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Structure and bioactivity of polysaccharide from a subseafloor strain of *Schizophyllum commune* 20R-7-F01

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ABSTRACT

Fungal polysaccharide is a kind of biomacromolecule with multiple biological activities, which has a wide application prospect and may play an important role in organisms to cope with extreme environments. Herein, we reported an extracellular polysaccharide (EPS) produced by *Schizophyllum commune* 20R-7-F01 that was isolated from subseafloor sediments at ~2 km below the seafloor, obtained during expedition 337. The monosaccharide of EPS was glucose and its molecular weight was 608.8 kDa. Methylation and NMR analysis indicated that the backbone of the EPS was (1 → 3)-β-D-glucan with a side chain (1 → 6) β-D-glucan linking at every third residue. Bio-active assays revealed that the EPS had potent antioxidant activity and could promote RAW264.7 cells viability and phagocytosis. These results suggest that fungi derived from sediments below seafloor are important and new source of polysaccharides and may be involved in the adaptation of fungi to anoxic subseafloor extreme ecosystem.

1. Introduction

Schizophyllum commune, a mushroom forming fungus, belongs to the phylum Basidiomycetes, order Agaricales and family Schizophyllaceae, is one of the most widely distributed fungi and has been cultivated for thousands of years because of its rich nutritional and medicinal value [1,2]. In particular, the extracellular polysaccharide (EPS) produced by *S. commune* has been concerned by scientists and enterprises due to its multiple bio-activities and wide applications in food preservative [3], mineral oil recovery [4], cosmetics production [5], and drug development [6]. For example, the β-D-glucan of *S. commune*, known as schizophyllan (SPG), has been used as an immunoadjuvant in the treatment of neoplasms, particularly cervical and head and neck cancer [7,8]. However, the bio-activity of these polysaccharides differs greatly with their chemical composition and chain conformation, as well as their physical properties such as solubility, molecular weight, branching ratio, and chemical structure [9–12]. Numerous studies have confirmed that the anti-tumor activity of polysaccharide is related to structural variability, such as the location of the monosaccharide residues, the position of glycosidic linkages, and the sequence of monosaccharide residues [13]. Polysaccharides with the main chain consisting of β-(1–3)-glucans and additional β-(1–6) branches, exhibit a prominent anti-tumor activity by promoting the interaction with specific receptors [14]. High molecular weight glucans appear to be more effective than those of low molecular weight [15]. Similarly, the presence of hydrophilic (polyhydroxylated) groups located outside on the surface of the triple helix of polysaccharide is also important for the anti-tumor activity [16]. Therefore, it is of great significance to discover and develop new natural polysaccharides with unique structure and biological activity.

Studies have showed that polysaccharides produced by *S. commune* from different habitats have different structures and molecular weights. For instance, the commercially used *S. commune* strain ATCC 38548 produces EPS with a molecular weight of 1.4×10^3 kDa, consisting of β-D-(1–3)-pyranose and β-D-(1–6)-pyranose groups [17]. Similarly, the EPS produced by *S. commune* ISTL04 is consist of β-D-glucopyranose, 2,4,6-tri-*O*-methyl as a predominant linkage originated from the 3-linked glucose residue [18]. While, the *S. commune* Fr. ACCC51174 produces heteropolysaccharide, mainly composed of glucose, fructose and arabinose, with a ratio of 81.9:10.51:7.56 and an average molecular weight of 4.65×10^4 kDa [19]. Another *S. commune* strain produced EPS with a molecular weight of 2,900 KDa, consisting of β-(1–3) glycosidic

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backbone, (1–4) and (1–6) glycosidic side chain, and containing a large amount of glucose [20]. As one of the most important EPS, SPG is a non-ionic water-soluble homoglucan with β-(1–3)-linked backbone and β-(1–6)-linked glucose side chains at approximately every third residue [21]. The degree to which the β-(1–3)-glucan backbone is substituted by single β-(1–6)-linked glycopyranosyl residues depends on the source of the strain and the culture conditions [22]. Thus, EPS produced by *S. commune* is a kind of fascinating macromolecules with structural and functional diversity. However, it remains unclear why fungi from different sources produce different EPS? And whether EPS is related to the adaptation of fungi to various environments? In this study, we report a neutral EPS produced by *S. commune* 20R-7-F01, which was isolated from coal-bearing sediments ~2 km below seafloor at Pacific off the Shimokita Peninsula of Japan [23]. The monosaccharide composition and chemical structure of the EPS were characterized, and its antioxidant activity and immunostimulatory activity were evaluated. Additionally, the potential role of the EPS in the adaptation of *S. commune* to anaerobic subseafloor environment was also discussed.

2. Materials and methods

2.1. Materials and reagents

DEAE-52 cellulose and Sephadex G-200 were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Standard monosaccharides, trifluoroacetic acid (TFA), fetal bovine serum (FBS) and lipopolysaccharide (LPS) were purchased from Sigma Chemicals Co., Ltd. (Missouri, USA). Antioxidant kit and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). RAW264.7 murine macrophage cells were obtained from the Type Cultural Collection of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified eagle's medium (DMEM) was purchased from Gibco-BRL (Maryland, USA). BCA protein assay kit, reducing sugar assay kit, and cell counting kit-8 (CCK-8) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). NO detection kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). TNF-α and IL-6 detection kits were purchased from Neo Bioscience Biotechnology Co. LTD (Shenzhen, China). All other chemicals and reagents used in this study were analytical grade.

2.2. Fungal strains and cultivation

Four strains of *S. commune* (20R-7-F01, 6R-2-F01, 15R-5-F01 and 24R-3-F01) were isolated from coal-bearing sediments below the seafloor obtained during the Integrated Ocean Drilling Program (IODP) Expedition 337 and maintained in a 4 °C refrigerator [23]. Strain 6R-2-F01 was isolated from sediment core Unit 2 at a depth of 1496 mbsf (meters below seafloor) with an estimated geological sediment age of Miocene, while strains 15R-5-F01/20R-7-F01/24R-3-F01 were derived from sediment core Unit 3 at depths of 1924 mbsf, 1966 mbsf and 1993 mbsf; respectively, having geological age of early to late Miocene [24]. *S. commune* CFCC-86616 and CFCC-87628 obtained from terrestrial wood were purchased from China Forestry Culture Collection Center. All strains of *S. commune* were cultured on potato dextrose agar (PDA) plates at 28 °C for 7 days. The growing margin of mycelia on the medium was homogenized in an autoclaved glass homogenizer, poured into a 500 mL conical flask containing 200 mL of the sterilized medium (30 g glucose, 3 g yeast powder, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 L double distilled water (ddH_2O)), and incubated at 28 °C, 170 rpm for 7 days. The culture broth was used to extract EPS.

