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Extracellular polysaccharides of endophytic fungus *Alternaria tenuissima* F1 from *Angelica sinensis*: Production conditions, purification, and antioxidant properties

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ABSTRACT

The yield, composition, structure and biofunction of microorganism exopolysaccharide (EPS) depended on their genetics and metabolic pathways. An EPS-producing endophytic fungus was isolated from *Angelica sinensis* planted in Dingxi, Gansu, China, and identified as *Alternaria tenuissima* F1. The production optimization, purification and characterization of the purified EPS from *A. tenuissima* F1 and the in-vitro antioxidant potentials was investigated. Results showed the optimal medium composition were 8% mannose, 2% yeast extract, 0.04% $MgSO_4 \cdot 7H_2O$, 0.005% Vc, and the optimal cultivation conditions were set at initial pH 7.0, culture temperature 30 °C, inoculum size of 3%, 180 r/min for 5 d. The EPS purified by ion exchange column chromatography and gel chromatography was a non-reducing sugar and glycoprotein with pyranoid ring by Fourier transform infrared spectroscopy (FT-IR) analysis, displayed a porous network structure by scanning electron microscope (SEM) observation and exhibited a high thermal stability with the degradation temperature of 303.9 °C by thermogravimetric analysis (TG/DSC). Molecular weight of the purified EPS was 3.246×10^4 g/mol, and EPS was composed of D-galacturonic acid, rhamnose, D-mannose, glucose, and D-galactose in ratio of 0.45:3.02:3.25:1.0:0.95. The EPS exhibited strong scavenging activity and may be a new source of natural antioxidant.

1. Introduction

Medicinal chemistry research showed that *Angelica sinensis* mainly contains volatile oil, carbohydrates, amino acids, organic acids and other chemical components [1,2,3]. Especially, polysaccharides of *A. sinensis* also have been confirmed to have functions of enriching blood, regulating immunity and promoting tumor vaccine's anti-tumor function [4–7]. Previous studies demonstrated endophytic microorganism living in medicinal plants exert many similar to/more than those of the host biological activities [8,9,10] and are the optimal producers of structurally novel and biologically active secondary metabolites, such as alkaloids, terpenoids, steroids, exopolysaccharide and other derivatives [11–17], and we have isolated more active compounds from different plant endophyte [18,19]. In recent years, fungi extracellular polysaccharides (EPS) were proved that not only play important roles in plant-endophyte interactions [8,9], but also exhibit several biological functions, such as antioxidant [21,22,23], antitumor [23,24,25], anti-inflammatory and anti-allergic activity [26], hypoglycemic activity [27], anticoagulant activity [28] and heavy metal ion removal [15]. Therefore, more and more endophytic fungal have been obtained and can be considered as important resources for structurally novel and biologically active EPS with potential applications in fields of nutraceuticals [21,22,23], pharmaceutical industries [23,24,25] and treatment and rehabilitation of environment [15].

Fungi extracellular polysaccharides (EPS) are kinds of biosynthetic polymers mainly composting of carbohydrates synthetised by intracellular or extracellular pathway during the process of growth and metabolism [15,20] and get attached to cell surfaces which help them to grow as colonies termed biofilms and released to extracellular environment [15]. Most endophytic fungi belong to the Ascomycota phylum and Fungi imperfecti [13]. Many researchers have described endophytic fungi can produce a variety of structurally different EPS, which depended on the genetics and metabolic pathways of the strains with

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different medium compositions and fermentation conditions [29–32]. In a previous research, the production of microbial polysaccharides is not only related to the intrinsic factors such as microbial genes, but also to the external factors such as the composition of the medium (nitrogen source, carbon source, trace elements, etc.) and culture conditions (liquid volume, temperature, pH, etc.) [31,32,33]. And a single factor design, orthogonal experiment, and response surface methodology have been used to optimized the EPS production [33–37]. Mahapatra and Banerjee [26] found the organism produced maximum exopolysaccharide from endophytic *Fusarium solani* SD5 after 13.68 days of incubation at 28 °C in potato dextrose broth supplemented with 9.8 g/L glucose, 0.69 g/L yeast extract, 0.05 g/L KCl, 0.05 g/L KH_2PO_4 with medium pH of 6.46. Wu et al. [34] found the optimized conditions of produced melanin-free exopolysaccharide (EPS) by *Aureobasidium pullulans* CJ001 were fermentation time 4 d, initial pH 6.0, and temperature 22 °C. Therefore, it can be seen that the strains producing extracellular polysaccharides are different, and their fermentation conditions are also different with the strains. Until now, RSM has been successfully applied in the optimization of medium compositions for EPS production [33–37].

As evident from the reported above, the culture conditions are very crucial to the yield, the composition, structure and biofunction of EPS for EPS-producing strains, and there are few reports on the EPS production and activities of endophytic fungi from *A. sinensis*. In our previous work, we investigated the EPS production of all the endophytic fungi isolated from *A. sinensis* according to the method described by Kanchan [38], and we found that EPS from the endophytic fungus *A. tenuissima* F1 has promising antioxidant activity and its yield is higher than other strains (Data not shown). Therefore, *A. tenuissima* F1 was chosen for further investigation to optimize the EPS production conditions including the culture medium (carbon, nitrogen source, metal ions and growth factors) and conditions (pH, temperature, time and inoculum size), and structures characteristics and antioxidant properties of EPS were evaluated by multiple spectroscopy techniques.

2. Materials and methods

2.1. Isolation and identification of microorganism

Healthy and mature *A. sinensis* was collected from a planting base in Minxian County, Dingxi City, Gansu Province, China. The roots of *A. sinensis* were surface sterilized by consecutive immersion for 90 s in 75% ethanol, 10 min in 2.0% sodium hypochlorite, rinsed six times in sterile distilled water and then dried with sterile paper towels. The sterilized roots were cut to approximate 5 mm in length and plated on potato dextrose agar (PDA) medium with streptomycin sulfate (50 mg/L) and ampicillin (100 μg/mL) to incubate at 28 °C in the dark and monitored regularly. Meanwhile, 100 μL of the final rinsing water also were plated onto PDA medium and incubated under the same conditions for testing the effectiveness of the surface sterilization. When microorganism colonies developed, the mycelium of different fungi were subcultured onto a new PDA plate respectively until the pure cultures were obtained. All pure culture fungi stored on PDA plates were then examined visually for macroscopic (morphology, size, coloration of the mycelium and agar medium) and microscopic (presence of spores or other reproductive structures) characteristics by light microscopes observation (SK200, Motic China Group Co., Ltd., Xiamen, China). Further identification of endophytic isolates representative of each morphotype was based on the nucleotide sequences analysis of the internal transcribed spacer region (ITS). In detail, DNA were extracted according to a modified CTAB extraction protocol [39], and the ITS sequences were amplified by polymerase chain reaction (PCR) using primers ITS1 and ITS4. The PCR conditions were set at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The amplified products were examined by 1% agarose gel electrophoresis with ethidium bromide staining and sequenced. The ITS sequences were compared with others sequences by Basic Local Alignment Search Tool search program (http://www.ncbi.nlm.gov/BLAST/) of the National Center for Biotechnology Information (NCBI) and multiple sequences were aligned in Clustal X 2.1. A phylogenetic tree was constructed with MEGA 6.06 by the neighbor-joining algorithm with 1000 bootstrap replicates.

2.2. Preparation and quantification of exopolysaccharides (EPS)

The endophytes *A. tenuissima* F1 in this study was inoculated into the yeast extract peptone dextrose medium (YEPD, 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, and natural pH) and enrichment cultured for 72 h, then inoculated with 2% inoculum into 250 mL flask containing 50 mL of basal sugar production medium (5% (w/v) glucose, 0.2% (w/v) ammonium sulfate, 0.1% (w/v) potassium dihydrogen phosphate, 0.1% (w/v) yeast extract, and pH 6.0). The culture was shaken at 28 °C with 180 r/min for 6 days. The fermented liquid was centrifuged for 10 min at 5000 r/min, and the supernatant was collected and concentrated up to a third of the original volume at 45 °C. The chilled ethanol was added into the concentrate with the final concentration of 80% and left overnight at 4 °C, the precipitates were collected and then dried by vacuum freeze dryer (FD-1A-50, Boyikang Instruments Co., Ltd., Beijing, China). The dried precipitates named EPS was obtained, the content of polysaccharide was measured by phenol-sulfuric acid method [40], and calculated according to the standard curve ($y = 7.21955x + 0.01513$, $R^2 = 0.99301$).

