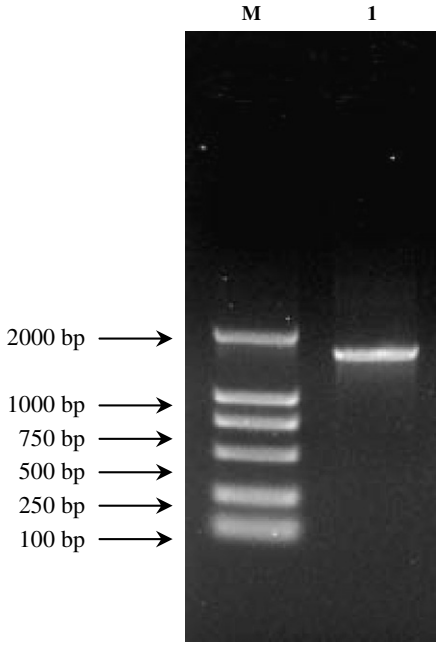

Fig. 1. Result of PCR product in 1% agarose gel electrophoresis. Lane M: DNA Marker DL2000; Lane 1: TJ-1 PCR product.

At the same time, the 16S rDNA of strain TJ-1 were sequenced directly following PCR amplification. The result of PCR product in 1% agarose gel electrophoresis is shown in Fig. 1. The 16S rDNA sequences of strain TJ-1 were determined to be 1462 bp long. The 16S rDNA sequences of strain TJ-1 was registered in GenBank and the accession number of strain TJ-1 is EF091150. Strain TJ-1 showed the highest degrees of relativity to the type strain *P. mirabilis* ATCC 29906T, sharing a 99% 16S rDNA similarity.

According to the 16S rDNA sequence and the biochemical and physiological characteristics, strain TJ-1 could be identified as *P. mirabilis*. And strain TJ-1 was named *P. mirabilis* TJ-1.

3.2. Factors affecting the bioflocculant production

The bioflocculant production is affected by many factors, such as the constituents of the culture medium and culture conditions (Nakata and Kurane, 1999; Zhou and Gao, 2002; He et al., 2004). The effects of the key factors, like culture time, initial pH of the production medium, carbon source, nitrogen source, C/N ratio, metal ion, ionic strength, culture temperature, shaking speed and inoculum size, on the flocculating activity of the bioflocculant by strain TJ-1, were investigated with an aim to identify the cost-optimal culture conditions.

3.2.1. Growth curve of TJ-1

Fig. 2 shows how the bioflocculant production varies with the growth curve of strain TJ-1. During the logarithm phase, the production of TJ-F1 was almost in parallel with

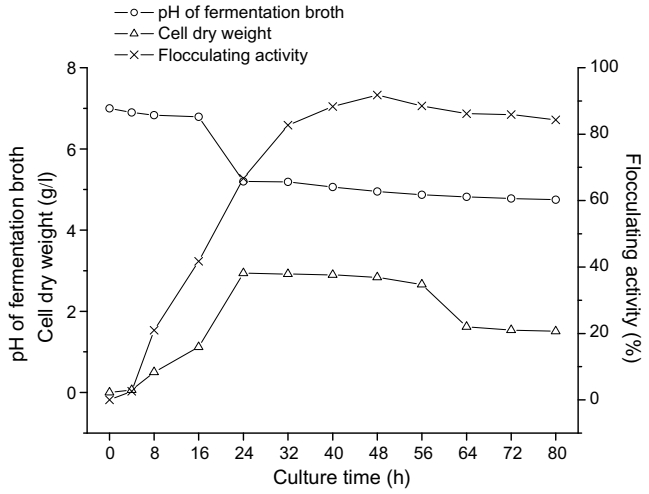

Fig. 2. Growth curve of strain TJ-1.

cell growth, and pH of the fermentation broth went down sharply. Cell production reached its maximum at 24 h (the stationary phase), while TJ-F1 reached its maximum flocculating activity at 48 h (the stationary phase). Both cell production and TJ-F1 production decreased in the death phase, while pH showed no obvious variation. To obtain the bioflocculant with high flocculating activity, we chose 48 h as culture time in the following studies.

