

Preliminary Characterization of Exopolysaccharides Produced by *Abortiporus biennis* in Submerged Fermentation

(Pencirian Awal Eksopolisakarida yang Dihasilkan oleh *Abortiporus biennis* dalam Fermentasi Tenggelam)

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ABSTRACT

Abortiporus biennis is a rare edible and medicinal mushroom that is valuable in traditional Chinese medicine. Exopolysaccharides (EPSs) as valuable metabolites have been harvested from fermentation broths of *A. biennis*. However, studies on preliminary characterisations and bioactivities of *A. biennis* EPSs are lacking. In this study, therefore, the production, characteristics and bioactivity of EPSs produced by *A. biennis* in submerged fermentation were investigated. An EPS-1 was isolated and purified from the culture broth of *A. biennis* using a Sepharose CL-6B column chromatography. The molecular weight, monosaccharide composition, structural characteristics, chain conformation and thermal property of EPS-1 were determined by gel-filtration chromatography, gas chromatography-mass spectroscopy, Fourier-transform infrared spectroscopy, Congo red test and thermogravimetric analysis, respectively. The *in vitro* antioxidant and immunoregulation activities of the EPS-1 were evaluated by adopting DPPH and hydroxyl radical-scavenging assays, and analysing NO production in macrophage RAW 264.7 cells, respectively. Results showed that the culture medium containing 30 g/L lactose and 3.0 g/L tryptone had the most suitable carbon and nitrogen sources, respectively, for EPS production in *A. biennis*. The maximum EPS production (18.29 g/L) was obtained after 6 days of cultivation in a 5 L stirred-tank reactor under the most suitable culture medium. After purification, EPS-1 with 85.4% yield and 96.1% carbohydrate content was obtained from the culture broth of *A. biennis*. EPS-1 was characterised as a neutral heteropolysaccharide with a molecular weight of 22.07 kDa and is composed of glucose, mannose and galactose at a molar ratio of 3.3:2.0:1.0. EPS-1 with a relatively high thermal stability existed as a random coil conformation in the aqueous medium and possessed prominent radical-scavenging ability and macrophage stimulation activity *in vitro*. Therefore, EPS-1 could be explored as a functional ingredient with potential applications in food, medical and cosmetic industries.

Keywords: *Abortiporus biennis*; bioactivity; exopolysaccharides; preliminary characterisation; submerged fermentation

ABSTRAK

Abortiporus biennis adalah cendawan yang boleh dimakan dan boleh digunakan dalam ubatan yang sangat berharga dalam perubatan tradisi Cina. Eksopolisakarida (EPS) sebagai metabolit berharga telah dituai daripada kaldu fermentasi *A. biennis*. Walau bagaimanapun, kajian mengenai pencirian awal dan bioaktiviti *A. biennis* EPS adalah agak kurang. Oleh itu, dalam kajian ini, pengeluaran, ciri-ciri dan bioaktiviti EPS yang dihasilkan oleh *A. biennis* dalam fermentasi tenggelam telah dilakukan. EPS-1 telah dipencilkan dan dituliskan daripada kultur kaldu *A. biennis* menggunakan kromatografi turus Sepharose CL-6B. Berat molekul, komposisi monosakarida, ciri struktur, penyesuaian rantai dan harta termal EPS-1 ditentukan menggunakan kromatografi penapisan gel, kromatografi gas-spektroskopi jisim, spektroskopi infra merah Fourier-tertransformasi, ujian Congo merah dan analisis termogravimetrik. Aktiviti antioksidan *in vitro* dan pengaturan imuno EPS-1 masing-masing ditentukan menggunakan asai DPPH dan hidroksil radikal skaveng serta menganalisis pengeluaran NO dalam sel makrofaj RAW 264.7. Hasil kajian menunjukkan bahawa kultur media yang mengandungi 30 g/L laktosa dan 3.0 g/L tripton mempunyai sumber karbon dan nitrogen yang paling sesuai untuk pengeluaran EPS dalam *A. biennis*. Pengeluaran EPS maksimum (18.29 g/L) diperolehi selepas penapaian selama 6 hari dalam reaktor tangki pengaduk 5L menggunakan kultur media yang paling sesuai. Selepas penulenan, EPS-1 dengan hasil sebanyak 85.4% dan kandungan karbohidrat sebanyak 96.1% telah diperolehi daripada kultur kaldu *A. biennis*. EPS-1 dicirikan sebagai heteropolisakarida neutral dengan berat molekul 22.07 kDa dan terdiri daripada glukosa, mannan dan galaktosa pada nisbah molar 3.3:2.0:1.0. EPS-1 dengan kestabilan termal yang agak tinggi wujud dalam konformasi gegelung rawak dalam media akuas dan mempunyai keupayaan radikal skaveng yang menonjol serta aktiviti rangsangan makrofaj *in vitro*. Oleh itu, EPS-1 boleh diterokai sebagai bahan berfungsi dengan potensi aplikasi dalam industri makanan, perubatan dan kosmetik.

