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Liquid-state fermentation with *Bacillus subtilis* Bs-07 to enhance anticoagulant function of *Auricularia auricula* polysaccharide

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Abstract: In order to improve the anticoagulant function of *Auricularia auricula*, *Auricularia auricula* polysaccharide (AAP) was converted into its derivatives by the microbial fermentation method and then polysaccharide derivatives with stronger anticoagulant activity were prepared. The optimal conditions for fermenting the polysaccharide from *A. auricula* were examined and the in vitro anticoagulant activities of transformed and untransformed polysaccharides were compared. Response surface tests and an orthogonal experiment indicated that the best conditions for microbial conversion of AAP_3 were an AAP_3 concentration of 4.0 mg/mL, a ratio of substrate (AAP_3) to donor (p-hydroxybenzoic acid) of 40∶1, and a pH of 6.0. *Bacillus subtilis* Bs-07 was inoculated and then placed on a rotary shaker (120 r/min), followed by fermentation for 48 h at 35 ℃. The conversion rate was found to be greater than 40%. The result of in vitro anticoagulant activity showed that the transformed polysaccharide improved activated partial thromboplastin time, prothrombin time, and thrombin time values and greatly enhanced anticoagulant activity compared to the untransformed polysaccharide.

Key words: *Auricularia auricula* polysaccharide; microbial fermentation; anticoagulation

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Derivatization of polysaccharides refers to using physical, chemical, biological, and other processes to transform a polysaccharide molecule to produce a molecule with new biological functions. In this study, we used microbial fermentation to transform a polysaccharide and found that the transformed polysaccharide had improved anticoagulant properties[1]. Microbial fermentation produces natural products through bioconversion and is one of the most effective methods for modifying polysaccharides. It is also useful for expanding production because enzymes produced during the growth and reproduction of a microbe have many advantages such as high activity, low cost, availability of different types, and good synergistic effects[2].

Auricularia auricula is a high-quality medicinal fungus. AAP_3, a polysaccharide from *Auricularia auricula*, has various properties such as anticoagulant, antiaging, antithrombotic, prevention of high cholesterol, and antihypertensive properties; in addition, this polysaccharide prevents coronary heart disease[3-5]. At present, heparin and coumarin drugs are primarily used as anticoagulant drugs in clinical treatment. However, they are associated with some side effects such as platelet disorders. Therefore, it is important to study the impact of the black fungus polysaccharides on the human circulatory system[6]. This study can serve as the theoretical basis to use black fungus polysaccharides and their derivatives as functional food additives or drugs[7-8].

1 Materials and Methods

1.1 Materials and equipment

All the materials used in this study, such as glucose, petroleum ether, acetone, ether, and all other reagents are of domestic analytical grade. Black fungus acid polysaccharide AAP_3 was obtained from the Food Microbiology Laboratory of the Northeast Forestry University[9]. The thrombin time reagent and thromboplastin-DS required for calculating activated partial thromboplastin time (APTT) were purchased from the Shanghai Sun Biotech Co. New Zealand white rabbits weighing 2-3 kg, *Bacillus cereus* Bc-01, *B. subtilis* Bs-07, *B. licheniformis* Bl-02, *Saccharomyces cerevisiae*, Rt-1, *Aspergillus niger* Wm-1, and *A. niger* Wm-3 were obtained from the Food Microbiology Laboratory of the Northeast Forestry University. In addition, the 722 Vis spectrophotometer and RE-52A rotary evaporator used in this study were obtained from the Shanghai Spectrum Instruments Manufacturing Co., Ltd. and the Shanghai Yarong Biochemical Instrument Factory, respectively.

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1.2 Test methods

1.2.1 Determination of concentration and conversion rate of the black fungus polysaccharide

The phenol-sulfuric acid method was used to determinate the concentration of the polysaccharide. The conversion rate r_c was calculated using the following formula:

$$r_c = \frac{\frac{c\Delta V}{1\,000n}M}{m_0}$$

where m_0 is the donor (in mg) added to the initial reaction; c is the concentration (in mol/L) of NaOH; ΔV is the difference in NaOH volume between the consumption of the added acid by the polysaccharide and the final titrated consumption volume after the transformation of the polysaccharide; M is the molecular weight of the donor; and n is the number of free donor H ions. NaOH volume (in mL) was also used for adjusting pH.

