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Characterization and flocculability of a novel proteoglycan produced by *Talaromyces trachyspermus* OU5

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A filamentous fungus strain OU5 was isolated from a soil sample for its ability to produce rich exopolymers (EPS), with high flocculation capability towards kaolin suspension and swine wastewater, at low-carbon source conditions. EPS from strain OU5 was extracted and characterized to determine its flocculating behavior and active constituents involved in the flocculation. Strain OU5 was identified as *Talaromyces trachyspermus* by 18S rDNA–ITS gene sequencing and morphological observation. The extracted EPS was a novel proteoglycan (designated as BF–OU5) composed of 84.6% (w/w) polysaccharides and 15.2% (w/w) proteins. The enzymatic digestion tests revealed that the polysaccharides in BF–OU5, composed of 67% glucose, 16.4% mannose, 8.6% xylose and 8% galactose, contributed to 99.7% of flocculating capacity and were the major active ingredients in the flocculation. By contrast, the proteins in BF–OU5 only had minor roles in the flocculation. The presence of hydroxyl, amide, carboxyl and methoxyl functional groups in BF–OU5, and the high molecular weight (1.053×10^5–2.970×10^5 Da) as well as the structure of a spherical conformation with inner pores and channels made of cross-linked netted textures contributed to the flocculation. A dosage of 20 mg/l BF–OU5 initiated more than 92.5% of flocculating efficiency towards kaolin suspension without any added coagulants; its flocculability was stable over a wide range of pH (4.0–8.0) and temperature (20°C–100°C). Treatment of swine wastewater using BF–OU5 achieved 52.1% flocculating removal for chemical oxygen demand, 39.7% for Kjeldahl nitrogen, 18.6% for NH_4^+–N, 21.5% for total phosphorus, and 75% for turbidity.

[**Key words:** Filamentous fungus; *Talaromyces trachyspermus*; Exopolymers; Flocculating efficiency; Active constituent; Low-nutrient; Ca^{2+}-independent; Kaolin suspension; Swine wastewater]

Extracelluar polymeric substances (EPS) are microbial metabolic products mainly composed of polysaccharides and proteins and have an average molecular weight of $>10^4$ Da (1). At certain cultivation conditions, these biomacromolecules in aqueous solution can exhibit the high aggregation ability for colloids, cells and suspended solids (2–4). Accordingly, microbial EPS may be utilized as an ideal candidate for replacing chemically synthesized flocculants because of their improved sustainability.

Over the past decades, the screening of EPS-rich microorganisms and extraction of their EPS as bioflocculants have attracted considerable attention. A variety of biological materials, including bacteria, yeast, fungi and algae, have been reported to produce EPS with high flocculation capability (5–7). The EPS extracted from various origins are generally heterogeneous (1). According to their different compositions, EPS flocculants are divided into proteins, polysaccharides, glycoproteins, proteoglycans and lipoproteins (8–10). It is well known that the successful application of EPS flocculants depends on many environmental and operating factors, including the type of EPS producers, composition of media, and dosage of flocculants as well as the ranges of pH and temperature (3,8,11). Some investigations suggested that the bioflocculation capability of EPS is also closely related to their spatial configuration (12,13). Recently, several EPS flocculants have been tested in bench- and pilot-scale studies to be effective for the removal of cell debris in fermentation broth, separation of oil from oil–water emulsions, and decrease of turbidity of micro-polluted source water (14–16).

The possibility of using microbial EPS to replace chemical flocculants has long been appreciated, but commercial applications of these biopolymers are few. The major reason is associated with the high production costs and low yield of bioflocculants, as compared with synthetic flocculants (2,9). For instance, 10 g/L–30 g/L of glucose or sucrose are commonly used in culture media for bioflocculants production, while the yield of bioflocculants that can be ultimately obtained from these high-nutrient media is only 0.4 g/L–1.5 g/L (5,14,17). There is an interestingly physiological phenomenon that in external nutrient-depleted environment, smart microbes are inclined to produce excessive EPS (serving as carbon and energy sources) for the survival in starvation (1). This finding triggers our interest to search novel bioflocculant producers from nutrient-poor conditions (e.g., intentionally constructed low-carbon media). Strains that are capable of producing more EPS with low-nutrient substrates would have a positive impact on the commercialization of bioflocculants. Meanwhile, although previous studies have examined the components and structure of EPS flocculants using various physicochemical methods (e.g., colorimetric analysis, X-ray photoelectron spectroscopy, Fourier transform

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infrared spectroscopy, gas chromatography–mass spectrometry) (7,17,18), the specific active constituents that are responsible for the flocculability of EPS are not fully understood. The elucidation of this information might favor a better understanding about the flocculating behavior of microbial EPS and the manipulation of bioflocculant production in the microbial growth.