2.3. Extraction and purification of EPS

After removing mycelia by centrifugation at 2000 rpm for 10 min, the filtrate was mixed with 4-fold ethanol (95 %), and precipitated overnight at 4 °C. The precipitate was washed 4 times with 95 % ethanol to obtain crude EPS, and its content was determined by phenol-sulfuric acid method [25]. The contents of protein and reducing sugar in the crude EPS were determined by BCA protein assay kit and reducing sugar assay kit, respectively. After deproteinization by Sevage method [26], the crude EPS was dissolved in ddH_2O and purified with DEAE-52 anion exchange column (2.6 cm × 50 cm), which was equilibrated with ddH_2O and eluded with 0–0.8 M NaCl gradient at a flow rate of 1.5 mL/min. The content of polysaccharide in each fraction (5 mL/tube) was determined by phenol-sulfuric acid method. The main polysaccharide fraction was further purified on Sephadex G-200 column (2.6 cm × 30 cm), eluted with ddH_2O at a flow rate of 0.5 mL/min, and freeze-dried at −40 °C for 24 h.

2.4. Monosaccharide analysis

Monosaccharide composition of the pure EPS was determined according to the method reported by Wang et al. [27]. Briefly, 5 mg of EPS was hydrolyzed with 1 mL of 2 M trifluoroacetic acid (TFA) at 105 °C for 6 h, dried with nitrogen, and washed with methanol for 3 times. The residue was dissolved in ddH_2O and filtered through 0.22 μm microporous membrane. The filtrate was then analyzed by high-performance anion-exchange chromatography (HPAEC) equipped with a pulsed amperometric detector (PAD; Dionex ICS 5000 system) and a CarboPac PA-10 anion-exchange column (4.6 mm × 250 mm; Dionex). 5 μL of filtrate sample was injected and eluted at 0.5 mL/min with mobile phase A (100 mM NaOH): B (200 mM sodium acetate) at 95:5 (0–30 min), 80:20 (30–45 min) and 95:5 (45–60 min), respectively. The standard monosaccharides (i.e., glucose, rhamnose, arabinose, xylose, mannose, galactose, fucose, fructose, ribose, glucosamine, D-glucosamine hydrochloride, galacturonic acid, guluronic acid, glucuronic acid, mannuronic acid) were also prepared and analyzed in the same way.

2.5. Determination of molecular weight

The weight-average molecular weight (MW) and polydispersity index (PI) of pure EPS were measured using SEC-MALLS-RI system, equipped with tandem columns (300 mm × 8 mm, Shodex OH-pak SB-805, 804 and 803; Showa Denko K.K., Tokyo, Japan), a DAWN HELEOS-II laser photometer (He—Ne laser, λ = 663.7 nm, Wyatt Technology Co., Santa Barbara, CA, USA), and a refractive index detector (Optilab T-rEX, Wyatt Technology Co., Santa Barbara, CA, USA). The system was injected with 100 μL of 1 mg/mL EPS solution containing 0.1 M $NaNO_3$ and 0.02 % NaN_3, and eluted with 0.1 M $NaNO_3$ at a flow rate of 0.4 mL/min. The column temperature was set to 45 °C [28].

2.6. Small angle X-ray scattering (SAXS) measurement

SAXS spectra was detected using a Nanostar SAXS (Bruker, Germany). The wavelength of the incident X-ray wavelength λ was fixed at 0.154 nm, and the sample-to-detector distance was 800 mm. The Vantec 2000 detector had 2048 × 2048 pixels with a pixel size of 68 × 68 μm. The correct path length was determined using silver behenate. Then the sample was placed in a 3-mm-diameter sample holder hole with the detection time of 600 s under vacuum conditions. The scattering intensities as 2D spectra were transformed into 1D profiles by circular averaging. The *d*-spacing was calculated using the Bragg equation ($d = 2\pi/q$), where $q = (4\pi/\lambda)\sin\theta$ (λ, wavelength; θ, scattering angle) [29].

2.7. Methylation analysis

The methylation of pure EPS was analyzed by the method described by Ciucanu et al. [30]. Briefly, 10 mg EPS was thoroughly dissolved in 3 mL dimethyl sulfoxide (DMSO) with 100 mg NaOH under nitrogen gas, ultrasonicated for 30 min at 450 W, and methylated with 2 mL CH_3I for 1 h. The methylated polysaccharide was extracted with 2 mL CH_2Cl_2,

washed three times with 2 mL ddH_2O, and blown dry with nitrogen. The dried samples were hydrolyzed with 1 mL TFA for 90 min at 121 °C, reduced with 3 mL $NaBH_4$ (10 mg/mL) in aqueous ammonia solution for 12 h at room temperature, followed by the addition of 1 mL glacial acetic acid to stop the reaction. The excess acid in the mixture was removed with 2 mL methanol and dried under vacuum. Then, 2 mL acetic anhydride and 1 mL pyridine were added and acetylated for 2 h at 120 °C. The acetylated samples were extracted with 2 mL CH_2Cl_2, dried and used for GC–MS (Shimadzu, Kyoto, Japan) analysis [31]. The carrier gas of GC–MS was high-pure N_2 at a flow rate of 1 mL/min. The split ratio was setting as 10:1, and the injection volume was 1 μL. The initial temperature was 140 °C and a hold time of 2 min, then a 3 °C/min was increased until 230 °C with a hold time of 3 min. Ion source and transmission line temperatures of MS were 230 °C and 280 °C, respectively. The detector was selected to operate in an electron impact mode of 70 eV with a scanning range of *m/z* 30–600 (Agilent 7890A, 5977B; Agilent Technologies, USA).