1.2.2 Infrared spectroscopy

The infrared (IR) spectra of the polysaccharide AAP_3 before the derivatization and of polysaccharide AAP_3-I after the derivatization were recorded using an Avatar IR spectrophotometer between 400 and 4 000 cm^{-1}, and were scanned 32 times. The samples were analyzed as KBr pellets.

1.2.3 Anticoagulation test

New Zealand white rabbits were fed black fungus polysaccharide and polysaccharide derivatives for a month, respectively, and then blood was withdrawn from the rabbit carotid artery. The blood was centrifuged at 3 000 r/min for approximately 15 min to obtain the plasma. Activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) were determined using a manual method based on the instructions mentioned in the kit[10].

1.3 Fungus polysaccharide microbial transformation experiments

1.3.1 Screening of bacteria

Approximately 5 mg/mL of the black fungus polysaccharide was mixed with p-hydroxybenzoic acid at a ratio of 50∶1. Next, the pH was adjusted to 5.0, and the mixture was shaken at room temperature to dissolve the polysaccharide completely. Next, the mixture was sterilized at 120 ℃ for 20 min and bacterial strains Bc-01, Bs-07, Bl-02, Rt-1, Wm-1, and Wm-3 were inoculated into the mixture. Fermentation was performed for 96 h by incubating the mixture in a rotary shaker at 120 r/min and 35 ℃. Data for each strain were measured every 12 h. We took the conversion rate as the vertical axis as well as regarding the conversion time as abscissa for generating the line chart, which is used to observe the best transformed bacteria and the best conversion time.

1.3.2 Selection of derivative donor groups

Approximately 5 mg/mL of the *A. auricula* polysaccharide was mixed with the donors l-lysine, l-cysteine, l-malic acid, succinic acid, malonic acid, and p-hydroxybenzoic acid at the ratio of 50∶1, and the pH was adjusted at 5.0. The solution was shaken at room temperature to dissolve the polysaccharide completely. The bacterial strain that provided the best results in the screening assay was inoculated in these solutions, and fermentation was performed for 48 h in a rotary shaker at 120 r/min and 35 ℃. Next, the conversion rate was measured to determine the best donor group.

1.3.3 Microbial transformation of the *A. auricular* polysaccharide

After preparation of 5 mg/mL black fungus polysaccharide solution, AAP and the donor were mixed in a certain proportion. Then we shook it to fully dissolve at room temperature, sterilizing the solution at 120 ℃, 20 min after adjusting its pH and inoculum. After incubation at an appropriate temperature for a certain period in a shaker, the solution was re-sterilized, and the conversion ratio was calculated using the NaOH titration method. After approximately 3 d of incubation, the fermented solution was centrifuged, and the supernatant was dialyzed with water. Next, it was condensed at a reduced pressure and was precipitated using alcohol. The obtained derivatized polysaccharide was then freeze-dried[11].

1.3.4 Response surface test of microbial transformation medium

Substrate concentration, ratio of substrate to donor, and pH were selected as independent variables to design three factors and three levels of response surface optimization experiments by using the Box-Behnken central composite method with Design-Expert 8.05 software (see Tab.1).

Tab.1 Response surface factors and levels

Factors	Levels		
	Polysaccharide concentration/(mg·mL^{-1})	Ratio of substrate to donor	pH
Upper level (+1)	3	30∶1	5
Reference level (0)	4	40∶1	6
Lower level (−1)	5	50∶1	7

1.3.5 Orthogonal experiment of microbial conversion process conditions

Conversion time, transformation temperature, and rotating speed were used as independent variables and conversion rate was used as index to design $L_9(3^4)$ for optimizing the conversion conditions with Minitab 16 software. Factors for orthogonal experiments are shown in Tab.2.

