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Preparation of alkali-soluble polysaccharide from culture mycelium of *Lentinula edobes* L0010 using mathematical model and associated physicochemical properties

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ABSTRACT

The extraction process of alkali-soluble polysaccharide from Lentinula edobes L0010 (ASP) and associated physicochemical properties were studied. Three independent variables including extraction time (X1), alkali concentration (X2) and ratio of solution to solid (X3) were selected to optimize the extraction process based on Box-Behnken design. The results demonstrated that the optimal conditions for the ASP extraction were extraction temperature 70.82 ℃, alkali concentration 8.95% and ratio of solid to liquid 35.81 mL/g. Under these conditions, experimental extraction of ASP was 23.45±0.019% (n= 5), similar to the predicted yield of 23.78%, the major ASP-2 fractionated from the crude ASP by DEAE-Sepharose and Sephacryl S-400 chromatography was analyzed to be composed of mannose, ribose and mannose in the molar ratio of 17.3: 28.3: 41.6. And the weight-average molecular weight and radius of gyration of ASP-2 in 0.1 M NaCl were determined by SEC-MALLS to be 3.56×106 g/mol and 40.3 nm, respectively. Further it exerted potent DPPH free radical scavenging activity in vitro with EC50 values of 48.5 μg/mL.

Keywords: *Lentinula edobes*, alkali-soluble polysaccharide, response surface methodology, physicochemical property

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INTRODUCTION

Lentinula edobes (Shiitake) is the second most popular culinary delicacy and has traditionally been used as health food in Asia dating back over 2000 years [1,2]. This fungi has attracted considerable attention because the extract from its fruiting body, mycelium or culture broth have been reported to present multiple therapeutic activities. Polysaccharides (lentinan), dietary fiber and ergosterol are of importance being attributed to its both nutritional value and medical value. Among those active ingredients, polysaccharides extracted from the mycelia of *L.edobes* showed significant immunomodulatory effect[3], ability of activating lympholutes, proliferative inhibition, function of promoting immune and free radical scavenging of tissue in immunosuppressive rates and obvious anti-fungal activities. However, despite this extensive utilization of *L.edobes*, no information has been published about the alkali-extractable polysaccharides.

Most bioactive polysaccharides have been found to be closely related with their various chain conformations in solution, such as the molecular weight and chain conformation [4]. Some valuable information on the molecular characteristics of the polymer molecules such as Z-average root-mean square radius of gyration $(R_g^2)_z^{1/2}$, weight-average molecular weight and polydispersity index could provide insights into the physico-chemical behavior and indicate effective application of the biopolymer [5,6]. Therefore, it is essential to acquire basic parameters of biomacromolecules in solution for the development of health food.

In the case of extraction of water-soluble carbohydrates, monosaccharide residues are bound into a polysaccharide molecule by glycosidic bonds, which are fairly stable in alkaline and neutral solution [7]. In contrast, the ester linkage in the alkali-soluble sugars can be hydrolyzed by alkali treatments. The other hydrogen bonds and van der Walls bound with macromolecules can be destroyed by heat without significant destruction of polymer structure. Box-Behnken design has been reported to be more efficient and easier to evaluate the model and determine multiple factors as well as possible interactions between independent variables with only three levels and fewer experiments involved [8,9].

In this work, Box-Behnken design, followed by canonical and ridge analyses, was employed to optimize the process parameters of alkali-soluble polysaccharide (ASP) extraction from the cultured mycelium of *L.edobes*-L0010. After the fractionation of the crude ASP by DEAE-Sepharose and Sephacryl S-400 column, the physicochemical properties of the major fraction were carried out by HPLC and SEC-MALLS analysis as well.

EXPERIMENTAL SECTION

2.1 Materials

Monosaccharide standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Sijiqing Biological Engineering Materials Co. (Hangzhou, China). DEAE-Sepharose FF column was purchased from Whatman Co. (Britain). Sephacryl S-400 and T-series Dextrans were purchased from Pharmacia Co. (Sweden). Glucose, peptone, ethanol, yeast extract, KH_2PO_4, $MgSO_4$, $FeSO_4$ and other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

The L0010 strain of *L. edobes* collected in Qingyuan County, Zhejiang province of China was used in this study. A seed culture was carried out in Erlenmeyer flasks (250 mL) with different volumes of fermentation medium according to a design in which the composition was 26.8 g of glucose, 3.9 g of peptone, 0.6 g of $FeSO_4$•$7H_2O$, 0.5 g of $MgSO_4$•$7H_2O$, and 1,000 mL of distilled water. After autoclaving at 121℃ for 25 min and cooling to 25℃, flasks were inoculated with mycelia from 10-day-old plates.