2.8. Detection of lipopolysaccharide (LPS) in EPS

The EPS was tested for possible contamination by LPS using the method described by de Santana-Filho et al. [32]. Briefly, 3 mg lyophilized EPS sample was dissolved in 400 μL MeOH and 100 μL MeOH-HCl (3 M) solutions, kept at 80 °C for 20 h, extracted three times with 1 mL hexane and 0.5 mL ddH_2O, and evaporated with a gentle N_2 stream. Then, 1 mL acetic anhydride and 1 mL pyridine were added, completely dried at 100 °C for 1 h, and re-dissolved in 70 μL acetone for GC–MS analysis.

2.9. Structure and morphology identification of EPS

The presence of major functional groups in EPS was detected by Fourier-transform infrared spectroscopy (FT-IR, FT-IR650, Tianjin, China) in the wavelength range of 400–4000 cm^{-1} [17]. The molecular structure of the EPS was elucidated by ^1H NMR, ^{13}C NMR, HSQC (heteronuclear singular quantum correlation) and HMBC (heteronuclear multiple bond correlation) spectra. 20 mg EPS was dissolved in 1 mL of mixed solvent Me_2SO-d_6/D_2O (6:1), and analyzed by NMR spectrometer (Bruker, Ascend 600 MHz, Germany) [33]. The morphology of lyophilized EPS was observed by scanning electron microscopy (Zeiss Merlin Compact, Germany, Oberkochen) [34].

2.10. In vitro free radical scavenging activity assay

Radical scavenging activity of pure EPS was determined at concentrations of 1–6 mg/mL by using ascorbic acid (Vitamin C, Vc) as a positive control. The total radical scavenging activity was determined by ABTS method [35]. The DPPH radical scavenging activity was assessed according to the method described by Xiao et al. [36] with few modifications. Briefly, 2 mL EPS of a given concentration (1–6 mg/mL) was fully mixed with 2 mL DPPH solution (0.2 mmol/L, formulated with absolute ethanol), and incubated in dark for 30 min. The absorbance was measured at 517 nm with Multimode Reader (TECAN, Switzerland, Männedorf) and DPPH radical scavenging activity was calculated. In addition, the hydroxyl radical scavenging activity of EPS was measured according to the method of Wang et al. [37]. Briefly, 1 mL $FeSO_4$ (1.367 mg/mL), 1 mL salicylic acid-ethanol (1.242 mg/mL), 1 mL H_2O_2 (0.272 mg/mL) and 1 mL EPS at a given concentration (1–6 mg/mL) were thoroughly mixed and incubated at 37 °C for 30 min. The absorbance at 510 nm was measured and the hydroxyl radical scavenging activity was calculated.

2.11. Determination of biological activity

2.11.1. Cell viability and phagocytosis assay

Cell viability was measured with CCK-8 assay method [38]. RAW264.7 cells were cultivated in DMEM medium containing 4.5 g/L glucose supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % penicillin and streptomycin. 100 μL RAW264.7 cells (10,000 cells/well) were seeded in each well of a 96-well plate, followed by the addition of 10 μL of each polysaccharide concentrations (6.25–200 μg/mL), and incubated for 24 h at 37 °C and 5 % CO_2 in a chamber. 10 μL of CCK-8 solution was added to each well, and the absorbance at 450 nm was measured by microplate reader after 1 h incubation. 10 μL LPS (1 μg/mL) was used as positive control, and endotoxin-free water was used as blank control.

Neutral red assay was used to evaluate the phagocytic activity of EPS on RAW264.7 cells [39]. Briefly, 100 μL of RAW 264.7 cells (10,000 cells/well) and 10 μL of a given EPS concentration (6.25–200 μg/mL) were added to each well of a 96-well plate, and incubated at 37 °C for 24 h. After removing the medium, 100 μL of 0.075 % neutral red staining solution was added to each well and incubated for 1 h. Then the plate was washed twice with PBS, and 100 μL lysis buffer (acetic acid: absolute ethanol = 1:1, *v*/v) was added to each well and incubated at 37 °C for 1 h to measure the absorbance at 540 nm. 10 μL LPS (1 μg/mL) was used as a positive control, and endotoxin-free water was used as blank control.

2.11.2. Nitric oxide (NO) production and cytokines secretion

100 μL of RAW 264.7 cells (10,000 cells/well) and a given EPS concentration were added to each well of a 96-well plate, and incubated at 37 °C for 24 h. 10 μL of LPS (1 μg/mL) and endotoxin-free water were used as positive and blank control, respectively. The contents of NO, TNF-α and IL-6 in each well were determined using the NO detection kit, TNF-α detection kit, and IL-6 detection kit, respectively, according to the manufacturer's instructions.

2.12. Comparison of crude polysaccharide production capacity of fungal strains

To understand the relationship between fungal strains in different habitats and their ability to produce EPS, we compared the production of crude polysaccharides produced by six *S. commune* strains isolated from terrestrial woods and subseafloor sediments under aerobic and anaerobic conditions [40]. Anaerobic culture conditions were created by adding Vc (100 μg/mL) and resazurin (10 μg/mL) to the medium and purging with 99.99 % pure nitrogen for 5 min [41]. All culture flasks were incubated at 28 °C, 170 rpm for 8 days to extract crude EPS.

2.13. Statistical analysis

Each experiment was performed in triplicate. Data were expressed as means ± standard deviations (SD), and analyzed with GraphPad Prism (version 3.02) software to estimate the significance of the differences ($p < 0.05$) among the treatments using one-way ANOVA and Dunnett test.