Kata kunci: *Abortiporus biennis*; bioaktiviti; eksopolisakarida; fermentasi tenggelam; pencirian awal

INTRODUCTION

Abortiporus biennis (Bull.) singer belongs to white rot Basidiomycetes and is mainly distributed in China, Japan and West Europe. This species is often parasitic on many types of broad-leaved trees or on the ground where wood was buried and thus causes white wood to decay. As a rare edible and medicinal mushroom, *A. biennis* has been used for hundreds of years due to its multiple pharmacological and biological activities (Zhang et al. 2011). Moreover, this organism is a fascinating producer of extracellular laccase and is viewed as an oxalate oxidase producing fungus (Jaszek et al. 2006), a unique feature among wood-degrading fungi (Graż et al. 2009). Submerged fermentation is a feasible, simple and efficient method for extracellular laccase production (Ding et al. 2014; He et al. 2014; Jaszek et al. 2006). For example, He et al. (2014) had optimised the culture conditions of laccase productions in a submerged culture of *A. biennis* in a pilot-scale bioreactor (300 L) using the rotating simplex method, and the maximum laccase production was 1361 U/L under the optimal culture medium. Exopolysaccharides (EPSs) as valuable metabolites have also been secreted in large quantities into the culture broth of *A. biennis*. Many studies reported that the EPSs harvested from fermentation broths of various fungi or mushrooms exhibit notable and excellent bioactivities, such as immunostimulatory, antitumor and antioxidant activities, and other pharmacological functions, thereby having great potential for applications in food, medicine and cosmetics (Cao et al. 2014; Chen et al. 2019; He et al. 2012; Meng et al. 2010). To the best of our knowledge, no or little information is available on the isolation, characterization and bioactivities of EPS produced from *A. biennis* in submerged fermentation.

In this study, the submerged culture conditions for the EPS production of *A. biennis* in shake-flasks and a 5 L stirred-tank reactor were investigated and screened. After isolation and purification, the physicochemical properties and preliminary structural characterizations of the purified EPS, namely EPS-1, were studied. The antioxidant and macrophage stimulation activities of the EPS-1 were also evaluated.

MATERIALS AND METHODS

CHEMICALS

Monosaccharide standards (arabinose, glucose, galactose, mannose, rhamnose and xylose), Congo red, glucuronic acid, trifluoroacetic acid, hydrogen peroxide (H₂O₂) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of laboratory grade and used without further purification.

MICROORGANISM, INOCULUM AND CULTURE CONDITIONS

Microorganism of *A. biennis* was collected by our own laboratory and was used throughout the study. The stock

culture was sustained by potato dextrose agar (PDA) slant and monthly subcultured at 25°C for 8 days and stored at 4°C. The seed culture was grown in a 250 mL conical flask containing 50 mL of glucose-peptone (GP) medium (3% glucose and 0.3% peptone, pH6.5) at 26°C for 4 days in a rotary shaker incubator at 150 rpm. *A. biennis* submerged cultures were conducted in 250 mL conical flasks with continuous stirring (150 rpm), containing 50 mL media after inoculating with 4% (v/v) of the seed culture. *A. biennis* was initially grown on PDA medium in a Petri dish and transferred into the seed medium by punching out 5 mm of the agar plate culture with a self designed cutter.

CULTIVATION IN A 5-L STIRRED-TANK REACTOR

A 5-L stirred-tank reactor with six-blade Rushton turbine impeller (Infors, Switzerland) was employed in this study for the batch submerged cultivation of *A. biennis* under the following conditions with inoculation amount of 4% (v/v), temperature at 26°C, aeration rate of 2 vvm, agitation speed at 150 rev/min, initial pH of 8.0, and working volume of 3.0 L. Samples were collected at 1, 2, 4, 6 and 8 days, and subjected to direct observation of morphology using an image analyzer (DT2000 System, China) with software linked to a light microscope (Nikon, Japan) via a CCD camera.

MEASUREMENT OF THE YIELDS OF MYCELIA AND EPS

A. biennis mycelia were separated by filtration and washed thrice with double distilled water, dried in a vacuum oven at 70°C overnight to a constant weight, and the mycelia dry weight was determined gravimetrically. After filtration, the culture broths were collected and precipitated by addition of 4 volumes of 95% ethanol overnight, and then the crude EPS was obtained by centrifugation at 10,000 g for 15 min. The EPS was redissolved in distilled water and the concentration of EPS was determined by the phenol-sulfuric acid method (Dubois et al. 1956).