2.2 Preparation of ASP

At the end of the *L.edobes*-L0010 submerged fermentation period, the brownish culture broth was centrifuged at 10,000×g for 15 min at 4℃. Mycelia were washed several times with distilled water and lyophilized to a constant weight. The lyophilized mycelia of *L.edobes* were ground in a sample mill to pass through No. 60 mesh after oven drying for 3 days at 60℃. The powdered material was refluxed in 80% ethanol for 6 h to remove some colored materials, monosaccharides, oligosaccharides, and small molecule materials. The residue was dried at room temperature for 24 h prior to extraction. Subsequently, the residue was blended with different concentration of NaOH (From 4.0% to 12.0%) at certain temperature for 2 h in a water bath and the residue was re-extracted under the same condition. The combined filtrate was neutralized by 20% H_2SO_4 until pH 5.0. Sediment of alkali-soluble polysaccharides (ASP) was filtered, washed with water until a neutral reaction, and then washed with 70-95% ethanol, acetone and ethyl acetate. The sediment was dissolved in distilled water to certain volume in which the ASP concentration was determined according to the classical method of Dubois *et al.*[10] using glucose solution as a standard reference.

2.3 Response surface methodology for extraction

A Box-Behnken design (BBD) (Design Expert Software, trial version 7.1.3; Stat-Ease Inc., Minneapolis, MN, USA) was used to determine the best combination of fermentation variables for production of ASP by *Lentinula edobes.* 3 variables were established on the basis of "one-factor-at-a-time" trials for ASP extraction (Table 1). The design included 17 experimental trials (Table 2). A total of 5 replicates at the center of the design were used to allow for estimation of a pure error sum of squares. Each experiment was performed in triplicate and the yield of ASP (%) was interpreted as the response (*Y*).

Table 1.Levels and coding of variables for a Box–Behnken design

Variable	Symbol		Coded level		
	Uncoded	Coded	-1	0	1
Extraction temperature (℃)	x_1	X_1	40	60	80
Alkali concentration (%)	x_2	X_2	4	7	10
Ratio of solution to solid (mL/g)	x_3	X_3	20	30	40

2.4 Fractionation of ASP

The crude ASP was dissolved in distilled water with ultrasonic treatment and then deproteined using Sevag method (n-buthanol: chloroform=1:4, *v*/*v*). Then the deproteined ASP was dissolved in distilled water and filtered with 0.45 μm membrane before it was loaded in a DEAE-Sepharose FF column (2.0 cm×35 cm). After the column was equilibrated with distilled water, it was washed with a range of 0.05 to 1.5 M NaCl containing 0.05 M PBS at a flow rate of 1.0 mL/min, with 3 mL fraction collected. The major peak was pooled, dialyzed completely and finally lyophilized. The obtained oligosaccharide was subjected to a Sephacryl S-400 column (1.5 cm×60 cm), which was eluted by a 0.1 M NaCl at a flow rate of 0.5 mL/min. Finally, the main fraction was obtained after desalt and lyophilization.

2.5 Physicochemical analysis

The monosaccharide components of ASP were analyzed by reverse-phase HPLC according to PMP (1-phenyl-3-methyl-5-pyrazolone) derivatization procedures with some modification [11]. Briefly, 11 standard monosaccharides or hydrolyzed sample were dissolved in 0.3 M NaOH (75 μL) and a 0.5 M PMP (50 μL) before the derivatization. Then the mixture was neutralized by 75 μL of 0.3 M HCl solution and was finally filtered through 0.22 μm membrane. 10 μL of the resulting solution was injected into the RP-C18 column. The wavelength for UV detection was 245 nm. Elution was carried out at a flow rate of 1.0 mL/min at 25℃. The mobile phase was a mixture of 0.05 M KH_2PO_4 (pH 10)-acetonitrile (83:17). Sugar identification was done by comparison with 11 reference sugars (rhamnose, ribose, arabinose, xylose, mannose, galactose, glucose, fucose, galacturonic acid, glucuronic acid and glucosamine).

