Carbohydrate Polymers 79 (2010) 700–704

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimization for the production of exopolysaccharides from *Morchella esculenta* SO-02 in submerged culture and its antioxidant activities *in vitro*

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ARTICLE INFO

Article history:
Received 28 March 2009
Received in revised form 30 August 2009
Accepted 28 September 2009
Available online 4 October 2009

Keywords:
Morchella esculenta SO-02
Exopolysaccharides
Orthogonal experiment
Antioxidant activity

ABSTRACT

The aims of this work were to optimize the conditions for exopolysaccharides (EPS) production by *Morchella esculenta* SO-02 in submerged culture, and to evaluate the antioxidant activities of EPS *in vitro*. The optimal medium constituents were determined as follows (g/l): bran 200, glucose 30, yeast extract 1, KH_2PO_4 2 and $MgSO_4{\cdot}7H_2O$ 1.5. The optimum parameters of liquid culture were temperature 25 °C, cultivation time 4 d, the volume of medium 100 ml, rotary speed 200 rpm and initial pH 6.5, respectively. Under the optimized conditions, the values of dry cell weight (DCW) and EPS were 9.2 ± 0.7 g/l and 2913 ± 262 mg/l, respectively, which were 42 ± 3% and 100 ± 7% higher than those in basal liquid medium, respectively. The EPS demonstrated positively antioxidant potential on superoxide anion radical scavenging, reducing power and hydroxyl radical scavenging.

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1. Introduction

Morchella esculenta Pers., a nutritional and medicinal mushroom with refreshing fragrance, contains many biological active materials, such as polysaccharides, protein, trace elements, dietary fiber and vitamins, etc. (Litchfteld, Vely, & Overbeck, 2006). The polysaccharides from *M. esculenta* fruiting body had the functions of antitumour, immunoregulation, fatigue resistance and antivirus (Nitha & Janardhanan, 2008; Rotaoll, Dunkel, & Hofmann, 2005; Wasser, 2002). Moreover, it also has been proven that the polysaccharides from *M. esculenta* mycelia had potentially antioxidant properties (Elmastas et al., 2006).

Compared with the polysaccharides from fruit bodies and mycelia, the exopolysacchairde (EPS) from fermentation broth with similar physiological and pharmacological functions is easily obtained. In order to harvest massive and effective EPS of mushrooms, many researchers have optimized the cultivation conditions for EPS production by *Pleurotus sajor-caju* (Confortin et al., 2008), *Fomes fomentarius* (Chen, Zhao, Chen, & Li, 2008), *Tremella fuciformis* (Cho, Oh, Chang, & Yun, 2006), *Pholiota squarrosa* (Wang, Lu, & Lv, 2004), *Agrocybe cylindracea* (Kim et al., 2005), *Collybia maculate* (Lim et al., 2004), *Cordyceps jiangxiensis* (Xiao et al., 2004), *Cordyceps militaris* (Kim et al., 2003), *Tremella mesenterica* (De Baets, Du Laing, Francois, & Vandamme, 2002) and *Pleurotus nebrodensis* (Jia, Hu, & Xu, 2007). However, it has not yet been reported on the optimization of producing EPS by *M. esculenta* SO-02 in submerged culture.

The antioxidant compounds play an important role in preventing and curing chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Kohen & Nyska, 2002). It is increasingly becoming attractive in employing antioxidants from natural sources by consumer preference for natural products (Safer & al-Nughamish, 1999). Therefore, the development and utilization of more effective antioxidants of natural origin are desired. However, there have been few reports about the antioxidant activities of EPS from mushroom. Dong et al. (2008) found that the EPS of *Marasmius androsaceus* had stronger superoxide radical (O_2^-) scavenging activity, hydroxyl radical (OH) scavenging capacity and reducing power *in vitro*. Similar results were obtained in *P. nebrodensis* and *Agaricus blazei* reported by Sheng, Wu, Guo, and Liu (2008) and Zhang, Li, and Liu (2004), respectively. Information is lacking with regard to the antioxidant activities of EPS *in vitro* by *M. esculenta* SO-02.

