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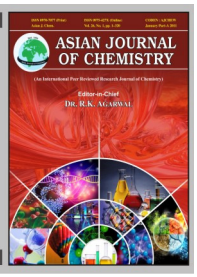

Composition of Extracellular Polymeric Substances Produced by *Geotrichum* sp. J-5

YINGXIN WAN*, LIPING WU, PEIJING TANG and JINGYI ZHANG

The College of Arts and Sciences of Beijing Union University, Beijing 100083, P.R. China

*Corresponding author: Tel/Fax: +86 10 62004523; E-mail: wyx@buu.edu.cn

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A fungus strain (J-5) which produces flocculating substances was isolated from activated sludge and identified as *Geotrichum candidum*. The growth of the isolated *Geotrichum candidum* was maximal after 3 days cultivation while the highest flocculating activity of the culture broth was highest after 1 day cultivation. The results of flocculating activity for *Geotrichum candidum* showed that the active ingredient of bioflocculant was existed in the supernatant of free-cell. The crude bioflocculant could be recovered from the supernatant of the culture broth by ethanol precipitation and dialysis against deionized water. The polymer was identified to be polysaccharide containing neutral sugar and uronic acid as its major and minor components, respectively. Infrared spectra showed the presence of hydroxyl, carboxyl and methoxyl groups in its molecules which could flocculate by bridging.

Keywords: Bioflocculant, Composition, Geotrichum candidum, Flocculating activity.

INTRODUCTION

Flocculating agents are widely used in various industrial fields including wastewater treatment, tap water production, dredging/downstream processing, food and fermentation processes[1]. A variety of flocculants, such as inorganic aluminum or ferric salts and organic synthetic high polymers, have been widely used due to their effective flocculating activity and low cost. However, they inherit the drawback of being less biodegradable and producing carcinogenic monomers during degradation[2]. In recent years, microbial-produced bioflocculants have received increasingly scientific and biotechnological attention due to their nontoxic, biodegradability and lack of secondary pollution of their degradation intermediates[3-5].

Bioflocculants are essentially polymers produced by microorganisms during growth, with flocculating activities that are dependent on the characteristics of the flocculants. Over the past decades, some microorganisms, including algae, bacteria, actinomyces and fungi, have been reported to produce bioflocculants[2,6-9]. The composition of bioflocculants has been reported to include polysaccharides, protein, nucleic acid or PHB[7,10-12]. Low flocculating capability and large dosage requirement, however, have been major problems in bioflocculant development for practical application[13]. Consequently, many researchers pay attention to discover novel efficient bioflocculants from microorganisms in varied environments and study their flocculating mechanisms over the past decades[14-16].

We have isolated several flocculant-producing strains from activated sludge of wastewater treatment plant. Among them, one strain (J-5) exhibited the excellent flocculating activity to kaolin suspension. It was selected for further studies and for the purification and partial characteristics for the biopolymer. The strain J-5 was identified as *Geotrichum candidum* by 18S rDNA sequence analysis and physiological characteristics. In this study, the main active components of MBFJ-5 were analyzed. In addition, the contribution of the active components to the flocculating activity of MBFJ-5 was discussed.

EXPERIMENTAL

Bioflocculant-producing strains were originally isolated from activated sludge that came from wastewater treatment plant by using kaolin clay as a flocculation test material. The composition of the screening and fermentation medium was as follows (per liter): 30 g sucrose, 0.01 g $FeSO_4$, 1g K_2HPO_4, 1g $(NH_4)_2SO_4$, 0.5g KCl, 1g $NaNO_3$, 0.5g $MgSO_4$, the pH was adjusted to 8. Fermentation was carried out on a rotary shaker (180 rpm) at 28 °C without control of pH during the operation. Culture broth propitious to flocculating rate was explored. Several strains were found to be flocculant-producing strains. Among them, one strain (J-5) exhibited the excellent flocculating activity to kaolin suspension. It was selected for further studies and for the purification and partial characteristics for the biopolymer. Biomass production was measured by reading optical density at 600 nm.

Identification of bioflocculant-producing strain J-5: Morphological and physiological properties of the isolated strain J-5 were observed according to Bergey's manual of

systematic bacteriology[17]. The 18S rDNA sequence of the purified product was finished using molecular biology. The common primers for amplification of 18S rDNA in fungi used were the primer NS1 (TAGTCATATGCTTGTCTC) and primer NS2 (GGCTGCTGGCACCAGACTTGC)$_{18}$. The 18S rDNA sequence data were compared with currently available microorganism sequences from NCBI GenBank.