Molecular weight of ASP was measured by means of size exclusion chromatography with a multi-angle laser light scattering system (DAWN HELEOS II, Wyatt Technology, USA). The system included a pump, a degasser, an injection valve fitted with a 100 μL loop, SEC columns, a multi-angle laser light scattering detector and a refractive index detector. Samples were dissolved directly in ultrapure water (1-3 mg/mL), and filtered through 0.22 μm filter membranes (Millipore, USA) prior to injection into the SEC/MALLS system. Nitrate buffer was used as the mobile phase, which containing 0.1 M NaCl containing 0.02% NaN_3, then filtered over a filter membrane with pore size 0.22 μm, and degassed by ultrasonic cleaner for several minutes [12].

2.6 Antioxidant activity *in vitro* by DPPH assay

The free-radical scavenging capacity of ASP was measured by 1,1-diphenyl-2-picryldydrazyl (DPPH) test according to the method of Blois [13] with some modifications. Briefly, 0.25 ml of various concentrations (0.05-1.0 mg/mL) of sample was added with 0.25 mL MeOH into a tube. Subsequently the solution was mixed by 2.5 mL of 0.075 mM DPPH in MeOH. Then the mixture was shaken vigorously and kept for 30 min in the dark, RT. The 517 nm absorbance was recorded against a blank. Ascorbic acid was used as positive control. The DPPH scavenging effect was calculated by the following equation:

$$S_C\% = [1 - (A_i - A_j)/A_c] \times 100\% \quad (1)$$

Where A_c is the absorbance of DPPH solution without sample (2.5 mL DPPH + 0.5 mL MeOH); A_i is the absorbance of the test sample mixed with DPPH solution (0.25 mL sample + 2.5 mL DPPH +0.25 mL MeOH); A_j is the absorbance of the sample without DPPH solution (0.25 sample + 2.75 mL MeOH).

2.7 Statistical analysis

Data were expressed as means standard error (SE) of three replicated determinations. The multiple regression analysis and analysis of variance (ANOVA) were carried out using a software Design-Expert 7.1.3 Trial to fit quadratic polynomial equations. The quality of the fitted model was expressed by the coefficient determination R^2, and its statistical significant was checked by *F*-test and *p*-value.

RESULTS AND DISCUSSION

3.1 Optimization of the ASP yield

On the basis of single factor experimental results, the 3 independent variables of extraction temperature, alkali concentration and ratio of solution to solid were analyzed to optimize the ASP yield from *Lentinula edobes* L0010. A total of 17 experimental runs were performed with different combinations of these 3 factors. Experimental and predicted values are shown in Table 2. There was a considerable variation in the ASP yield depending on different extraction conditions. The maximum ASP yield was 22.52% in run number 8, in which the conditions of extraction were a extraction temperature of 60.00℃, alkali concentration of 7.0%, and a ratio of solution to solid of 30.0 mL/g. While the minimum ASP yield was 11.47% in run number 12, and the center point in the design was repeated five times to estimate the error.

Table 2.The Box–Behnken design matrix with experimental and predicted values of the ASP yield

Run order	Extraction temperature (X_1)	Alkali concentration (X_2)	Ratio of solution to solid (X_3)	The ASP yield (g/L)	
				Experimental	Predicted
1	-1(40)	0(7)	1(40)	15.36±0.049	15.39
2	0(60)	1(10)	-1(20)	18.74±0.006	18.90
3	1(80)	1	0(30)	22.17±0.021	22.29
4	1	-1(4)	0	12.46±0.036	12.50
5	0	-1	-1	12.57±0.019	12.56
6	-1	0	-1	12.87±0.042	12.75
7	0	0	0	21.79±0.009	21.68
8	0	0	0	22.52±0.005	21.68
9	1	0	-1	16.98±0.027	16.94
10	0	-1	1	15.49±0.011	15.33
11	1	0	1	21.48±0.076	21.11
12	-1	-1	0	11.47±0.034	11.60
13	0	0	0	21.26±0.017	21.28
14	-1	1	0	14.56±0.042	14.52
15	0	0	0	21.55±0.018	21.68
16	0	1	1	21.43±0.023	21.69
17	0	0	0	21.25±0.015	21.68

Using multiple regression, data obtained were analyzed. The predicted response of Y for ASP was as follows:

$$Y\%=21.68+2.35X_1+3.11X_2+1.58X_3+1.66X_1X_2+0.50X_1X_3-0.059X_2X_3-3.45X_1^2-3.06X_2^2-1.56X_3^2 \quad (2)$$

Where Y is the predicted response variable (the yield of ASP as a %), and X_1, X_2, and X_3 are coded values of the independent variables of temperature, alkali concentration, and ratio of solution to solid, respectively.