3. Results and discussion

3.1. Comparison of crude polysaccharide produced by different *S. commune* strains

As shown in Fig. 1, the amount of crude EPS produced by subseafloor strains (i.e., 6R-2-F01, 15R-5-F01, 20R-7-F01 and 24R-3-F01) were far more than those produced by terrestrial strains (i.e., CFCC-86616 and CFCC-87628) under both aerobic and anaerobic conditions for 7 days at 28 °C. The EPS produced by subseafloor strains was 5.9–7.7 g/L, as compared to that produced by *S. commune* ISTL04 (4.2 g/L) [18]. In addition, the EPS produced by strains under aerobic conditions was 2–4 times more than that under anaerobic conditions, indicating that O_2 plays an important role in the synthesis of polysaccharide in *S. commune*. In fact, our previous studies have shown that *S. commune* buried in subseafloor sediments for >20 million years have developed unique

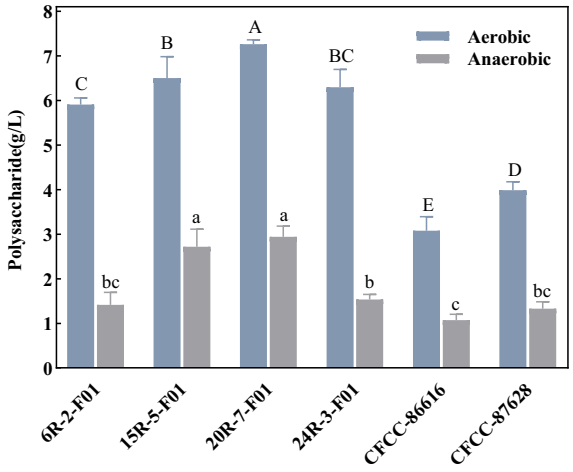

Fig. 1. Polysaccharide produced by different *S. commune* strains under anaerobic and aerobic conditions. Strains 6R-2-F01, 15R-5-F01, 20R-7-F01 and 24R-3-F01 were isolated from deep subseafloor sediments, and strains CFCC-86616 and CFCC-87628 were collected from terrestrial woods. The upper and low case letters represent the significant differences ($p < 0.05$) in the amount of polysaccharides produced by different strains under aerobic and anaerobic conditions, respectively.

strategies such as extracellular production of amino acids especially branched-chain amino acids (BCAAs), ethanol production, increase in mitochondrial number, to produce energy (ATP) and cope with hypoxia/anaerobic conditions [42]. Whether exopolysaccharide secretion is one of the strategies for fungi to cope with anaerobic extreme environment is worth studying.

3.2. Structural characterization of EPS

3.2.1. Isolation, purification and composition analysis of EPS

The crude EPS extracted from culture broth of *S. commune* 20R-7-F01 on the 7th day after inoculation was composed of polysaccharide (90.04 %), protein (4.25 %) and reducing sugar (1.87 %). DEAE-52 cellulose gel chromatography showed that there was a major component in crude EPS (Fig. 2A), which was further purified by Sephadex G-200 column to obtain a pure homogeneous EPS (Fig. 2B). HPAEC analysis revealed that the EPS was mainly composed of glucose (99.38 %) and traces of galactose (0.43 %) (Fig. 2C), which was similar to the documented SPG [43]. These results suggest that the EPS produced by *S. commune* is highly conserved regardless of the habitat of the strain.

3.2.2. Molecular weight

SEC-MALLS-RI analysis showed that the MW of the pure EPS produced by *S. commune* 20R-7-F01 was about 608.8 kDa, which was different from that produced by other strains, such as strain ATCC 20165 with a MW of 4.6×10^3 kDa [44] and strain IBRC-M 30213 with a MW of 370 kDa [34]. It has been reported that the MW of polysaccharides is affected by the extraction process and detection method. For example, ultrasonic treatment of samples resulted in a decrease in the MW of polysaccharides [45]. The mobile phase during high-performance gel permeation chromatography (HPGPC) detection also affected the MW of polysaccharides [46]. Moreover, the MW of polysaccharides is also affected by differences between strains and culture conditions. In addition, the PI of the EPS produced by strain 20R-7-F01 is low (1.12), indicating that it has a narrow molecular weight distribution [47].

3.2.3. FT-IR analysis

FT-IR spectrum (Fig. 3A) of the pure EPS showed a strong and broad peak between 3500 cm^{-1} and 3200 cm^{-1}, indicating the existence of O—H in sugar residue [26]. The peaks around 2931.3 cm^{-1} indicated the existence of C—H stretching of CH_2 groups in carbohydrates [48]. The bands at 1632.5 cm^{-1} (C═O stretching vibration), 1414.1 cm^{-1} (CH_2 bending and C—O—O stretching vibrations), 1390.0 cm^{-1} (bending vibration of C − H), 1152.3 cm^{-1} (C—O—C stretching), and 1028 cm^{-1} (C—O stretching) were due to sugar ring vibrations [49,50]. The weak absorption peaks around 900 cm^{-1}, and 1147 cm^{-1} corresponded to vibrations of C—O, and C—O—C, respectively [51]. The peak at 1050.8 cm^{-1} reflected the presence of β-configuration of glucan due to O-substituted glucose residues in the polysaccharide structure [52]. Additionally, the peak at 892.5 cm^{-1} suggested the presence of β-pyranoside in the molecular structure of polysaccharide [53]. All these characteristics confirm the identity of EPS as a polysaccharide.

3.2.4. SEM analysis

The photograph of scanning electron microscopy (SEM) showed that the pure EPS produced by strain 20R-7-F01 had a unique and complex network structure composed of irregular filaments and strips (Fig. 3B). This structure is distinct from the cylindrical or triple helix structures of

