Original Research Paper

Optimization of Chelating Process of Polysaccharides of *Lyophyllum decastes* with Zinc and Its Antioxidant Activity

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Abstract: In this study, polysaccharides of *Lyophyllum decastes* (LDS) were used as raw material. The chelating rate was set as the index, the effect of chelating time, mass ratio and initial mass concentration on chelating rate was investigated. Response surface method was used to optimize the preparation technology of LDS and Zinc (II) chelate (LDS-Zn) based on single factor experiments. LDS-Zn was analyzed by FTIR. Antioxidant activity in vitro of LDS and LDS-Zn was investigated. Research results showed that optimal preparation conditions of LDS-Zn were as follows: Chelating time was 4 h, mass ratio (LDS with zinc) was 6:1, initial mass concentration (zinc) was 4 mg/mL. LDS achieved the best chelating rate as high as 87.08%. Infrared spectroscopy analysis showed that Zinc (II) successfully chelated with LDS. Compared with LDS, LDS-Zn had better scavenging effect on DPPH free radicals and hydroxyl radicals with an increase of antioxidant activity by 23.46 and 16.77%, respectively. These results indicated that LDS-Zn can be expected to serve as a nutritional Zn supplement with antioxidant activity.

Keywords: *Lyophyllum decastes*, Polysaccharide, Single Factor, Chelation, Response Surface, Antioxidant

Introduction

Lyophyllum decastes is a delicious food with medicinal properties (Cheng, 2014). Its fruit body is rich in anticancer polysaccharides and has adjuvant treatment effects on six major diseases, such as diabetes and hypertension. It is a low-calorie, low-fat health care product that has the unique effects of preventing constipation, nourishing beauty, preventing and fighting cancer, improving immunity, increasing intelligence and prolonging life (Shang, 2014). In recent years, polysaccharide Zn-chelate has become a research hotspot as an organic Zn supplement. Some synthetic polysaccharide Zn-chelate included *Flammulina velutipe* polysaccharide-Zn^{2+} chelate (Zhao *et al*., 2016; He and Lu, 2011), *Pleurotus eryngii* polysaccharide-Zn^{2+} chelate (Gu *et al*., 2019), black fungus polysaccharide Zn complex (Li and Wang, 2006), d-glucosamine Zn complex (Raikwar *et al*., 2019) and chitosan Zn complexes (Ding *et al*., 2003). These synthetic polysaccharide Zn complexes can be developed into a new Zn supplement (Debon and Tester, 2001; Hedemann *et al*., 2006; Namkung *et al*., 2006). However, few studies on the chelation and bioactivity of polysaccharides of

Lyophyllum decastes (LDS) and trace elements have been conducted.

In this study, polysaccharide (LDS) was selected as raw material. The chelation of polysaccharides (LDS) and Zn(II) were studied. Furthermore, the effect of chelation time, mass ratio and initial Zn(II) concentration on the chelation of polysaccharide (LDS) and Zn(II) were studied. Optimal chelation parameters were determined by response surface experiment. The chelated product was analyzed by Fourier transform infrared spectroscopy. So far, some polysaccharide zinc complexes such as *Dictyophora indusiata* polysaccharide-zinc complex with antiproliferative activity (Liao *et al*., 2015) and *Prunella vulgaris* polysaccharide-zinc complex with antiproliferative activity (Li *et al*., 2016) have been successfully prepared. Fructus Mori polysaccharide and zinc complex(MFP4P-Zn) had higher antioxidant and hypoglycemic activities than MFP4P at the same concentration (Wang *et al*., 2019). The integration of *Fritillaria ussuriensis* polysaccharide and Zn greatly enhanced the antioxidant activities due to the synergistic effect, which provided vital theoretical basis for the promising antioxidant and zinc supplement

(Zhang *et al.*, 2020). In the present study, optimization of chelating process of LDS with Zinc (LDS-Zn) and its antioxidant activity were investigated. LDS-Zn could be used as an emerging Zn supplement with antioxidant activity in the future. Therefore, the study can provide a theoretical basis including preparation and antioxidant activity for the development of a novel natural zinc supplement (LDS-Zn).