The statistical significance of Eq. (2) was checked using Fisher's statistical ANOVA test (F-test) and results are shown in Table 3. The F-test indicated that the second model had a high model F value of 118.57 and a low p value of $p < 0.0001$, indicating the model was well suited for the experimental data. The adjusted coefficient of determination (R^2_{adj}) indicated that the sample variation of 98.51% for the ASP yield was attributable to the independent variables. The value of R(0.9967) shows a close agreement between the experimental results and the theoretical values predicted by the polynomial model [14]. A relatively low value of CV (2.78) illustrated further the experiments were practical with a better precision and reliability. The coefficient estimates of Eq. (2), along with the corresponding p-values are presented in Table 3 as well. It can be seen that most regression coefficients are significant except for two linear coefficients (alkali concentration X_2 and ratio of solution to solid X_3).

Table 3.ANOVA results of the fitted quadratic polynomial model for optimization of ASP yield[1)]

Source	Sum of squares	DF	Mean square	F-value	Probability $p>F$
Model	263.81	9	29.31	118.57	<0.0001
Lack-of-fit	0.63	3	0.21	0.76	0.5725
Pure error	1.10	4	0.28		
Corrected total	265.54	16			

[1)] R^2=0.9935; adj-R^2=0.9851;R=0.9967;CV=2.78%

Table 4.Results of regression analysis of a full second-order polynomial model for optimization of the ASP yield

Model term	Coefficient estimate	SE	Sum of squares	F-value	Probability $p>F$
Intercept	21.68	0.22			
X_1 (Extraction temperature)	2.35	0.18	44.34	179.36	< 0.0001
X_2 (Alkali concentration)	3.11	0.18	77.58	313.82	< 0.0001
X_3(Ratio of solution to solid)	1.58	0.18	19.87	80.36	< 0.0001
X_1X_2	1.66	0.25	10.97	44.38	0.0003
X_1X_3	0.50	0.25	1.02	4.13	0.0818
X_2X_3	-0.059	0.25	0.014	0.056	0.8202
X_1^2	-3.45	0.24	50.05	202.46	< 0.0001
X_2^2	-3.06	0.24	39.44	159.55	< 0.0001
X_3^2	-1.56	0.24	10.20	41.27	0.0004

3.2Analysis of response surface

The fitted response surface plots and their corresponding contour plots for ASP extraction by the previous model were shown in Fig.1, in which it provides a method to visualize the relation between the response and experimental levels of each variable, and the type of interaction among the testing variables with an aim of optimum conditions.