Tab. 2 Factors and levels for orthogonal experiments

Factors	Levels		
	Conversion time/h	Transformation temperature/℃	Rotational speed/(r · min^{-1})
1	36	30	100
2	48	35	120
3	60	40	140

2 Results and Analysis

2.1 Microbial transformation of the fungal polysaccharide

2.1.1 Screening of the strain

Fig. 1 shows the conversion ratios of the six strains at different periods. The figure indicates that all the conversion rates increased to a maximum value and then decreased subsequently. Comparison of the conversion rates of the six strains showed that Bs-07 had the highest conversion rate followed by Wm-3, Wm-1, Bc-01, Bl-02, and Rt-1. Therefore, Bs-07 was selected as the best strain for microbial transformation.

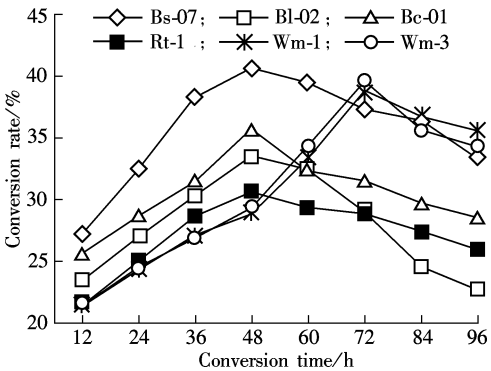

Fig. 1 Effect of bacteria screening on conversion rate

2.1.2 Selection of donor groups

Fig. 2 shows that p-hydroxybenzoic acid provided the highest conversion rate compared with that obtained using other donor groups. Therefore, p-hydroxybenzoic acid was selected as the best donor group for transforming the polysaccharide.

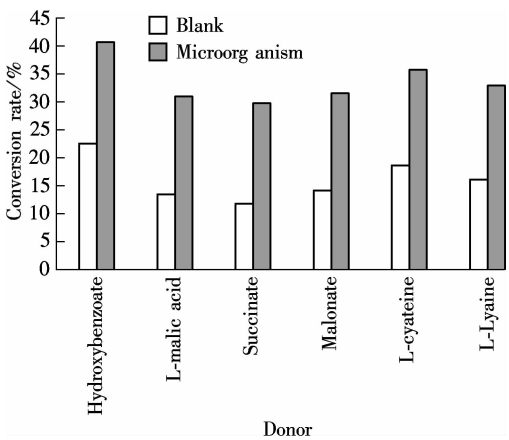

Fig. 2 Effect of donor groups on conversion rate

2.2 Response surface test of microbial transformed medium

2.2.1 Response surface test of program and results

According to the single-factor experimental results, the Box-Behnken central composite method was used to design programs of response surface. Conversion rate was used as the response value. The experimental design is shown in Tab. 3. The fitting regression equation based on the Box-Behnken method is as follows:

$$Y = 40.88 + 0.34A - 0.089B + 1.12C - 1.16A^2 - 1.01B^2 - 2.20C^2 + 1.08AB - 0.25AC - 0.14BC$$

where A is the conversion time; B is the transformation temperature; C is the rotational speed.

Tab. 3 Experimental design and estimated values of the response surface test

Run	Polysaccharide concentration	Ratio of substrate to donor	pH	Conversion rate/%
1	0	0	0	40.96
2	0	0	0	41.05
3	0	0	0	40.01
4	−1	0	1	37.89
5	0	1	1	39.01
6	1	1	0	39.54
7	0	−1	−1	36.03
8	−1	−1	0	40.03
9	1	0	1	38.77
10	1	0	−1	37.64
11	0	0	0	41.45
12	0	0	0	40.91
13	1	−1	0	37.86
14	−1	1	0	37.39
15	0	−1	1	39.16
16	0	1	−1	36.43
17	−1	0	−1	35.76

2.2.2 Variance analysis of the response surface test

Variance analysis of the above equation model (see Tab. 4) indicated that this model was the most significant ($p < 0.001$) in accordance with the data presented in Tab. 3. This model appears to be a better fitting degree. The analysis showed that pH had the greatest impact because its Prob > F value was 19 (< 0.05), which was significant. Thus, pH had the highest impact followed by polysaccharide concentration. The minimum factor belongs to the ratio of substrate to donor. From Figs. 3 to 5, they also directly reflected the interaction among different factors as well as the impact on the response value[12]. According to the prediction regression model, the optimum conditions for conversion of the polysaccharide were a polysacchride concentration of 4.12 mg/mL, a ratio of substrate to donor about 40.25, and a pH of 6.25.