The G + C content of the genome of strain J-5 was calculated by determining the melting temperature of the genomic DNA according to Mandel *et al*.[19]. Two parallel tests of the sample were performed, together with an *Escherichia coli* control.

Determination of flocculating activity: Flocculating activity was used as a measurement of the flocculating activity of the bioflocculant using the Kaolin clay suspension method according to the method of Kurane *et al*.[20]. A 4g Kaolin clay and 0.2g $CaCl_2$ was suspended in 1000 mL deionized water, Culture broth (0.01 mL) was then added to the 50 mL kaolin suspension (4 g l^{-1}), also, pH value of that was adjusted to 7 with diluted HCl or NaOH solution. The mixture was stirred at 200 rpm for 1 min with a vortex mixer and then kept still for 5 min. The absorbance (OD_{550}) of the supernatant (A) and the blank control without bioflocculant (B) were measured at 550 nm with a spectrophotometer. The flocculating rate was calculated according to the following formula:

$$\text{Flocculating rate (\%)} = (B\text{-}A)/B \times 100\ \%$$

Distribution of flocculating activity in the culture: Culture broth was centrifuged at 8000 rpm for 0.5 h. The supernatant was collected. The cell suspension was obtained after precipitated cells were washed twice with distilled water and resuspended in equal volum distilled water. Flocculating activity of the culture broth, supernatant and washed cells were assayed, respectively.

Bioflocculant purification: Purification of the bioflocculant is followed by Kellems and Lion[21]. The culture broth was centrifuged at 12000 rpm for 20 min to remove cells. The supernatant was used as the source of bioflocculant. Three volumes of cold ethanol (4 °C) were added to the supernatant and left overnight at 4 °C followed by recovery of the precipitate by centrifugation (4000 rpm, 15 min). The precipitate obtained was rinsed by ethanol (75 %) repeatedly, then dialyzed in distilled water for 24 h and freeze-dried to obtain the partially purified biopolymer. The crude bioflocculant produced by J-5 was named as MBFJ-5 in this paper. The MBFJ-5 was used to further studied.

Composition of purified bioflocculant MBFJ-5: The total sugar content of the bioflocculant was determined by the phenol-sulfuric acid method using glucose as standard solution[22]. The total protein content was measured by the bradford method, with bovine serum albumin as the standard[23]. The contents of neutral sugar, uronic acid and amino sugar in the bioflocculant were determined after hydrolysis with sulfuric acid. The methods of phenol-sulfuric and carbazole-sulfuric acid were used to determine the contents of uronic acid and neutral sugar, respectively and amino sugar was measured by the Elson-morgan method[22].

The crude bioflocculant was resuspended in double-distilled water and diluted to a predetermined volume. The UV absorption spectra of the suspended solution was recorded by a UV-visible spectrophotometer (UV-2102PC, Unico Co., China) in the range of 200-400 nm.

The functional groups of bioflocculant were determined by IR spectrophotometer using an IR spectrophotometer (Nexus 670, Nicolet, US). The dried sample was ground with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 4000-400 cm^{-1}.

Scanning electron microscopy on surface structure of floc: A drop of Kaolin suspension or MBFJ-5-induced floc was added to a slide and it was fixed by air drying. The fixed specimen was coated with osmium and examined with a LEO1450VP scanning electron microscope (Leo, German).

RESULTS AND DISCUSSION

The colony of strain J-5 was found to be white, tiled, powdery and with diameter 50-60 mm. Table-1 shows J-5's morphological and physiological characteristics. It was determined that strain J-5 resembled Geotrichum sp. from these characteristics. The 18S rDNA Sequence of the purified product was finished using molecular biology. The partial 18s rRNA sequences of this strain were compared to other sequences in the NCBI GenBank and we found a 99 % comparability to *Geotrichum candidum*. The molar concentration of G + C was 42.57 %. Based on the morphological 18S rDNA sequence data and molar concentration of G + C, the original strain could be identified as *Geotrichum candidum*. The strain has now been preserved in China General Microbiological Culture Collection Center, serial number CGMCC5066.