2.3. Optimization for the production of EPS

2.3.1. Medium optimization for EPS production by orthogonality experiment

In this study, the medium compositions optimization of EPS production firstly entailed identifying the preferred nutrients, including carbon sources (fructose, xylose, sucrose, lactose and glucose), nitrogen sources ($NaNO_3$, NH_4NO_3, yeast extract, $(NH_4)_2SO_4$, peptone and NH_4Cl), metal ions (magnesium sulfate, sodium chloride, calcium chloride, ferrous sulfate, manganese sulfate and copper sulfate), and growth factor (Vc, V_{B1} and glycine), and then varying one factor at a time while keeping the others constant. Based on the collected results, the effect of the selected nutrients (and their concentrations) on EPS yield was also decided by single-factor experiments, and a four-factor-three-level orthogonality experiment was designed for the medium optimization with the EPS yield as an evaluation index. Meanwhile, the EPS production of the strain under the optimization medium was carried out to verify the agreement between the model predictive value and the experimental actual value.

2.3.2. Cultivation conditions optimization for EPS production by Box-Behnken experimental design

The culture conditions for EPS production were screened. In details, cultivation duration (3, 4, 5, 6, 7, 8 d), initial pH (4.0, 5.0, 6.0, 7.0, 8.0), inoculum size (1%, 2%, 4%, 6%, 8%, 10%) and culture temperature (28, 30, 32, 34 °C) were respectively investigated by single-factor tests. All experiments were performed in the optimum liquid medium obtained from the former step. According to the results of single-factor tests, Box-Behnken experimental design with four factors and three levels was used to optimize the fermentation conditions for EPS production. The DesignExpert Software (Version 8.0.5, Stat Ease Inc., Minneapolis, USA) was used to perform quadratic multiple regression fitting on the experimental data to establish the quadratic response surface regression model and find out the optimal impact factor level, and the verification experiment under the optimum fermentation conditions also was performed to evaluate the reliability of the model.

2.4. Isolation and purification of EPS

The EPS of *A. tenuissima* F1 were obtained by inoculating the strain into the optimized medium and culturing under optimal conditions, and isolated and were purified by the method of Wang et al. [41]. Elution curve was plotted based on phenol-sulfuric acid assay, and the homogeneous fractions were gathered, concentrated, and lyophilized for further studying. The purity of the final purified EPS sample was checked. The polysaccharide content of the sample was determined by the phenol-sulfuric acid method with glucose as standard, and others physicochemical properties of purified EPS was determined by iodination reaction, Fehling's test and ninhydrin reaction [41].

2.5. Determination of molecular weight and monosaccharide composition

The molecular mass of the purified EPS was analyzed by high-performance size-exclusion chromatography using a TSK-GEL G60000PWXL column (HPSEC) with detection by multiple-angle laser light-scattering (MALLS, $\lambda = 690$ nm; DAWN EOS, Wyatt Technology Co., USA) and refractive index (RI, Optilab Wyatt, USA) at an internal temperature of 30 °C. The column was eluted with 0.2% NaN_3 solution at a flow rate of 1.0 mL/min, and the injection volume of sample filtered through 0.2 μm filter was 20 μL, and dn/dc of 0.135 at 690 nm and 25 °C as a refractive index increment was used for polysaccharides solution. The data was collected and further analyzed with Wyatt Astra software (Version 5.3.4.14, Wyatt Technology, USA).

5 mg of the purified EPS was hydrolyzed with 2 mL trifluoroacetic acid (TFA, 4 mol/L) at 120 °C for 10 h. TFA was then removed by evaporation under N_2 gas, 10 mg of hydroxylamine hydrochloride and 1.0 mL of pyridine was added into the hydrolysate and reacted at 90 °C for 30 min. After cooling down to room temperature, 1 mL of acetic anhydride was used for acetylation at 90 °C for 30 min and concentrated by the pressured N_2 gas blowing concentrators. The whole reaction procession was carried out under the protection of N_2 gas. Then, samples were dissolved with 1.0 mL of chloroform, and transferred to a eppendorf tube. A small amount of distilled water was added in. After thoroughly shaking, the aqueous phase at the upper layer was removed. This extraction procedure was repeated three times. The chloroform layer was dried with an appropriate amount of anhydrous sodium sulfate, pre-filtrated with 0.22 μm millipore filtration, and then analyzed on a GC ITQ 1100 gas chromatography - mass spectrometry platform (Thermo, USA) fitted with an HP-5 column (0.32 mm × 0.25 μm × 30 m) and a flame ionization detector (FID). The GC–MS operation was performed under the following conditions: initial column temperature was kept at 160 °C, then programmed at a rate of 10 °C/min to reach at 240 °C and the temperature of inlet was hold at 220 °C, FID detector temperatures was set at 250 °C with N_2 as carrier gas (1.0 mL/min). The distribution ratio of flow was 1:10. Sugar identification was confirmed by comparison with standard rhamnose, arabinose, galactose, glucose, mannose, and fructose.

2.6. FT-IR analysis

The mixture of the purified EPS and dry KBr were prepared and the fingerprint regions of the purified EPS were recognized by a Fourier transform infrared spectrophotometer (Thermo Scientific Nicolet iN10) in the range of 500–4000 cm^{-1} in transmission mode with an infrared spectral resolution of 4 cm^{-1}. The number of scans was no <10 for all spectra [41].

2.7. Scanning electron microscope observation

The purified EPS was coated with a layer of gold with a carbon coater at 10 Pa vacuum and ion current of 15 mA, and the surface morphology of EPS was observed by scanning electron microscope (JSM-6701F, Japan Electronic Optics Co, Japan) [41].

2.8. Thermogram analysis (TGA) and differential scanning calorimeter (DSC)

3 mg of EPS sample placed in crucible made of Al_2O_3 was heated from room temperature to 500 °C at the heating rate of 10 °C/min under the protection of nitrogen and the pyrolysis and combustion were performed in TGA/DSC simultaneous thermal analyzer (STA449C, Netzsch, Germany). Meanwhile, the melting point and enthalpy changes were determined [42].

2.9. Antioxidant activity of EPS

The different concentration of the purified EPS (0.1, 0.25, 0.5, 0.75, 1.25, 2.5 and 5 mg/L) were prepared, and the antioxidant activity were determined [41], including the hydroxyl radical scavenging activity, the superoxide anion scavenging activity and the reducing power.

2.10. Statistical analysis

All data obtained were based on the average of three measurements. The difference was considered to be significant when $P < 0.05$. The Origin Pro Software Package 8.0 (Origin Lab. Corp.) and DesignExpert Software Version 8.0.5 (Stat-Ease Inc.) were employed for the statistical analysis.

3. Results and discussion

3.1. Identification of the tested strains and phylogenetic analysis

An endophytic fungi marked F1 was isolated from the root of healthy *A. sinensis* plants, and the morphotypes was recognizable on the basis of their morphological characteristics (colonies, hypha and conidia). The early colonies of strain F1 illustrated white and slimy, and then gradually transformed into olive-green colonies like wrinkles with various shades of yellow, brown, or green in the centre of the colony (Fig. 1A), and the microscopic morphology elucidated strain F1 has yeast-like cells and mycelia morphologies, both branched and unbranched chains was of 5–10 conidia with very short apical secondary conidiophores. Branching was often seen via formation of secondary conidiophores from the conidium body (Fig. 1B). All these phenotypic characteristics of the isolate are identical to those of *Alternaria tenuissima* [32,43]. The ITS sequence (542 bp) of strain F1 was obtained and compared with the BLAST in GenBank (NCBI database), the closest matches revealed at least 99% similarity to known reported sequences of *Alternaria tenuissima*, which strongly indicated that F1 belongs to *A. tenuissima* family. More sequences of different strains with high similarity (≥99%) downloaded from the GenBank database were used to multiple alignment and phylogenetic trees construction as shown in Fig. 1C, which revealed the strain F1 and other strains were clustered into one class, further proved the strain F1 belonged to *A. tenuissima*. The sequence was submitted to the GenBank (Accession number MH035972.1), and the strain was named *A. tenuissima* F1.

