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Characterization of a bioflocculant from a newly isolated *Vagococcus* sp. W31*

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Abstract: Screening of microorganisms producing flocculating substances was carried out. A strain secreting a large amount of bioflocculant was isolated from wastewater samples collected from the Little Moon River in Beijing. Based on the morphological properties and 16S rDNA sequence analysis, the isolate (designated W31) was classified as *Vagococcus* sp. A bioflocculant (named MBFW31) produced by W31 was extracted from the culture broth by ethanol precipitation and purified by gel chromatography. MBFW31 was heat-stable and had strong flocculating activity in a wide range of pH with relatively low dosage requirement. MBFW31 was identified as a polysaccharide with molecular weight over 2×10^6. It contained neutral sugar and uronic acid as its major and minor components, respectively. Infrared spectra showed the presence of hydroxyl, carboxyl and methoxyl group in its molecules. The present results suggested that MBFW31 had potential application in wastewater treatment.

Key words: *Vagococcus* sp., Bioflocculant, Characterization, Flocculating activity
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INTRODUCTION

Bioflocculation is a dynamic process resulting from the synthesis of extracellular polymer by living cells. Since flocculation in microbial system was first reported by Louis Pasteur (Salehizadeh and Shojaosadati, 2001), bioflocculation has been investigated extensively and a correlation was established between the accumulation of extracellular bioflocculants and cell aggregation (Tenney and Verhoff, 1973). Bioflocculants are essentially polymers produced by microorganism during their growth, with their flocculating activity being dependent on the characteristics of the flocculants. Compared with conventional synthesis flocculants, bioflocculants has special advantages such as safety, strong effect, biodegradable and harmlessness to humans and the environment, so they may potentially be applied in drinking and wastewater treatment, downstream processing, and fermentation processes (Salehizadeh and Shojaosadati, 2001).

The interest in biotechnological methods for production of bioflocculant lies in the possibility of using different microorganism to synthesize extracellular substances with different compositions. In recent years, many bioflocculant-producing microorganisms including bacteria, fungi and actinomyces have been reported to produce extracellular polymeric substances, such as polysaccharides, functional proteins and glycoprotein, which function as bioflocculant. Flocculants produced by a haloakalophilic *Bacillus* sp. I-471 (Kumar *et al*., 2004), *Alcaligenes cupidus* KT201 (Toeda and Kurane, 1991) and *Bacillus subtilis* IFO3335 (Yokoi *et al*., 1996) are polysaccharides. *Nocardia amarae* YK-1 (Koizumi *et al*., 1991), *Bacillus licheniformis* (Shih *et al*., 2001) and *Rhodococcus erythropolis* (Takeda and Kurane, 1991) produce protein flocculant, while *Arcuadendron* sp. TS-4 (Lee *et al*., 1995) and *Arathrobacter* sp. (Wang *et al*., 1995) produce glycoprotein biofloccu-

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lant, although few of these bioflocculant has been practically applied in industry because of their low flocculating capability and large dosage requirement. To utilize bioflocculants widely in industrial fields, it is desirable to find various microorganisms with high bioflocculant-producing ability and improve the flocculating efficiency of the bioflocculant.

In this study, screening of a new flocculant-producing strain was carried out. The characterization and biochemical analysis of its bioflocculant are discussed in detail.

MATERIALS AND METHODS

Screening and identification of bioflocculant-producing microorganisms

More than 20 wastewater samples including industry wastewater and sewage were collected from the Gaobeidian Factory of Wastewater Treatment, the Little Moon River and many other places in Beijing. Isolation of bioflocculant-producing microorganisms was carried out using an agar plate culture containing YPG medium with composition of peptone 20.0 g, yeast extract 10.0 g, glucose 20.0 g and agar 15.0 g per litre of deionized water at pH 6.5 (Chen and Zhao, 2003). Bioflocculant-producing microorganisms were originally screened based on colony morphology (mucoid and ropy). The isolated strains were grown in 50 ml of YPG medium on a rotary shaker (120 r/min) at 25 °C for 3 d, and the resultant culture broths were examined for their flocculating activity. Finally, one strain with high and stable flocculating activity for kaolin was selected for further study.

