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Production, characterization and antioxidant activities *in vitro* of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3

Jun Liu, Jianguang Luo, Hong Ye, Yi Sun, Zhaoxin Lu, Xiaoxiong Zeng *

College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, Jiangsu, China

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ABSTRACT

The production, characterization and antioxidant activities *in vitro* of exopolysaccharides (EPS) from endophytic bacterium *Paenibacillus polymyxa* EJS-3 were investigated. For EPS production, the preferable culture conditions were 24 °C and pH 8 for 60 h with sucrose and yeast extract as the carbon and nitrogen sources, respectively. Notably, sucrose concentration was the prominent factor, and the maximum yield of EPS (22.82 g/L) was obtained at a sucrose concentration of 160 g/L. The crude EPS was purified by chromatography of DEAE-52 and Sephadex G-100, affording EPS-1 and EPS-2 with molecular weights of 1.22×10^6 and 8.69×10^5 Da, respectively. They were composed of mannose, fructose and glucose in a molar ratio of 2.59:29.83:1 and 4.23:36.59:1, respectively. In addition, both crude and purified EPS showed strong scavenging activities on superoxide and hydroxyl radicals, and their antioxidant activities decreased in the order of crude EPS > EPS-2 > EPS-1.

1. Introduction

There are increasing evidences indicating that reactive oxygen species (ROS) and oxygen-derived free radicals may contribute to a variety of pathological effects (e.g. DNA damages, carcinogenesis and cellular degeneration) and induce many diseases including aging, cancer, atherosclerosis, diabetes and rheumatoid arthritis (Seifried, Anderson, Fisher, & Milner, 2007; Valko et al., 2007). In order to reduce ROS-induced oxidative damage, both synthetic and natural antioxidants are used. However, synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene are considered to be responsible for liver damage and carcinogenesis (Grice, 1988). Therefore, it is essential to develop natural non-toxic antioxidants to protect human body from free radicals and retard the progress of many chronic diseases. In the search of new natural antioxidants, a number of polysaccharides obtained from plants, animals and microorganisms have been demonstrated to possess potent antioxidant activities and potential applications as natural antioxidants (Luo & Fang, 2008; Sun, Wang, Fang, Gao, & Tan, 2004; Yu et al., 2007).

As a slightly opened reservoir of special bio-resources, endophytes (microorganisms that reside in the internal tissues of living plants without causing any overt negative effects) have been demonstrated to be excellent producers of bioactive and structurally novel metabolites (Strobel, 2003). A lot of novel bioactive products such as antibiotics, antiviral, anticancer and antidiabetic agents have been isolated from endophytes (Guo, Wang, Sun, & Tang, 2008). It has also been reported that endophytes can produce useful antioxidants (Liu et al., 2007; Strobel et al., 2002). Strobel et al. (2002) obtained isopestacin, an isobenzofuranone with antifungal and antioxidant activities, from the culture broths of the endophytic fungus *Pestalotiopsis microspora*. Liu et al. (2007) isolated an endophytic fungus *Xylaria* sp. from *Ginkgo biloba* and found that the methanol extract of fruit bodies of *Xylaria* sp. exhibited strong antioxidant activity. However, no report on the antioxidant activities of polysaccharides from endophytes is available to date.

Paenibacillus polymyxa (previously *Bacillus polymyxa*) EJS-3 was an endophytic bacterium strain isolated from the root tissue of *Stemona japonica* (Blume) Miquel, a traditional Chinese medicine (Lu et al., 2007). We also noted that *P. polymyxa* EJS-3 could produce viscous exopolysaccharide (EPS) (Sun et al., 2006). Till now, there have many reports on the culture conditions for the production of EPS from *P. polymyxa* (Han & Clarke, 1990; Lee et al., 1997). However, most of these *P. polymyxa* strains were isolated from soil. In addition, studies on the biological activities of EPS from *P. polymyxa* are mainly focused on its thickening, stabilizing and gelling properties (Madden, Dea, & Steer, 1986; Mitsuda, Miyata, Hirota, & Kikuchi, 1981). Their antioxidant activities have not been investigated yet. Therefore, the objectives of present study were to investigate the culture conditions and the antioxidant activities *in vitro* of EPS from *P. polymyxa* EJS-3. Firstly, single factor experiments were carried out to investigate the effects of medium and culture conditions (initial pH, temperature, carbon source and concentration, nitrogen source) on cell growth and EPS production. Then, the crude EPS obtained from the culture was sequentially

* Corresponding author. Fax: +86 25 84396791.
E-mail address: zengxx@njau.edu.cn (X. Zeng).