3.2. Medium optimization for EPS production

3.2.1. One-factor-at-a-time optimization of medium for EPS production

The microorganism, types and contents of nutrients play very significant roles in determining EPS production [15,24,29,30]. There is no general cultivation medium suitable for all microbial producers that will guaranty high EPS productivities [29,30]. In submerged cultures of fungi, different nutrients such as carbon sources, nitrogen sources and other factors have been reported responsible for maximum EPS formation [31]. In this study, the effect of different carbon sources including glucose, xylose, mannose and galactose on the production of EPS from *A. tenuissima* F1 were evaluated, and mannose was found to be the most efficient carbon source for EPS production, followed by xylose,

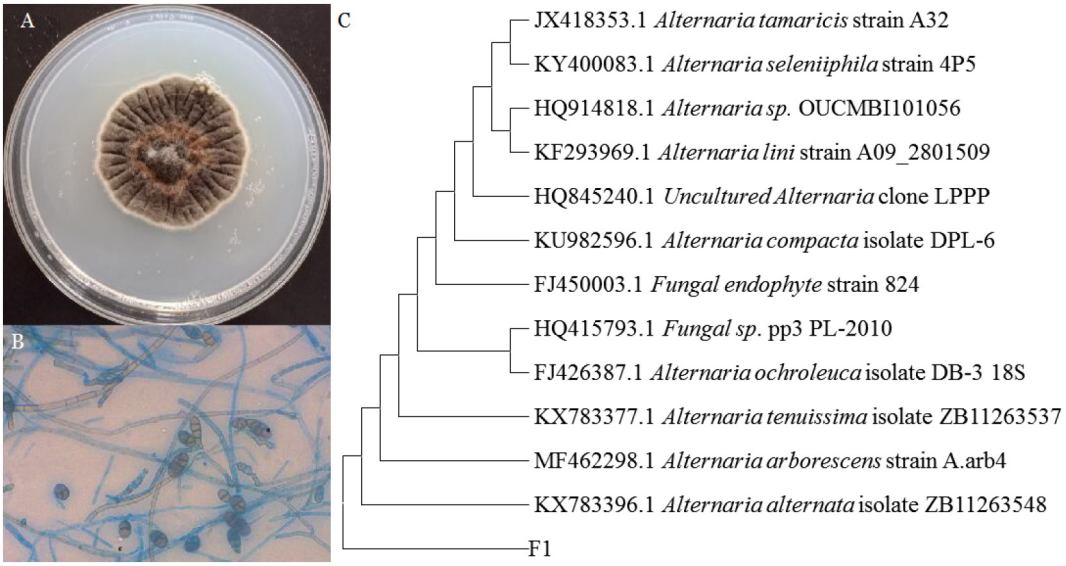

Fig. 1. The morphologies and phylogenetic tree of *A. tenuissima* F1 (A. colonies; B. microscopic morphology, ×40; C. phylogenetic tree constructed based on neighbor-joining method).

glucose and galactose, successively (Fig. 2A). The EPS production displayed a statistically significant ($P < 0.05$) difference for the strain with different carbon at 28 °C, 180 r/min for 6 days, and mannose concentration had a marked effect on EPS yield ($P < 0.05$) as rising the concentration, and the maximum EPS production were obtained at the concentration of 8.0%. However, EPS yield was decreased, when the concentration of mannose was higher than 8.0% (Fig. 2B). In order to screen out the most effective nitrogen source for the EPS production, different nitrogen sources (peptone, carbamide, ammonium sulfate, yeast extract, 0.2% ammonium sulfate with 0.1% yeast extract, 0.1% ammonium sulfate with 0.2% yeast extract, and 0.1% ammonium sulfate with 0.1% yeast extract) were tested in the submerged culture at 28 °C with 180 r/min for 6 days. The results presented in Fig. 2C, indicated that the highest EPS production (1.6 mg/mL) was observed in yeast extract group. When the concentration of yeast extract was increased from 0.2% to 2.0%, the EPS yield was significantly increased ($P < 0.05$) and reached up to 2.1113 mg/mL (Fig. 2D). The effects of six kinds of metal ions on EPS production also were considered, respectively (Fig. 2E), when the metal ions (Fe^{2+} and Na^{+}) was added into the basal medium, the production of EPS reduced significantly ($P < 0.05$), Ca^{2+} and Cu^{2+} had no significant effect on the yield of EPS ($P > 0.05$). However, Mg^{2+} significantly increased the yield of EPS ($P < 0.05$) and the yield of EPS could reach up to 2.1878 mg/mL at the concentration of 0.03% (Fig. 2F), which can be explained that Mg^{2+} could significantly promote the growth of *A. tenuissima* F1 or the enzyme activity involved in the EPS biosynthesis [15]. The results of the various growth factors (V_C, V_{B1} and glycine) affecting the EPS content were described in Fig. 2G, and V_C was determined as the optimal growth factor, which could increase the production of EPS ($P < 0.05$) significantly, and the highest production of EPS (2.7044 mg/mL) achieved at the concentration of 0.01% exhibited in Fig. 2H. The possible reason of this phenomenon can be explained by the contribution to the different metabolic pathways employed by the microorganism to metabolize different nurtures including the diverse carbon sources or nitrogen sources (and other compositions) in the cultivation media [29,30]. Extracellular polysaccharide synthesis by microorganisms is accomplished by a specific secreted enzyme (polymerization and precursor synthesis enzymes), which occurs either outside the cell or within the cell wall [29]. Cations are known as the subsidiary groups or activators/inhibitors of enzymes to affect EPS synthesis, especially magnesium and V_C are the cofactors in hundreds of enzymatic reactions in microorganisms. Similar studies conclusion also have been reported by many researchers, previous workers have studied the kinds of EPS by the utilization of different carbon sources in 13 species of *Aspergillus* [44], and showed the different preference of nutrients in the production procession of EPS. Goatley reported [45] the biomass of *Alternaria solani* on fructose was maximum at NH_4NO_3 concentrations of 1.5 to 2.5 g/L, but EPS production was maximum at 0.8 g/L. However, the growth and polysaccharide production of this strain were greatest with fructose as the carbon source, and showed the decreasing levels of growth occurred with mannitol, glucose, and sucrose, respectively. Especially, EPS were not detected with mannitol as carbon source, which further implied the EPS production was not correlation to the biomass yield of strain [38].

3.2.2. Medium optimization for EPS production by orthogonal matrix method

Based on the abovementioned results of the single factor test, the L9 (3^4) orthogonal experiment was designed to further optimize the nutrient concentration of the medium. Table 1 illustrated the factor allocations of the orthogonal matrix, respectively. In the optimization experiments, the culture temperature, initial pH, rotation rate, and culture duration were fixed at 28 °C, 7.0, 180 rpm, and 6 d, respectively. The effects of different test combinations on EPS production were calculated and the results were shown in Table 1. To obtain the optimum levels or composition of each factor, the analysis of variance was conducted (Table 2) based on the data in Table 1, and the optimization results were as follow: (1) the mannose and yeast extract had the significant effect on EPS production ($P < 0.05$), following with magnesium and V_C, and the mannose had the most significant effect on EPS production; (2) the optimal medium combination ($A_2B_2C_3D_1$) for EPS production was 8% mannose, 2% yeast extract, 0.04% magnesium and 0.005% V_C. Our results were in an agreement with many others reports, which have stated that medium composition such as carbon source or nitrogen sources are important parameters in EPS biosynthesis and production [30,31].

To verify the credibility of experiment results, *A. tenuissima* F1 was cultured in the optimized medium for EPS production at 28 °C, 180 rpm for 6 d in shake flask. EPS production reached at 2.6159 mg/mL, 2.6184 mg/mL and 2.4628 mg/mL in the optimal medium, respectively, which was in good agreement with the predicted value and indicated that the best medium formula is reliable and practical.