The objectives of this study were to optimize the medium composition and the cultivation conditions of producing EPS and mycelial biomass by *M. esculenta* SO-02 in submerged culture on the basis of the orthogonal experimental design and single-factor tests, and determine the antioxidant activities of EPS *in vitro* with the superoxide radical scavenging assay, hydroxyl radical scavenging assay and reducing power as main index.

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), methionine (MET) and riboflavin (RF) were from Sigma Chemicals Co (St. Louis, USA). All other chemicals used in this experiment

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doi:10.1016/j.carbpol.2009.09.032

were analytical reagent grade and purchased from local chemical suppliers in China.

2.2. Microorganism and liquid culture

A strain of *M. esculenta* SO-02 was provided by our laboratory and used in this experiment. It was incubated on synthetic potato dextrose agar (PDA) plates for 7 days at 25 °C, maintained at 4 °C and subcultured every 2 months.

All experiments of liquid cultivation were carried out in 250-ml Erlenmeyer flasks. The basal liquid medium composition (g/l) was bran 150, sucrose 25, peptone 2, KH_2PO_4 2 and $MgSO_4{\cdot}7H_2O$ 1 with natural pH. Each flask containing 100 ml basal medium was inoculated with a 0.5-cm^2 mycelial block of *M. esculenta* SO-02 from above maintained plates and incubated in a rotary shaker (Anting, Shanghai, China) at 160 rpm, 25 °C for 4 days after being laid still for 24 h.

2.3. Medium optimization for EPS production

Bran was used a basic substrate in medium composition. The addition of carbon (Fructose, Glucose, Lactose, Sucrose, Xylose) and nitrogen sources (Peptone, Yeast extract, NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$, NH_4Cl) were primarily selected and screened by single-factor experiments. A five-factor-three-level orthogonal test was subsequently applied to optimize the medium composition for EPS production after finding out the optimal carbon and nitrogen sources, while the cultivation conditions were 160 rpm, 25 °C for 4 days with natural pH. The orthogonal experimental design with the name and symbol is shown in Table 2.

2.4. Cultivation conditions optimization for EPS production

Cultivation time (2, 3, 4, 5, 6, 7, 8 d), temperature (20, 25, 30 °C), initial pH (4.5, 5.5, 6.5, 7.5, 8.5, 9.5), rotary speed (100, 150, 200, 250 rpm) and volume of medium (50, 100, 150, 200 ml) in 250 ml-sized flasks were investigated in this experiment by single-factor tests for EPS production. All of trials were carried out in the optimum liquid medium obtained from the former step.

2.5. Measurement of DCW and EPS content

The originally mycelial block of inoculation in liquid medium was discarded and the mycelia of *M. esculenta* SO-02 were obtained by centrifugation (3000*g*, 20 min) in a centrifuge (Beckman Instruments, Inc., Palo Alto, CL, USA) at the end of cultivation time according to the experiment design. The dry cell weight (DCW) of *M. esculenta* SO-02 mycelia was measured after rinsing the mycelia precipitate with distilled water for three times and then drying to a constant weight at 60 °C for about 48 h in an oven.

Table 1
Effect of carbon and nitrogen sources on DCW and EPS content of *M. esculenta* SO-02.

Source	DCW (g/l)	EPS content (mg/l)
Carbon sources		
Fructose	4.4 ± 0.2	1033 ± 847
Glucose	7.3 ± 0.5	2301 ± 212
Lactose	5.0 ± 0.4	584 ± 51
Sucrose	5.9 ± 0.4	2244 ± 209
Xylose	7.5 ± 0.6 *	838 ± 70 **
Nitrogen sources		
Peptone	6.3 ± 0.5	1514 ± 138
Yeast extract	6.3 ± 0.4	17332 ± 157
NH_4NO_3	5.3 ± 0.4	247 ± 22
$(NH_4)_2SO_4$	6.5 ± 0.4	1203 ± 115
$NaNO_3$	6.2 ± 0.3	622 ± 51
NH_4Cl	6.0 ± 0.5	568 ± 44 *

* $P < 0.05$.
** $P < 0.01$.