Many reported bioflocculants were collected in the latter logarithmic growth phase and the early stationary phase because the flocculants production would not increase after those stages and at the worst case it would decrease (Kurane and Nohata, 1991; Shih et al., 2001; Lu et al., 2005). For example, the maximum flocculant production of *Alcaligenes latus* was achieved at the middle and latter stage of the logarithmic growth phase (2–3 days), and flocculating activity began to decrease at the latter stationary phase because of deflocculation enzymes (Kurane and Nohata, 1991). The flocculant production of *Bacillus licheniformis* and the cell growth simultaneously reached their maximums at the stationary phase (at 96 h) (Shih et al., 2001). The bioflocculant production of *Enterobacter aerogenes* was in parallel with cell growth and reached its maximum flocculating activity in the early stationary phase (at 60 h), which indicated that the bioflocculant was produced by biosynthesis during its growth, not by cell autolysis (Lu et al., 2005). From Fig. 2, the pH decreased from 7.0 to 5.2 within 24 h, which could be caused by the strain TJ-1 using the nutrients in the culture medium fleetly to realize its logarithmic growth. The flocculating activity of TJ-F1 also reached its peak in the stationary phase (at 48 h), but produced the bioflocculant in a shorter time compared with other strains, showing that strain TJ-1 had a higher productivity in the bioflocculant production than other strains. After 56 h, as the death rate of the strain began to exceed its birth rate, the strain entered the decline phase, and the flocculating activity of the bioflocculant decreased gradually.

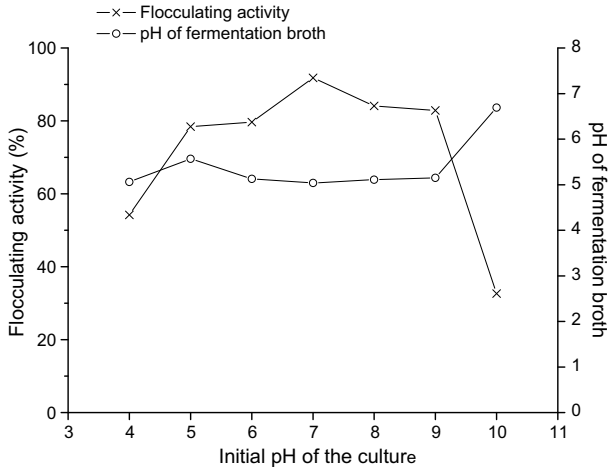

Fig. 3. Effect of the initial pH of the production medium on the flocculating activity of bioflocculant TJ-F1.

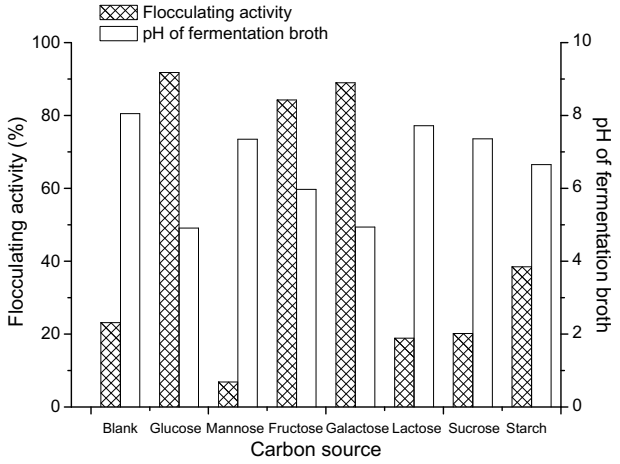

Fig. 4. Effect of carbon source in the production medium on the flocculating activity of bioflocculant TJ-F1.