3.3. Cultivation conditions optimization for EPS production

3.3.1. Single-factor experiment

Microorganisms growth and metabolism are obvious significance by culture conditions except the nurtures, such as pH, temperature, initial

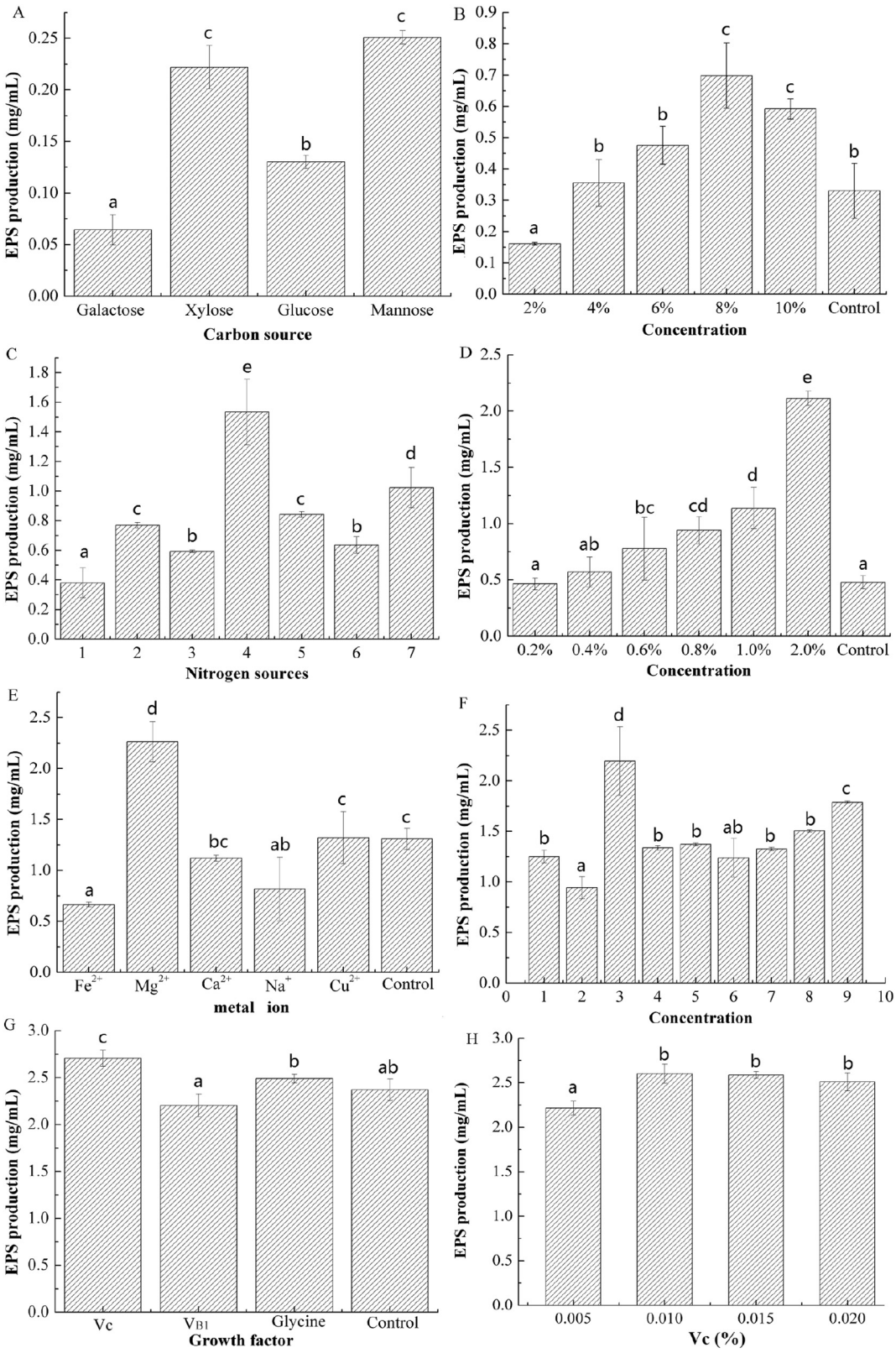

Fig. 2. Medium optimization for EPS production (A. The effects of different carbon sources on EPS production; B. The effects of concentration of mannose on EPS production; C. The effects of different nitrogen sources on EPS production (1. peptone; 2. carbamide; 3. ammonium sulfate; 4. yeast extract; 5. 0.2% ammonium sulfate with 0.1% yeast extract; 6. 0.1% ammonium sulfate with 0.2% yeast extract; 7. 0.1% ammonium sulfate with 0.1% yeast extract); D. The effects of concentration of yeast extract on EPS production; E. The effects of different metal ions on EPS production; F. The effects of concentration of magnesium on EPS production (1, 2, 3, 4, 5, 6, 7, 8 and 9 represented 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.1%, 0.2%, 0.4% and 0.6%, correspondingly); G. The effects of different growth factors on EPS production; H. The effects of concentration of Vc on EPS production) Note: values are the means of three replication ± SD. The same letter means no significant difference among the different groups at the 5% level.

Table 1
L9 (3^4) orthogonal design and results of EPS production in shake flask culture.

Run	A	B	C	D	EPS production (mg/mL)
1	1 (6)	1 (1)	1 (0.02)	1 (0.005)	0.8242
2	1 (6)	2 (2)	2 (0.03)	2 (0.01)	1.2485
3	1 (6)	3 (3)	3 (0.04)	3 (0.015)	0.9215
4	2 (8)	1 (1)	2 (0.03)	3 (0.015)	1.8682
5	2 (8)	2 (2)	3 (0.04)	1 (0.005)	2.6271
6	2 (8)	3 (3)	1 (0.02)	2 (0.01)	2.1213
7	3 (10)	1 (1)	3 (0.04)	2 (0.01)	1.4628
8	3 (10)	2 (2)	1 (0.02)	3 (0.015)	1.8231
9	3 (10)	3 (3)	2 (0.03)	1 (0.005)	1.2642

Note: the arrangement of columns A, B, C, and D is decided by orthogonal design for 4 (factor) × 9 (run number). Every row of run number represents one experimental replicate and every run was performed in triplicate. Values are the mean of three determinations. A, B, C, and D represents the concentration of mannose (%), yeast extract (%), magnesium (%) and V_C (%), respectively.

inoculum size, culture duration and other factors, which could affect enzyme activity (inhibition or stimulation), carbohydrate synthesis (induction and repression), etc. [31,33,34]. In this work, *A. tenuissima* F1 was cultured in the optimized medium for the cultivation conditions optimization. With the prolongation of culture duration, the EPS production significantly increased until to the 6th day, and then the yields of EPS decreased gradually after 6 days (Fig. S1A). The results maybe due to some strains could use the EPS as carbon source during carbon deficiency, which induced extracellular enzymes or enzyme complex to complete degradation of EPS at the late stage of fermentation [46]. EPS production of *A. tenuissima* F1 cultured with initial pH values ranging from 2 to 7 was investigated (Fig. S1B). Maximum EPS production (2.9099 mg/L) was obtained at an initial pH 7.0, indicating that the biosynthesis of EPS could maybe up-regulated in a neutral environment, the initial pH is an important factor that may affect cell membrane, cell morphology and structure, the uptake of various nutrients as well as EPS biosynthesis [33]. Inoculum size is also an important parameter in fermentation to obtain secondary metabolites. In this investigation, 2% (v/v) inoculum was the best inoculum size to support the maximal production of EPS (Fig. S1C). On the other hand, it was noticed that EPS production started to decrease when increased the inoculums' proportion, which may be elaborated the nutrients were used to the growth rather than the synthesis of EPS. Similar results have been reported that the yield of EPS did not show the linear correlation with the growth of microorganism [38]. The effect of temperature on EPS production was displayed in Fig. S1D. With the increase of culture temperature from 28 to 32 °C, the growth metabolism of the strain was accelerated and the synthesis rate of EPS was improved, and EPS production increased significantly. While the yield of EPS decreased significantly at 34 °C, indicating that the activity of EPS synthetase reduced and resulted in the anabolism of EPS decreasing gradually. According to the abovementioned results, the initial pH, inoculum size, culture temperature and culture time were obtained as 7.0, 2.0%, 32 °C and 6 d, respectively.