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purified through anion-exchange and gel filtration chromatography, and the purified fractions were characterized by high performance liquid chromatography (HPLC) and gas chromatography (GC). Finally, the antioxidant activities *in vitro* of EPS including reducing power, scavenging activity on superoxide radical and scavenging activity on hydroxyl radical were determined.

2. Materials and methods

2.1. Microorganism and seed culture

Paenibacillus polymyxa EJS-3 used in the present study was provided by Laboratory of Enzyme Engineering, Nanjing Agricultural University. The strain was cultured on potato dextrose agar (PDA) slant at 28 °C for 1 d, and then maintained at 4 °C. Seed culture was carried out in a 250-ml flask containing 50 ml basal medium (sucrose 30 g/L, yeast extract 5 g/L, meat peptone 5 g/L, K_2HPO_4 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ 0.5 g/L, and initial pH 7.0) at 28 °C on a rotary shaker incubator at 180 rpm for 18 h.

2.2. Flask culture conditions

Flask cultures were performed in 250 ml flasks containing 50 ml of the basal medium after inoculating with 6% (v/v) of the seed culture, and cultivated at 150 rpm for 72 h. Factors affecting cell growth and EPS production were investigated using one-factor-at-a-time method. To determine the optimum initial pH for EPS production, medium was adjusted to the required pH by addition of 1 M HCl and 1 M NaOH before sterilization. To find out the optimum temperature for EPS production, flasks cultures were incubated at 21, 24, 27, 30 and 33 °C, respectively. To optimize the medium composition, different kinds of carbon and nitrogen sources were chosen to substitute the corresponding components in the basal medium. Time course experiment was carried out in a 1-L flask containing 200 ml of the screened culture medium based on the results of single factor experiments.

2.3. Determination of cell growth and EPS production

Samples collected at various intervals from shake flasks were properly diluted and centrifuged at 10,000 rpm for 15 min. The biomass was obtained by washing the resulting precipitated cells with distilled water repeatedly and drying at 80 °C to a constant weight. The supernatant was filtered through a 0.45-μm membrane filter, mixed with four volumes of anhydrous ethanol, stirred vigorously and kept overnight at 4 °C. The precipitate from the ethanol dispersion was collected by centrifugation at 10,000 rpm for 15 min, redissolved in distilled water and followed by deproteinization with 1/5 volume of Sevag reagent ($CHCl_3$–BuOH, v/v = 5/1) for seven times (Staub, 1965). The deproteinized solution was then dialyzed against distilled water, concentrated and lyophilized to afford the crude EPS.

2.4. Purification of crude EPS

The crude EPS was purified sequentially by chromatography of DEAE-52 and Sephadex G-100 according to our reported method (Qiao et al., 2009). Crude EPS solution (10 mg/ml, 5 ml) was applied to a column of DEAE-52 (2.6 × 30 cm), and the column was stepwise eluted with distilled water, 0.1, 0.3 and 0.5 M sodium chloride solutions at a flow rate of 60 ml/h. Eluate (10 ml/tube) was collected automatically and the carbohydrates were determined by the phenol–sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). As results, two fractions of polysaccharides (F-1 and F-2) were obtained, concentrated, dialyzed and further purified through a column of Sephadex G-100 (2.6 × 50 cm) to afford EPS-1 and EPS-2, respectively. The resultant EPS-1 and EPS-2 were then concentrated, dialyzed and lyophilized for further study, respectively.

2.5. Determination of molecular weight

The molecular weight of EPS-1 and EPS-2 were determined on an Agilent 1100 HPLC system equipped with a refractive index detector (RID) and a TSK-GEL G3000SW_{xl} column (7.5 × 300 mm, Tosoh Corp., Tokyo, Japan). The column was eluted with 0.1 M Na_2SO_4 solution at a flow rate of 0.8 ml/min. Pullulan P-800, P-400, P-200, P-100, P-20, P-10 and P-5 (Shodex standard P-82, Showa denko, Japan) were used as standards for molecular weight measurement.

2.6. Analysis of monosaccharide compositions of purified EPS

For monosaccharide composition analysis, EPS-1 and EPS-2 (5 mg, each) were hydrolyzed with 2 ml of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h or 0.2 M TFA at 80 °C for 30 min. The hydrolyzate was repeatedly co-concentrated with methanol to dryness and converted to its trimethylsilyl (TMS) derivative. The TMS derivative of hydrolyzate was prepared by adding 1 ml pyridine, 0.4 ml hexamethyldisilazane and 0.2 ml trimethylchlorosilane and heating at 80 °C for 30 min. The TMS derivatives of standard sugars were prepared in the same way. After cooling, samples were analyzed on HP 6890 GC equipped with flame ionization detector (FID) and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The following chromatographic conditions were used: nitrogen gas was used as the carrier gas at a flow rate of 1 ml/min; the temperature of injector and detector were set at 250 °C and 280 °C, respectively; initial column temperature was held at 100 °C for 5 min, then programmed at a rate of 5 °C/min to 150 °C and held at 150 °C for 5 min, subsequently programmed at 5 °C/min to 240 °C and held at 240 °C for 2 min.