3.2.2. Effect of the initial pH on TJ-F1 production

The effect of the initial pH of the culture medium on TJ-F1 production was investigated (Fig. 3). When the initial pH was 7.0, the flocculating activity of TJ-F1 reached a peak (91.78%). The initial pH of the culture medium determines the electric charge of the cells and the oxidation–reduction potential which can affect nutrient absorption and enzymatic reaction (Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001). For different strains, their requirement for the initial pH varied greatly. The alkaline pH, especially pH 9.5, effectively stimulated the flocculant production of *R. erythropolis* (Kurane et al., 1986b). At pH 8.0, the bioflocculant was possibly produced in large amounts by *Aspergillus sojae* and liberated to the culture broth (Nakamura et al., 1976a). In the case of strain TJ-1, pH 7.0 is the optimum for the bioflocculant production, which is a neutral pH and can save large numbers of acid and alkali used to adjust pH. This value was chosen as the initial pH in the following studies.

3.2.3. Effect of carbon source, nitrogen source and C/N ratio on TJ-F1 production

The effects of carbon source, nitrogen source and the C/N ratio on the bioflocculant production were investigated. As shown in Fig. 4, mannose, lactose, sucrose, and starch were not favorable carbon sources for the bioflocculant production. Glucose was the most preferred and cheap carbon source, and was used in the following experiments. The effect of nitrogen sources was investigated by cultivating the strain in the same medium, except that the nitrogen source was changed (Fig. 5). Strain TJ-1 was able to effectively use beef extract, yeast extract, and peptone as nitrogen source. Peptone was the most cost-effective nitrogen source and used in the following experiments. At a fixed peptone concentration of 1 g/l, lower or higher C/N ratio than 10 would go against the bioflocculant production, and then this C/N ratio was chosen for the next experiments.

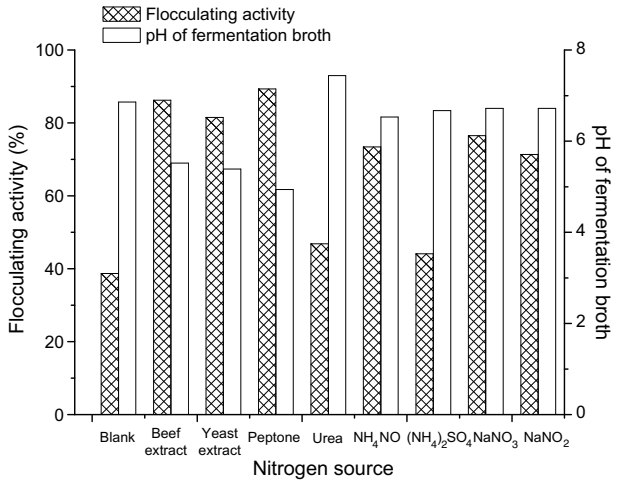

Fig. 5. Effect of nitrogen source in the production medium on the flocculating activity of bioflocculant TJ-F1.

The importance of carbon source and nitrogen source as well as that of the C/N ratio has been emphasized for bioflocculant production (Nakamura et al., 1976b; Kurane et al., 1994). For example, flocculant production of *R. erythropolis* was more in the medium using 0.5% glucose and 0.5% sucrose as carbon sources than in the medium using 1% sucrose or 8% waste sugar syrup (containing glucose, fructose and sucrose) (Kurane and Nohata, 1991). Urea and ammonium sulphate as inorganic nitrogen sources appeared favorable for both flocculant production and cell growth of *R. erythropolis* (Kurane et al., 1986b). The yield of the flocculant produced by *Zoogloea ramigera* could be increased by decreasing the C/N ratio (<38) at a fixed glucose concentration of 25 g/l (Norberg and Enfors, 1982). In the case of strain TJ-1, the most preferred carbon source, nitrogen source and C/N ratio (w/w) were found to be glucose, peptone and 10, respectively. Furthermore, 10 g/l of glucose and 1 g/l of peptone in the production medium are relatively economical compared with 20 g/l of glucose,

0.5 g/l urea, 0.5 g/l yeast extract, and 0.2 g/l $(NH_4)_2SO_4$ in the screening medium.