3.3.2. Establishment of regression model and analysis of variance (ANOVA)

The single factor method is tedious and ignores the interactions among different variables involved. On the basis of single-factor experiments results, an response surface methodology was carried out to further optimize the fermentation conditions. A total of twenty-one experimental runs were set by the software and the values of EPS production in different levels combinations of the variables also were shown in Table 3. Based on multivariate regression analysis of experimental data, the relationship between the response variables and the experimental variables were expressed by the following second-order polynomial equation:

$$Y = -98.99222 + 5.58266A + 1.85237B + 4.57264C - 3.68596D - 2.6795 \times 10^{-3}AB - 8.4352 \times 10^{-2}AC + 5.0795 \times 10^{-2}AD + 8.0619 \times 10^{-2}BC + 0.29228BD + 0.10944CD - 8.3 \times 10^{-2}A^2 - 0.29294B^2 - 0.18817C^2 - 2.0486 \times 10^{-2}D^2$$

where Y denoted EPS production, A, B, C and D denoted temperature, culture duration, pH and inoculum proportion, respectively.

The statistical significance of the model was checked by F-test, and the ANOVA for response surface quadratic model were summarized in Table 4. The value of the determination coefficient ($R^2 = 0.9958$) from the ANOVA of the quadratic regression model indicated that 99.58% of the variability could be explained by this model between the observed and predicted value. In addition, the P-value for the lack of fit ($P = 0.0888$) implied that the pure error was insignificant and the model equation was suitable for predicting EPS production under any combination of variables. The relatively low coefficient of variation (cv, 1.69%) further showed good accuracy and reliability of the experiment performed. The parameter optimization estimated by the coefficient suggested that the independent variables (A), cross coefficient (AC, AD, BC, BD, CD), and quadratic terms (A^2, B^2 and C^2) significantly affected the EPS production ($P < 0.01$, $F \approx 101.0673$). The results in Table 4 exhibited that the temperature was the most significant factor which influenced on EPS production, and followed by pH, inoculum size and culture time.

Table 2
Analysis of L9 (3^4) orthogonal test on EPS production in shake flask culture.

Factor	SS	Df	F ratio	F critical value	Significance
A	2.201	2	275.125	19.000	*
B	0.482	2	60.250	19.000	*
C	0.067	2	8.375	19.000	
D	0.008	2	1.000	19.000	

SS: the sum of deviation squares; Df: degree of freedom.
* Significant ($0.01 < P < 0.05$), significance test at $\alpha = 0.05$ level.

3.3.3. Interaction analysis of regression model

The interaction among four factors (temperature, culture time, pH and inoculum size) were displayed as the three-dimensional response surface (Fig. 3) and contour plots (Fig. S2), which illustrated the interaction of the different paired factors and determined the optimum of each paired factor for maximum EPS production. The data in Fig. 3 indicated that the EPS yield increased to a maximum with the value of factors increasing, and the combined effect of the two different factors on the EPS yield also showed the yield increased up to a certain value and then decreases, indicating that the model was stable and the stationary point was the maximum. Meanwhile, the interaction exhibited the significant effects on the output of EPS. As previously noted, The shape of the contour plots can further prove whether the interactions among corresponding variables are significant [41]. When the contour shape is circular contour curve, indicating the interactions between the corresponding variables can be neglected, while elliptic or saddle shape of contour curve denotes that the interactions between the corresponding variables is remarkable. According to the rules, these interactions shown in Fig. S2B–F. between temperature and pH (temperature and inoculum size; culture time and pH; culture time and inoculum size; pH and inoculum size) significantly effected on the EPS yield. However, the interactions between temperature and culture time had no prominent influence on EPS production (Fig. S2A). By analyzing the response surface contour plots, the maximum predicted EPS production (3.34 mg/mL) was obtained at the optimal levels of variables appeared at 30.93 °C, 5.28 d, pH 6.95 with inoculum size of 2.87%.

When the error is taken into account in the actual operation, the optimal fermentation condition should be modified appropriately as cultivation for 5 d at 30 °C, pH 7 with inoculum size of 3%. Under the conditions, three additional experiments in shake flasks were performed and EPS production could reach at 3.25, 3.28 and 3.42 mg/mL, respectively, which was in good agreement with the predicted value

Table 3
Design and results of response surface methodology (RSM).

No.	A (Temperature, °C)	B (Culture duration, d)	C (pH)	D (Inoculum size, %)	Y (EPS production, mg/mL)
1	+1 (32)	+1 (6)	+1 (8)	−1 (1)	2.0713
2	+1 (32)	+1 (6)	−1 (6)	−1 (1)	2.4280
3	+1 (32)	−1 (4)	+1 (8)	+1 (3)	2.3267
4	−1 (28)	+1 (6)	−1 (6)	+1 (3)	2.0323
5	+1 (32)	−1 (4)	−1 (6)	+1 (3)	2.5682
6	−1 (28)	−1 (4)	+1 (8)	−1 (1)	2.6505
7	−1 (28)	+1 (6)	+1 (8)	+1 (3)	2.7881
8	−1 (28)	−1 (4)	−1 (6)	−1 (1)	2.6549
9	−1 (28)	0 (5)	0 (7)	0 (2)	2.7172
10	+1 (32)	0 (5)	0 (7)	0 (2)	3.1188
11	0 (30)	−1 (4)	0 (7)	0 (2)	2.9656
12	0 (30)	+1 (6)	0 (7)	0 (2)	2.9486
13	0 (30)	0 (5)	−1 (6)	0 (2)	2.9904
14	0 (30)	0 (5)	+1 (8)	0 (2)	3.1332
15	0 (30)	0 (5)	0 (7)	−1 (1)	3.2461
16	0 (30)	0 (5)	0 (7)	+1 (3)	3.2130
17	0 (30)	0 (5)	0 (7)	0 (2)	3.2717
18	0 (30)	0 (5)	0 (7)	0 (2)	3.3207
19	0 (30)	0 (5)	0 (7)	0 (2)	3.2447
20	0 (30)	0 (5)	0 (7)	0 (2)	3.3038
21	0 (30)	0 (5)	0 (7)	0 (2)	3.3167

(3.34 mg/mL) and further indicated that the fermentation process parameters and model were accurate, reliable and practical.

3.4. Characterization of the purified EPS

The EPS solution was prepared and purified by a DEAE-52 cellulose column, the main fraction was collected, concentrated and further purified by Sephadex G-100 column. Subsequently, the content of polysaccharide of purified EPS was quantified with the recovery rates of 51.3% by phenol-sulfuric acid method. Physicochemical properties tests indicated that the purified EPS was non-reducing sugar combined with amino acid or protein, yellowish powder, soluble in water at room temperature instead of insoluble in ethanol and didn't contain starch.

Table 4
Analysis of variance for fitted quadratic polynomial model.

Source	SS	DF	MS	F-value	P-value	Significance
Model	3.344334	14	0.238881	101.0673	< 0.0001	**
Residual	0.014181	6	0.002364			
Lack of fit	0.009955	2	0.004978	4.710858	0.0888	
Pure error	0.004226	4	0.001057			
Cor. total	3.358515	20				
A	0.080657345	1	0.080657345	34.1250446	0.0011	**
B	0.000144364	1	0.000144364	0.061078493	0.8130	
C	0.008764442	1	0.008764442	3.708118202	0.1025	
D	0.000547673	1	0.000547673	0.231712961	0.6473	
AB	4.59502E-05	1	4.59502E-05	0.01944092	0.8937	
AC	0.227691016	1	0.227691016	96.33302587	< 0.0001	**
AD	0.01651252	1	0.01651252	6.986226469	0.0384	*
BC	0.051995385	1	0.051995385	21.9985526	0.0034	**
BD	0.136688834	1	0.136688834	57.83121881	0.0003	**
CD	0.095822162	1	0.095822162	40.54107608	0.0007	**
A^2	0.281382082	1	0.281382082	119.048998	< 0.0001	**
B^2	0.219067048	1	0.219067048	92.68434024	< 0.0001	**
C^2	0.090395222	1	0.090395222	38.24501027	0.0008	**
D^2	0.001071407	1	0.001071407	0.453298042	0.5258	

$R^2 = 0.9958$, $R^2_{Adj} = 0.9859$. Note: SS represents the sum of deviation squares; DF denotes degree of freedom; MS represents mean square.
** Very significant ($P < 0.01$).
* Significant ($0.01 < P < 0.05$).