2.7. Assay of antioxidant activities in vitro of EPS

2.7.1. Assay of reducing power

The reducing power was determined according to the method of Oyaizu (1986) with some modifications. Reaction was carried out in the mixture containing 2.5 ml of sample (0.05–1 mg/ml), 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1%, w/v) by incubating at 50 °C for 20 min. After addition of 2.5 ml trichloroacetic acid (10%, w/v), the mixture was centrifuged at 5000 rpm for 10 min. The upper layer (5 ml) was mixed with 0.5 ml of fresh $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. Deionized water and ascorbic acid were used as the blank and control, respectively.

2.7.2. Assay of superoxide radical scavenging activity

The superoxide radical scavenging activity was performed by the method of Liu, Ooi, and Chang (1997) with some modifications. The superoxide radical was generated in 3 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 156 μM β-nicotinamide adenine dinucleotide (NADH), 52 μM nitroblue tetrazolium (NBT) and 20 μM phenazine methosulfate (PMS). After addition of 1 ml sample (0.05–1 mg/ml), the mixture was incubated at 25 °C for 5 min. The absorbance of the mixture was measured at 560 nm. The scavenging activity on superoxide radical (%) = $(1 - A_{sample}/A_{blank}) \times 100$. Deionized water and ascorbic acid were used as the blank and control, respectively.

2.7.3. Assay of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by the method of Jin, Cai, Li, and Zhao (1996). The hydroxyl radical was

generated in the mixture of 1 ml of 0.75 mM 1,10-phenanthroline, 1.5 ml of 0.15 M sodium phosphate buffer (pH 7.4), 1 ml of 0.75 mM $FeSO_4$ and 1 ml of H_2O_2 (0.01%, v/v). After addition of 1 ml sample (0.05–1 mg/ml), the mixture was incubated at 37 °C for 30 min. The absorbance of the mixture was measured at 536 nm. The scavenging activity on hydroxyl radical (%) = $(A_{sample} - A_{blank})/(A' - A_{blank}) \times 100$, where A' was the absorbance of the deionized water instead of H_2O_2 and sample in the assay system. Deionized water and ascorbic acid were used as the blank and control, respectively.

2.8. Statistical analysis

All data were analyzed by one-way ANOVA. Tests of significant differences were determined by Turkey-HSD at ($P < .05$). In all cases, there were three replicates ($n = 3$).

3. Results and discussion

3.1. Production of EPS from P. polymyxa EJS-3

3.1.1. Effect of initial pH and temperature on cell growth and EPS production

The initial culture pH is an important factor that may affect cell membrane, cell morphology and structure, the uptake of various nutrients and EPS biosynthesis (Kim et al., 2005). To find out the optimal pH for *P. polymyxa* EJS-3, various pH values ranging from 5 to 9 were applied. As shown in Fig. 1A, the optimal pH value for cell growth and EPS production were 6 and 8, with the corresponding cell biomass and EPS production of 1.93 g/L and 6.24 g/L, respectively. Unlike *P. polymyxa* strains from soil (Han & Clarke, 1990; Lee et al., 1997), the optimal pH for EPS production by *P. polymyxa* EJS-3 was slightly alkaline. This might be due to the specific growth condition of *P. polymyxa* EJS-3 within the living tissues of plant.

Incubation temperature is also a critical factor in EPS biosynthesis. As shown in Fig. 1B, the optimal temperature for cell growth and EPS production were 27 °C and 24 °C with the corresponding yields of 1.92 g/L and 6.42 g/L, respectively. This is consistent with the report that bacteria favor lower temperature for EPS production comparing with that for cell growth (Fett, 1993).