Materials and Methods

Material and Reagent

LDS was purchased from Fujian Gutian Rongxiang Biological Technology Co., Ltd.; DPPH (1,1-diphenyl-2-brothquinone) was purchased from Kuer Chemical Technology (Beijing) Co., Ltd.; Tris (tris hydroxy methyl aminomethane) was purchased from Wuhan Benjamin Pharmaceutical Co., Ltd.; $CH_3CH_2CH_2CH_2OH$, CH_3CH_2OH, NaOH, H_2O_2, $FeSO_4 \cdot 7H_2O$, HCl, H_2SO_4, $C_7H_6O_3$, $ZnSO_4 \cdot 7H_2O$, $ZnCl_2$ and $Zn(CH_2COO)_2 \cdot 2H_2O$ are all of analytical purity.

Instruments and Equipment

722N visible spectrophotometer was from Beijing Kangfulai Technology Co., Ltd.; Fourier infrared spectrometer was from Tianjin Gangdong Technology Development Co., Ltd.; SP-3520 Atomic Absorption Spectrophotometer was from Shanghai Zhongyong Testing Equipment Co., Ltd.; HH-6 water bath was from Changzhou Kaihang Instrument Co., Ltd.; N-1300D-W Rotary Evaporator was from Shanghai Zhucheng Industrial Co., Ltd.; CAV264 electronic precision balance was from Shenzhen Huaiyi Electronic Technology Co., Ltd.; UV-6300 UV-visible spectrophotometer was from Shandong Chentuo Scientific Instrument Co., Ltd.; NHWY-111B constant temperature shaker was from Shenzhen Yixin Instrument Equipment Co., Ltd.; TD5A-WS centrifuge was from Xiangyi Centrifuge Instrument Co., Ltd.; XY-10 crusher was from Hebei Baoer Technology Development Co., Ltd.; DF-101S constant temperature heating magnetic stirrer was from Shanghai Tin, Scientific Instrument Co., Ltd.; pH meter was from Shandong Detian Environmental Protection Equipment Co., Ltd.

Methods

Preparation of Chelate of Polysaccharide and Zinc (LDS-Zn)

LDS powder was dissolved with water, stirred and adjusted for pH. Zn sulfate was added according to the preferred ratio, stirred and controlled for temperature for chelation. The solution was filtered, concentrated and then precipitated (Yang *et al.*, 2004; Hu *et al.*, 2007; Wang,

2008; Jung and Kim, 2007). The solution was freeze-dried under vacuum environment to obtain LDS-Zn.

Calculation of Chelating Rate

The chelating rate was calculated according to the following equation (Yang *et al.*, 2017a):

$$Chelating \ rate(\%) = (C_0 - C) / C_0 \times 100$$

where, C_0 is the mass concentration (g/L) of Zn(II) in the solution before chelation. C is the mass concentration (g/L) of Zn(II) in the solution after chelation.

Single-Factor Experiment

Effect of Chelating Time on Chelating Rate

0.4 g of $ZnSO_4 \cdot 7H_2O$ was dissolved in 100 mL of distilled water to prepare a solution with a mass concentration of 4 mg/mL. LDS and Zn(II) solution were added with a mass ratio of 4:1. Chelation was performed under neutral pH, 30°C at a speed of 150 r/min. The specimens were collected at 1, 2, 4, 6 and 8 h, respectively. After alcohol precipitation, the supernatant was obtained. The Zn concentration of these specimens was determined and the chelating rate was calculated (Qin *et al.*, 2009). The best chelating time was determined.

Effect of Mass Ratio on Chelating Rate

0.4 g of $ZnSO_4 \cdot 7H_2O$ was dissolved in 100 mL of distilled water to prepare a solution with a Zn concentration of 4 mg/mL. LDS and Zn (II) solution were added with a mass ratio of 1:1, 2:1, 3:1, 4:1, 5:1 and 6:1, respectively. Chelation was performed under neutral pH, 30°C at a speed a speed of 150 r/min for 4 h. After alcohol precipitation, the supernatant was obtained. The Zn concentration of these specimens was determined and the chelating rate was calculated. The best mass ratio was determined.

Effect of Initial Mass Concentration on Chelating Rate

0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 g of $ZnSO_4 \cdot 7H_2O$ were added to 100 mL of distilled water to prepare a solution of Zn(II) with an initial concentration of 1, 2, 3, 4, 5 and 6 mg/mL, respectively. LDS and Zn(II) were added to the solution with a mass ratio of 5:1. Chelation was performed under neutral pH, 30°C at a speed of 150 r/min for 4 h. After alcohol precipitation, the supernatant was obtained. The Zn concentration of these specimens was determined and the chelating rate was calculated. The best initial mass concentration was determined.

