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Revealing a novel natural bioflocculant resource from *Ruditapes philippinarum*: Effective polysaccharides and synergistic flocculation

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ABSTRACT

A novel natural bioflocculant resource of *Ruditapes philippinarum conglutination mud* (RPM) with the effective flocculation components was firstly reported in this study. Experimental results showed that the maximum flocculation activity (FR) of RPM to kolin clay reached 86.7% in deionized water assay system and 91.8% in sea water assay system. RPM could flocculate marine microalgae *Chlorella salina* with a FR of 74.1%. The crude RPM polysaccharides extract(RPMP) were composed of 97.8% (w/w) polysaccharides and 2.2% (w/w) protein and the functional components of pure RPMP were firstly discovered to be two complex heteropolysaccharides of RPMP-1 and RPMP-2 with similar monosaccharides composition except glucose content. The molecular weights of RPMP-1 and RPMP-2 were 5.7 kDa and 18.0 kDa, respectively. It is interesting to find that RPMP-1 and RPMP-2 exhibited synergistic flocculation activity of 65.6% at a mass ratio of 2:1 as in crude RPMP, suggesting the original proportion is significant to synergistic flocculation.

1. Introduction

Extracellular biopolymer flocculants (EBFs) are widely used in a variety of industries, including wastewater and drinking water treatment, decolorization, heavy metal removal, cell removal and biomass recovery, synthesis of nanoparticles, emulsification, cryoprotection and mining (Aljuboori, Uemura, Osman, & Yusup, 2014; Devi & Natarajan, 2015; Guo & Ma, 2015; Pu, Qin, Qin, Zhang, & Xu, 2014; Sathiyanarayanan, Kiran, & Selvin, 2013; Sathiyanarayanan et al., 2015; Subramanian, Yan, Tyagi, & Surampalli, 2010; Zhao et al., 2016). Compared with conventional synthetic flocculants, EBFs are nontoxic and environmentally friendly. However, high production cost and complicated fermentation/recovery process have become the bottlenecks restraining their widespread commercial application (Salehizadeh & Yan, 2014; Ummalyma et al., 2017).

Ruditapes philippinarum is one of the most exploited marine bivalves worldwide with a production around 4 million tons annually (FAO, 2017; Nie et al., 2016). From our previous study, *R. philippinarum* conglutination mud (RPM) refers in particular to the settled sludge from *R. philippinarum* when freshly harvested clams are kept in clean seawater for mud spitting (Gao, Zhu, Mu, Zhang, & Dong, 2009; Wei, Mu, Zhu, Gao, & Zhang, 2011). RPM from clam siphon appears sticky flocs and showed some good bioflocculant-like activity, as could decolorize methylene blue, crystal violet, malachite green, and ink blue, with decolorization efficiencies of above 90% under the optimal conditions (Wei et al., 2011). Moreover, researches showed that bioflocculation components of RPM may have microbial origin, which means there are probably EBFs functioning in RMP. In our previous study, seventeen bioflocculant-producing bacteria strains have been isolated from RPM, and novel bioflocculant-producing bacteria including *Rothia* sp. ZHT4-13 and *Arthrobacter*sp. ZHT3-9 have been found (Gao et al., 2009; Wei et al., 2011). *Rothia* sp. ZHT4-13 produced bioflocculant MBF4-13, which were useful for the treatment of kaolin clay suspension, decoloration of dye solutions, and removal of heavy metal ions including $Cr_2O_7^{2-}$ and Ni^{2+} (Gao et al., 2009).

In the culture broth of many microorganisms, EBF consisting of polysaccharides, proteins or lipids were secreted (Salehizadeh & Yan, 2014), and polysaccharide was proved to be the main component of fermentation bioflocculant MBF4-13 (Gao et al., 2009). However, there has been no knowledge about whether the microbial fermentation products like MBF4-13 do occur in RPM and what are the main effective components of RPM contributing to the removal of turbidity. Therefore, it is a necessity to uncover the effective bioflocculant compounds profile in RPM for interpreting the role of bioflocculant-producing bacteria within RPM and exploiting natural RPM bioflocculant. In the present study, field *R. philippinarum* was collected and fresh RPM was obtained

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for the flocculant experiment. The purposes are to investigate the flocculation activity of RPM, the polysaccharide ingredients within RPM and probable flocculation mechanism. As far as we know, this is the first time to explore the flocculation activity of RPM and uncover the molecular synergistic flocculation mechanism.

2. Materials and methods

2.1. Materials and chemicals

DEAE-52 Cellulose and Sephadex G-100 were purchased from Solarbio (Beijing, China). The standard bovine serum albumin (BSA) was purchased from Shanghai Jinsui Co. Monosaccharides (D-glucosamine, D-galactose, D-arabinose, D-xylose, D-galactosamine, D-glucuronic acid, L-fucose, D-galacturonic acid) were purchased from Shanghai Yuanye Biotech. Co. (China). The other standard monosaccharides (D-mannose, D-glucose and L-rhamnose) were obtained from Dr. Ehrenstorfer GmbH (Germany). 1-Phenyl-3-methyl-5-pyrazolone (PMP) and HPLC-grade acetonitrile were purchased from USA Tedia Co. Water used throughout the experiments was deionized, and for HPLC process, deionized water was further purified on a Milli-Q system (Millipore Inc., Milford, MA, USA). Ethanol, sulfuric acid, phenol, chloroform, *n*-butyl alcohol and other chemicals were all of analytical grade and purchased from Shanghai Chemical Reagents Co. (China). DextranT-150 (MW133800), DextranT-40 (MW36800), Dextran T-10 (MW9700), Dextran T-5 (MW2700) were purchased from Sigma-Aldrich Co. (Germany).