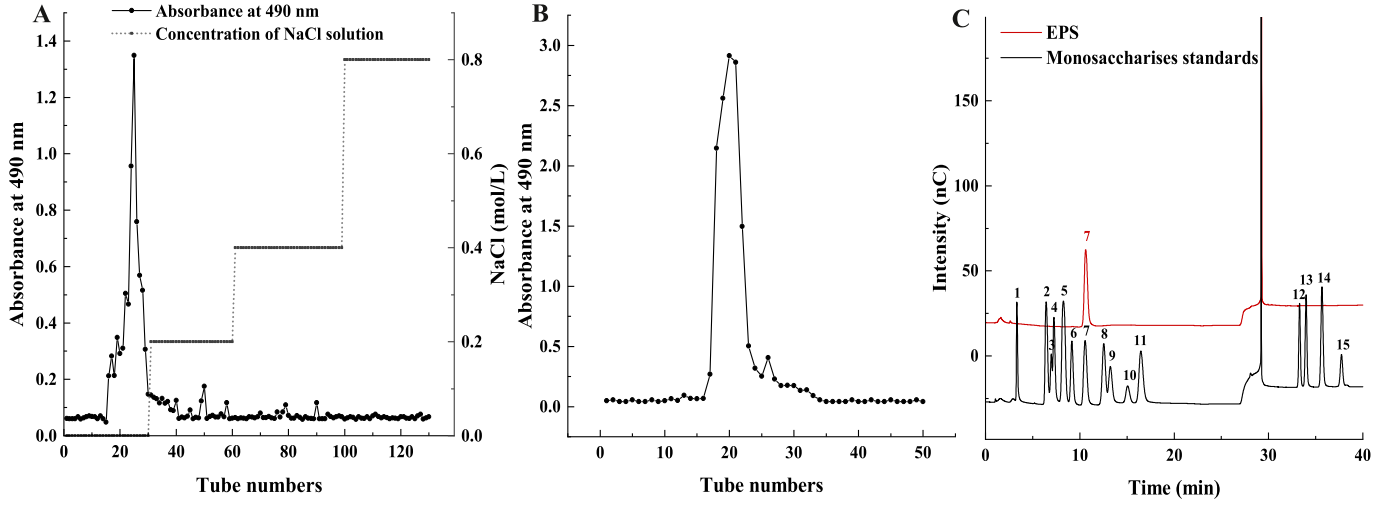

Fig. 2. Purification of EPS by DEAE-52 (A) and Sephadex G-200 columns (B) and identification of monosaccharide components (C). The polysaccharide content in each tube (A, B) was detected by phenol-sulfuric acid method. The numbers on the peaks in C from 1 to 15 represent standard fucose, D-glucosamine hydrochloride, rhamnose, arabinose, glucosamine, galactose, glucose, xylose, mannose, fructose, ribose, galacturonic acid, guluronic acid, glucuronic acid, and mannuronic acid, respectively. Red 7 indicates pure EPS peak.

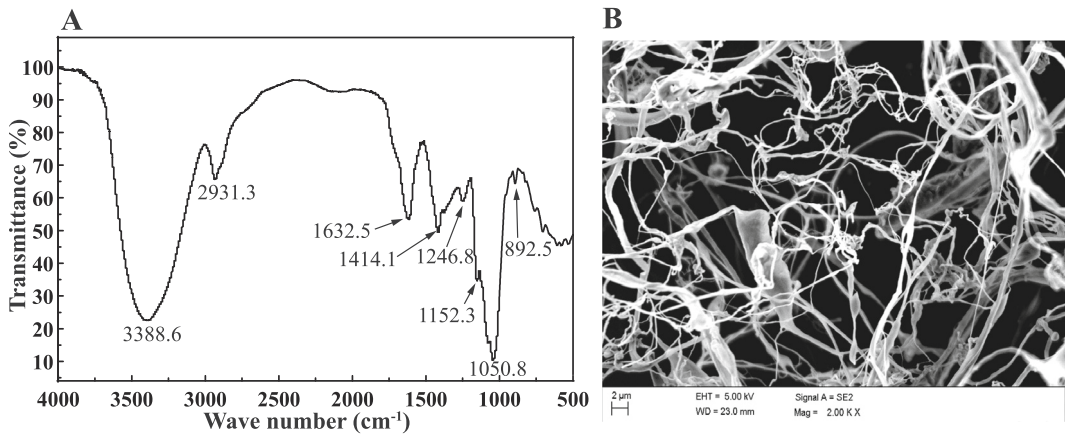

Fig. 3. FT-IR (A) and SEM image (B) of *S. commune* 20R-7-F01 polysaccharide.

SPG [34,54]. Although it is not clear why the apparent structure of exopolysaccharide produced by *S. commune* strains is different, it may be related to the living environment of the strain, and the molecular weight of the polysaccharide. For example, the network structure of polysaccharide can be distorted at high temperature to form filamentous and strip like structure [55]. Given that strain 20R-7-F01 was isolated from a relatively high temperature habitat (~47 °C) [56], the unique structure of EPS may help the strain to persist in the deep subseafloor environment for millions of years.

3.2.5. SAXS analysis

SAXS was used to determine the precise helical pitches of the EPS produced by strain 20R-7-F01. We found a SAXS diffraction peak at q = 3.82 nm^{-1}, corresponding to *d*-spacing of 1.64 nm (Fig. S1), which is consistent with the triple helix pitch of SPG [29]. SPG contains a repeating tetrasaccharide unit of three β-(1 → 3)-linked glucose residues together with a β-(1 → 6)-linked glucose residue and exists as a rigid triple helix in H_2O [57,58]. Thus, EPS produced by strain 20R-7-F01 may have the same triple-helical conformation as SPG.

3.2.6. Methylation analysis

GC–MS analysis indicated the presence of three methylated and acetylated derivatives in EPS, including 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol (A), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol (B), and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol (C), respectively (Table 1 and Fig. S2). Compound A reflected the presence of terminal residues of T-Glc*p*-(1→, compound B indicated the presence of linear residues of →3)-Glcp-(1→, and compound C showed the presence of branched residues of →3,6)-Glcp-(1 → in polysaccharide [17]. The molar ratio of A, B, and C was 1.1: 1.9: 1.0. And the ratio of branch points (C) to terminal units (A) was about 1:1, indicating that the number of branch points roughly equals to the number of terminal units [59]. These results confirmed that the structure of EPS produced by subseafloor strain 20R-7-F01 is a homoglucan with β-1,3-linked backbone and β-1,6-linked glucose side chains at every third residue, i.e., schizophyllan [17].

Table 1
Linkage patterns and mass spectra of acetylated methyl glycosides of EPS.