Determination of flocculating activity

Using a suspension of kaolin clay as test material, flocculating activity was measured according to the method of Kurane *et al*.(1986) with minor modification. The kaolin clay (average diameter 4 μm) was suspended in distilled water at a concentration of 5.0 g/L at pH 7. In every 10 ml of test tube, 9 ml kaolin clay suspension, 0.1 ml bioflocculant solution and 0.25 ml of $CaCl_2$ solution (100 mmol/L) were added. A reference in which distilled water instead of bioflocculant was also measured under the same conditions. The final volumes of all mixtures were made up to 10 ml with distilled water. After magnetic stirring, the test tubes were allowed to settle for 5 min. The absorbance of the upper phase was measured at 550 nm using an ultraviolet spectrophotometer (Cintra 10e, Australia). The flocculating activity was calculated as $[(B-A)/B]\times100\%$, in which A and B were the absorbances at 550 nm for sample and reference, respectively. The flocculating activity was measured and calculated using this uniform method in this study.

16S rDNA sequence determination and phylogenetic analysis

Genomic DNA preparation, PCR amplification of 16S rDNA, and sequencing of the PCR products were carried out as described previously (Rainey *et al*., 1996). The 16S rDNA sequence primers were rDNA37f (5′-AGAGTTTGATCCTGGCTCAGG-3′, positions 8~37) and rDNA1479r (5′-ACGGCAA CCTTGTTACGAGTT-3′, positions 1506~1479), which were designed according to Gupta *et al*.(1983). Purification of the desired PCR products and the determination of sequences were carried out by Sangon Biotechnology Co. Ltd. (Shanghai, China). The 16S rDNA sequence data were compared with currently available microorganism sequences in GenBank. Phylogenetic analysis was performed according to the methods reported previously (Yoon *et al*., 2000). The GenBank accession numbers of reference strains 16S rDNA sequences in this study were as follows: X54258, *Vagococcus fluvialis*; AY943820, *Enterococcus caccae*; AY179329, *Vagococcus carniphilus*; AY028437, *Enterococcus phoeniculicola*; AJ888906, *Enterococcus canintestini*; AJ420804, *Enterococcus casseliflavus*; AJ420801, *Enterococcus durans*; AJ420800, *Enterococcus faecium*; AJ420799, *Enterococcus hirae*; AF286831, *Enterococcus moraviensis*; AB022027, *Abiotrophia para-adiacens*; Y18341, *Enterococcus sulfurous*; Y18295, *Enterococcus flavescens*; Y18161, *Enterococcuscas seliflavus*; X54272, *Vagococcus salmoninarum*.

Time course of bioflocculant production

The composition of the medium for bioflocculant production was follows: glucose 20.0 g, KH_2PO_4 2.0 g, K_2HPO_4 5.0 g, $(NH_4)_2SO_4$ 0.2 g, NaCl 0.1 g, urea 0.5 g and yeast extract 0.5 g per litre of deionized water with initial of pH 6.5 (Wang *et al*., 1995). The selected strain was pre-cultured in 50 ml medium in 250 ml flasks on a rotary shaker (120 r/min) at 25 °C

for inoculation preparation. After 16 h of cultivation, the culture broth was used as seed culture and 1% of it was inoculated into 400 ml of medium in 1000 ml flask. Batch fermentations were carried out under the same cultivation conditions as those of the pre-cultivation. Medium samples were drawn at appropriate time intervals and monitored for pH, cell growth (dry cell weight, DCW) and flocculating activity. Five milliliters of culture broth was centrifuged at 8000 g for 15 min, and the cell-free supernatant was used as the test bioflocculant to study the flocculating activity. The precipitate was washed twice with distilled water and dried at 105 °C to constant weight. The dry cell weight (DCW) was used to measure the cell growth (He *et al*., 2004).

Extraction and purification of the bioflocculant

After 60 h of fermentation, the culture solution was centrifuged at 8000×g for 30 min to remove bacterial cells. One volume of distilled water was added to the super phase and centrifuged at 8000×g for 15 min to remove insoluble substances. To the supernatant, two volumes of ethanol were added, and then the mixed solution was stirred and left to stand at 4 °C for 12 h. The precipitate was vacuum dried to obtain crude biopolymer. The crude product was directly dissolved in distilled water to yield a solution, to which one volume of the mixed solution of chloroform and *n*-butyl alcohol (5:2, *V*/*V*) was added. After stirring, the mixture was set aside for 12 h at room temperature (about 20 °C). The upper phase was centrifuged at 3000×g for 15 min and the supernatant was concentrated at 40 °C. Then two volumes of ethanol were added to recover the precipitate, which was vacuum dried and re-dissolved in distilled water, and then was applied to a Sephadex 4B column (1.6 cm×80 cm). The column was previously balanced with phosphoric acid buffer solution (0.02 mol/L, pH=7.2). Elution was carried out with the same buffer at 0.5 ml/min. The flocculating activity of the eluate solution was detected respectively. The active fraction was collected and dialyzed against distilled water for 24 h, followed by vacuum drying (Chang *et al*., 1998; Chen *et al*., 2002).