ISOLATION AND PURIFICATION OF EPS

After a series of deproteinisation by Sevag method, dialysis and lyophilisation, the pretreated EPS was redissolved in 0.2 M NaCl buffer and loaded onto a pre-equilibrium Sepharose CL-6B column (2.4 cm × 100 cm, Sigma Chemical Co. St. Louis, MO) and eluted with the same buffer at a flow rate of 0.6 mL/min under room temperature. An automatic fraction collector was used to collect the eluate at a frequency of 5 mL/tube. Carbohydrates and proteins in each tube were monitored by the phenol-sulfuric acid method and the Bradford method at 490 nm and 280 nm, respectively (Bradford 1976; Dubois et al. 1956). The leading peak was collected, concentrated, dialysed and lyophilised to yield purified EPS, designated as EPS-1.

The total carbohydrate, uronic acid and protein contents of EPS-1 were determined by the phenol-sulfuric acid method using glucose as a standard (Dubois et al. 1956), via sulfuric acid-carbazole method using glucuronic

acid as a standard (Bitter & Muir 1962), and via the Bradford method using bovine serum albumin as a standard (Bradford 1976), respectively.

PRELIMINARY STRUCTURAL CHARACTERISATIONS

The gel-filtration chromatography on a Sepharose CL-6B column was applied to determine the molecular weight (MW) of EPS-1 according to our previous study (Cao et al. 2014). The calibration equation for average MW against retention time was prepared with Dextran MW standards ranging from 10 kDa to 150 kDa (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The experimental conditions were the same as described in Isolation and Purification of EPS section.

The monosaccharide composition of EPS-1 was analysed using gas chromatography-mass spectroscopy (GC-MS) on a Varian Star 3600 CX instrument (Varian Co., Lexington, MA, USA) with a fused silica capillary column (Na form, 30 m × 0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector with the conditions as reported before (Zheng et al. 2014).

Fourier-transform infrared (FT-IR) spectrum of EPS-1 was determined using a FT-IR spectrometer (Bruker Tensor 27) in the wave number range of 400-4000 cm⁻¹ with KBr pellets and referenced against air.

Thermogravimetric analysis (TGA) of the EPS-1 was carried out in a TA Q5000IR TGA apparatus. TGA curve plot was for the TGA signal (converted to percent weight change on the *Y*-axis) against the reference material temperature (on the *X*-axis).

The triple helix conformation or random coil structures of polysaccharide chains in an aqueous alkaline solution was investigated using Congo red test (Cao et al. 2014). Briefly, 5.0 mg of EPS-1 was dissolved in 2.0 mL distilled water and mixed with an equal volume of 80 μM Congo red solution, together by drop-wise addition of 1 M NaOH solution to 0-0.5 M final concentrations. UV-visible spectra of the resultant mixture at various concentration of the NaOH were scanned with the UV-visible spectrophotometer (TU-1800, China) at 400-800 nm and the maximum absorption wavelength (λ_{\max}) was recorded.

ANTIOXIDANT ACTIVITY ASSAYS

Concerning the DPPH scavenging assay, the EPS-1 was previously dissolved in distilled water at various concentrations (0-3.0 mg/mL). A volume of 2 mL of the EPS-1 solution was mixed with an equal volume of 0.1 g/L DPPH solution in 50% ethanol. The reaction mixture was stirred and incubated at 25°C for 20 min in the dark. The absorbance of the resulting solution was measured at 517 nm by using UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The DPPH radical scavenging activity was calculated according to the following equation: scavenging activity (%) = $[1 - (A_1 - A_2)/A_0] \times 100\%$, where A_0 , A_1 and A_2 are the respective absorbance values of the blank control (distilled water), the EPS-1 with DPPH

solution, and the EPS-1 with distilled water. Vitamin C (V_c) was used as a positive antioxidant reference.

For the adapted hydroxyl radical ($\cdot\text{OH}$) method, the EPS-1 was previously dissolved at various concentrations (0 to 3.0 mg/mL) in distilled water. EPS-1 or control solution (0.2 mL) was added into an equal volume of an aqueous solution of 5 mM FeSO₄. Then, 0.2 mL of 1% (v/v) H₂O₂ was added into the mixture under continuous stirring. After stirring and incubation at 25°C for 60 min, the absorbance of the resulting solution was measured at 510 nm using UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The $\cdot\text{OH}$ scavenging activity (%) was estimated by the following formula: scavenging activity (%) = $(1 - A/A_0) \times 100\%$, where A , and A_0 are the absorbance values of the sample and blank control (distilled water), respectively. V_c was used as a positive antioxidant reference.