3.2.4. Effect of metal ion and ionic strength on TJ-F1 production

The production of bioflocculant is influenced by metal ions in the culture medium. The flocculant production of *Flavobacterium* sp. was stimulated in the presence of Ca^{2+}, Mn^{2+}, and Ba^{2+}, but was inhibited by Mg^{2+} (Gonzalez and Hu, 1991). The Ca^{2+} ion enhanced both the cell growth and the flocculating activity of *Paecilomyces* sp., but Fe^{2+} and Cu^{2+} inhibited the growth of cells (Takagi and Kadowaki, 1985). For strain TJ-1 (Fig. 6), some cations like K^+, Na^+ and Fe^{2+} have no obvious connection with the process of TJ-F1 production, some cations like Al^{3+} can inhibit its production, and other cations like Ca^{2+}, Mg^{2+}, and Fe^{3+} can be assimilated as an micronutrient by the strain and be propitious to its growth and bioflocculant production. The last cations may also become a part of the structure of TJ-F1, which can result in neutralization of the zeta potential and enforcement of the bridge between bioflocculant and suspended materials. Mg^{2+} was the most favorable metal ion and used in the following studies.

We studied the influence from ionic strength as well. $MgSO_4$ functioned as a micronutrient of strain TJ-1, while K_2HPO_4 and KH_2PO_4 were used as a buffer to stabilize pH of the production medium during the fermentation process. The optimum concentrations of them were 0.3 g, 5 g and 2 g per liter production medium, respectively.

3.2.5. Effect of culture temperature and shaking speed on TJ-F1 production

Various culture temperatures were tested in order to investigate their effect on the bioflocculant production. When the culture temperature was 25 °C, the flocculating activity of TJ-F1 was up to 92.28%. The optimal temperature for TJ-F1 production was 25 °C, which was used in the following studies. The metabolism of microorganisms has direct relationship with culture temperature (Salehizadeh and Shojaosadati, 2001; Zhang et al., 2007). Maximum enzymatic activation can only be obtained at an optimum temperature (Nakata and Kurane, 1999). A lower culture temperature might make strain TJ-1 hibernate partially, and its enzyme system for bioflocculant production couldn't be activated completely. On the other hand, a higher culture temperature may have an adverse effect on the nucleiu acid and the enzyme system of the strain, further on the bioflocculant production. At the temperature between 25 °C and 30 °C, the strain can grow well, and the enzyme system can show its maximal activity.

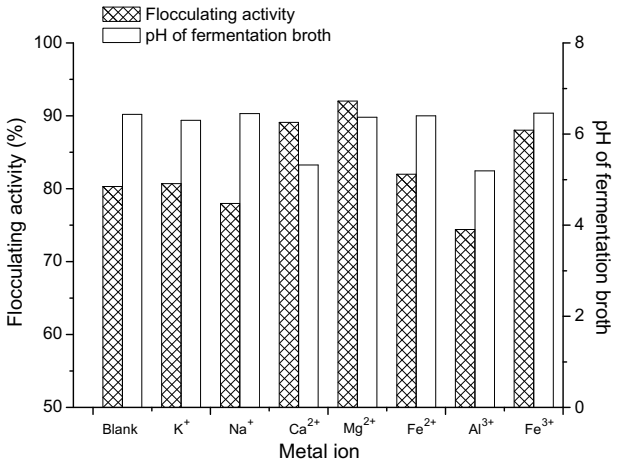

Fig. 6. Effect of the presence of metal ion in the production medium on the flocculating activity of bioflocculant TJ-F1.