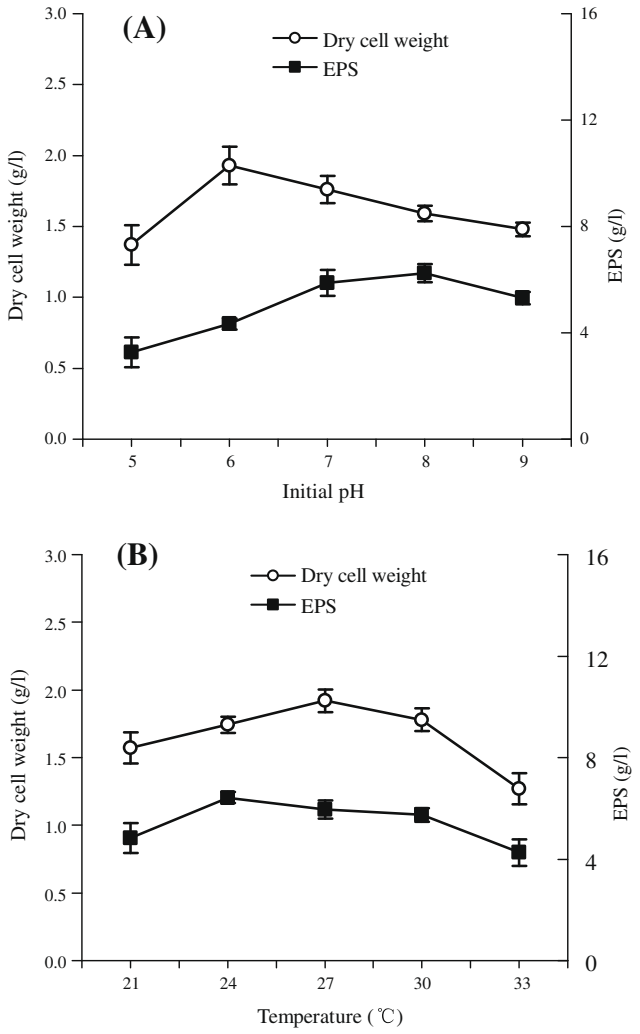

Fig. 1. Effects of initial pH (A) and temperature (B) on biomass and EPS production by *P. polymyxa* EJS-3. Data are means ± *SD* of triplicates.

3.1.2. Effect of carbon and nitrogen sources on cell growth and EPS production

In general, carbon source is the most critical nutrient and energy source for cell growth, and different carbon sources may have different effects of catabolic repression on cellular secondary metabolism (Kim et al., 2005). To select the suitable carbon source for cell growth and EPS production, various carbon sources were separately provided at 30 g/L in place of sucrose in the basal medium. As shown in Table 1, sucrose was the most suitable carbon source for EPS production. This is consistent with the finding of many other investigators (Han & Clarke, 1990; Lee et al., 1997). It has been reported that levansucrase with strong sucrose hydrolyzing activity is involved in many *P. polymyxa* strains, which may be responsible for the high yield of EPS with sucrose as carbon source (Park, Choi, & Oh, 2005).

To select the optimal sucrose concentration for cell growth and EPS production, 40–200 g/L sucrose was added into the basal medium. As shown in Table 1, the increase of the initial sucrose concentration in the medium led to the increases of both cell growth and EPS production. However, only small amounts of

Table 1
Effects of carbon and nitrogen sources on biomass and EPS production by *P. polymyxa* EJS-3 in shake flask cultures.

Culture conditions	Final pH	Dry cell weight (g/L)	EPS (g/L)
Carbon sources (3%)			
Xylose	5.12	1.63 ± 0.09 bc[a]	3.34 ± 0.22 de
Fructose	5.56	1.76 ± 0.17 b	2.79 ± 0.03 f
Glucose	5.53	1.73 ± 0.07 bc	3.78 ± 0.14 bc
Galactose	5.24	1.95 ± 0.04 a	3.11 ± 0.25 ef
Sucrose	5.67	1.62 ± 0.10 bc	7.08 ± 0.33 a
Lactose	5.31	1.66 ± 0.02 bc	4.09 ± 0.17 b
Maltose	6.02	1.57 ± 0.05 c	3.61 ± 0.15 cd
Sucrose concentration (%)			
4	5.78	1.91 ± 0.08 e	8.87 ± 0.62 e
8	5.29	2.13 ± 0.15 d	14.28 ± 0.51 d
12	5.00	2.66 ± 0.26 c	17.72 ± 1.08 c
16	4.71	3.28 ± 0.29 b	19.38 ± 0.87 a
20	4.58	3.58 ± 0.13 a	18.65 ± 0.08 b
Nitrogen sources (1%)			
Beef extract	5.70	1.69 ± 0.08 a	4.67 ± 0.51 b
Yeast extract	5.68	1.89 ± 0.10 a	7.98 ± 0.21 a
Meat peptone	6.01	1.46 ± 0.09 b	4.59 ± 0.37 b
$NaNO_3$	6.54	0.97 ± 0.15 cd	2.55 ± 0.09 d
KNO_3	6.70	1.40 ± 0.20 b	3.70 ± 0.52 c
$(NH_4)_2SO_4$	4.47	0.81 ± 0.06 d	1.61 ± 0.06 ef
NH_4Cl	4.44	1.07 ± 0.10 c	1.50 ± 0.10 f
Glutamine	6.58	0.97 ± 0.14 cd	2.08 ± 0.22 e