Table 2
Five-factor-three-level design of orthogonal experiment.

Variable	Levels		
	1	2	3
(A) Bran (g/l)	100	150	200
(B) Glucose (g/l)	20	25	30
(C) Yeast extract (g/l)	1	2	3
(D) KH_2PO_4 (g/l)	1	2	3
(E) $MgSO_4$ $7H_2O$ (g/l)	0.5	1.0	1.5

The supernatant fluid (5 ml) from former step was mixed with 3 times volume of 95% ethanol (v/v), stirred vigorously and kept at 4 °C for 18 h. After centrifugation (3000*g*, 15 min), the precipitate of EPS was dissolved with distilled water (60 °C) and the EPS content was determined by phenol–sulfuric acid method using glucose as the standard (Chaplin & Kennedy, 1994).

2.6. Preparation of EPS

Under the optimal conditions of producing EPS, the culture filtrates of *M. esculenta* SO-02 were collected. After one liter of the culture filtrate was concentrated fivefold under vacuum, it was mixed with 3 times of 80% ethanol (v/v) and left for overnight at −20 °C. The precipitate (EPS) was obtained after centrifugation (3000*g*, 15 min) and lyophilized to a powder form, which was applied to detect the antioxidant activities *in vitro*.

2.7. Superoxide radical (O_2^-) scavenging assay by EPS

The superoxide anion scavenging activity of EPS was determined according to the method of Stewar and Beewley (1980) with slight modification. The reaction mixture (3 ml) contained 13 mM methionine (MET), 10 mM riboflavin (RF), 75 μM nitroblue tetrazolium (NBT), 100 mM EDTA, 50 mM phosphate buffer (pH 7.8), and the EPS (5–250 mg/l). After illuminating the reaction mixture with a fluorescent lamp at 25 °C for 30 min, the absorbance of the EPS was measured at 560 nm, using BHT for a positive control. The whole reaction was assembled in a box lined with aluminium foil. The inhibition percentage of superoxide anion generation was calculated using the following formula:

$$\%\ \text{Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the EPS/BHT.

2.8. Hydroxyl radical (OH) scavenging assay by EPS

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton (1984). The reaction mixture contained 1 ml of 0.15 M phosphate buffer saline (pH 7.4), 1 ml of 40 μg/ml safranin, 1 ml of 0.945 mM EDTA–Fe (II), 1 ml of 3% (v/v) H_2O_2, and 0.5 ml of the EPS (5–250 mg/l). After incubating at 37 °C for 30 min, the absorbance of the EPS was measured at 560 nm, using BHT for a comparison. The EC_{50} value (mg/l) of EPS or BHT is the effective concentration at which the hydroxyl radicals were scavenged by 50%. The hydroxyl radical scavenging activity was expressed as:

$$\text{Scavenging activity}\ (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of EPS/BHT.

2.9. Determination of reducing power of EPS

The reducing power of EPS was evaluated according to the method of Oyaizu (1986) with slight modification. The reaction mixtures contained 2.5 ml phosphate buffer (pH 6.6, 0.2 M), 2.5 ml potassium ferricyanide (1%, w/v) and the EPS (5–250 mg/l). After incubating at 50 °C for 20 min, 2.5 ml of trichloroacetic acid (10%, w/v) was added to the mixture for terminating the reaction, and then centrifuged at 1200*g* for 10 min. An aliquot of 2.5 ml supernatant was collected and mixed with 2.5 ml deionized water and 0.5 ml $FeCl_3$ (0.1%, w/v). After incubating at room temperature for 15 min, the absorbance of the EPS was measured at 700 nm, using BHT for a comparison.