Characteristics of the bioflocculant

The purity of the flocculant was originally evaluated by ultraviolet spectrophotometer (Cintra 10e, Australia) and electrophoresis was also done according the reported method (He *et al*., 2002). The molecular weight of the purified biopolymer was measured by gel-filtration chromatography using Sephadex 4B column (1.6 cm×80 cm). NaCl of 0.5 mol/L was used as an eluant at a flow rate of 0.5 ml/min and blue dextran was used as molecular weight standard (average molecular mass of 2×10^6).

The culture broth together with the purified flocculant was used as test bioflocculant to estimate the effect of flocculant dosage and pH on the flocculating activity. The culture broth and the purified flocculant were obtained at the same time of cultivation. Various amounts of culture broth and purified flocculants were added to a constant concentration of kaolin suspension (5.0 g/L) at pH 7 containing 2.5 mmol/L $CaCl_2$. A control was prepared with distilled water in place of different amount of flocculants. The flocculating activity was measured and calculated using the procedure described above. To estimate the influence of the pH value on the flocculating activity, the reaction mixture composed of kaolin suspension, flocculant (culture broth or purified flocculant) and $CaCl_2$ (2.5 mmol/L) was adjusted to pre-determined pH value using HCl or NaOH, and the flocculating activity was measured.

The purified flocculant was dissolved into a suitable volume of distilled water to yield a flocculant solution. Ten millilitre of flocculant solution and culture broth of W31 were heated at 100 °C for 1 h, and then the temperature dependence was determined by measuring the residual flocculating activity for kaolin suspension (5 g/L, pH 7) at room temperature.

Composition analysis of the bioflocculant

The total protein content of the flocculant was investigated by the Bradford (1976) method with bovine serum albumin as standard. The total sugar was determined by phenol-sulfuric acid method with glucose as a standard. Neutral sugars, uronic acids and amino sugars were determined with anthrone method, carbazole sulfate acid method and the Elson-Morgan method, respectively, using the procedure of Chaplin (1986). The dried product was analyzed using a Fourier transform infrared (FT-IR) spectrophotometer (AVATAR 360, USA). The spectrum of the sample was recorded on the spectrophotometer over a wavenumber range of 4000~400 cm^{-1}.

RESULTS AND DISCUSSION

Screening and identification of bioflocculant-producing microorganism

A total of 206 colonies were isolated from wastewater samples, and twelve pure culture strains with slimy or mucoid appearance were screened on the basis of kaolin suspension flocculating activity over 75% (data not shown). Among them, one strain having kaolin at flocculating activity exceeding 90% was selected for further study. This strain was originally isolated from the sewage of Little Moon River and designated W31. The colonies of W31 were round, cream-coloured, viscous, smooth and elastic when cultivated on YPG agar plate. The bacterium was Gram-positive. The 16S rDNA sequence obtained in this study has been deposited in the GenBank database with accession number DQ118031. Phylogenetic tree based on 16S rDNA sequence was constructed using the neighbor-joining method (Fig.1). Based on the morphological characteristics and 16S rDNA sequence data analysis, W31 was identified as *Vagococcus* sp.

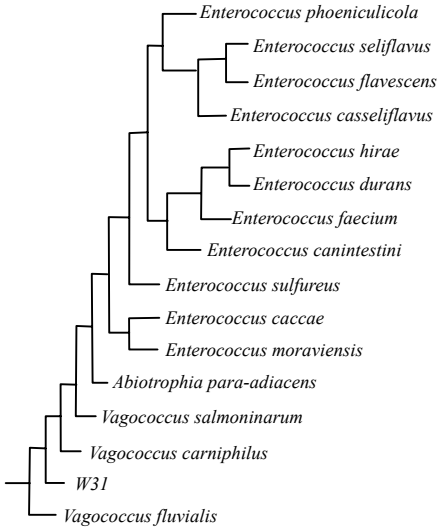

Fig.1 Phylogenetic tree based on the nucleotide sequences of 16S rDNA

Time course of bioflocculant production from W31

The growth curve of the strain, the flocculating activity and pH variation of the culture broth are shown in Fig.2. During the fermentation, the flocculating activity increased with culture time at first, and after reaching maximum value of 90% at 60 h, decreased slowly thereafter. The cell grew rapidly with increase of culture time in the first 60 h of cultivation, and then leveled off. The flocculating activity curve was parallel to the cell growth curve and the flocculating activity increased with increasing culture time, indicating that the flocculant was produced by biosynthesis during the growth of W31, not by cell autolysis. However, the slight decrease of flocculating activity may indicate this strain has a bioflocculant-degrading enzyme (Tago and Aida, 1977). The results in Fig.2 also showed that the pH of the culture broth decreased from 6.5 to 4.9 with increase of cultivation time from 0 to 96 h, which may be due to the production of organic acid from the metabolism of glucose or to the production of organic acid components of the polymer produced (Dermlim *et al*., 1999). The bioflocculant produced during the fermentation was named MBFW31.