[a] Data are means ± *SD* of triplicates and alphabet letters indicate the same letters in the same column are not statistically significantly different according to Turkey-HSD ($P < .05$).

sucrose residues (less than 10% of initial sucrose) could be detected in the final culture broth, indicating that most of the sucrose in the medium had been utilized for cell growth and EPS production. A similar conclusion has been drawn from culture studies of various *P. polymyxa* strains (Han & Clarke, 1990; Lee et al., 1997). The highest EPS production (19.38 g/L) was obtained at the sucrose concentration of 160 g/L. And it should be noted that the cell growth increased continuously even when sucrose concentration reached 200 g/L. This was also probably due to the specific growth condition within the living tissues of plant that *P. polymyxa* EJS-3 could survive in such high sucrose concentration as mentioned above.

To investigate the suitable nitrogen source for cell growth and EPS production, various nitrogen sources were separately provided at 10 g/L in place of meat peptone and yeast extract in the basal medium. As shown in Table 1, yeast extract yielded the highest cell biomass (1.89 g/L) and EPS production (7.98 g/L). The stimulatory effect of yeast extract may be due to its rich contents of protein, amino acid and vitamin (Pokhrel & Ohga, 2007). In comparison with organic nitrogen sources, inorganic nitrogen sources resulted in relatively lower cell biomass and EPS production, a phenomenon also reported by other investigators (Kim et al., 2005; Pokhrel & Ohga, 2007).

3.1.3. Time course of cell growth and EPS production

To investigate the time course of cell growth and EPS production, *P. polymyxa* EJS-3 was cultivated in a 1 L flask containing 200 ml of the screened culture medium (sucrose 160 g/L, yeast extract 10 g/L, K_2HPO_4 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ 0.5 g/L, and initial pH 8.0) based on the single factor experiments at 24 °C for 108 h. As shown in Fig. 2, the bacterium grew rapidly in the first 40 h, which was correlated with the rapid decline of pH value. We also found that EPS production was closely related to the cell growth. The EPS yield reached the maximum level (22.82 g/L) after the cell growth reached the stationary phase in 60 h. The results indicate that *P. polymyxa* EJS-3 is a promising EPS producer with potential industrial application.

3.2. Purification and characterization of EPS

3.2.1. Purification of EPS

In the present study, crude EPS was prepared from the fermentation broth of *P. polymyxa* EJS-3 by centrifugation, ethanol precipitation and deproteinization. Furthermore, the crude EPS solution was firstly separated through an anion-exchange chromatography of DEAE-52, affording two independent elution peaks (F-1 and F-2) as detected by the phenol-sulfuric acid assay (Fig. 3A). The two fractions were collected, concentrated and purified by gel filtration chromatography of Sephadex G-100, respectively. As a result, each fraction generated one single elution peak (Fig. 3B and C), named as EPS-1 and EPS-2, respectively. The recovery rates of EPS-1 and EPS-2 based on the amount of crude EPS were 53.6% and 4.8%, respectively. The results indicate that EPS-1 is the major polysaccharide produced by *P. polymyxa* EJS-3.

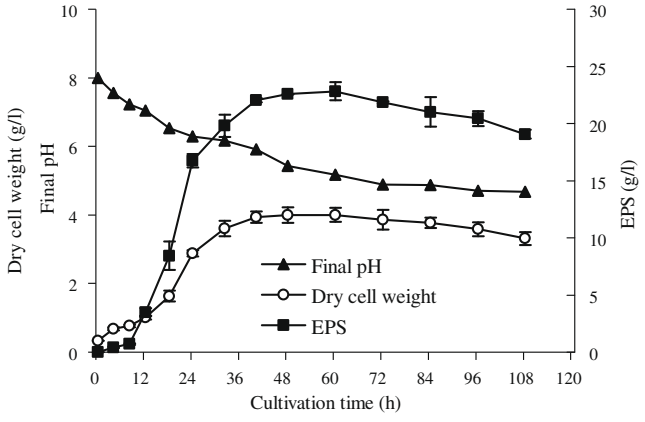

Fig. 2. Time course of cell growth and EPS production by *P. polymyxa* EJS-3 in a 1 L shaking culture under the screened culture condition. Data are means ± *SD* of triplicates.