TABLE-1
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF J-5

Characteristics	J-5
Shape	Rod ((4.0-44.2) μm × (2.5–5.8) μm
Glucose	+
Fructose	+
Mannitol	+
Glycerol	+
Ethanol	+
Sorbitol	+
Sucrose	-
Maltose	-
Hydrolysis of starch	-
Hydrolysis of protein	+
Liquefaction of gelatin	+
Ammonium sulfate	+
Urea	+
Milk peptonized	+
Mol % G + C of DNA	42.57 %

Bioflocculant production: Fig. 1 shows the flocculant activity and cell growth with respect to culture time for 5 days. The flocculating activity increased in parallel with cell growth within 1 day's cultivation, indicating that bioflocculant is produced by strain J-5 during its growth. Then flocculating activity leveled off and followed decreased rapidly after 2 day's cultivation. Nevertheless, the cell growth never decreased during 5 days' culture. The cell growth log phase appeared within 3 days, yet no normally occurring stationary phase was observed even though cell growth moderately varied during the culture time. The rapid decline of the flocculating activity

may indicate that the strain also possessed polymer-degrading enzymes as reported by others[3]. These results illustrated that J-5 after 1day cultivation was the most suitable strain due to its high flocculating activity (90 %) and relative high cell growth.

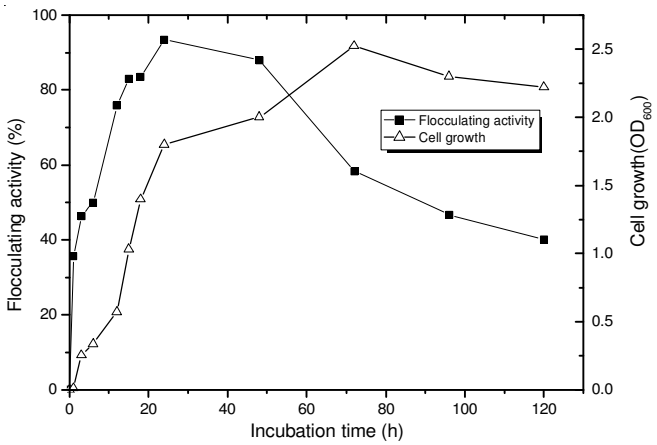

Fig. 1. Time course of a batch culture of the strain J-5 grown under standard conditions

Distribution of flocculating component in the culture: The distribution of the flocculating activity of the culture broth was examined and the major constituent with flocculating activity was identified. Fig. 2 shows the distribution of the flocculating activity in the culture broth. Most of the flocculating activity was found in the supernatant, while nearly nothing remained in the cells, which indicate that MBFJ-5 is an extracellular flocculant and the predominated flocculating substances are in the fermentation medium. Culture supernatant, therefore, was used to purify bioflocculant.

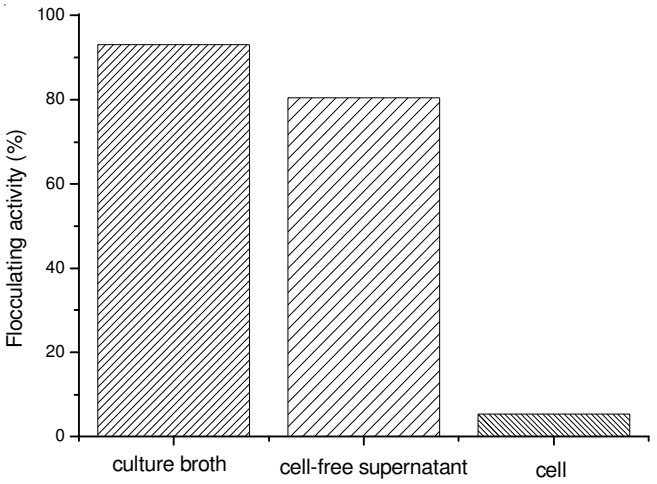

Fig. 2. Distribution of flocculating activity

Characteristics of purified bioflocculant MBFJ-5: From the study of the components of bioflocculant, total sugar content was found to be 80.6 % (w/w) by the phenol-sulfuric acid method and no protein was detected by the Bradford method, indicating that the bioflocculant was mainly polysaccharide. Since polysaccharides can consist of many saccharides including neutral sugar, uronic acid and amino sugar, bioflocculant was hydrolyzed with sulfuric acid to determine the content of different sugars. The analyses showed that the contents of neutral sugar, uronic acid were 39.4 and 21.7 % respectively. Amino sugars was under the detectable level.

The UV-visible scanning spectra of MBFJ-5 is illustrated in Fig. 3. Absorption spectra of crude bioflocculant suspended in double-distilled water had an absorption peak at 200 nm characteristic for polysaccharide 24 and a faint peak at 340 nm characteristic, suggesting the presence of many double bonds. Instead, no absorption peak was observed at 254 and 280 nm, which indicated that there was no nucleic acid and protein in this bioflocculant.