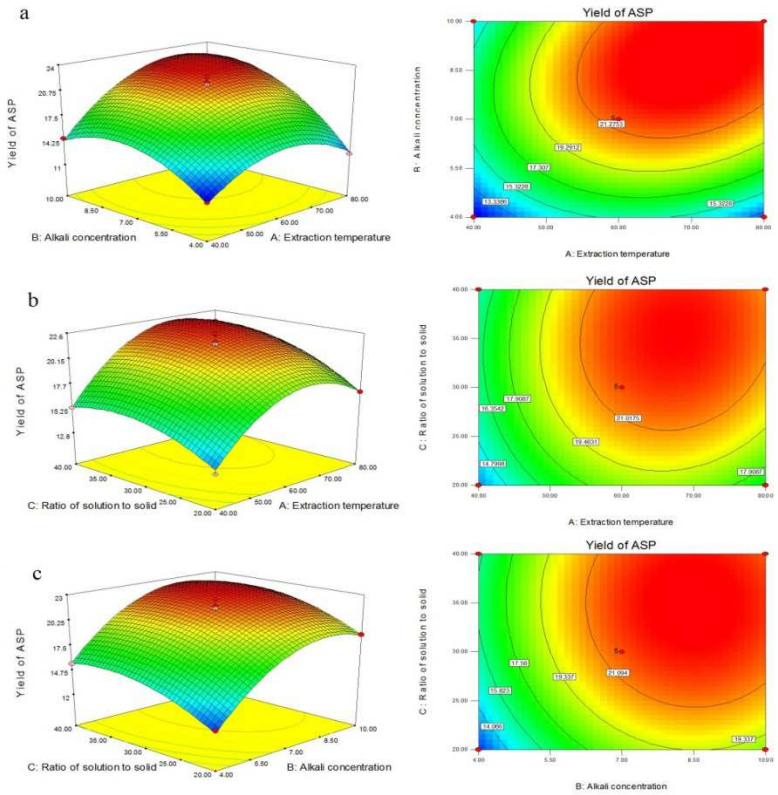

Figure 1.Response surface (left) and contour (right) plots of the effects of 3 variables on ASP extraction from *Lentinulaedobes* L0010. (A) Response surface plot showing the effects of extraction temperature and alkali concentration, and their mutual effect on yield of ASP. (B) Response surface plot showing the effect of extraction temperature and ratio of solution to solid, and their mutual effect on yield of ASP. (C) Response surface plot showing the effect of alkali concentration and ratio of solution to solid, and their mutual effect on yield of ASP

Fig. 1a indicated that an interaction between X_1 and X_2 was found to contribute to the yield of ASP at a significant level. This fact could be well accounted for by *p*-value (0.0001<0.05) in Table 3. It was evident that when the extraction temperature was from 55 to 68℃, and the alkali concentration was from 6.5% to 8.2%, the ASP yield was over 21.27% and then decreased slowly beyond this range. These facts were important in making the whole extraction process economically more feasible and made it possible to save energy in the future industrial application. Those were consistent with the results of *Lyciumbarbarum* polysaccharides [15]. The reason could probably be explained that the increasing energy can promote the process of extraction when the temperature and alkali concentration are at relatively low value. While high concentration of alkali could provide a possibility of degradation among the extracted carbohydrate polymers.

Fig. 1b showed that the yield of ASP climbed up continuously when the value of X_1 increased from 40℃ to 55℃. Then it went down slightly outside that optimum point. The curve of ratio of solution of in the contour plot was slightly inclined to horizontal showing that the yield of ASP was up to a saturated value regardless of the increasing X_3. With the assistance of ratio of solution to solid on the initial process of ASP from *Lentinula edobes*, it enhanced the diffusion process and provided a greater space of components into solvent during the extraction. However it had no effect on the yield of ASP at relatively high ratio, which might be explained by decreasing the available surface area between alkaline solvent and the cells. Those findings could also be found in the report made by Sun *et al*. [16]. While Fig. 1c described that ASP yield increased gradually with the increasing alkali concentration and ratio of solution to solid. While the alkali concentration was at a low level, the effect of extraction temperature on the response was insignificant. It was obvious that the yield of ASP was higher than 21.09% when alkali concentration was in the range of 7.3% to 9.0% (actual value) and ratio of solution to solid in the range of 32 mL/g to 38 mL/g. Miyajima*et al.*[7] reported that alkali treatment played a critical role in the extraction of polysaccharides by easily hydrolyzing the O-glycosidic bonds.

3.3.Optimization of extracting parameters and validation of the model

By applying the software of Design-Expert (7.1.3), the maximum predicted value and the predicted optimum conditions for ASP extraction could be obtained quietly. Those were as following: extraction temperature 70.82℃,

alkali concentration 8.95% and ratio of solid to liquid 35.81 mL/g, and a maximum response of 23.78% was predicted by the model equation. To confirm the predicted value was not bias toward the practical result, further experiments were carried out under the slightly modified optimal conditions. A mean value of 23.45±0.019% (*n*=5) acquired from real experiments, demonstrated the validation of the RSM model. It drew a conclusion that the model of Eq. (3) developed was considered to be satisfactory and accurate for predicting the yield of ASP from *Lentinula edobes*.