EFFECT OF EPS-1 ON NO PRODUCTION IN MACROPHAGE RAW 264.7 CELLS

Mouse macrophage RAW264.7 cells were cultured in Dulbecco's modified Eagle (Gibco Co., Carlsbad, CA, USA) supplemented with 10% (w/v) fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Amersco Co., Solon, OH, USA) maintained at 37°C in an incubator with 5% CO₂ and a humidified atmosphere. The RAW264.7 cell suspension (180 μL; 5×10^5 cells/mL) and the EPS-1 (20 μL) at different concentrations (100, 200, and 500 μg/mL) were added to a 96-well plate and incubated at 37°C in a 5% CO₂ atmosphere for 48 h. The supernatants were collected, and NO production was determined by mixing with 50 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) for 10 min at 25°C. NO production was measured by comparing the absorbance at 543 nm against a standard curve generated using NaNO₂. PBS and lipopolysaccharide (LPS, 1 μg/mL) were used as negative and positive controls, respectively.

STATISTICAL ANALYSIS

All experiments were conducted in three replicates and the mean ± standard deviation (SD) was used in the analysis. The statistical analysis was performed by Student's *t*-test. Data Processing System (DPS Version 3.0) was used for the experimental designs and statistical differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

EFFECTS OF CARBON AND NITROGEN SOURCES FOR EPS PRODUCTION

Different carbon and nitrogen sources were selected and kept at constant concentration levels of 30 g/L and 3 g/L, respectively, for 8 days in the basal medium to screen for the most suitable carbon and nitrogen sources for EPS production in *A. biennis* (Figure 1). Among the

carbon sources examined, the culture medium with 30 g/L lactose could produce the highest yields of biomass (1.47 g/L) and EPS (14.44 g/L) (Figure 1(A)). In addition, the EPS production was almost close to 0 in the presence of sorbitol as the carbon source in the culture medium. This result indicated that sorbitol can inhibit the EPS synthetic pathway, which was in agreement with our previous study (Zheng et al. 2014). Hence, we viewed lactose as the best carbon source for the EPS production in *A. biennis*. This finding was opposite to the results for the carbon source requirements reported by other researchers in different fungi or mushrooms (Chen et al. 2019; Meng et al. 2010). Figure 1(B) shows that among the six different nitrogen sources, organic nitrogen sources produced the relatively highest mycelial growth and EPS production in *A. biennis*. Notably, the maximum production of EPS (8.07 g/L) was obtained when tryptone was used as nitrogen source, and the yield of biomass was 1.13 g/L, which was lower than that in medium containing yeast extract (3.73 g/L). Considering that the EPS production was established in the current work, we then selected lactose and tryptone as the best carbon and nitrogen sources for *A. biennis* submerged fermentation.

EPS PRODUCTION IN A 5 L STIRRED-TANK REACTOR

Figure 2 presents the typical time profile of EPS production by *A. biennis* in a 5 L stirred-tank reactor under the most suitable culture conditions (lactose 30 g/L, tryptone 3 g/L, 26°C and pH8.0). The EPS production reached a maximum concentration of 18.29 g/L at day 6, which was considerably higher than that in shake-flask cultures (14.44 g/L, Figure 1(A)). This phenomenon occurred because the high shear force provided by stirred-tank reactor could improve the mass transfer, thus maintaining a satisfactory supply of sugars and other nutrients to the cells, accelerating the EPS anabolic synthesis, and contributing to the enhanced EPS production in the submerged fermentation of *A. biennis* (Fontana et al. 2009). Figure 3 displays the typical morphology changes during the entire submerged cultivation period in the stirred-tank reactor. Fresh cell showed a fungus pellet with a number of fluffy hairiness, which was a complex of EPS and mycelium that existed in and around the fungal pellet core region. In the submerged cultures, the fungal pellets became clear, and the outer hairy region of the fungal pellets thinned out. These phenomena occurred because the high shear force in the stirred-tank