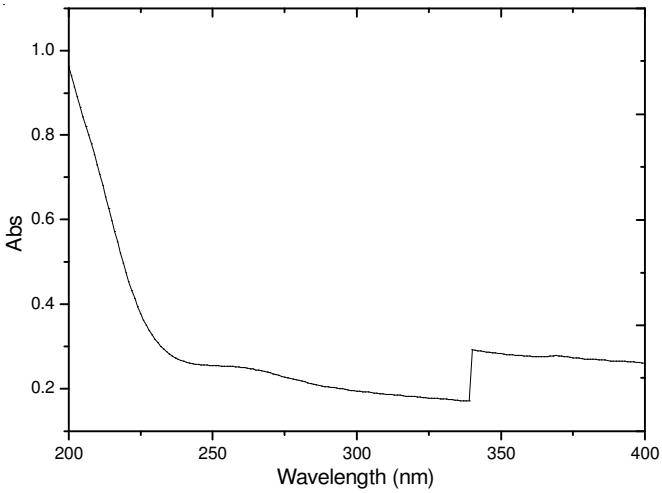

Fig. 3. UV-visible spectra of the MBFJ-5

The infrared spectrum of MBFJ-5 (Fig. 4) was taken by a spectrophotometer. The peak at 3392.57 cm^{-1} is characteristic of -OH stretching vibration, indicating the presence of broad hydroxyl and amino groups. The peak at 2928.99 cm^{-1} represents aliphatic C-H band. The bands at 1655 cm^{-1} and 1400 cm^{-1} could be assigned to the C=O (carboxylic acid) antisymmetrical and symmetrical stretchings in the carboxylate respectively, which indicates the presence of uronic acids in polysaccharides. The bands of 1307 cm^{-1} and 1044 cm^{-1} were regarded as the C-O stretching vibration and the presence of methoxy. The 1250 cm^{-1} band is due to C-O stretching in the free carboxylic acid. The absorption peak presented in the range from 1200-1000 cm^{-1} which were generally known to

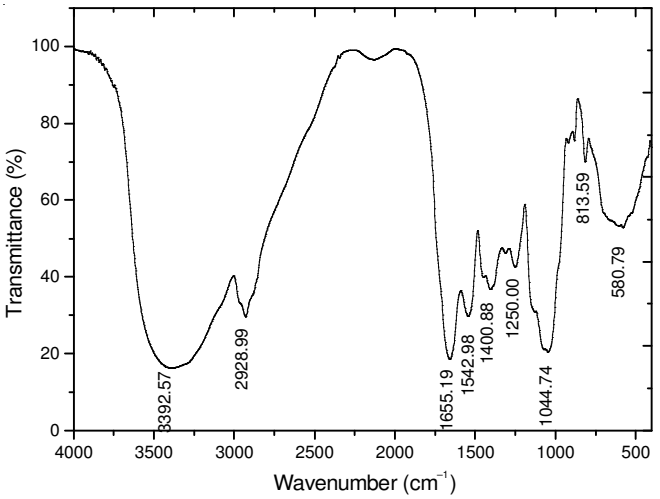

Fig. 4. Infrared spectra of the MBFJ-5

be typical characteristics of all sugar derivatives[25], Furthermore, the absorption peaks of C-H at 813.59 cm^{-1} are known to be characteristic of mannoside, in addition, the peak at 580 cm^{-1} indicated C=O twisting vibration[15].

Based on the above proofs of the carboxyl and hydroxyl groups and methoxyl groups, namely required groups for flocculation in polyelectrolytes[26], it was deduced that the main component of MBFJ-5 should be polysaccharide.

SEM observation on surface structure of the floc: SEM is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals which contain information about the sample's surface structures. SEM observations were performed to aid in interpreting the mechanism of Kaolin suspension flocculating. Fig. 5a shows Kaolin clay before the addition of MBFJ-5. Fig. 5b shows floc from the precipitated Kaolin clay after the addition of MBFJ-5. Previously, it had been reported that biopolymers can form floc with Kaolin particles by bridging[7,12,26]. The comparison of Fig. 5a, b illustrate that MBFJ-5 molecules connected the scattered Kaolin particles to form floc, consequently the floc settled down from suspension. Therefore, bridging of MBFJ-5 played a key role in the flocculating process.