Response Surface Test

The BBD center combination test design principle was used in this experiment (Yang *et al*., 2017b; Wu *et al*., 2014). Based on the results of all single-factor experiments, chelation time, mass ratio and initial mass concentrations were selected as independent variables. The response surface test was performed with the chelation rate of the chelate as the response value. The experiment was carried out under three factors and three levels.

A total of 15 trials were conducted including 12 factorial points and 3 zero points. Test error was estimated by repeating the test at the center point as shown in Table 1. The optimal process conditions for preparation technology of LDS-Zn were optimized by regression analysis of the test results. When $p < 0.05$, the significance of all terms in the polynomial was considered statistically different.

Structural Characterization of LDS and LDS-Zn

Dry potassium bromide solid was added to an appropriate amount of sample. They were fully grounded in an agate mortar under the illumination of an infrared lamp. The sample was compressed into a transparent sheet. The infrared spectrum was measured in the wavenumber range of 400-4000 cm^{-1} by an infrared spectrometer.

Determination of Antioxidant Activity

Determination of DPPH Free Radical Scavenging Capacity

0.004 g of DPPH was dissolved in 100% ethanol to a constant volume of 1L. 0.1 mmol/L DPPH solution was prepared and stored away from light. Different concentrations of LDS and LDS-Zn with 2.0 mL each were obtained, placed in different tubes and added with 2.0 mL of DPPH solution. The tubes were then stirred to homogenize the solution. Their reaction lasted 30 min under an environment without sunlight. Absorbance value was measured at 517 nm. The blank control group was replaced with distilled water. The recorded value was set as A1. DPPH free radical scavenging rate was calculated as follows and the measurement was repeated three times (Chen and Zhang, 2016):

$$Clearance\ rate\ /\ \% = \left[1 - \left(A2 - A1\right) / A0\right] \times 100$$

where $A1$ represents the absorbance measured by different concentrations of polysaccharide (LDS). $A1$ represents the absorbance measured by using distilled water instead of DPPH and $A0$ represents the absorbance measured by replacing distilled polysaccharide (LDS) with distilled water.

Table 1: Level of response surface factors

A	B	C
Initial mass concentration (mg/mL)	Chelating time (h)	Mass ratio (mg/mg)
3	2	5:1
4	4	6:1
5	6	7:1

Determination of Hydroxyl Radical Scavenging Ability

0.3 mL salicylic acid solution (20 mmol/L), 1.0 mL of $FeSO_4 \cdot 7H_2O$ solution (1.5 mmol/L), 2.0 mL different concentrations of LDS and LDS-Zn solution and 0.7 mL H_2O_2 (6 mmol/L) were added together to form a 3 ml reaction solution. The solution was then stirred to homogenize it and placed in a constant temperature water bath at 37°C for 60 min. Absorbance was measured at 517 nm. The blank control group was replaced with distilled water. The measurement was repeated three times and the average value was obtained. The formula was performed as follows to calculate the clearance rate. The measurement was repeated three times:

$$Clearance\ rate\ /\ \% = \left[1 - \left(A2 - A1\right) / A3\right] \times 100$$

where $A1$ is the absorbance measured by replacing distilled water with salicylic acid. $A2$ is the absorbance measured for different concentrations of polysaccharide. $A3$ is the absorbance measured by replacing different concentrations of polysaccharide with distilled water.

Results and Discussion

Preparation Process of LDS-Zn

Single Factor Experiment

Effect of Chelating Time on Chelating Rate

As can be seen from Fig. 1, the chelating rate increased with the chelating time and the chelating reaction became more sufficient (Dong *et al*., 2016). With the increase of time, the chelation of LDS with Zn(II) was more sufficient. The chelating rate increased with time. The chelating rate was the highest when the time was 4 h. After 4 h, too long time caused the instability of LDS structure, resulting in a decrease in chelation rate. Therefore, the optimal chelating time was 4 h.
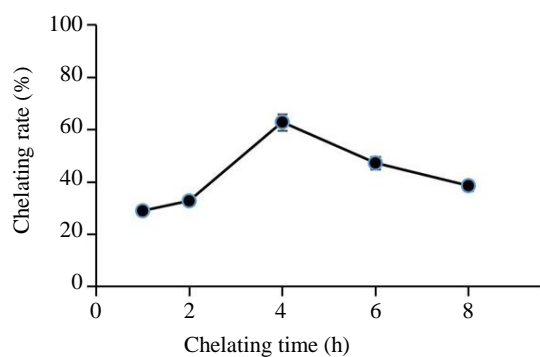
Effect of Mass Ratio on Chelating Rate