3.2.2. Molecular weights of EPS-1 and EPS-2

The molecular weights of EPS-1 and EPS-2 were detected by HPLC with size exclusion column. As shown in Fig. 4A and B, both of EPS-1 and EPS-2 showed only one symmetrical peak on HPLC, indicating that no other polysaccharide was present in the sample. According to the calibration curve of the elution times of standards, the molecular weights of EPS-1 and EPS-2 were estimated to be 1.22×10^6 and 8.69×10^5 Da, respectively.

3.2.3. Monosaccharide compositions of EPS-1 and EPS-2

To determine the monosaccharide composition, EPS-1 and EPS-2 were first hydrolyzed with 2 M TFA at 120 °C for 2 h, respectively. As results, mannose, fructose and glucose were detected in both EPS-1 and EPS-2 by GC (Table 2). However, it should be noted that

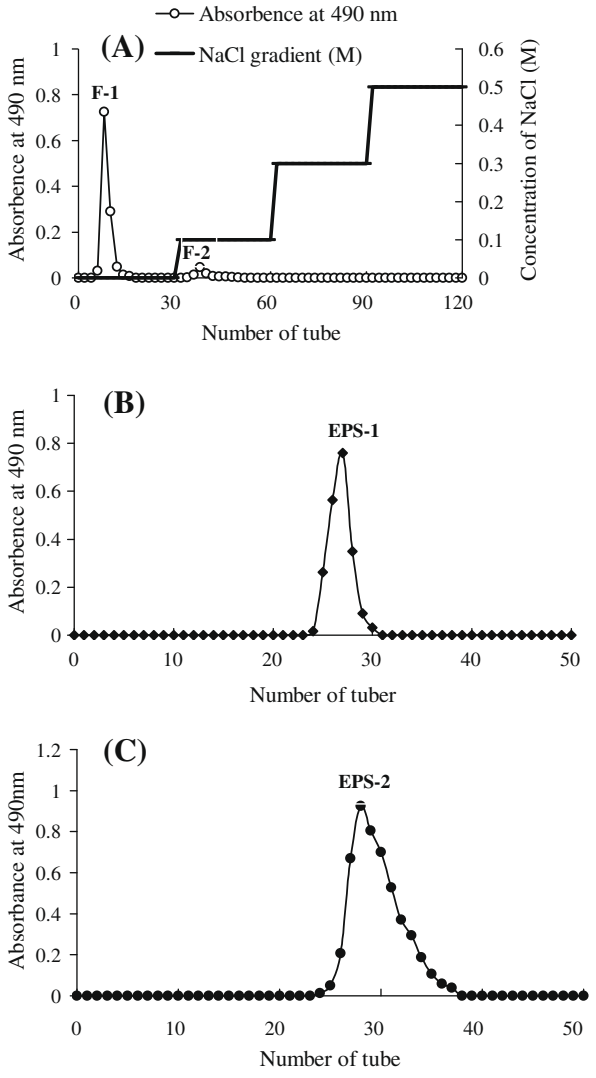

Fig. 3. Stepwise elution curve of crude EPS on DEAE-52 column (A) and elution curve of polysaccharides fractions (F-1 and F-2) on Sephadex G-100 column (B and C).

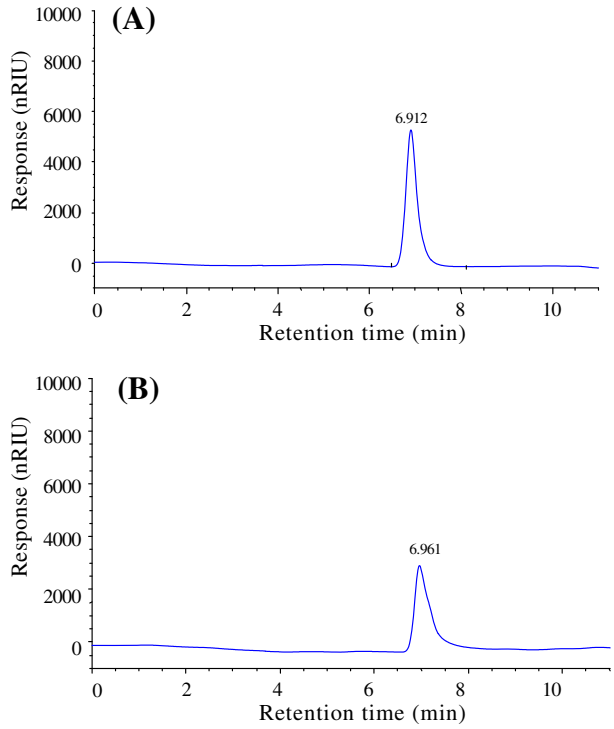

Fig. 4. HPLC chromatograms of EPS-1 (A) and EPS-2 (B) on TSK-GEL G3000SW$_{xl}$ column.