In this study, a novel bioflocculant producer *Talaromyces trachyspermus* OU5 was isolated from a soil sample using a low-carbon source medium. This strain grew well at low-nutrition conditions and produced rich proteoglycans flocculants (BF–OU5) with good flocculation capability. The compositions of BF–OU5 as well as its active ingredients involved in the flocculation were determined. The flocculation performance of BF–OU5 in kaolin suspension and a real swine wastewater was examined, respectively.

MATERIALS AND METHODS

Isolation and identification of EPS flocculants producers A nutrient-poor soil sample that was collected from the abandoned Zhaoyuan mine tailing region in Yantai City, China was used for the isolation of oligotrophic bioflocculant producers. A low-carbon source screening medium was selected for microbial growth and EPS production, which contained (per liter): 0.2 g glucose (the available carbon concentration was ~1/100 of the regular dosage), 0.02 g urea, 0.01 g KH_2PO_4, 0.03 g K_2HPO_4, 0.01 g $MgSO_47H_2O$ with an initial pH of ~7.2. Serial dilutions of soil suspensions were plated on the low-carbon agar plates. After incubation at 30°C for 96 h, several culturable microbial colonies were found. After purification of single colonies, each isolated strain was inoculated into 50 ml of screening medium in 150 ml Erlenmeyer flasks, which were then incubated in a shaker at 150 rpm for 72 h at 30°C. Flocculating efficiencies of culture broth from different isolates were examined using kaolin suspension. The strain with the highest flocculating efficiency was selected as a target strain for further studies.

The DNA of the target strain was extracted by fungal DNA kit. The 18S rDNA–ITS gene fragment of the strain was amplified using individual fungal colony PCR. PCR amplification of 18S rDNA–ITS was performed with a forward primer (5′–TCCGTAGGTGAACCTGCGG–3′) and a reverse primer (5′–TCCTCCGCTTATTGATATGC–3′). The obtained PCR products were sequenced by Beijing Sunbiotech Company. The resulting sequence was compared with the available 18S rDNA sequences in the GenBank database of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence accession number The 18S ITS rDNA gene sequence for the target strain was deposited into the GenBank database with accession number EU076917.1.

Flocculating efficiency measurement Kaolin suspension was used as a model system for the evaluation of the flocculation capability of EPS (11). To prepare the kaolin suspension, 4 g of kaolin clay (Merck, Germany) was suspended in 1 L of deionized water. Subsequently, 1 ml of the culture broth was added to 99 ml of the kaolin suspension in a 250 ml beaker. The mixture was stirred at 200 rpm for 1min, then slowly stirred at 60 rpm for 5 min, and allowed to settle for 5 min. Images of the formed floc materials were observed using a Nikon ECLIPSE 80i optical microscope (Nikon Co., Japan). In addition, 5 ml of the supernatant was removed from 2 cm below the upper layer. The optical density (OD) was measured with a Unic-7200 spectrophotometer (Unico Co., China) at 550 nm. For the controls, 1 ml of the culture broth was replaced with 1 ml the fresh culture medium. The flocculating efficiency was calculated according to the following equation:

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

where A and B are the absorbance values of the sample and control at 550 nm, respectively.

Purification of EPS flocculants Purification of EPS flocculants was conducted after incubating the target strain for 72 h at 30°C. The culture broth was centrifuged at 12,000 ×g for 20 min at 4°C to remove the cell debris. Two volumes of cold ethanol were added to the supernatant and left overnight at 4°C. The formed precipitate was collected and resuspended in deionized water, dialyzed against deionized water at 4°C for 24 h (molecular weight cut off: 8000–14,000 Da), and lyophilized to prepare the purified EPS flocculants, which was designated as BF–OU5.