The effect of shaking speed on TJ-F1 production was also investigated. Shaking speed of 130 r/min is the optimum. Either higher or lower shaking speed than this speed caused a decrease in the flocculating activity of the bioflocculant. The shaking speed of 130 r/min was used in the following studies. The shaking speed determines the concentration of dissolved oxygen which can also affect nutrient absorption and enzymatic reaction of strain TJ-1 (Salehizadeh and Shojaosadati, 2001). The optimum shaking speed for strain TJ-1 was 130 r/min, lower than the speed in many reports (Shih et al., 2001; Zhang et al., 2007). Because of the culture temperature close to the room temperature and lower shaking speed, TJ-F1 production can save a large amount of power.

3.2.6. Effect of inoculum size on TJ-F1 production

The effect of inoculum size of strain TJ-1 on the bioflocculant production was also investigated. When the inoculum size of the strain was 0.1 ml pre-culture per 50 ml of production medium, namely, when the inoculum size of the strain was 2‰ (v/v), the flocculating activity of TJ-F1 was up to 93.13%. A small inoculum will prolong the stagnant phase, whereas a large inoculum size will make the niche of strain TJ-1 overlap excessively and inhibit the bioflocculant production (Salehizadeh and Shojaosadati, 2001); 2‰ (v/v) was found to be the optimal inoculum size for strain TJ-1, which could acclimatize strain TJ-1 to the production medium, shorten the lag phase, and promote the productivity of bioflocculant TJ-F1.

Based on the above experiments, the optimal culture conditions for TJ-F1 production induced were listed as follows. The bioflocculant production medium consisted of (per liter): 10 g glucose, 1 g peptone, 0.3 g $MgSO_4 \cdot 7\ H_2O$, 5 g K_2HPO_4, 2 g and KH_2PO_4, pH 7.0. In terms of the inoculum size of 2‰ (v/v), the preculture of strain TJ-F1 was inoculated into the production medium. The inoculated medium was cultivated in a rotatory shaker at 25 °C, 130 r/min for 48 h. Under these conditions, the flocculating activity of TJ-F1 was up to 93.13%.

3.3. Characterization of the bioflocculant TJ-F1

About 1.33 g purified TJ-F1 was recovered from 1.0 l of fermentation broth. Chemical analysis of the bioflocculant

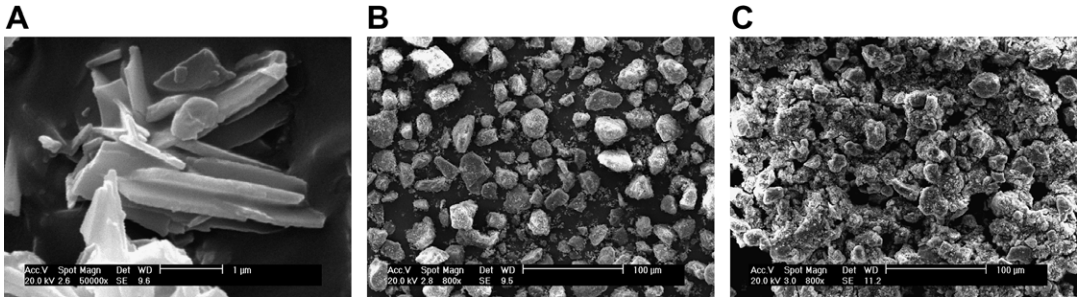

Fig. 7. SEM images of (A) the purified solid-state TJ-F1, (B) Kaolin clay, and (C) Kaolin clay flocculated by TJ-F1.

revealed that the proportions of the total sugar content and the total protein content were found to be 63.1% and 30.9% (w/w), respectively. The purified biopolymer was hydrolyzed with trifluoroacetic acid to determine the content in various sugars, and was found to be a mixture of many saccharides including neutral sugar, glucuronic acid and amino sugar (approximate weight ratio of 8.2:5.3:1).