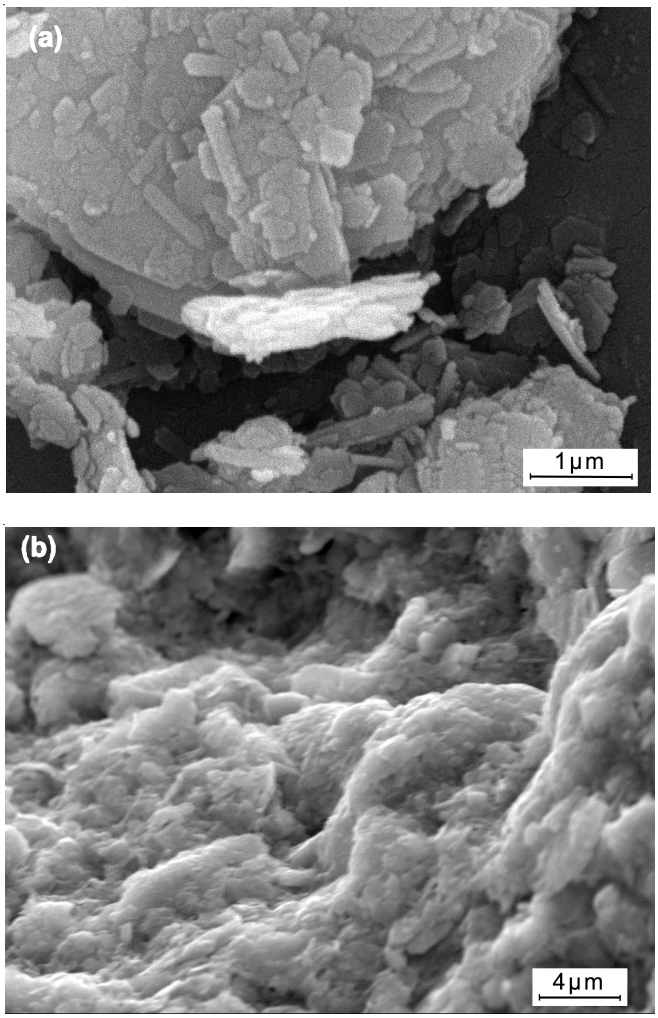

Fig. 5. SEM images of a Kaolin clay (a), Kaolin clay flocculated by MBFJ-5 (b)

It is determined that the MBFJ-5 is polysaccharides by chemical analysis. Polysaccharides can play an important role in removal of stable coagulation of colloid because of its significant emulsification. The FT-IR spectra of the MBFJ-5 showed the presence of carboxyl groups and hydroxyl groups, which may result in flocculating process involve ionic interactions between MBFJ-5 molecules and Kaolin particles. The net effect of such interactions could be explained that MBFJ-5 serving as bridging agents resulted in aggregation of Kaolin particles. The force of adsorption may come from hydrogen bonds that are formed between OH groups and suspended substances and chemical bonds between the COO- groups and suspended substances. Bridging mechanisms occur after the particles have adsorbed onto the chains of bioflocculant. Many particles could adsorb to a long molecular chain and the particles adsorbed on the chain could be adsorbed simultaneously by other flocculant chains, leading to the formation of three-dimensional flocs that are capable of settling fast.

The SEM observations indicated that the individual Kaolin clay particles were visible in Kaolin suspension and the particles were interlaced with MBFJ-5 in floc from the SEM images, which showed MBFJ-5 could absorb multiple particles and entangle to form a larger floc so that the floc could be precipitated from aqueous phase.

Conclusion

This study attained a bioflocculant MBFJ-5 from an indigeneous flocculant-producing strain J-5 identified as *Geotrichum candidum*. The growth of the isolate strain showed highest flocculation activity within 1 day of cultivation. This study also demonstrated that most of the flocculating activity in the culture broth of *Geotrichum candidum* was present in the supernatant. The polymer was identified to be polysaccharide containing neutral sugar and uronic acid as its major and minor components, respectively. The infrared spectrum of the bioflocculant indicated the presence of hydroxyl, carboxyl and methoxyl groups in its molecules which could flocculate by bridging. The SEM observations illustrate that bridging of MBFJ-5 played a key role in the flocculating process. Bioflocculant MBFJ-5 may find possible application as an alternative for environmental bioremediation and other bio-technological processes. Further studies on its flocculation mechanism and comparative cultivation study of bioflocculant are in progress in order to improve the production, decrease the cost and realize the utility in industry.

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