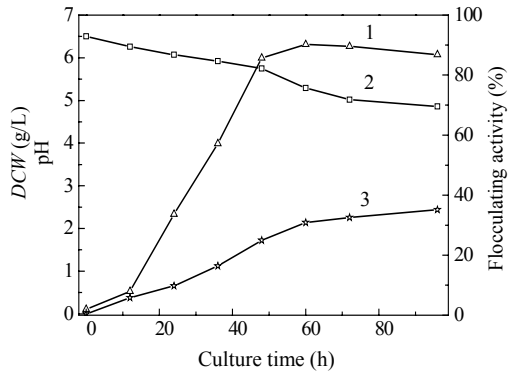

Fig.2 Time course of flocculant production by W31
1: Curve of flocculating activity; 2: Curve of pH; 3: Curve of cell growth

Characteristics of bioflocculant MBFW31

During the whole process of purification, the flocculating activity of the eluate appeared at the active peak, which was detected at 208 nm. There was no obvious absorption of protein or amide in the active fraction and the electrophoresis chromatogram of the purified product also indicated its homogeneous quality (data not shown). Finally, the yield of the purified bioflocculant was more than 2.3 g/L. The gel-filtration chromatogram indicated the polymer was eluted earlier than the blue dextran. Therefore, its molecular mass was higher than 2.0×10^6, which is

beneficial for flocculation. It was reported that the molecular weight of a flocculant is usually related to the chain length of the polymer, in that a large molecular-weight flocculant usually is long enough and has sufficient number of free functional groups, which can act as bridges to bring many suspended particles together (Michaels, 1954).

Fig.3 shows the relationship between the flocculant dosage and flocculating activity. When the culture broth in kaolin suspension (5.0 g/L) was tested in the dosage range of 0.5~30.0 ml/L, it was apparent that the flocculating activity increased proportionally to the flocculant dosage of 0.5 to 10.0 ml/L and was highest around 10 ml/L. In the same way, when the concentration of the purified MBFW31 was 25~30 mg/L, the flocculating activity was much higher. In view of the purified flocculant yield (2.3 g/L), it seemed that the amount of the purified MBFW31 was a little higher than that of the culture broth (10 ml) with similar flocculating efficiency. This could be explained by the loss of a small quantity of flocculating substances in the culture broths during the process of extraction and purification, because the flocculants content and the percentage of the main components were greatly dependent on the extraction method (Sheng *et al*., 2005). In recent reports, the dosage of culture broths and purified bioflocculant used for kaolin sedimentation was usually about 0.1 to 150 ml/L and 1 to 700 mg/L, respectively (Suh *et al*., 1997; Deng *et al*., 2003). Compared with these reported bioflocculants, MBFW31 was an effective flocculant with relatively low dosage requirement.

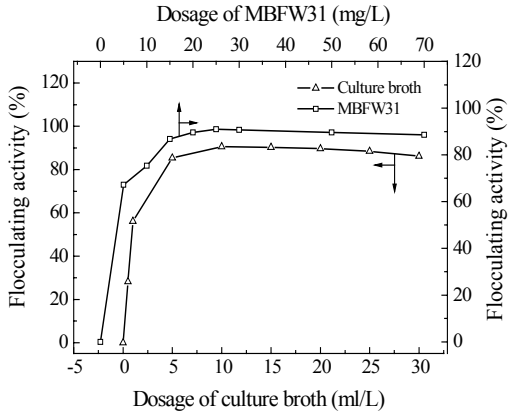

Fig.3 Relationship between dosage of MBFW31 and its flocculating activity

Flocculating activity of MBFW31 varied with pH. Keeping the dosage of culture broth of W31 fixed at 10 ml/L and the purified flocculant at 25 mg/L in kaolin suspensions (5 g/L), the effect of pH on the flocculating activity is shown in Fig.4. Either using culture broth or purified flocculant for test, the flocculating activity was high and stable at pH of 7~10. Most reports indicated that the pH affected the flocculating efficiency of the bioflocculant.