As shown in Fig. 2, when the mass ratio of LDS and Zn(II) was 6:1, the chelating rate was the highest. If the mass ratio severely dropped, the ring structure of LDS was not sufficiently stable. Thus, the chelating rate became small. The increase in the mass ratio adversely

affected the progress of the chelating reaction and thus decreased the chelating rate. Therefore, the optimal mass ratio was 6:1.

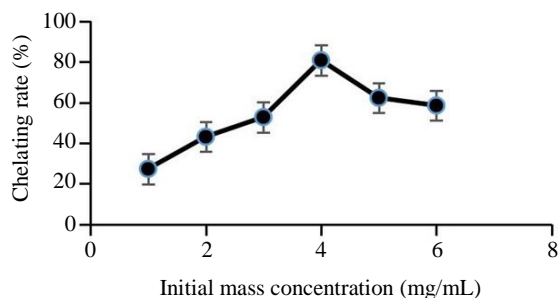### Effect of Initial Mass Concentration on Chelating Rate

Figure 3 showed that the chelating rate increased when the concentration of Zn(II) increased. However, the excessive concentration of Zn(II) adversely affected the progress of the chelating reaction, resulting in a decrease in the chelating rate. At a concentration of 4mg/mL, chelating rate was the highest. However, when the concentration of Zn(II) increased further, the chelation rate decreased. Therefore, the optimum initial mass concentration of Zn(II) was 4 mg/mL.

Fig. 1: Effect of chelating time on chelating rate
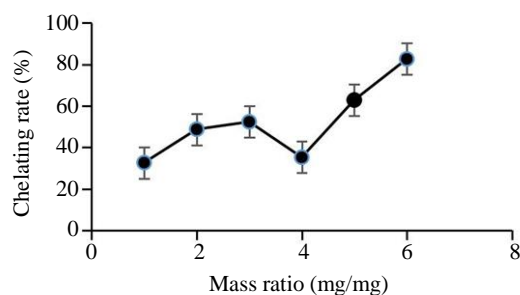
Fig. 2: Effect of mass ratio on chelating rate

Fig. 3: Effect of initial mass concentration on chelating rate

Optimization of LDS-Zn Preparation Process Conditions

Establishment and Analysis of Response Models

The results of the single-factor test were entered into the Design-Expert software and the response surface experimental design and data were obtained (Table 2). The test results of the table design test, the regression coefficient of the test model (Table 3) and the response surface map (Fig. 4 to 6) were listed.

Using the chelating rate of LDS-Zn(II) as response value, the response surface analysis was performed by using Design-Expert 8.0 software (Table 2). A quadratic multiple regression equation was obtained for initial mass concentrations (A), chelating time (B) and mass ratio(C):

$$Y = +81.63926 - 0.1286 * A + 0.05537 * B + 0.17202$$
$$*C - 8.21595E - 00 * B * C + 1.16287 - 003 *$$
$$A^2 - 9.87456 - 004 * C^2$$

As shown in Table 3, the quadratic polynomial model indicated that the effect was extremely significant ($p<0.01$). The missing term ($p>0.05$) indicated that the effect was not significant. The effect of A^2 was extremely significant for chelating rate ($p<0.01$). The effect of B^2 was significant for chelating rate ($p<0.05$). Similarly, the effect of C^2 was not significant for chelating rate ($p>0.05$). The p value of the misfit term of the equation was 0.2939, indicating that the influence was not significant and the model was accurate.

Response Surface Analysis

The response surface contour line represented the interaction between the factors. The steep surface indicated that the factor greatly influenced the chelation rate. The gentleness could be judged that the effect was not significant. The shape and intensity of the contours could also reflect the extent to which factors affect the chelation rate. The oval shape indicated that the effect was significant and the circle was not significant. Figure 4 indicated that the initial mass and chelation time greatly influenced the chelation rate. Figure 5 indicated that the contour was elliptical and the pattern was steep, indicating that the initial mass concentration and mass ratio greatly influenced the chelation rate. Figure 6 indicated that the contour line was dense and the graph was steep, indicating that the mass ratio and the chelation time greatly influenced the chelation rate.