3.5. Monosaccharide composition and molecular weight of the purified EPS

The fungal exopolysaccharides represent a wide range of chemical structures and properties, are rich in high molecular weight polysaccharides and mostly have heteropolymeric composition [15,38]. In addition to yield, molecular mass, monomer composition between the polymers also play important roles for determining the viscosity and application of EPS. In this study, measurement of weight-average molecular weight (Mw) and number-average molecular weight (Mn) were carried out by HPSEC-MALLS (Fig. 4). The EPS molecular mass and its distribution was analyzed simultaneously by two differential and light scattering detectors, and polydispersion (PD, Mw/Mn) of 1.613 was used as a tool to measure the distribution of molecular weight. The chromatograms of the purified EPS exhibited a single peak, implying that was the homogeneity. The Mw and Mn were individually 3.246×10^4 g/mol and 2.012×10^4 g/mol. The retention times of the purified EPS after hydrolysis and derivatization analyzed by GC–MS chromatogram were 6.44, 12.00, 19.54, 19.94, and 20.90 min (Fig. 5), and the derivatization of different standard sugars was also analyzed with the retention time of 6.08, 6.59, 11.95, 13.07, 19.58, 20.21 and 21.22 min, respectively. Compared with the retention time of the standard monosaccharides, the purified EPS was primarily consisted of five kinds of different monosaccharides including D-galacturonic acid, rhamnose, D-mannose, glucose, and D-galactose in a ration of 0.45:3.02:3.25:1.0:0.95. Goatley [45] purified two main fractions of EPS from *Alternaria solani*, and that fraction I contained glucose, galactose, and glucosamine in a 2.4:1:1 ratio whereas fraction II contained glucose, galactose, and mannose in a 6.4:1:1.2 ratio by chromatography analysis. Several previous reports described EPS production is widely distributed among fungi, which indicated molecular weight of EPS producing with different strains (*Aureobasidium*, *Candida*, *Cryptococcus*, and many others) showed distinct differences, and most of them were homopolysaccharides but heteropolysaccharides were also very common [15]. EPS biopolymers contained D-mannose, either alone or in combination with other sugars (e.g. galactose, xylose), together with uronic acids and non-sugar substituents [47]. Cheng et al. reported the EPS from *Antrodia cinnamomea* was composed of Fuc, GlcN, Gal, Glc, Man in different ration with the molecular mass and its distribution of 1.0×10^3–1.0×10^5 [48]. Prajapati et al. revealed that pullulan producing by *Aureobasidium pullulans* was

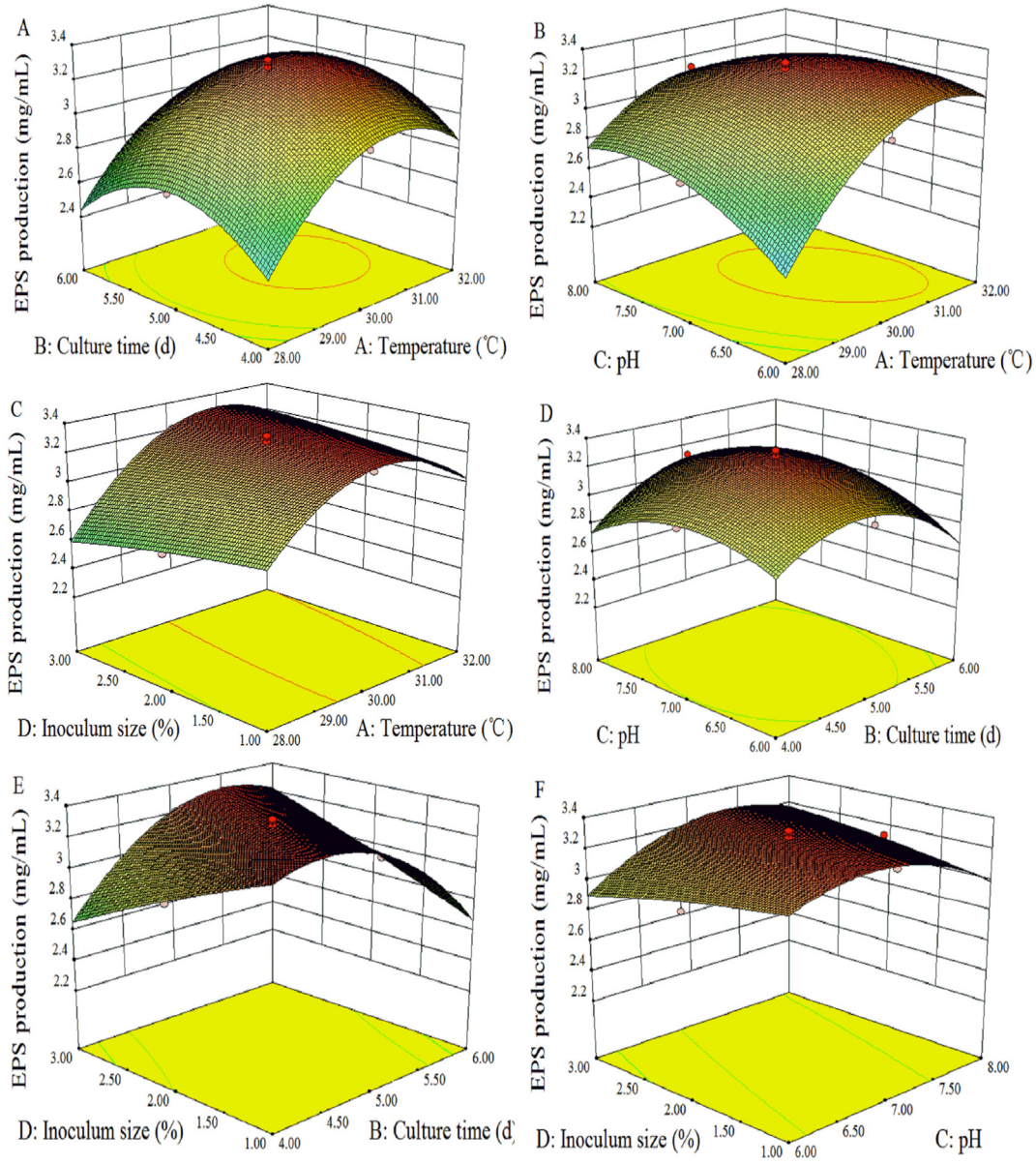

Fig. 3. Response surface plots for the mutual effects of (A) temperature and culture time; (B) temperature and pH; (C) temperature and inoculum size; (D) culture time and pH; (E) culture time and inoculum size; (F) pH and inoculum size on EPS production.

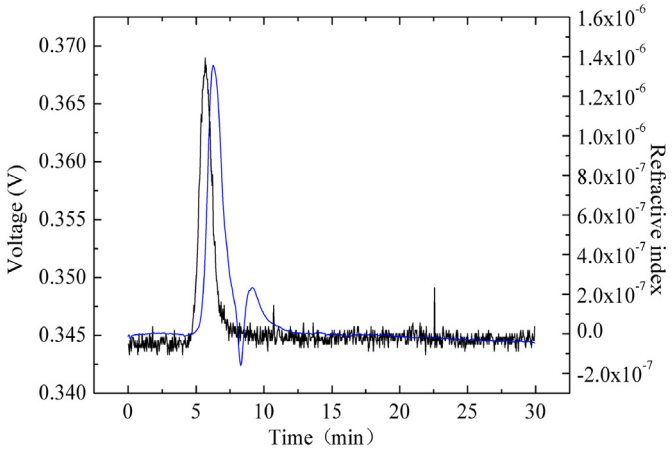

Fig. 4. HPSEC-MALLS chromatogram of the purified EPS.

made up of a series of similar units joined together, which was made up of glucose units [49]. The molecular weight of EPS produced by *Streptococcus thermophilus* 05-34 under the optimal fermentation condition was 4.7×10^5 Da [50]. The various monosaccharide composition and molecular weight reported in the literature may be due to the differences in the types of strain, composition of fermentation medium and culture conditions used.