3.4. Physicochemical properties of ASP

The crude ASP obtained from the mycelium of *Lentinula edobes* L0010 was subjected to fractionation on DEAE-Sepharose FF column(2.8×45 cm) with gradient elution to give two elution peaks (Fig. 2), as detected by the phenol-sulfuric acid assay. Among them, ASP-1 was not further investigated here due to its small amount. The other major fraction of ASP-2 (80.6%) was then pooled and further purified by gel chromatography on a Sephacryl S-400 (60×1.6 cm) column. The process was repeated several times until it presented one symmetrical peak with a purity of ＞99.6% in ASP-2, using the phenol-sulfuric acid method. Herein, we came to a conclusion that there was none of protein existed in ASP-2, verified further by no absorbance peak at 280 nm in UV spectrum.

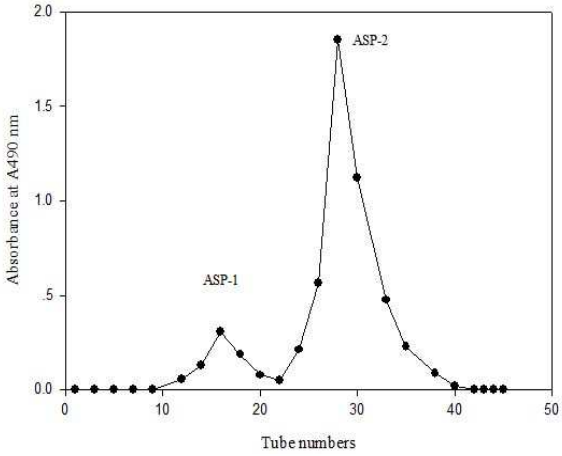

Figure 2.Elution profiles of the crude ASP from *L.edobes* by DEAE-Sepharose FF column

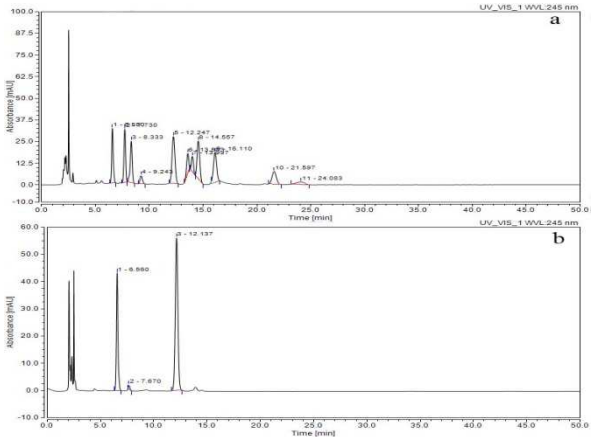

Figure3.HPLC chromatograms of PMP derivatives of component monosaccharides released from (a)ASP-2and (b) sugar standards

The composition of ASP-2 shown in Fig. 3, determined by HPLC analysis as PMP derivatives, indicated that it was composed of mannose, ribose and mannose in the molar ratio of 17.3: 28.3: 41.6. In term of peak area, the two predominant monosaccharides were glucose and mannose, which was similar with the results of alkali-extract polysaccharide from cultured mycelia of *Cordycepsmilitaris* made by Yu *et al*. [17]. They reported that D-Mannose, D-Galactose and D-Glucose were identified in the hydrolysate of CBP-1 in a ratio of 2.81: 4.01: 1.00, which was slightly different with the major component in ASP-2.

The SEC/MALLS approach can provide great insight into the characterization of biomacromolecules by automatically measuring the various physical properties of every sample including molar mass, hydrodynamic sizes and distribution. And the advanced conformation of biopolymers has been reported to be closely related to their biological activities [3]. Figure 4 shows the elution profile of ASP-2 for the determination of molecular mass, in which it presented a single peak monitored both by both LLS and RI detectors. After the calculation of its molar mass with the formula of Rayleigh-Debyte, the number-average molecular weight (M_n), weight-average molecular weight (M_w) and z-average molecular weights (M_z) of ASP-2 in 0.1 M NaCl were determined to be 3.13×10^6 g/mol, 3.56×10^6 g/mol and 4.05×10^6 g/mol, respectively. Interestingly, Zhang *et al.*[18] reported that the triple-helix lentinan with Mw of 1.49×10^6 g/mol was obtained from water-soluble polysaccharides in *Lentinus edobes*, which was quite similar with that of alkali-soluble polysaccharide. The $(R_g^2)_z^{1/2}$ value, one of parameters of macromolecules, was usually a measure regarding how far from the center of mass and how the mass of the polymer chains was concentrated. The bioactive lentinan has been found to have a potent antitumor activity due to its advanced conformation with an $(R_g^2)_z^{1/2}$ of 60.5 nm [19,20]. A $(R_g^2)_z^{1/2}$ value of 40.3 nm for ASP-2 reflecting its polymer chains with more compact conformation showed that it might have a similar antitumor activity with lentinan. Furthermore, the 1.138 of polydispersity (M_w/M_n) indicated that ASP-2 has a relatively narrow molecular-weight distribution.