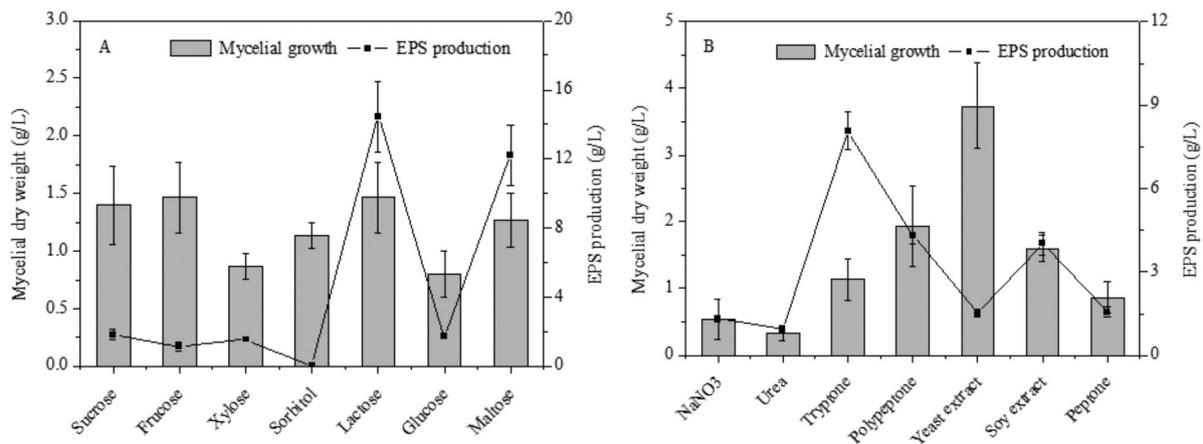


FIGURE 1. Effect of (A) carbon and (B) nitrogen on mycelia growth and EPS production by *A. biennis* in shake flask cultures. Each value is expressed as a mean \pm standard deviation (SD, $n=3$)

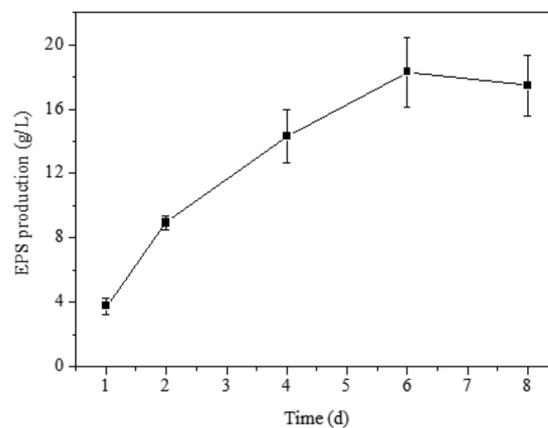


FIGURE 2. Time profile of EPS in submerged culture of *A. biennis* in a stirred-tank reactor

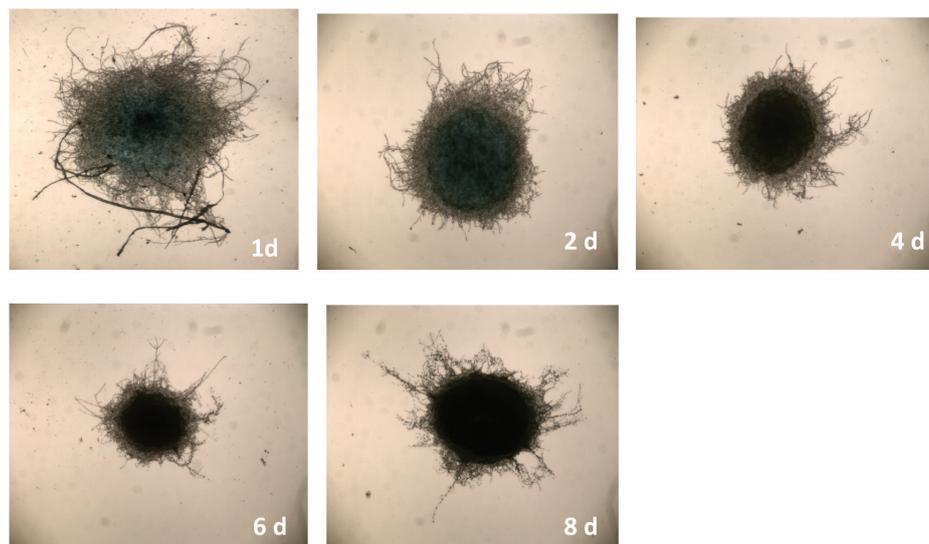


FIGURE 3. The morphological changes in *A. biennis* in a 5-L stirred-tank reactor. All representative images were taken at 4-fold magnification, at 1, 2, 4, 6 and 8 d mean cultivation period in day

reactor could easily destroy the hairiness of the fungal pellet and consequently lower the EPS production after 8 days of cultivation.