2.10. Statistical analysis

All experiments were carried out in triplicate and all the data were expressed as means ± SD (standard deviation). The statistics significance was evaluated using Student's *t*-test and $P < 0.05$ was taken as significant.

3. Results and discussion

3.1. Medium optimization for EPS production

Some researchers have optimized the liquid medium component for EPS production from *P. sajor-caju* (Confortin et al., 2008), *C. militaris* (Kim et al., 2003) and *T. mesenterica* (De Baets et al., 2002), but the optimal carbon and nitrogen sources and environmental conditions of producing EPS from *M. esculenta* SO-02 have not been reported. The results of the various carbon and nitrogen sources affecting the DCW and EPS content are described in Table 1. The highest production of EPS (2301 ± 212 mg/l) was obtained in the presence of glucose as carbon source in medium, while the DCW was 7.3 ± 0.5 g/l. The DCW (7.5 ± 0.6 g/l) in medium containing xylose was a little higher than that in medium containing glucose, but the EPS content was only 838 ± 70 mg/l, significantly lower than that in medium containing glucose. For the EPS content examined in the present study, glucose was determined as the best carbon source, which was in accordance with the results reported by other researchers in different mushrooms (De Baets et al., 2002; Kim et al., 2003). ANOVA showed the different effect of the five carbon sources on DCW ($P < 0.01$) and on EPS content ($P < 0.05$) (Table 1). When yeast extract was used as nitrogen source, the maximum production of EPS (1733 ± 157 mg/l) was obtained, and the DCW was 6.3 ± 0.4 g/l, slightly lower than that in medium containing peptone (6.3 ± 0.5 g/l) and ammonium sulfate (6.5 ± 0.4 g/l), respectively. ANOVA revealed the diverse effects of six nitrogen sources on biomass ($P > 0.05$) and on EPS content ($P < 0.05$) (Table 1). It is known that the organic nitrogen sources were generally better than inorganic nitrogen sources for EPS production in submerged culture of mushrooms (Confortin et al., 2008; De Baets et al., 2002).

Table 3
Results of orthogonal experiments for medium optimization.

No.	(A) Bran (g/l)	(B) Glucose (g/l)	(C) Yeast extract (g/l)	(D) KH_2PO_4 (g/l)	(E) $MgSO_4{\cdot}7H_2O$ (g/l)	DCW (g/l)	EPS content (mg/l)
1	1	1	1	1	1	5.4 ± 0.4	190 ± 19
2	1	1	1	2	2	6.3 ± 0.6	473 ± 41
3	1	1	1	3	3	6.6 ± 0.5	457 ± 39
4	1	2	2	1	2	7.5 ± 0.6	835 ± 76
5	1	2	2	2	3	6.9 ± 0.6	774 ± 61
6	1	2	2	3	1	7.8 ± 0.6	819 ± 79
7	1	3	3	1	3	8.2 ± 0.6	2263 ± 206
8	1	3	3	2	1	8.4 ± 0.7	1108 ± 106
9	1	3	3	3	2	8.7 ± 0.7	1308 ± 125
10	2	1	3	2	3	7.2 ± 0.7	1295 ± 115
11	2	1	3	3	1	6.6 ± 0.5	812 ± 65
12	2	1	3	1	2	6.3 ± 0.5	800 ± 60
13	2	2	1	2	1	8.6 ± 0.6	1136 ± 102
14	2	2	1	3	2	7.1 ± 0.6	1358 ± 117
15	2	2	1	1	3	8.1 ± 0.6	777 ± 62
16	2	3	2	2	2	8.0 ± 0.7	1206 ± 110
17	2	3	2	3	3	7.8 ± 0.7	1577 ± 142
18	2	3	2	1	1	7.3 ± 0.7	1231 ± 116
19	3	1	2	3	2	7.9 ± 0.6	2281 ± 210
20	3	1	2	1	3	8.4 ± 0.7	1806 ± 182
21	3	1	2	2	1	7.4 ± 0.7	2504 ± 237
22	3	2	3	3	3	8.0 ± 0.7	1692 ± 145
23	3	2	3	1	1	7.5 ± 0.6	1539 ± 139
24	3	2	3	2	2	8.1 ± 0.7	1365 ± 128
25	3	3	1	3	1	8.5 ± 0.7	1752 ± 134
26	3	3	1	1	2	8.5 ± 0.7	2501 ± 233
27	3	3	1	2	3	8.6 ± 0.7	2766 ± 230
K_1	7.32	6.91	7.66	7.43	7.58		
K_2	7.84	7.72	8.05	7.96	7.77		
K_3	8.11	8.65	7.62	7.87	8.04		$\Sigma = 20.85$
R	0.81	1.72	0.47	0.53	0.56		
$P > F$[a]	0.04295*	0.00011**	0.35678	0.30357	0.21159		
K'_1	958.41	1213.09	1523.49	1438.13	1232.49		
K'_2	1132.66	1143.95	1481.52	1591.52	1447.30		
K'_3	2311.85	2045.89	1397.92	1372.86	1723.14		$\Sigma = 39626.35$
R′	1353.44	901.94	125.57	219.05	490.65		
$P > F$[b]	0.00077**	0.01617*	0.91663	0.76610	0.30091		