In theory, the conversion rate of the acylated polysac-

charide was 41% under these conditions. The correct optimum technical conditions were a polysaccharide concentration of 4.0 mg/mL, a ratio of substrate to donor of 40∶1, and a pH of 6.

Tab. 4 Variance analysis of factors of the response surface test

Source of variance	Sum of squares	DOF	Mean square	*F* value	*P* value
Model	49.460	9	49.460	12.75	0.001 4 *
A	0.940	1	0.940	2.18	0.183 6
B	0.063	1	0.063	0.15	0.713 6
C	10.060	1	10.060	23.33	0.001 9
A^2	5.630	1	5.630	13.07	0.008 6
B^2	4.330	1	4.330	10.05	0.015 7
C^2	20.460	1	20.460	47.47	0.000 2
AB	4.670	1	4.670	10.82	0.013 3
AC	0.250	1	0.250	0.58	0.471 2
BC	0.076	1	0.076	0.18	0.687 9
Residual	3.020	7	0.430		
Loss-faulty	1.900	3	0.630	2.27	0.223 0 **
Pure error	1.120	4	0.280		
Total	52.470	16			

Note: * significant; ** insignificant.

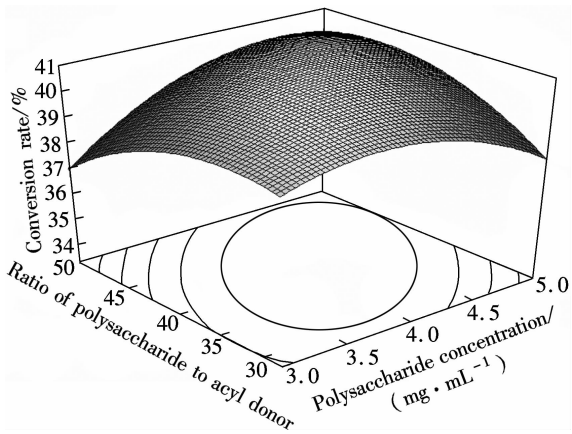

Fig. 3 Response surface graph of polysaccharide to acyl donor ratio and polysaccharide concentration to conversion rate

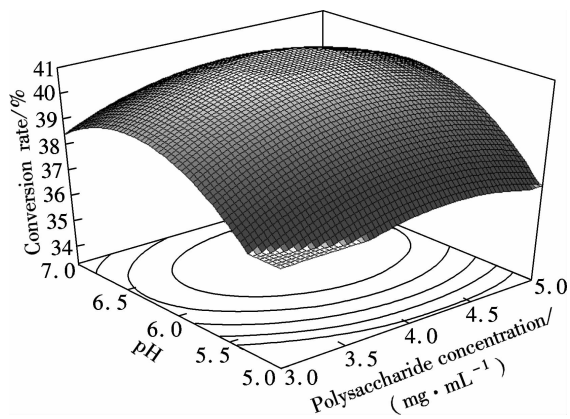

Fig. 4 Response surface graph of pH and polysaccharide concentration to conversion rate

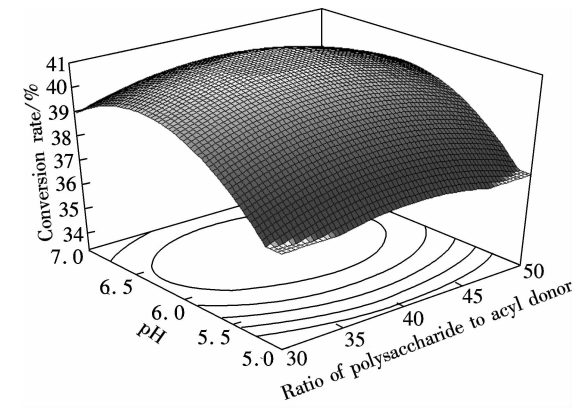

Fig. 5 Response surface graph of pH and polysaccharide to acyl donor ratio to conversion rate