PURIFICATION AND PRELIMINARY STRUCTURAL CHARACTERISATIONS OF EPS-1

In this work, the crude EPS harvested from the culture broth of *A. biennis* was purified by Sepharose CL-6B column chromatography to obtain a purified polysaccharide (Figure 4(A)), designated as EPS-1, with a yield of 85.4%. In Figure 4(A), the elution peak showed only one absorbance at 490 nm but not at 280 nm, indicating that the EPS-1 is a polysaccharide. The carbohydrate content of EPS-1 was 96.1%, and no protein or uronic acid was present in EPS-1. This result suggested that EPS-1 is a neutral polysaccharide. Moreover, no characteristic absorption peaks at 250 and 280 nm were observed in the UV-visible

absorption spectrum of EPS-1 (data not shown), thus confirming the absence of nucleic acids and proteins in EPS-1. This result was consistent with those in Figure 4(A). Gel-filtration chromatography on Sepharose CL-6B column was applied to determine the MW of the EPS-1 (Figure 4(B)). The MW of EPS-1 was 22.07 kDa according to the calibration equation derived from the linear regression of the calibration curve with standard dextrans (Figure 4(B)). Based on the monosaccharide composition analysis, EPS-1 is a heteropolysaccharide mainly composed of glucose, mannose and galactose in a molar ratio of 3.3:2.0:1.0. Collectively, these results indicated that EPS-1 is a neutral heteropolysaccharide.

Figure 5(A) displays the FT-IR spectrum of EPS-1. Two characteristic absorption peaks of polysaccharides were observed, a strong and broad absorption peak at 3324.5 cm^{-1} corresponding to the O-H stretching vibration and a weak

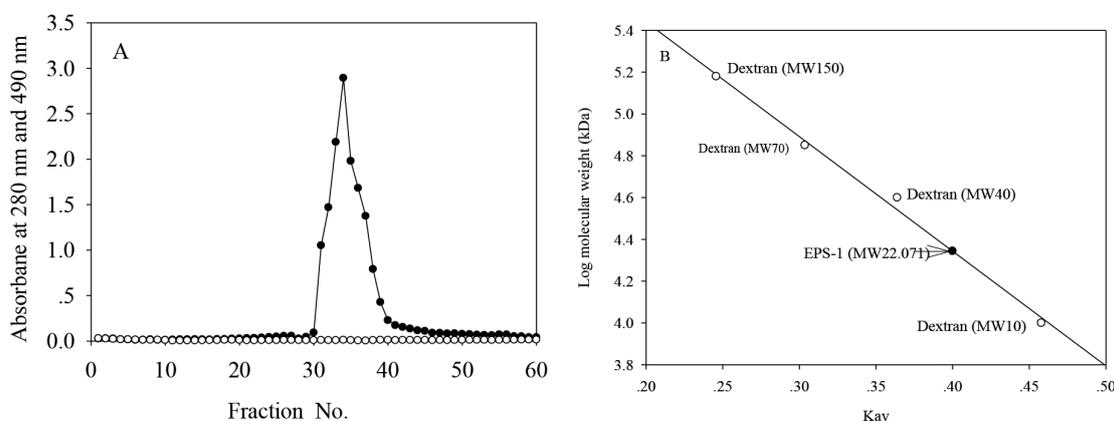


FIGURE 4. (A) Elution profile of the EPS-1 from *A. biennis* in Sepharose CL-6B column chromatography. Elutes were analysed by measuring the absorbance at 490 nm for carbohydrate (black circle) and the absorbance at 280 nm for protein (blank circle). (B) Determination of molecular weight of EPS-1 in Sepharose CL-6B column chromatography. $K_{av} = (V_c - V_o)/(V_t - V_o)$ (V_o : void volume, V_t : total volume, V_c : elution volume). EPS-1 was denoted as a closed circle.