No.	Retention time (min)	Methylated and acetylated derivatives	Major mass fragments (*m/z*)	Molar ratio	Linkage pattern
A	9.335	1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	59, 71, 87, 102, 129, 145, 161, 205	1.1	T-Glc*p*-(1→
B	12.644	1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	59, 87, 101, 118, 143, 161, 174, 203, 234, 277	1.9	→3)-Glc*p*-(1→
C	18.292	1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol	59, 87, 101, 118, 129, 139, 160, 189, 202, 234, 305	1.0	→3,6)-Glc*p*-(1→

3.2.7. NMR analysis

The ^{1}H and ^{13}C NMR spectra showed that the EPS produced by *S. commune* 20R-7-F01 contained two anomeric proton (H-1) signals at δ_H 4.53 ppm and 4.21 ppm and correlated with the anomeric carbon signals at δ_C 103.44 ppm in Fig. 4A and B [60]. By comparing with previous reports, the anomeric carbon/proton signals at δ 103.44/4.53 ppm were identified as those originated from O-3 substituted glucose residues (β-1,3-linked-Glcp) [61]. And the correlation at δ 103.44/4.21 ppm were identified as the anomeric signals of T-β-Glcp [29]. Characteristic methylene -CH_2 signals were apparent at δ_H 3.41 and 3.64 ppm by combining with the carbons signal at δ_C 61.38 ppm [61]. After comparing the spectrum data of our compound with the reported molecule in literature, it was finally deduced that sugar residues in EPS were arbitrarily labeled **A**, **B1**, **B2**, and **B3** (Fig. 4B). The chemical shifts of the full assignments of different residues were assigned by HSQC (Fig. 4C). The cross peaks of anomeric carbons/protons of residues were detected at δ 103.15/4.21 ppm (assigned for residue **A**) and at δ 103.50/4.53 ppm (assigned for residue **B1, B2, B3**) [29]. The sequences of sugar residues were also analyzed according to HMBC spectrum (Fig. 4D). As assigned by arrows in Fig. 4D, the signal at δ 4.21/68.90 ppm showed that the anomeric proton C6 of residue **B2** correlated with the H1 of residue **A**, which indicated that the connection between residue **A** and **B2** was β-1,6-glycoside bonds [33]. The overlapping peaks containing several signals at around δ 4.53/86.84 ppm showed that the residue **B1** (H1) corresponded to the residue **B2** (C3), the residue **B2** (H1) corresponded to the residue **B3** (C3), and the residue **B3** (H1) corresponded to the residue **B1** (C3). This indicated the connection between residue **B1**, **B2** and **B3** was β-1,3-glycoside bonds [33]. Based on GC/MS and NMR data, it was finally deduced that the EPS produced by subseafloor *S. commune* is composed of a β-(1–3)-glucan backbone with one side-branching units by every three residues. These data are consistent with the documented polysaccharide being schizophyllan [21].

3.3. Free radical scavenging activity assay

The scavenging ability of polysaccharide produced by *S. commune* varies from strain to strain. Here we found that the EPS produced by *S. commune* 20R-7-F01 had broad and concentration-dependent free radical scavenging activity. At 6 mg/mL, the scavenging activities of EPS on DPPH, hydroxyl and ABTS radicals were 60.05 %, 70.43 % and 59.67 %, respectively (Fig. 5), which was equivalent to or higher than that of

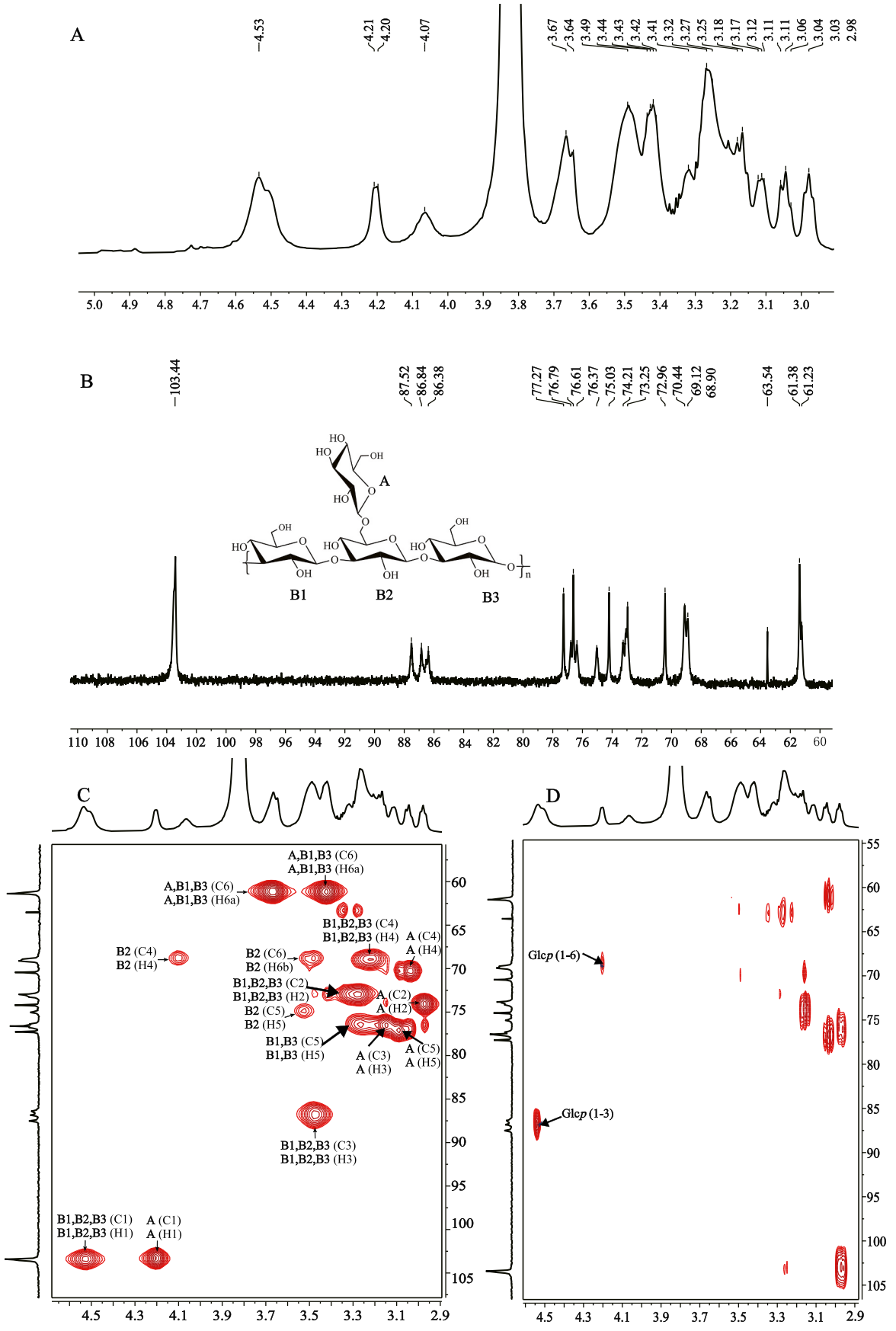

Fig. 4. ^{1}H NMR (A), ^{13}C NMR (B), HSQC (C) and HMBC (D) spectra of *S. commune* 20R-7-F01 polysaccharide.