2.2. RPM preparation

Fresh field *R. philippinarum* and clean sea water were collected from an aquiculture farm located in Zhoushan, Zhejiang Province of China. Five kilograms *R. philippinarum* were put into the clean sea water for obtaining the activated mud for 24 h under room temperature (around 25 °C). The upper sea water was discarded and approximately 500 g fresh mud was obtained.

The solid content and total organic carbon (TOC) of fresh RPM were measured according to the methods from "water and wastewater monitoring analysis method" (SEPAC, 2002). And TOC was determined using an elemental analyzer (vario TOC cube, German elementar).

2.3. Assay of RPM flocculation activity

2.3.1. Assay of ordinary kaolin clay flocculation

Flocculation activity was calculated as flocculation rate (FR) and measured using the kaolin clay suspension method with slight modifications (Kurane, Toeda, & Takeda, 1986). Briefly, 93 mL Kaolin clay suspension (4 g L^{-1}), 5 mL $CaCl_2$ (10 g L^{-1}), and 2 mL bioflocculant sample (make complete with deionized water if volume is insufficient) were successively added, and the pH was adjusted to 7.5 with 2.0 mol L^{-1} hydrochloric acid and 2.0 mol L^{-1} sodium hydroxide solution. The mixture solution was quickly stirred at 200 rpm for 1 min, and slowly stirred at 80 rpm for 2 min, followed by a standing period for 10 min. The optical density (OD value) of the clarifying liquid was measured at 550 nm using a spectrophotometer (DR 1900-05, Hach, Shanghai, China). A control experiment with 2 mL deionized water instead of sample was conducted in the same way. The flocculation rate was calculated by the following equation:

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

Where *FR* is the flocculation rate; (A and B are OD values of the control and sample, respectively).

Fresh RPM initially spanning the range from 0.2 g to 2.0 g (final reaction concentration of 2.0 g L^{-1}–20.0 g L^{-1} correspondingly) was added into kaolin clay flocculation system for the analysis of flocculation activity.

2.3.2. Assay of seawater-based kaolin clay flocculation

Since *R. philippinarum* lives in sea, with an aim at evaluating flocculation activity of fresh RPM for the removal of kaolin clay in a seawater background, an assay of seawater-based kaolin clay flocculation was performed. In this test, the Kaolin clay suspension and $CaCl_2$ solution were both prepared with artificial seawater (ASW) and 2 mL ASW was also used as a control substitution for deionized water, while the experiment procedure followed the ordinary kaolin clay flocculation assay. Seawater was prepared according to the ASW recipe (Goldman & McCarthy, 1978).

2.3.3. Assay of marine microalgae Chlorella salina flocculation

Marine microalgae strain *Chlorella salina* MU101 was stocked in our laboratory, and cultivated in the medium composed of: NH_4Cl 0.2 g L^{-1}, K_2HPO_4 0.05 g L^{-1}, Ferric citrate 0.01 g L^{-1}, prepared with artificial seawater and pH adjusted to 8.0. The microalgae strain MU101was inoculated into a 1000 mL Erlenmeyer flask with 200 mL medium, cultivated under a 12:12 h light–dark cycle with a light intensity of 80 μmol photons m^{-2} s^{-1} at 25 °C for two weeks.

0.2–1.0 g Fresh RPM (final reaction concentration of 2.0–10.0 g L^{-1} correspondingly) was added into 100 mL prepared *Chlorella salina* culture. The mixture was gently stirred for 2 min and settled for 30 min. The OD value of the supernatant was measured at 675 nm using a spectrophotometer (DR 1900-05, Hach, Shanghai, China). A control experiment was carried out without addition of RPM. The FR for *Chlorella salina* was calculated according to the Eq. (1).

2.4. Extraction of crude polysaccharide

The crude polysaccharide from RMP (crude RPMP) was extracted based on water extraction method (Xu et al., 2015; Zheng, Zhang, Liu, Ma, & Lai, 2016), meanwhile modified by Box-Behnken design (BBD) and Design Expert 8.0.6 software for analysis (reported elsewhere). The optimal conditions for the extraction of crude RPMP provided by Response Surface Methodology (RSM) model are presented as follows: extraction time, 4.0 h; extraction temperature, 92 °C; water-solid ratio, 20 mL g^{-1} for dried mud or 100 mL g^{-1} for fresh mud; twice extraction. RPMP was extracted into the liquid phase under the optimal condition, and then three folds volume of dehydrated cold alcohol was added into the liquid, mixed and stayed at 4 °C overnight. After centrifuging at 6000 × *g* for 20 min (Thermofisher, USA), lyophilized the precipitate then obtained the crude RPMP.

2.5. Purification of polysaccharides

The crude RPMP extract was dissolved in distilled water and mixed with Sevag reagent (trichloromethane:n-butyl alcohol = 4:1, v/v) to remove the associated proteins. The deproteined water phase was further thoroughly dialyzed against distilled water at 4 °C for 48 h (Mw cut-off 2 kDa), condensed and lyophilized.

RPMP was further purified by chromatography method provided by Jiang, Wang, Liu, Gan, and Zeng (2011) with slight modifications. The RPMP solid was redissolved in deionized water (3.0 mL) and loaded onto an anion-exchange chromatography column of DEAE-52 Cellulose (1.5 × 50 cm), and then stepwise eluted with 0, 0.1, 0.3 M sodium chloride (NaCl) solution at a flow rate of 1.0 mL min^{-1}. The eluent was collected (5 mL/tube) with an automatic fraction collector (Jiapeng DBS-100, Shanghai, China) and polysaccharides were traced by the phenol-sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). Main fractions containing carbohydrates were collected, dialyzed and concentrated separately. Further purification involved a Sephadex G-100 gel permeation chromatography (1.5 × 50 cm), eluted with deionized water, lyophilized and yielded pure fractions.