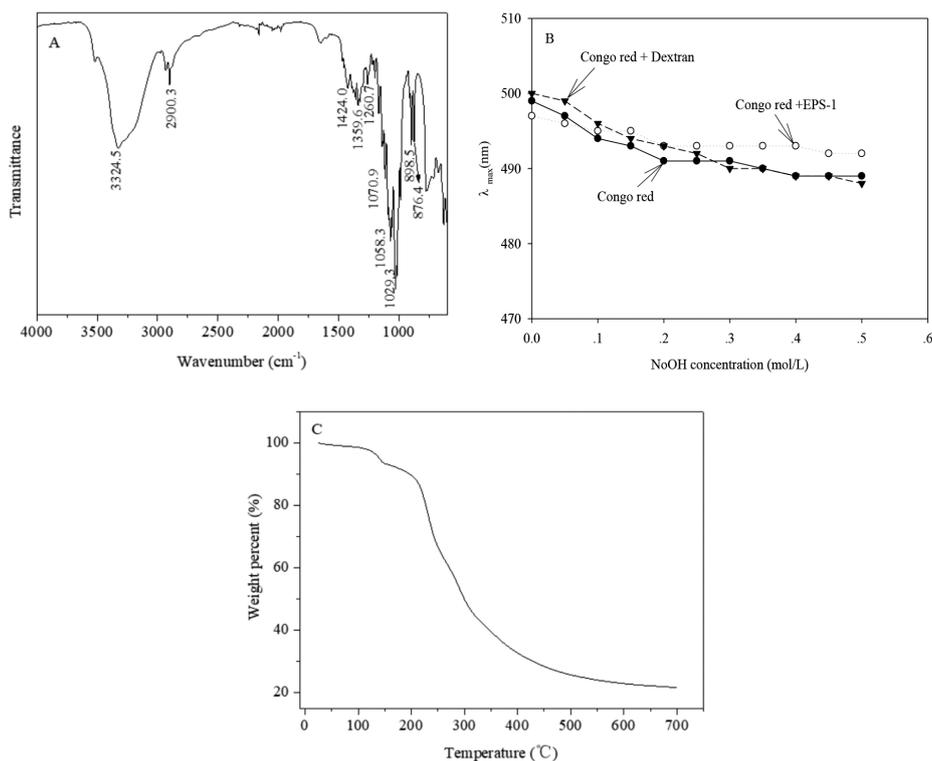


FIGURE 5. (A) FT-IR spectrum of EPS-1. (B) TGA thermogram of EPS-1. (C) Helix-coil transition analysis of EPS-1 based on the maximum absorption wavelength of the Congo red-EPS-1 complex at various concentrations of NaOH. Congo red and dextran viewed as controls

absorption peak at 2900.3 cm^{-1} due to the C-H stretching vibration. No absorption peak was observed at 1730 cm^{-1} , which suggested the absence of uronic acid in the EPS-1 structure (Jing et al. 2014). The absorption band at 1652.7 cm^{-1} was due to the bound water. Strong absorption bands in the range of $1000\text{--}1200\text{ cm}^{-1}$ were associated with the stretching vibrations of C-O-C and C-O-H groups (Zhang 1999). Three stretching peaks at 1070.9 , 1058.3 and 1029.3 cm^{-1} further showed the presence of pyranoside in EPS-1. In addition, characteristic peaks at 876.4 and 898.5 cm^{-1} suggested that α - and β -anomeric configurations existed in EPS-1 structure.

Figure 5(B) shows the changes in the maximum absorbance (λ_{max}) of Congo red-EPS-1 solution with NaOH concentration ranging from 0 M to 0.5 M in comparison with dextran, a known random coil conformer. Congo red-EPS-1 and -dextran solutions did not change the λ_{max} between 500 and 490 nm compared with that of Congo red alone within the 0–0.5 M NaOH concentration range, suggesting that similar to dextran, the EPS-1 chain exhibits a random coil conformation instead of a helix structure in the aqueous medium.

Figure 5(C) shows the TGA curve of EPS-1. The degradation temperature (T_d) of EPS-1 was 115°C , which suggested that the thermogravimetric stability of EPS-1 was decomposed at temperatures above the observed T_d . Furthermore, we noted a significant mass loss in EPS-1 at 250°C , and this mass loss gradually decreased to leave a final residue of ca. 21.61% of the original EPS-1 mass.

Thus, the TGA analysis in this work confirmed that the EPS-1 possesses a relatively high thermal stability.

In vitro ANTIOXIDANT ACTIVITY

The EPS derived from fungi or mushrooms as excellent free radical scavengers exerted important roles in the prevention of oxidative stress in living organism, thereby having great potential as natural antioxidants. In this study, free radical-scavenging abilities on DPPH and $\cdot\text{OH}$ were adopted to evaluate the *in vitro* antioxidant activities of EPS-1 and compare them with those of the positive control, Vc. As shown in Figure 6(A)–6(B), EPS exhibited strong DPPH and $\cdot\text{OH}$ radical-scavenging abilities in dose-dependent manners at 0–3.0 mg/mL. At 3.0 mg/mL, the DPPH and $\cdot\text{OH}$ radical-scavenging abilities of EPS-1 were 70.78% and 64.55%, respectively, which were weaker than those of Vc (90.59% and 72.58%, respectively, 0.5 mg/mL). Its noticeable antioxidant activities might be related to carbohydrate content, monosaccharide composition, MW, chemical structure and chain conformation. In particular, the MW of EPS-1 plays an important role in its antioxidant activity, and a relatively low MW appears to increase the radical-scavenging ability. For example, Xing et al. (2005) have confirmed that these free radical-scavenging capacities for low MW polysaccharides were more pronounced than those for high MW polysaccharides, because low MW polysaccharides have many reductive $-\text{OH}$ terminals that