Optimal Process Conditions Determination and Verification Test

The response surface test results were analyzed using the Design-Expert software. Based on the response

surface plots, contour plots and analysis of variance, effect of chelation time, initial mass concentration and mass ratio on chelating rate was optimized and analyzed. The optimal preparation conditions of LDS-Zn were as follows: Chelating time was 4 h, mass ratio (LDS with zinc) was 6:1, initial mass concentration (zinc) was 4 mg/mL. LDS achieved the best chelating rate as high as 87.08%. To verify the adequacy of the model equation, three verification experiments were performed under optimal conditions as described above. Chelating rate was 85.04, 86.42 and 85.65% in three parallel experiments. An average chelating rate was 85.70%, which was close to predicted value. The result indicated that the chelating process condition parameters obtained by the response surface method could be used. Therefore, it was achievable to predict the model based on the response surface method.

Table 2: Response surface experimental design and data

| Test number	A	B	C	Chelating rate (%)
1	-1	1	0	84.67
2	0	0	0	87.08
3	-1	-1	0	84.08
4	0	1	-1	86.82
5	0	0	0	87.09
6	1	0	-1	85.31
7	1	0	1	85.63
8	0	-1	0	85.03
9	0	0	-1	85.03
10	0	1	1	85.93
11	-1	0	1	83.43
12	-1	0	0	85.67
13	0	-1	1	84.61
14	0	1	0	86.55
15	0	0	-1	84.62
16	0	0	1	85.43
17	0	0	0	87.27

Table 3: Variance analysis of regression models

Source	Sum of squares	Degrees of freedom	Mean square	F value	P value
Model	26.150	9	2.910	2.590	0.0002
A	1.070	1	1.070	0.960	0.0004
B	0.670	1	0.670	0.600	0.0009
C	3.260	1	3.260	2.910	0.0001
AB	0.810	1	0.810	0.720	0.4237
AC	0.042	1	0.042	0.037	0.8521
BC	2.430	1	2.430	2.170	0.1844
A^2	2.530	1	2.530	2.260	0.0019
B^2	7.370	1	7.370	6.570	0.0374
C^2	6.170	1	6.170	5.500	0.0515
Residual error	7.860	7	1.120		
Lack of fit	4.470	3	1.490	1.760	0.2939
Pure error	3.390	4	0.850		
Sum total	34.010	16			