Table 2
Monosaccharide composition analysis of EPS-1 and EPS-2 by GC[a].

Monosaccharide component (mol)	EPS-1		EPS-2	
	A	B	A	B
Mannose	2.76	2.59	4.19	4.23
Fructose	9.95	29.83	2.67	36.59
Glucose	1.00	1.00	1.00	1.00

[a] Hydrolysis of EPS was performed with 2 M TFA at 120 °C for 2 h (A) or 0.2 M TFA at 80 °C for 30 min (B).

fructose was instable and might be degraded in strong acids (Wack & Blaschek, 2006). Furthermore, it was observed that the final hydrolyzate turned yellowish, which was probably due to the fructose degradation. Thus, EPS-1 and EPS-2 were subsequently hydrolyzed in a relatively mild acidic condition according to the method of Wack and Blaschek (2006). As shown in Table 2, the content of fructose detected increased significantly when EPS-1 and EPS-2 were hydrolyzed with 0.2 M TFA at 80 °C for 30 min. The results indicated that both EPS-1 and EPS-2 were mainly composed of fructose.

It has been reported that EPS produced by *P. polymyxa* are in a great variety, depending on the type of *P. polymyxa* strains, culture conditions and medium composition. Mitsuda et al. (1981) reported that EPS from *P. polymyxa* 458 was composed of glucose, mannose and glucuronic acid. Madden et al. (1986) reported that EPS produced by *P. polymyxa* NCIB 11429 was composed glucose, mannose, galactose, glucuronic acid and pyruvate. While Han & Clarke (1990) found that EPS from *P. polymyxa* NRRL B-18475 was β-(2→6) linked fructan. And Lee et al. (1997) reported that EPS produced by *P. polymyxa* KCTC 8648P was composed of glucose, galactose, mannose, fucose and glucuronic acid. In contrast, our results showed that the monosaccharide compositions of EPS-1 and EPS-2 were very similar to fructan.

3.3. Antioxidant activities in vitro of EPS

3.3.1. Reducing power of EPS

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. In the reducing power assay, antioxidants are able to reduce Fe^{3+}/ferricyanide complex to its ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung, Chang, Chao, Lin, & Chou, 2002).

Fig. 5A shows the reducing power of the crude EPS, EPS-1, EPS-2 and ascorbic acid. The reducing powers of all samples and ascorbic acid increased with the increase of concentrations. And the reducing power of the crude EPS was higher than that of its purified fractions (EPS-1 and EPS-2). This was probably due to the presence of other antioxidant components in the crude EPS, such as proteins, amino acids, peptides, organic acids and microelements. Among these antioxidant components, there may be some interactions and synergistic effects for antioxidant properties. In addition, the reducing power of EPS-2 was higher than that of EPS-1, which was probably due to the differences in the monosaccharide compo-