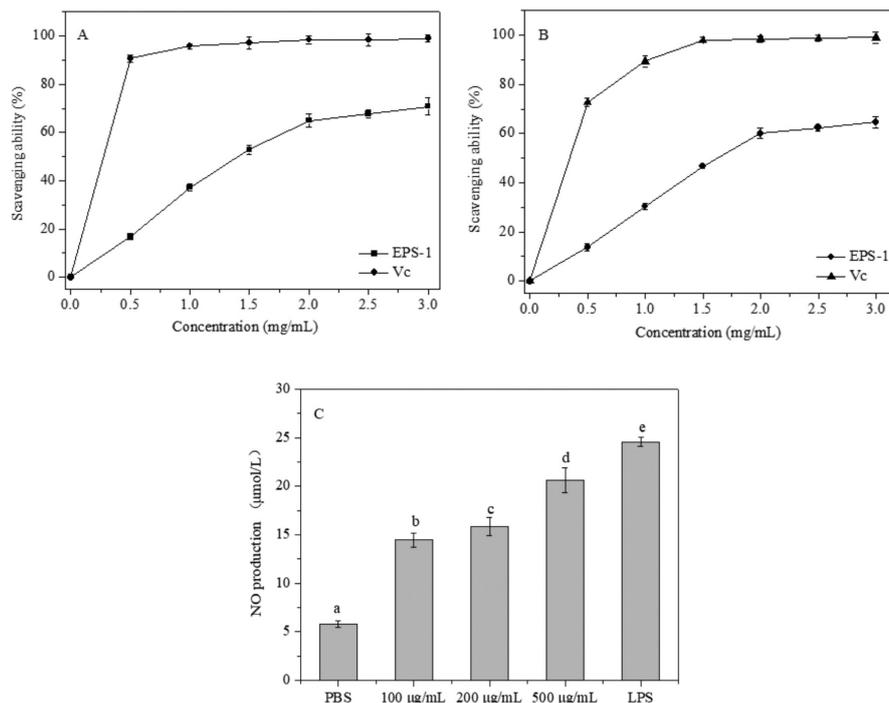


FIGURE 6. Antioxidant activities *in vitro* of EPS-1 in terms of its scavenging abilities on (A) scavenging abilities on DPPH and (B) ·OH. (C) Effect of EPS-1 on NO production in RAW 264.7 cells. RAW 264.7 cells were treated with various concentrations of EPS-1, or LPS (1 µg/mL) for 48 h. Each value is presented as mean \pm SD (n=3). Bars with different letters are statistically different ($P < 0.05$)

can react with radical series. In summary, these results indicated that EPS-1 with high carbohydrate content (96.1%) and low MW (22.07 kDa) exhibits potential antioxidant capacities.

EFFECT OF EPS-1 ON NO PRODUCTION IN MACROPHAGE RAW 264.7 CELLS

In immunopharmacology, NO as key molecule exhibits beneficial biological effects on various normal cells that are mostly connected with immunomodulatory or inflammatory or physiological processes (Nathan 1992). As an important cytotoxic mediator, this molecule is conducive to the antimicrobial and tumoricidal activities of the macrophages and plays a vital role in the pathogenesis of several infectious and inflammatory diseases (Bogdan 2001; Nathan 1992). Figure 6 presents the effect of EPS-1 on NO production in macrophage RAW 264.7 cells. The NO production of RAW 264.7 cells was increased after the incubation of different concentrations of EPS-1 in a dose-dependent fashion. In comparison, the NO generation at the measured concentration range was significantly higher than that of the negative control ($P < 0.05$). This finding indicated that EPS-1 can stimulate the release of NO by macrophage RAW 264.7 cells, thus showing its underlying immune function. Similar results were confirmed by Hu et al. (2013), who found that the levels of NO produced by macrophages were increased by high polysaccharide concentration. At 500 µg/mL, the NO secretion of EPS-1 was 24.56 µmol/L, which was nearly

close to that of the LPS group (24.56 µmol/L, 1 µg/mL). The prominent NO stimulating activity of EPS-1 might be due to its high carbohydrate content and low MW.

CONCLUSION

In this study, we investigated the production, physicochemical characterization and biological activities of EPS from the submerged culture of *A. biennis*. Lactose (30 g/L) and tryptone (3 g/L) were identified as the best carbon and nitrogen sources, respectively, for EPS production in *A. biennis* submerged fermentation. The maximum EPS production achieved was 18.29 g/L at day 6 in a 5 L stirred-tank reactor under the most suitable culture conditions. After purification using gel-filtration chromatography, EPS-1 with 85.4% yield and 96.1% carbohydrate content was obtained from the crude EPS, which was harvested from the culture broths of *A. biennis*. EPS-1 is a neutral heteropolysaccharide mainly composed of glucose, mannose and galactose at a molar ratio of 3.3:2.0:1.0 and has a MW of 22.07 kDa. EPS-1 with a relatively high thermal stability existed as a random coil conformation in the aqueous medium, and exhibited noticeable radical-scavenging ability and NO stimulating activity in macrophage RAW 264.7 cells. Further investigations on its bioactivity *in vivo* and detailed structural characterization are currently underway in our laboratory.

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