3.5.1. FT-IR analysis

In order to illuminate the major functional groups of the purified EPS, FT-IR was performed and the result was shown in Fig. 6. The EPS contained hydroxyl groups, which displayed a peak at 3310 cm^{-1} (range 3600–3200 cm^{-1}) [51]. The peak around 2940 cm^{-1} was due to C-H stretching vibration. The absorption band from 1800 cm^{-1} to 600 cm^{-1}, called "finger print" region, was related to conformation and surface structure of molecule. The relative absorption peak at 1670 cm^{-1} (around 1600–1680 cm^{-1}) indicated the characteristic of C=O [52], meanwhile, the existence of C=O indicated EPS contained amide bond or carboxylic acid. At 1550 cm^{-1}, a peak corresponded

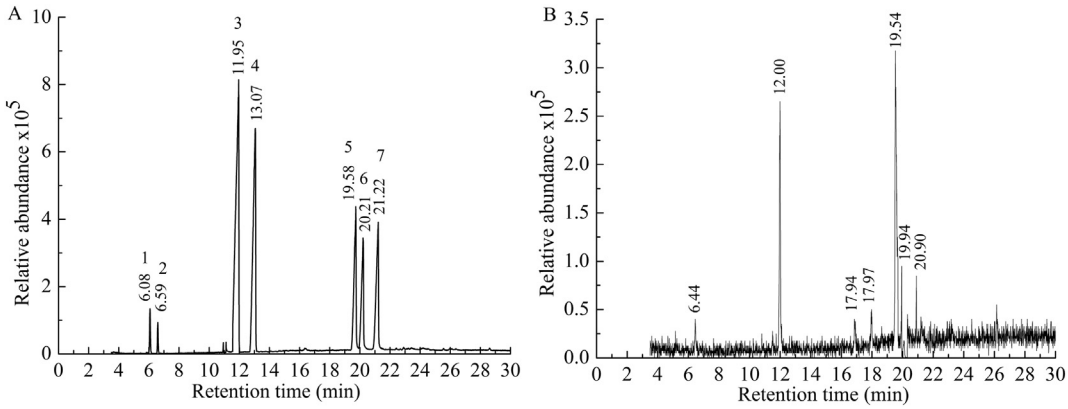

Fig. 5. GC–MS spectrum of the standard monosaccharides (A: 1, 2, 3, 4, 5, 6, 7 represented D-glucuronic acid, D-galacturonic acid, rhamnose, D-arabinose, D-mannose, glucose, and D-galactose, respectively) and the purified EPS (B).

the N-H deformation vibration of an amine group [53]. The peak at 1460 cm^{-1} was corresponded to asymmetric deformation of -CH_3 and -CH_2- in proteins. Besides, the peak at 1390 cm^{-1} could be attributed to >C=O stretch of the COO^- groups and C-O bond from COO^- groups [52]. The peak at 1320 cm^{-1} may be due to symmetric -CH_3 bending. The absorption peak at 1230 cm^{-1} demonstrated the presence of sulfate groups as S-O and C-O-S [54]. It suggested the presence of C-O-C, C-O-H link bonds and hydroxyl of pyranose ring under the region ranging from 950 cm^{-1} to 1200 cm^{-1} [51,52], so a peak at 1060 cm^{-1} indicated the presence of pyranose. The FT-IR results were consisted with the physicochemical property of the purified EPS.

3.5.2. Scanning electron microscope observation

Scanning electron microscopy (SEM) is a powerful tool for the study of the surface morphology of macromolecules, which helps to understand about its common physical properties. The microstructures of the purified EPS were observed by SEM (Fig. 7). The EPS seemed to be a porous network showing a relatively stable three-dimensional structure. Pore structure indicated that the existence of molecular repulsion among the polymer. The surface of the purified EPS was rough with many detrital and branched structures. The above-observed structure is similar to that of most reported microbial EPS [42], and sheet-like compact morphology also existed in many EPSs. The different microstructure implied that EPS exhibited distinct physical properties such as viscosity, water-holding capacity, which may determine its application in foods and medicine.

3.5.3. Thermogravimetric (TGA) and differential scanning calorimeter (DSC) analysis

The industrial and commercial utility of polysaccharides are closely related to their thermodynamic properties. Thermogravimetric analysis (TGA) is an analytical technique that determines the relationship between material weight loss and temperature [42,53,55]. The result of TGA of the purified EPS was shown in Fig. 8. As can be seen from the figure, with the increase of temperature, the loss weight of EPS increased, and two distinct stages of weight loss were displayed. The first stage (100–245 °C) weight loss may be related to the loss of free water and bound water, indicating that a high carboxyl content in EPS molecules, which is consistent with the previous reports [42]. The weight loss in the second stage (245–400 °C) is caused by the degradation of the sample itself. The degradation temperature (Td) of EPS is approximate 303.9 °C. Then the weight loss gradually decreased to leave a final residue 5.04% of the total EPS. Wang et al. [42] and Wang et al. [55] found that Td of EPS produced by *Lactobacillus plantarum* KF5 and YW11 isolated from Tibet kefir were 279.59 °C and 287.7 °C, respectively. Undoubtedly, all kinds of the EPSs displayed the different thermostability and degradation behavior, which could be attributed to the different structure (carbohydrate composition) of the EPS. Meanwhile, the chemical properties and applicability of polysaccharides is largely dependent on its thermal behavior. The heat absorption and emission are accompanied with physical changes by deformation of the polymer structure or the melting of the crystallized polysaccharide [33]. The variation of energy level and the endothermic enthalpy change (ΔH) of the purified EPS with heat flow from 0 °C to 350 °C were determined by DSC (Fig. 8).

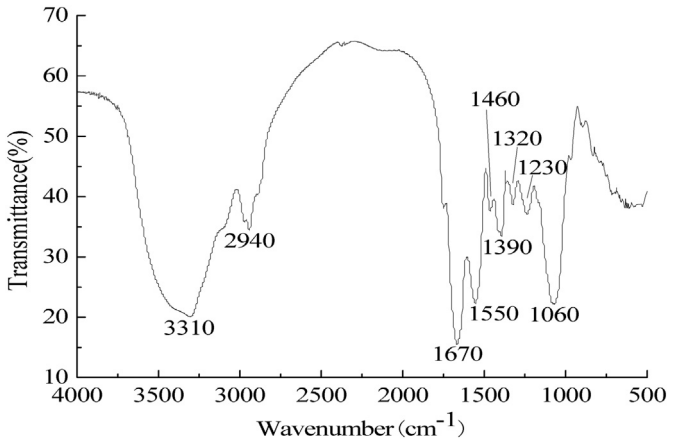

Fig. 6. Infrared absorption spectra of the purified EPS.

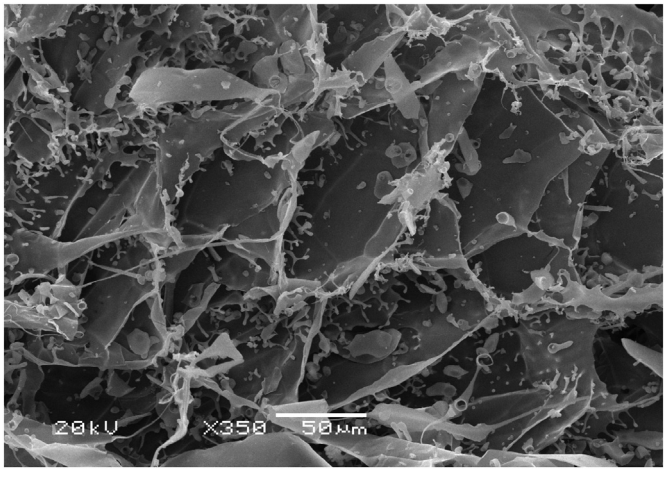

Fig. 7. The microscopic structure of the purified EPS (20 KV, ×350, 50 μm).