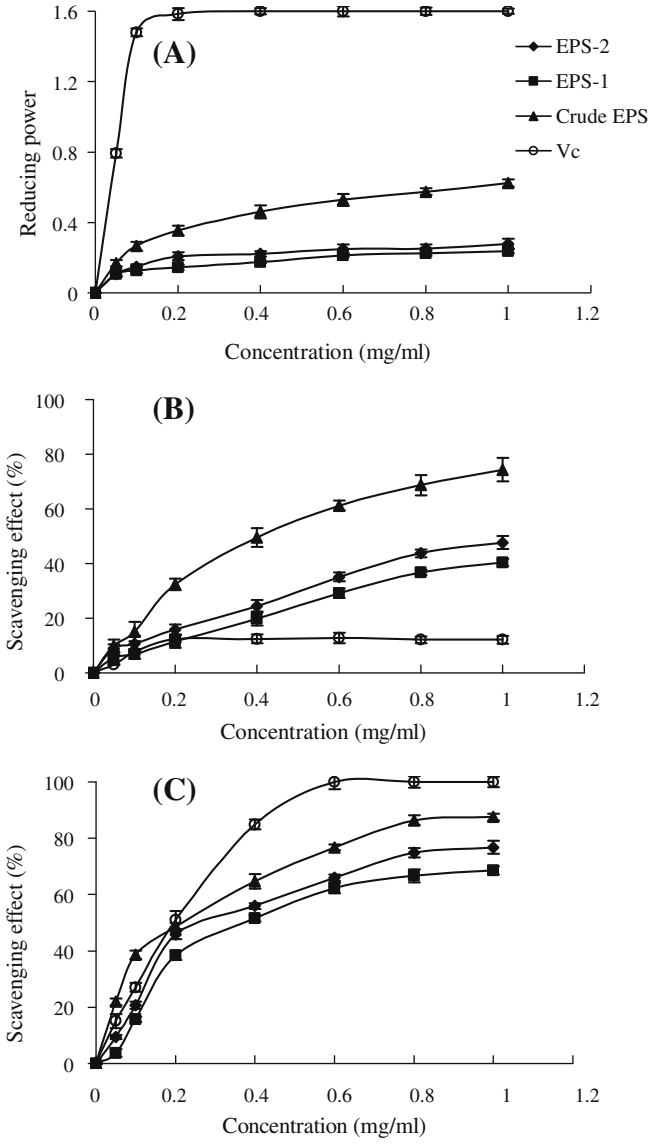

Fig. 5. The reducing power (A), scavenging activity on superoxide radical (B) and scavenging activity on hydroxyl radical (C) of EPS from *P. polymyxa* EJS-3. Data are presented as means ± *SD* of triplicates.

sition and molecular weight between them. However, for all the samples tested, the reducing powers were lower than that of ascorbic acid. The results indicate that EPS have moderate reducing power.

3.3.2. Scavenging activity on superoxide radical of EPS

Superoxide radicals were generated in a PMS/NADH system for being assayed in the reduction of NBT. Superoxide radicals would play important roles in the formation of other ROS, such as hydrogen peroxide, hydroxyl radical and single oxygen, which induce oxidative damage in lipids, proteins and DNA (Wickens, 2001).

Fig. 5B shows the scavenging effects of crude EPS, EPS-1, EPS-2 and ascorbic acid on the superoxide radicals. The scavenging effects of EPS were correlated well with the increase of concentrations. And the scavenging effect of the crude EPS was more pronounced than that of EPS-1 or EPS-2, which was in accordance with the results in Section 3.3.1. At a concentration of 1 mg/ml, the scavenging effect of the crude EPS was 74.38%. The scavenging activity of EPS-2 was relative higher than that of EPS-1. However, the scavenging activity of ascorbic acid was much lower than that of EPS-1 or EPS-2, with the scavenging effect of 12.11% at 1 mg/ml. These results indicate that EPS have strong scavenging activity on superoxide radical.

3.3.3. Scavenging activity on hydroxyl radical of EPS

Except for superoxide radical, hydroxyl radical is also an important free radical, which can react with all bio-macromolecules in living cells (Gülçin, 2006). The hydroxyl radical, generated by the Fenton reaction in the system, was scavenged by the crude EPS, EPS-1 and EPS-2. The scavenging effects of EPS and ascorbic acid are shown as in Fig. 5C. The crude EPS exhibited a stronger scavenging effect against hydroxyl radical than EPS-1 and EPS-2, which was in accordance with the results in Sections 3.3.1 and 3.3.2. Moreover, the scavenging effect of EPS-2 was stronger than that of EPS-1. However, ascorbic acid showed higher hydroxyl radical scavenging activity than EPS. At a concentration of 1 mg/ml, the scavenging activities for the crude EPS, EPS-1, EPS-2 and ascorbic acid were 87.58%, 76.73%, 68.55% and 100%, respectively. These results indicate that EPS have strong scavenging activity on hydroxyl radical.

4. Conclusions

In the present study, the production, characterization and antioxidant activities *in vitro* of EPS from endophytic bacterium *P. polymyxa* EJS-3 were investigated. We found that sucrose concentration was the prominent factor affecting the cell growth and EPS production. The maximum yield of EPS (22.82 g/L) was obtained based on the screened culture medium (sucrose 160 g/L, yeast extract 10 g/L, K_2HPO_4 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ 0.5 g/L, and initial pH 8.0) and culture conditions (24 °C, 60 h). The results indicate that *P. polymyxa* EJS-3 is a promising EPS producer with potential industrial application. The crude EPS obtained from the fermentation broth was purified by chromatography of DEAE-52 and Sephadex G-100, affording two fractions of EPS-1 and EPS-2. And characterization of the two fractions demonstrated that they were mainly composed of fructose. In addition, the assay of the antioxidant activities *in vitro* demonstrated that both the crude and purified EPS had strong scavenging activities on superoxide and hydroxyl radicals. And the antioxidant activities decreased in the order of crude EPS > EPS-2 > EPS-1, according to the assays of reducing power, scavenging activity on superoxide radical and scavenging activity on hydroxyl radical. These results indicate that EPS from *P. polymyxa* EJS-3 may be a new source of natural antioxidants with potential value for health food and therapeutics. And further works on the structure and antioxidant activities *in vivo* of EPS is in progress.

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