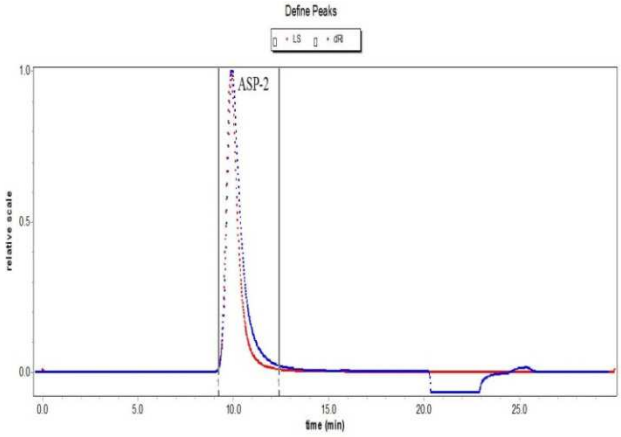

Figure 4.Elution profile of ASP-2 for the determination of molecular mass in a SEC-MALLS system

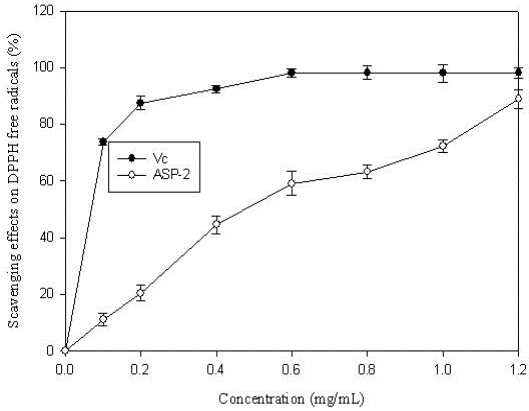

Figure 5.Scavenging effects of ASP-2 and control standard on DPPH free radicals; data are presented as mean value (n=3)

3.5. *In Vitro* antioxidant assay

The DPPH is a stable organic free radical with a maximum absorption band around 515-528 nm and thus, it is widely accepted as a tool for estimating the free radical scavenging activities of antioxidants [21,22]. Fig. 5 illustrates that a concentration-dependent, radical-scavenging ability at a series of concentration of Vc and ASP-2, the scavenging effects of them were 98.7% and 72.4% at the dose of 1.0 mg/mL respectively. These results suggested that ASP-2 has an obvious effect on scavenging free DPPH radical at relatively low amount of addition. And the EC50 value of ASP-2 was found to be 48.5 μg/mL. From dose of 0.1 to dose of 0.6 mg/mL, ASP-2 could donate more hydrogen to combine with DPPH radical when it was purified completely, which was fairly higher than that of water-soluble polysaccharide obtained from the same mycelium of *Lentinus edobes*[23].

CONCLUSION

It was valuable for the preparation of alkali-soluble polysaccharide (ASP) from the mycelium of *Lentinula edobes* L0010 by using response surface methodology and regression analysis. All coefficients had a significantly positive influence on the response values, regardless of the interaction between extraction temperature and ratio of solution to solid ($p<0.8202$). The optimal conditions for the ASP extraction were as following: extraction temperature 70.82 ℃, alkali concentration 8.95% and ratio of solid to liquid 35.81 mL/g. Under these conditions, experimental extraction of ASP was 23.45±0.019% (n=5), similar to the predicted yield of 23.78%. Further, ASP-2, the major fraction obtained from the crude ASP by DEAE-Sepharose and Sephacryl S-400 chromatography was analyzed to be composed of mannose, ribose and mannose in the molar ratio of 17.3: 28.3: 41.6. And the weight-average molecular weight and radius of gyration of ASP-2 in 0.1 M NaCl were determined by SEC-MALLS to be 3.56×10^6 g/mol and 40.3 nm, respectively.

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