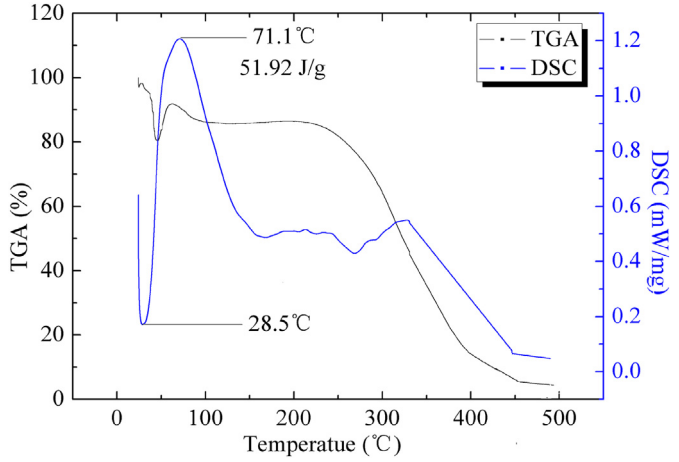

Fig. 8. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of EPS.

The peak temperature of EPS was about 71.1 °C with ΔH of 51.92 J/g, which showed some difference with previous reports. Ahmed et al. [53] reported that the peak temperature of the EPS from *Lactobacillus kefiranofaciens* ZW3 was 97.38 °C and ΔH was about 249.7 J/g. Wang et al.[55] reported that the peak temperature of the EPS-producing *Lactobacillus plantarum* YW11 was about 143.6 °C and ΔH to melt 1 g EPS was about 217.8 J. Thus, the EPS from different strain showed a different thermal behavior.

3.5.4. Antioxidant activity of EPS

Compared to polysaccharides isolated from other natural sources, microbial EPS show more and more novel and distinct properties including the chemical and structural diversity and give a great potential for development. However, their biological activity are still studied due to their complexities [15,21,22,23]. In this trial, different biochemical methods of reducing power activity, superoxide anion radical and hydroxyl radical scavenging assay were used to evaluate the antioxidant activities of the purified EPS from *A. tenuissima* F1 in vitro. As shown in Fig. 9A, EPS has a significant hydroxyl radical scavenging activity, and it can be used as a good free radical scavenger. The hydroxyl radical scavenging activity of EPS was concentration-dependent and weaker than that of Vc within the dosage range of 0–5 mg/mL, the scavenging capacity of EPS rapidly increased and be closed to that of Vc with the continually increasing its concentrations. Fig. 9B showed that the inhibition percentages of superoxide anion of EPS and Vc scavenging superoxide radical were directly proportional to their concentrations. The inhibition percentage of EPS was lower than that of Vc within 0–5 mg/mL, indicating that the EPS slightly affects the scavenging of the superoxide radical. It can be seen from Fig. 9C that the reducing capacity of EPS was obviously lower than that of Vc, and the reducing power expressed by absorbance at 700 nm (OD_{700}) of EPS was 0.58 at 5 mg/mL. In a word, EPS has strong antioxidant activities and can be applied as an antioxidant.

4. Conclusion

In this study, this is the first report of an endophytic fungus *A. tenuissima* F1 isolated from *A. sinensis*, the EPS production optimized by orthogonal experiment and response surface methodology and its

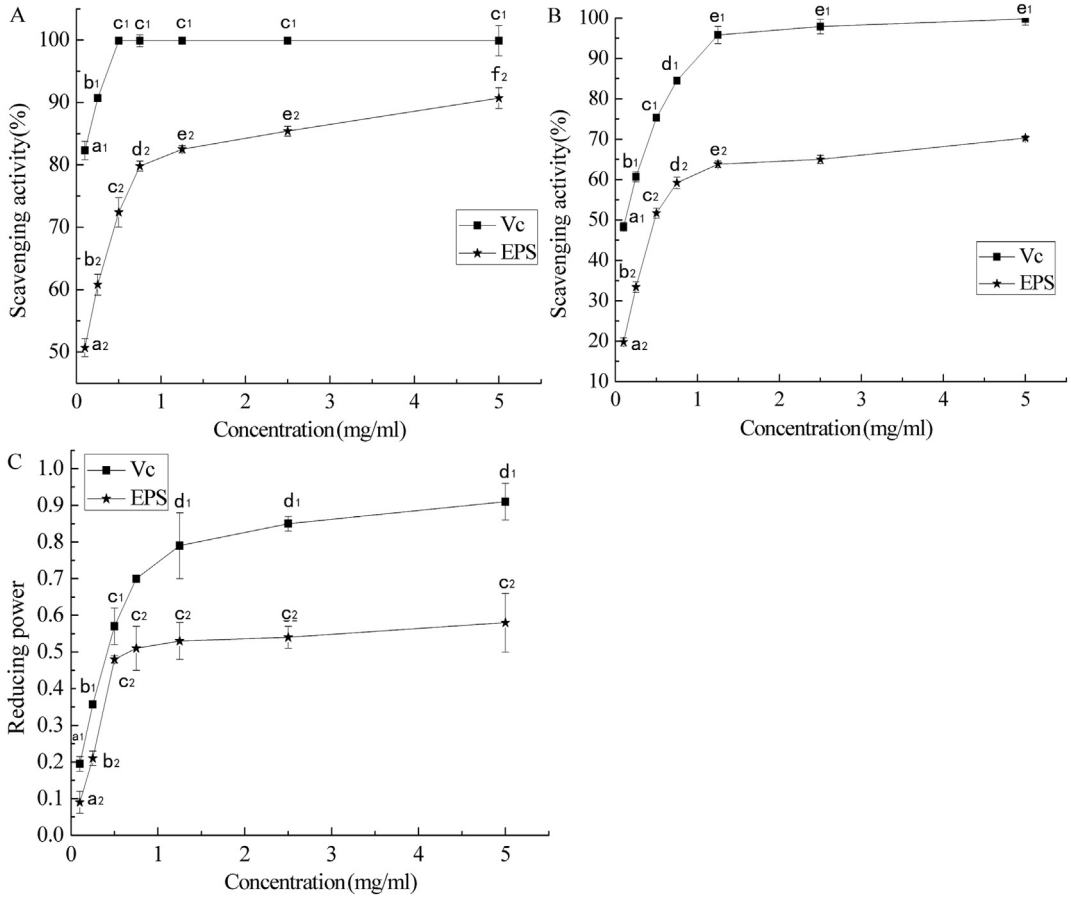

Fig. 9. Antioxidant activities of the different concentration of EPS and Vc. A. Scavenging activity of hydroxyl radical; B. Scavenging ability of superoxide anion; C. Reducing power. Data were shown as mean (n = 3). Note: values are the means of three replication ± SD. The same letter means no significant difference among the different groups at the 5% level.

antioxidant activity in vitro was been studied. The highest yield was obtained under the optimal medium constituents as follows: 8% mannose, 2% yeast extract, 0.04% $MgSO_4 \cdot 7H_2O$, 0.005% Vc, and the optimal cultivation conditions (initial pH 7.0, 30 °C, 180 r/min with inoculum size of 3% for 5 d). The EPS was purified by DEAE-52 chromatography and Sephadex G-100, respectively, and was proved to be a glycoprotein with the structure of pyranose analysis by FT-IR. The purified EPS mainly contains D-galacturonic acid, rhamnose, D-mannose, glucose, and D-galactose in a ratio of 0.45:3.02:3.25:1.0:0.95, and its molecular weight is 3.246×10^4 g/mol. The porous network structure, good thermostability and strong antioxidant activity of the EPS exhibited potential application in the biomedical engineering and food science. Further studies are required to evaluate the molecular mechanisms on biological activities of EPS in more detail.

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Authors' contribution

YG Wang and YL Li contributed equally to this work. YG Wang and JX Chen designed and revised the manuscript. YG Wang and YL Li performed the experiments, analyzed data and wrote the manuscript. Lormoua Kiatoukosin collected data. SW Li and WG Fang revised the manuscript. QY Li was responsible for drawing TOC graph. All authors have read and approved the manuscript.

Compliance with ethical standards.

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.03.246.

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