SEM observations were carried out to elucidate the surface morphology of the biopolymer and its flocculation to Kaolin clay. As we can see from Fig. 7A, TJ-F1 shows a crystal-linear structure, and its length is about 4.0 μm. Fig. 7B shows the state of suspended Kaolin clay before the addition of TJ-F1, and Fig. 7C shows the state of precipitated Kaolin clay after the addition of TJ-F1. From the comparison of Fig. 7B and C, we can easily find TJ-F1 makes scattered Kaolin clay connected together like a well-knit net and separate from water, which reveals the excellent flocculating performance of TJ-F1 to Kaolin clay. The FTIR spectrum of TJ-F1 displayed a broad stretching intense peak at around 3400 cm^{-1} characteristic for hydroxyl and amino groups. Further, the asymmetrical stretching peak was noticed at 1700 cm^{-1}, suggesting the presence of carboxyl groups. The absorption peaks around 1000–1100 cm^{-1} and 980 cm^{-1} are known to be characteristic for all sugar derivatives.

The standard curve between retention time (T) and Log(MW) was acquired with GPC. The regressed equation was as follows: $\log(MW) = -0.3587T + 10.2078$, and the average molecular weight of TJ-F1 was determined to be 1.2×10^5 Da.

Several types of bioflocculants have been reported including polysaccharides, proteins, lipids, glycolipids, and glycoproteins (Zhang et al., 2002; Li et al., 2003). Both protein and acid polysaccharide were found to be the major components of bioflocculant TJ-F1. From the SEM images, TJ-F1 shows a crystal-linear structure and excellent flocculating performance. The FTIR spectrum of TJ-F1 shows that it contains carboxyl, hydroxyl and amino groups. The molecular weight of TJ-F1 is about 1.2×10^5 Da, similar to what has been previously reported (Zhang et al., 1999; Nakamura et al., 1976b). During the flocculating process, the efficiency of the bridging mechanism in flocculation is related to the functional groups, the molecular weight, and the structure of the bioflocculant. Carboxyl, hydroxyl and amino groups are the preferred groups for the flocculation process similar to what was observed in polyelectrolytes (Zajic and Knetting, 1971). The flocculation with a high-molecular-weight bioflocculant involves more adsorption points, stronger bridging ability, and higher flocculating activity than in the case of the flocculation with a low-molecular-weight flocculant (Zhang et al., 2007). For a flocculant, the linear structure can assure more adsorption points to be functional and bridge more particles to form flocs (Salehizadeh and Shojaosadati, 2001; Zhang et al., 2007). Bioflocculant TJ-F1 possesses all the characteristics favoring the flocculation process, which makes it become a strong flocculating agent.

4. Conclusions

In the present study, strain TJ-1 producing bioflocculant TJ-F1 with high flocculating activity was screened out and identified as *P. mirabilis*. The most preferred carbon source, nitrogen source and C/N ratio (w/w) for strain TJ-1 were glucose, peptone and 10, respectively. The production of bioflocculant TJ-F1 could be stimulated by cations Ca^{2+}, Mg^{2+} and Fe^{3+}. The optimal conditions for TJ-F1 production were inoculum size 2‰ (v/v), initial pH 7.0, culture temperature 25 °C, and shaking speed 130 r/min for 48 h, under which the flocculating activity of bioflocculant TJ-F1 reached 93.13%. The yield and the molecular weight of bioflocculant TJ-F1 were 1.33 g/l and 1.2×10^5 Da, respectively. Bioflocculant TJ-F1 contained acid polysaccharide (63.1%, w/w) and protein (30.9%, w/w). SEM image indicated that the purified solid-state TJ-F1 has a crystal-linear structure. Spectroscopic analysis of the biopolymer by FTIR revealed the presence of carboxyl, hydroxyl and amino groups.

Bioflocculant TJ-F1 may find possible application as a polymer for environmental bioremediation and other biotechnological processes. Further studies on its flocculation mechanism and scaling-up culture process are in progress in order to enhance the bioflocculant production and realize its industrial utilization.

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