* $P < 0.05$.
** $P < 0.01$.
[a] DCW.
[b] EPS content.

Based on the results of glucose and yeast extract as best carbon and nitrogen sources, they were selected and applied to optimize the medium composition by orthogonal experiments in the submerged culture. The design of five-factor-three-level orthogonal test is shown in Table 2 and the results are described in Table 3. Among these substrates, bran showed significant influence on EPS content ($P < 0.01$), and glucose had the same effect on DCW ($P < 0.01$). The optimal medium composition was obtained as follows (g/l): bran 200, glucose 30, yeast extract 1, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 1.5. Under this condition, the DCW and EPS content were 8.6 ± 0.7 g/l and 2766 ± 230 mg/l, respectively.

3.2. Cultivation conditions optimization for EPS production

Table 4 showed that *M. esculenta* SO-02 could grow at initial pH value ranging from 4.5 to 9.5, and the DCW (6.5 ± 0.5 g/l) and EPS content (1977 ± 170 mg/l) reached their maximum at initial pH 6.5. By ANOVA, the initial pH value had a significant influence not only on mycelial biomass but also on EPS content ($P < 0.01$). Being the critical factor in biomass accumulation and metabolite formation, the lower pH value was favorable for higher production of EPS in some basidiomycetes (Fang & Zhong, 2002; Lee, Lee, & Lee, 1999; Winder, 2006). The DCW was 6.4 ± 0.5 g/l at 20 °C, slightly higher than that at the others, but the EPS content (1498 ± 128 mg/l) was obviously lower than that at 25 °C (1664 ± 142 mg/l). We chose 25 °C as optimal culture temperature in submerged culture by *M. esculenta* SO-02, in agreement with the result of Wu and An (2004). But it was unknown that the effect of culture temperature on biomass and EPS content had no statistical significance ($P > 0.05$).

With the prolongation of culture time, the DCW remarkably increased until day 4 ($P < 0.01$), and the maximal EPS content (1994 ± 107 mg/l) was obtained at the same time ($P < 0.01$). The DCW and EPS content decreased gradually after 4 days, similar to the results reported by Yang, Chen, Shi, and Wang (2006) in *Pleurotus ferulae*. The volume of liquid medium had a significant effect on biomass ($P < 0.05$) and EPS content ($P < 0.01$) (Table 4). The maximum DCW (6.6 ± 0.5 g/l) and EPS production (2314 ± 201 mg/l) were obtained in 250-ml Erlenmeyer flask containing 100 ml liquid medium, which was essentially in keeping with our previous report in *P. nebrodensis* (Jia et al., 2007). The DCW of *M. esculenta* SO-02 increased gradually (Table 4) with the rotary speed from 100 to 250 rpm, and the EPS content (2472 ± 217 mg/l) reached maximum at 200 rpm. The results showed the cultivation conditions of temperature 25 °C, cultivation time 4 days, initial pH 6.5, volume of medium 100 ml and rotary speed 200 rpm.