2.3 Single-factor experiment of microbial conversion process conditions

2.3.1 Effect of transformation temperature on the conversion ratio of the polysaccharide

As shown in Fig. 6, the graph indicates that the impact of different temperatures on the conversion rate was situated under the condition of a rotational speed of about 120 r/min. The conversion rate gradually increased from 20 to 35 ℃ and decreased subsequently. The best transformation temperature was found to be 35 ℃.

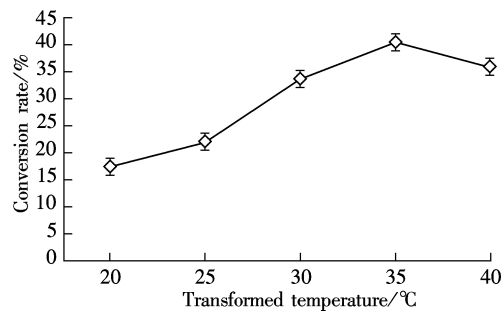

Fig. 6 Effect of transformation temperature on conversion rate

2.3.2 Impact of rotational speed on the conversion rate of the polysaccharide

When the rotational speed was increased, the conversion rate increased initially and then decreased; the highest conversion rate was achieved at the rotational speed of 120 r/min (see Fig. 7). This is mainly because very large or very small amounts of ventilation change the microbial metabolic pathways. It also affects the yield and activity of enzymes. The experiment also shows that the best rotational speed condition is 120 r/min.

2.3.3 Orthogonal experiment of microbial conversion process' conditions

Optimal conditions for microbial conversion determined using the orthogonal experiment are presented in Tab. 5 and the analysis of variance conversion rates is shown in Tab. 6. Minitab 16 software was used to analyze the results. The main order of factors affecting the experiment index was B > A > C. This indicates that conversion tem-

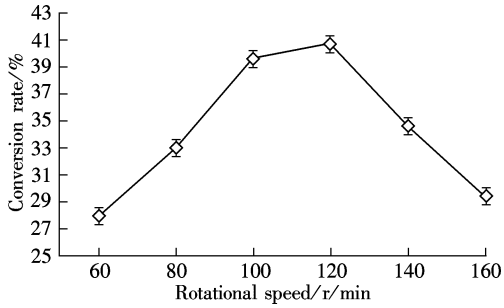

Fig. 7 Effect of rotational speed on conversion rate

perature was the most influencing factor followed by conversion time while the rotational speed was the least influencing factor. The best combination was A2B2C2, i. e., a conversion temperature of 35 ℃, a rotational speed of 120 r/min, and a conversion time of 48 h. We conducted experiments that had been paralleled three times to obtain the value of the conversion rate (40.96%, 41.02%, and 41.12%).

Tab. 5 $L_9(3^4)$ orthogonal table

Test No.	Factors				Conversion rate/%
	A	B	C	D	
1	1	1	1	1	19.96
2	1	2	2	2	34.69
3	1	3	3	3	29.21
4	2	1	2	3	28.66
5	2	2	3	1	37.52
6	2	3	1	2	36.57
7	3	1	3	2	25.42
8	3	2	1	3	37.79
9	3	3	2	1	38.99
k_1	27.95	24.68	31.44		
k_2	34.25	36.67	34.11		
k_3	34.07	34.92	30.72		
R	6.30	11.99	3.40		

Tab. 6 Analysis of variance conversion rates

Source	Degree of freedom	Seq SS	Adj SS	Adj MS	F	P
A	2	77.054	77.054	38.527	398.42	0.003
B	2	251.645	251.645	125.823	1 301.16	0.001
C	2	19.207	19.207	9.604	99.31	0.010
Error	2	0.193	0.193	0.097		
Total	8	348.100				

Note: $S = 0.310\,966$, $R^2 = 99.94\%$, $R^2(\text{adj}) = 99.78\%$.