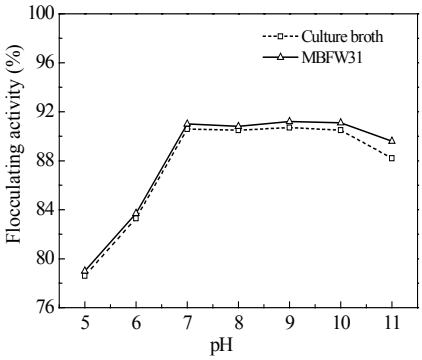

Fig.4 Effect of pH on the flocculating activity of MBFW31

The temperature dependence of the flocculant was investigated using culture broth (10 ml/L) and the purified flocculant (25 mg/L) as test bioflocculants. The flocculating activity was relatively stable at 100 °C with the residual flocculating activity of culture broth and purified flocculant being 86.5% and 87.2%, respectively. This suggested that MBFW31 was a heat-stable bioflocculant.

Composition analysis of MBFW31

The phenol-sulfuric acid method showed that the flocculant contained more than 90% of total sugar and no protein content being detected by the Bradford method, indicating the main composition of MBFW31 was polysaccharide. Since polysaccharides are mixtures of many saccharides, the analysis showed that the polymer contained neutral sugar (71.5%) and uronic acid (15.4%), respectively. MBFW31 had appropriate content of uronic acid, which can provide a certain amount of carboxyl. The carboxyl groups presented on the molecular chain provided more effective sites for particles attachment, so many particles can be adsorbed to the long molecular chain.

The IR spectrum (Fig.5) of MBFW31 was ana-

lysed as follows (Dermlim *et al.*, 1999). The absorption peak at 3363 cm^{-1} suggested the presence of −OH and the C-H stretching band caused the absorption peak at 2980 cm^{-1}. The spectrum also displayed an asymmetrical stretching band at 1633 cm^{-1} and a symmetrical stretching band near 1400 cm^{-1}, which were consistent with the presence of carboxylate ion. The peak at 1115 cm^{-1} was caused by C-O stretching, thus indicating the presence of a methoxyl group. The absorption peak of CH (840 cm^{-1}) showed that MBFW31 was composed of sugar derivatives. There were also minor bands that were not identified in Fig.5 and they will be studied further.

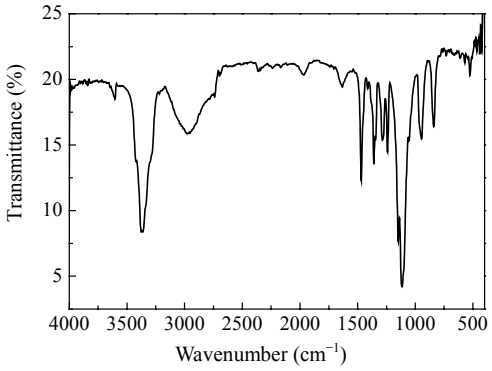

Fig.5 Infrared spectra of the purified MBFW31

The presence of hydroxyl groups evidenced by the IR spectra within the polymer favored the possibility of hydrogen bonding with one or more water molecules, so MBFW31 exhibited high solubility in aqueous solutions, following the solubility principle "like dissolves like" (James, 1986). A major condition for flocculation is that the molecules of flocculants could adsorb onto the surface of particles. The surface charge of kaolin particles in aqueous solution is negative. When MBFW31 is approaching particles in solution, an attractive force must exceed the electrostatic repulsion force. The calcium ion is necessary for the flocculating activity of MBFW31 on kaolin. This can be explained in that Ca^{2+} stimulates flocculating activity by neutralizing and stabilizing the residual charge of functional groups as the binding distance is shortened (Kwon *et al*., 1996). Then OH, COOH or COO^- group of the bioflocculant and H^+, OH^- group on the surface of particles might form hydrogen bonds as the bioflocculant chains approach the surface of particles. Bridging mechanism occurs after the particles have adsorbed onto the bioflocculant chains. Many particles could adsorb to a long molecular chain and the particles adsorbed onto the chain could be adsorbed simultaneously by other flocculant chains, leading to the formation of three-dimensional flocs capable of settling rapidly (Deng *et al*., 2003). Thus, the bioflocculant MBFW31 has good flocculating capability.

CONCLUSION

To our knowledge, this is the first report on the bioflocculant from *Vagococcus* sp. The bioflocculant from W31 is heat-stable and has strong flocculating activity in a wide range of pH with relatively low dosage requirement. It is anticipated that MBFW31 would be utilized not only in the area of wastewater treatment, but also in drinking water processing, and food and fermentation industry because of its effective flocculation and harmlessness towards humans and the environment.

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