Table 4
Effect of cultivation conditions on DCW and EPC by *M. esculenta* SO-02 in submerged culture.

Cultivation condition	DCW (g/l)	EPS content (mg/l)
Cultivation time (d)	**	**
2	3.1 ± 0.2	994 ± 82
3	4.4 ± 0.4	1397 ± 125
4	6.9 ± 0.5	1994 ± 107
5	6.2 ± 0.5	1645 ± 141
6	5.7 ± 0.4	1281 ± 116
7	5.9 ± 0.4	1075 ± 877
8	5.9 ± 0.5	664 ± 59
Temperature (°C)	*	
20	7.4 ± 0.5	1498 ± 128
25	6.2 ± 0.5	1664 ± 142
30	6.0 ± 0.5	1470 ± 122
Initial pH		**
4.5	5.4 ± 0.5	1218 ± 106
5.5	5.6 ± 0.4	1836 ± 168
6.5	6.5 ± 0.5	1977 ± 170
7.5	6.1 ± 0.6	1805 ± 167
8.5	5.9 ± 0.4	1355 ± 111
9.5	5.5 ± 0.4	1240 ± 104
Volume of medium (ml)	*	**
50	5.6 ± 0.4	1622 ± 142
100	6.6 ± 0.5	2314 ± 201
150	4.3 ± 0.4	1386 ± 115
200	3.7 ± 0.3	1323 ± 116
Rotary speed (rpm)	**	*
100	4.7 ± 0.4	1736 ± 150
150	5.3 ± 0.4	2055 ± 186
200	6.2 ± 0.5	2472 ± 217
250	6.6 ± 05	2376 ± 219

* $P < 0.05$.
** $P < 0.01$.

3.3. Validation of the model

Under the optimal medium composition and cultivation conditions, the DCW and EPS content determined were 9.2 ± 0.7 g/l and 2913 ± 262 mg/l, respectively, which were 42 ± 3% and 100 ± 7% than those (6.5 ± 0.4 g/l, 1455 ± 128 mg/l) in basal liquid medium, respectively. The model was optimal for EPS production by *M. esculenta* SO-02.

3.4. Antioxidant activity of EPS

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, binding of transition metal ion catalysts, etc. (Frankel & Meyer, 2000). In this experiment, the antioxidative activities of EPS from *M. esculenta* SO-02 *in vitro* were evaluated using different biochemical methods of reducing power activity, superoxide anion radical and hydroxyl radical scavenging assay.

Fig. 1 showed that the inhibition percentages of superoxide anion of EPS and BHT to scavenge superoxide radical were directly proportional to their concentrations. The EC_{50} values of superoxide radical scavenging activity were 105 ± 2 mg/l for EPS ($P < 0.01$) and 111 ± 4 mg/l for BHT ($P < 0.05$). Although the inhibition percentage of EPS was slightly lower than that of BHT at the dosage range of 5–250 mg/l, but it reached 36 ± 2% at 20 mg/l, much higher than 11 ± 1% of *P. nebrodensis* (Sheng et al., 2008) and 10% of *A. blazei* (Zhang et al., 2004), respectively, indicating that the EPS significantly affects the scavenging of the superoxide radical.

As shown in Fig. 2, the hydroxyl radical scavenging activity of EPS was concentration-dependent and weaker than that of BHT at the dosage range of 0–175 mg/l, the scavenging capacity of EPS rapidly increased and exceeded that of BHT with the continually increasing its concentrations. The EC_{50} value of EPS for hydroxyl radical scavenging activity was 103 ± 4 mg/l ($P < 0.01$), which has no significant difference from scavenging effect of BHT (99 ± 4 mg/l, $P < 0.05$).