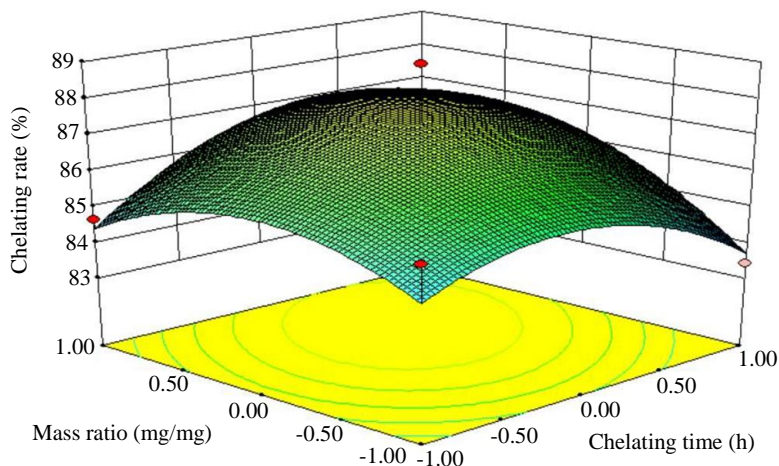(Note: $p < 0.05$ means the difference is significant; $p < 0.01$ means the difference is extremely significant; $p > 0.05$ means the difference is not significant.)
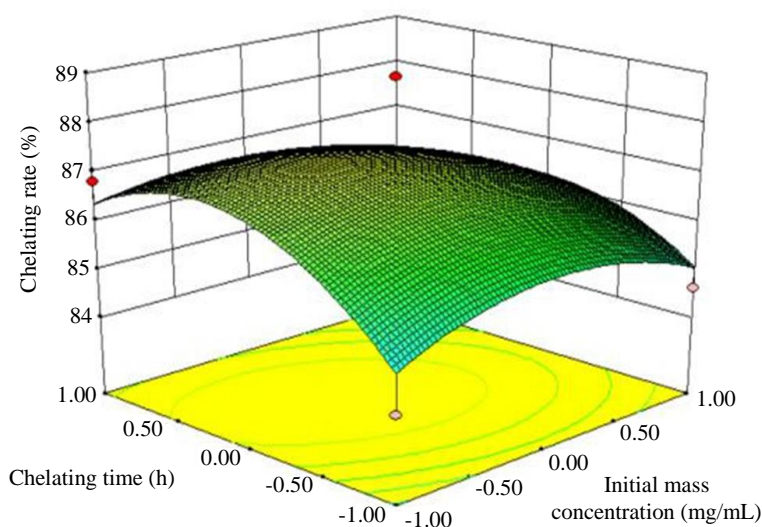
Fig. 4: Interaction of initial mass concentration and chelating time
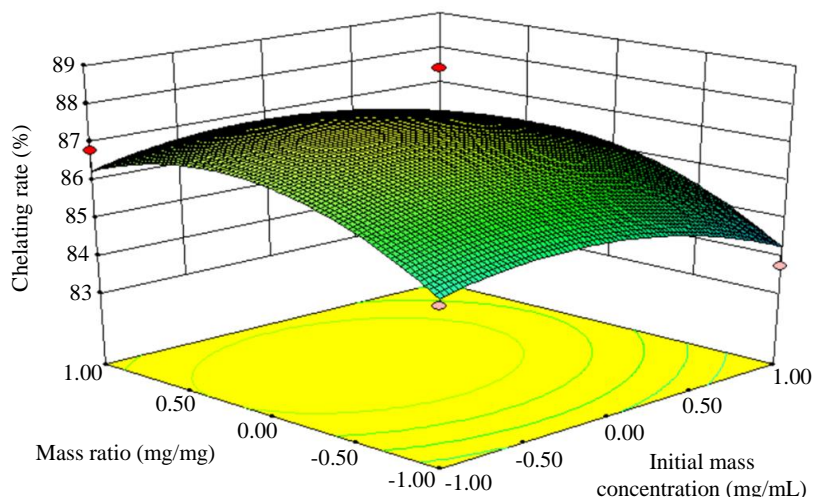
Fig. 5: Interaction of initial mass concentration and mass ratio

Fig. 6: Interaction of mass ratio and chelation time

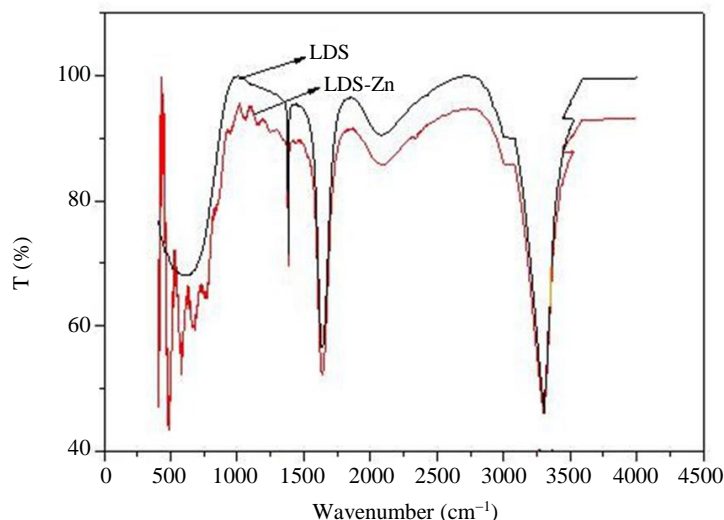### Infrared Spectroscopy Analysis of LDS and LDS-Zn