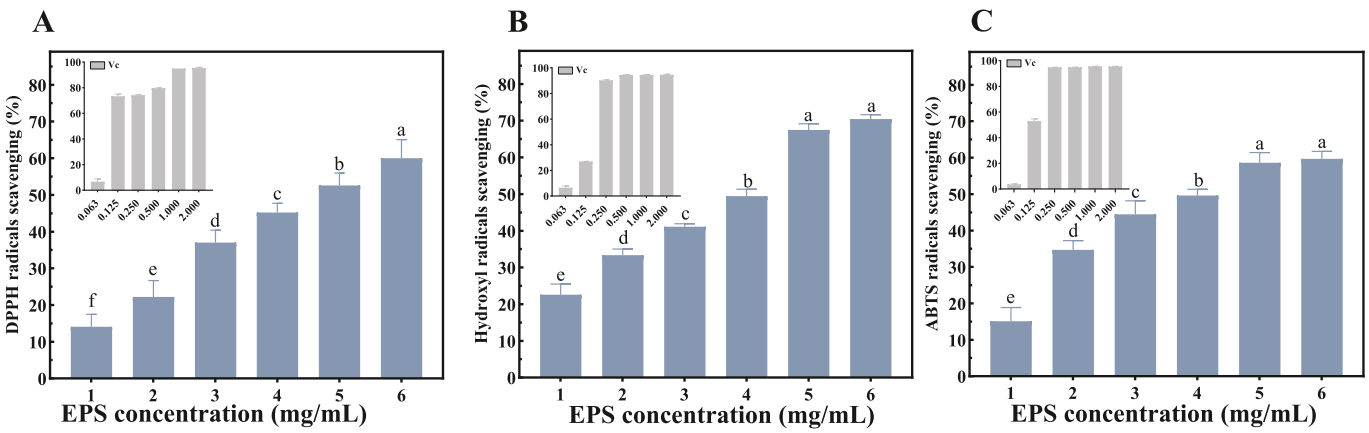

Fig. 5. The scavenging abilities of EPS on DPPH (A), hydroxyl (B) and ABTS (C) Hydroxyl radicals. Vc was used as a positive control. Lowercase letters represent significant differences between concentrations ($p < 0.05$).

SPG. For instance, the SPG derived from *S. commune* Fr. can scavenge DPPH radicals by 40 % [62], while those obtained from *S. commune* Fries 1815 has strong anti-oxidative activity, with IC_{50} of 16.03 to 72.65 μg/mL [63]. The EPS or SPG derived from diverse strains of *S. commune* have distinct free radical scavenging activities, which may be due to their different molecular weight. This broad bio-activity indicates that the EPS produced by strain 20R-7-F01 not only has potential applications in the biomedical field, but may also contribute to the adaptation of fungi to the extreme environment of the deep biosphere.

3.4. Immunoactivity

3.4.1. Effects of EPS on the viability of RAW264.7 cells

Macrophage, an important and common cell in the immune system, plays a vital role in the congenital and adaptive immune regulation [64]. The effect of EPS at different concentration (6.25–200 μg/mL) on the viability of RAW264.7 cells were detected using CCK-8 method. The results showed that the EPS produced by subseafloor *S. commune* could significantly enhance the proliferation activity of RAW264.7 cells at a concentration higher than 25 μg/mL (Fig. 6A).