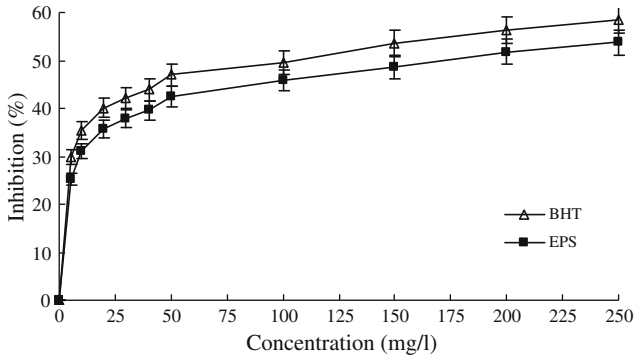

Fig. 1. Scavenging effects on superoxide anion radicals.

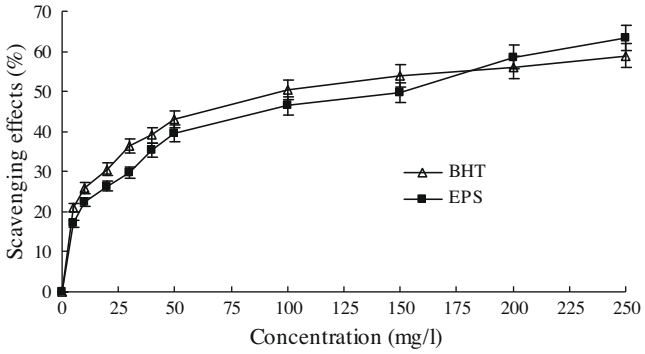

Fig. 2. Scavenging effects on hydroxyl radicals.

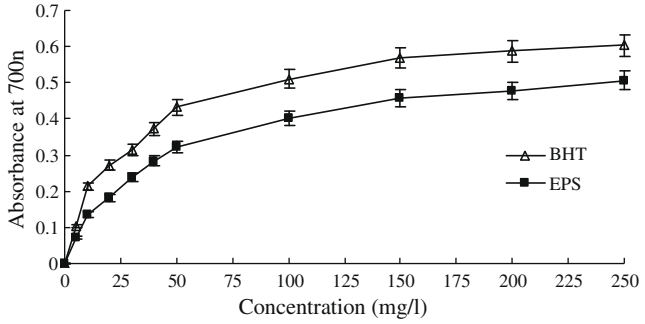

Fig. 3. Reducing power of EPS/BHT.

Sheng et al. (2008) reported that the scavenging ability of EPS from *P. nebrodensis* on hydroxyl radicals was 19 ± 1% at 20 mg/l. In this study, the hydroxyl radical scavenging activity was 26 ± 1%, which was higher than that reported earlier.

It can be seen from Fig. 3 that the reducing capacity of EPS was slightly lower than that of BHT. The reducing power (absorbance at 700 nm) of EPS was 0.48 ± 0.02 at a dose of 200 mg/l, remarkably higher than that (0.13 ± 0.05) at the same dosage in *P. nebrodensis* (Sheng et al., 2008), showing that the EPS from *M. esculenta* SO-02 has potential antioxidant activities.

4. Conclusion

To date, no reports are available in the literature regarding the optimization of EPS production by *M. esculenta* SO-02 in submerged culture and its antioxidant activities *in vitro*. The results obtained would provide references for large-scale production of EPS by *M. esculenta* SO-02 as potentially functional food or antioxidants. However, the biological activities and antioxidant mechanism of EPS are areas for future studies.

Acknowledgment

The authors gratefully acknowledge the financial supports by Natural Science Fund Program of Shandong (Y2006D08) and Doctoral Fund Program of Shandong (2007BS02021).

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