A comparison of the infrared spectra of LDS and LDS-Zn (Fig. 7) showed that LDS had a typical polysaccharide characteristic absorption peak. If the Zn substance was complexed with polysaccharide (LDS), the stretching vibration absorption peak (1600 cm^{-1}) of the hydroxyl group (-OH) in the polysaccharide molecule and its variable angle vibration (1384 cm^{-1}) might shift towards the red direction. Given the influence of moisture in the KBr tablet, the change of the hydroxyl absorption peak of the hydroxy group was used as the basis to investigate the chelation of the sample polysaccharide. The direction of the LDS-Zn wave shifted and the stretching vibration peak of -OH was at 3300cm^{-1}. The absorption intensity was weak. Its variable angle vibration was weaker than that of

unchelated form. The C=O vibration absorption peak was at 2900 cm^{-1} and the absorption intensity was lowered. This red shift phenomenon and change in the absorption intensity of the group indicated that the Zn ion was chelated with the -OH of LDS (Cui *et al.*, 2018).
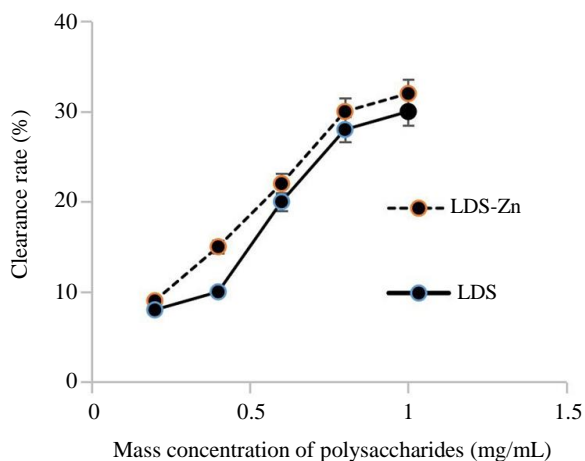
Antioxidant Activity of LDS and LDS-Zn

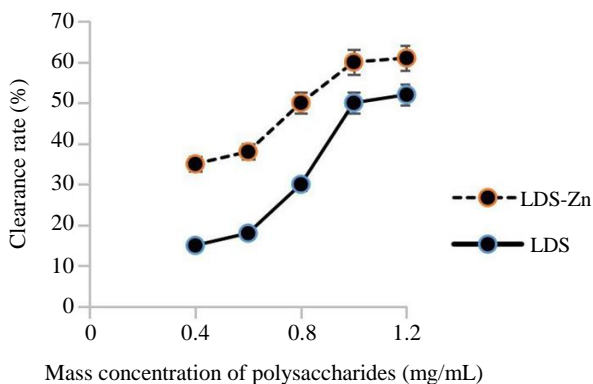### Scavenging Effect of LDS and LDS-Zn on DPPH Free Radicals

The antioxidant activity of LDS and LDS-Zn was compared (Fig. 8). LDS had strong effect of scavenging DPPH free radicals with a linear relationship. LDS-Zn had better antioxidant activity than LDS. The IC50 of LDS was 0.81 mg/mL. The IC50 of LDS-Zn was 0.62 mg/mL. The antioxidant activity increased by 23.46%.

Fig. 7: Infrared spectroscopy of LDS and LDS-Zn

Fig. 8: Scavenging Effect of LDS and LDS-Zn on DPPH Free Radicals

Fig. 9: Scavenging Effect of LDS and LDS-Zn on Hydroxyl Radicals

Scavenging Effect of LDS and LDS-Zn on Hydroxyl Radicals

Figure 9 indicated that LDS-Zn had a significantly higher clearance rate for hydroxyl radicals than LDS. The IC50 of LDS was 0.83 mg/mL. The IC50 of LDS-Zn was 0.75 mg/mL. The antioxidant activity increased by 16.77%.

Conclusion

According to the response surface method, the optimal preparation conditions of LDS-Zn were as obtained as follows: Chelating time was 4 h, mass ratio (LDS with zinc) was 6:1, initial mass concentration (zinc) was 4 mg/mL. LDS achieved the best chelating rate as high as 87.08%. Infrared spectroscopy analysis showed that zinc (II) successfully chelated with LDS.

Compared with LDS, LDS-Zn had better scavenging effect on DPPH free radicals and hydroxyl radicals and antioxidant activity increased by 23.46 and 16.77%, respectively. These results indicated that LDS-Zn could be expected to serve as a nutritional Zn supplement with antioxidant activity. Meanwhile, the results can provide some scientific basis for developing new Zn supplement. In future research, the mechanism of change in the antioxidant activity of LDS after chelating zinc as well as advantages of LDS-Zn as zinc supplement is not yet clear, which will be further studied.

Acknowledgment

Author's Contributions

Bing Xu: Participated in all experiments and article writing.

Yi Dai and Jing Hong: Participated in all experiments and data analysis.

Yiyong Chen: Participated in research plan and experimental guidance of this manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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