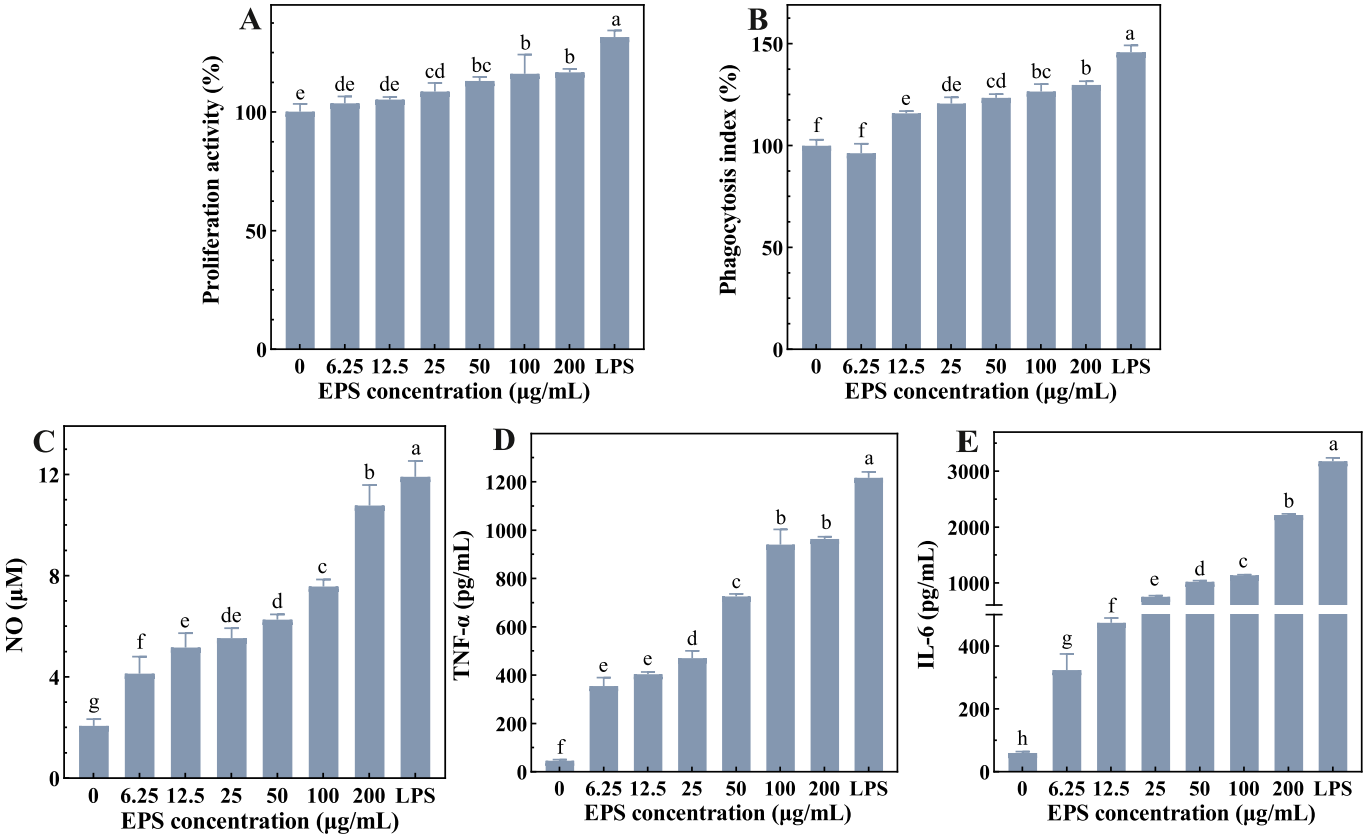

Fig. 6. Immunoactivities of different concentrations of EPS on RAW264.7 cells. (A) Proliferation activity; (B) Phagocytosis index; (C) NO production activity; (D) TNF-α production activity; (E) IL-6 production activity. Lowercase letters represent significant differences between EPS concentrations ($p < 0.05$).

3.4.2. Effects of EPS on the phagocytosis of RAW264.7 cells

Phagocytosis is an important indicator to evaluate the immune response of macrophages [65]. Macrophages act as scavengers to remove foreign bodies and pathogens. The effect of EPS on phagocytosis of RAW264.7 was assayed by neutral red method. As shown in Fig. 6B, the EPS produced by strain 20R-7-F01 had a dose-dependent promoting effect on the phagocytic activity of RAW264.7 cells at 6.25–200 μg/mL. Generally, biological activities of polysaccharide greatly depend on their chemical composition, chain configuration and physical properties. It has been reported that SPG has antitumor activity against Sarcoma 180 only when its MW is higher than 50 kDa and it exists as a triple helical structure in aqueous solution [66]. Thus, the phagocytic effect of EPS produced by strain 20R-7-F01 on RAW264.7 cells may be ascribed to its high MW (608.8 kDa).

3.4.3. Effects of EPS on NO and cytokines secreted by RAW264.7 cells

Upon invasion by pathogens, activated macrophages secrete important chemicals called cellular messengers, including inflammatory mediators NO and cytokines such as IL-6, TNF-α etc., to regulate immune response against the pathogens [67]. As shown in Fig. 6C, D and E, EPS produced by *S. commune* 20R-7-F01 significantly enhanced the secretion of NO, TNF-α, and IL-6 by RAW264.7 cells. Similar results were reported in EPS produced by terrestrial strains of *S. commune* [68]. It has been well known that β-glucans possess a variety of biological activities and applications in drugs, healthcare products, adjuvants in anti-infection vaccines, and immunomodulators in anti-cancer immunotherapy [69]. As immunostimulants or adjuvants, they can recognize various membrane receptors such as dectin-1, complement receptor 3 (CR3), TLR2, CD5, lactosylceramide, and trigger multiple signaling pathways [70]. Recent studies have reported that SPG can modulate the effective response of macrophage cells by binding to the dectin-1 receptor [71]. Thus it is speculated that EPS produced by strain 20R-7-F01 may activate the signaling pathway and induce cytokines production by binding to the dectin-1, which needs more extensive researches to be confirmed.

To prove that EPS samples were not contaminated by LPS, we conducted GC–MS analysis on the tested polysaccharide. As shown in Fig. S3, no signal of LPS marker 3-hydroxy tetradecanoic acid was detected [32], indicating that the EPS used for biological activity test was not contaminated by LPS. Therefore, the effects observed in the above experiments were purely produced by the polysaccharide.

4. Conclusions

Fungi are one of the major microbial group producing a variety of bioactive extracellular polysaccharide, which might help fungal adaptation to various extreme environments. Here we report the extraction, purification, structure and biological activities of EPS produced by *S. commune* 20R-7-F01, which was isolated from 1966 mbsf sediment with an estimated geological age of early to late Miocene (approx. 20 million years ago). The EPS is composed of 99.38 % glucose and a trace of galactose, with a molecular weight of 608.8 kDa, and has a well-known structure of schizophyllan (a β-1, 3-linked backbone with a β-1, 6-linked glucose side chain at every third residue). Importantly, this EPS has strong antioxidant activity, enhances the viability and phagocytosis of RAW264.7 cells, and promotes the secretion of NO and cytokines by macrophages, which shows its great potential in the healthcare, pharmaceutical and cosmetic industries. In addition, we have proved for the first time that fungi can produce polysaccharides under both aerobic and anaerobic conditions. Moreover, subseafloor strains of *S. commune* produce more polysaccharides than terrestrial strains under anaerobic productions, suggesting a possible ecological role of polysaccharide in fungal adaptability to low oxygen conditions, which need to be elucidated in the future.

CRediT authorship contribution statement

Yunan Ma: Methodology, Formal analysis, Data curation, Writing – original draft. **Zhen Wang:** Validation. **Muhammad Zain Ul Arifeen:** Writing – review & editing. **Yarong Xue:** Methodology, Writing – review & editing. **Sheng Yuan:** Methodology, Writing – review & editing. **Changhong Liu:** Project administration, Supervision, Conceptualization, Investigation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors report no declarations of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.09.189.

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