Characterization of EPS flocculants The total sugar content of BF–OU5 was determined via the phenol–sulfuric acid method using glucose as the standard solution (19). The total protein content was measured by the Bradford method with bovine serum albumin as the standard (20). The nucleic acid content was measured by the diphenylamine colorimetric method with salmon DNA as the standard (21). The monosaccharide composition was determined by hydrolyzing 1 mg of BF–OU5 with 4 M trifluoroacetic acid at 121°C for 2 h. The resulting sugars were analyzed with a Waters 1525–2487 high performance liquid chromatography (HPLC) (Waters Co., USA) equipped with a Rezex RCM monosaccharide Ca^{2+} column.

The molecular weight distribution was measured with a Waters–Alliance 2695 gel permeation chromatography (GPC) (Waters Co., USA) equipped with a Waters 2410 Reactive Index (RI) detector and a Waters Ultrahydrogel linear column. The column was calibrated by standard dextrans.

The functional groups were determined with a Nicolet 500 FT–IR spectrometer (Thermo Co., USA) over a wave number range of 4000 cm^{-1}–500 cm^{-1}.

A Hitachi S-4800 scanning electron microscopy (SEM) (Hitachi Co., Japan) was used to visualize the morphology of BF–OU5.

Determination of active ingredients in EPS flocculants for flocculation An enzymatic digestion method using pronase and papain was used to remove proteins in BF–OU5 (19). First, 50 mg of BF–OU5 was dissolved in 10 ml of 50 mM phosphate buffer at pH 7.0; 1% (w/w) of pronase was then added to the BF–OU5 solution and incubated at 37°C for 24 h. Subsequently, 1% (w/w) of papain was added, and the mixture was incubated at 60°C for another 24 h. The enzyme-digested BF–OU5 solution was dialyzed against deionized water, filtered and lyophilized to obtain a solid sample (protein-deficient BF–OU5). The flocculating efficiencies of BF–OU5 and protein-deficient BF–OU5 were evaluated, as the method described above, to differentiate the contributions of polysaccharides and proteins to the flocculation.

Treatment of real swine wastewater A swine wastewater sample rich in chemical oxygen demand (COD), nitrogen, phosphorus and suspended solids, which was taken from the Xingli pig farm in Yixing City, China, was used to examine the validation of BF–OU5 for the flocculation treatment of real wastewater. The selected physicochemical properties of the wastewater are listed in Table 1. All gravels and litters were removed from the wastewater and the sample was stored at 4°C before use.

Five milliliters of dissolved BF–OU5 was added to 100 ml of swine wastewater. After 15 min of flocculating reaction, the supernatant was collected for the determination of COD, Kjeldahl nitrogen (TKN), ammonia nitrogen (NH_4^+–N), total phosphorus (TP) and turbidity. The removal efficiency of the pollutants was calculated by the following equation:

$$\text{Removal efficiency} = \frac{C_0 - C_f}{C_0} \times 100\% \quad (2)$$

where C_0 is the initial value and C_f is the value after flocculation.

Statistical analysis All experiments were performed in triplicate throughout the present study, and the data presented are the mean values of the triplicate samples with standard deviation. Microsoft Excel 2003 was used for all statistical procedures.

TABLE 1. Flocculation performance of swine wastewater treated by 5% (v/v) of BF-OU5.

Parameter	Raw wastewater	Treated wastewater	Removal (%)
COD (mg/l)	6746	3238	52.1
NH_4^+-N (mg/l)	785	639	18.6
TKN (mg/l)	1158	698	39.7
TP (mg/l)	153	120	21.5
Turbidity (NTU)	35742	8935	75

COD, chemical oxygen demand; TKN, Kjeldahl nitrogen; TP, total phosphorus.

RESULTS AND DISCUSSION

Identification of EPS flocculants producer A total of 7 different microbial strains were isolated from the collected soil sample. Among these isolated strains, strain OU5 was found to grow faster at the low-carbon sources conditions, yield more EPS and exhibit the best flocculation performance towards kaolin suspension. Thus, it was selected as a target EPS flocculants producer. When grown on the medium (at 30°C for 60 h), the colonies of strain OU5 had center raised, with distinctly funiculose, dense, velutinous, low-margin and greenish–white mycelium (Fig. S1). The phylogenetic tree based on ITS rDNA sequence of strain OU5 was constructed by the neighbor-joining method (Fig. S2). A BLAST search against GenBank indicated that the ITS rDNA gene nucleotide sequences of strain OU5 had 99% similarity with the *T. trachyspermus* type strain ASR–167 (GenBank accession number GU973722.1). Based on both morphological and phylogenetic analyses, strain OU5 was identified as *T. trachyspermus*.