2.4 Infrared spectroscopy

IR spectra of polysaccharide AAP_3 (before derivatization) and polysaccharide AAP_3-I (after derivatization) were recorded using the Avatar IR spectrophotometer between 400 and 4 000 cm^{-1}, and were scanned 32 times.

The band at 3 400 cm^{-1} in Figs. 8 and 9 corresponds to the strong stretching vibration of the —OH group. This result indicates that hydroxyl groups in the polysaccharide chains were not completely substituted by acyl groups. At the same time, hydroxyl absorption peaks of polysaccharide AAP_3-I decreased at 3 430.75 cm^{-1}, indicating that the part of hydroxyl may have been substituted. The band at 2 927 cm^{-1} corresponded to the asymmetric vibration absorption of —CH_2 group in the polysaccharide. The bands at 1 546.27, 1 460.64, and 1 457.45 cm^{-1} in Fig. 9 may correspond to the stretching vibration absorption of C═C group in the aromatic skeleton. This band was not observed in Fig. 8. These results indicated that polysaccharide AAP_3 was connected to p-hydroxybenzoic acid. The band at 1 738.53 cm^{-1} corresponded to the absorption peak of the C═O group in the transformed polysaccharide, indicating that the polysaccharide was completely acylated.

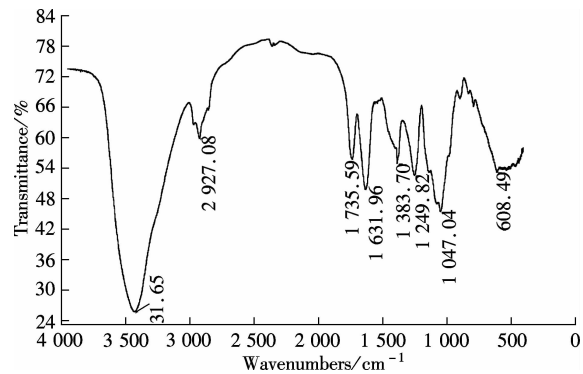

Fig. 8 IR analysis of polysaccharide AAP_3 (before conversion)

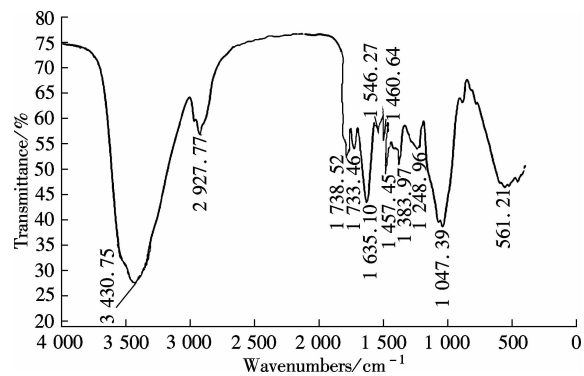

Fig. 9 IR analysis of polysaccharide AAP_3-I (after conversion)

2.5 Comparison of converted polysaccharide derivatives (AAP_3-I) and unconverted polysaccharides' anticoagulant effect in vitro

From the data of Tab. 7, it could be clearly observed that the derived polysaccharide in vitro could greatly enhance the anticoagulant effect. The reason may be that the increase in the solubility of the converted polysaccharide and the reduction in viscosity of the polysaccharide improve the activity of the polysaccharide to some extent.

Tab. 7 Effect of conversion of the polysaccharide on PT, APTT, and TT s

Groups	PT	APTT	TT
AAP_3	$18.91 \pm 0.57^{**}$	$47.96 \pm 2.09^{*}$	$24.17 \pm 1.03^{**}$
AAP_3-I	$26.25 \pm 0.37^{**}$	$56.15 \pm 1.33^{**}$	$30.88 \pm 1.05^{*}$

Note: Compared with normal saline, $^{**}p < 0.01$ and $^{*}p < 0.05$, mean ± SD of values indicates $n = 4$.

3 Conclusion

In this study, *B. subtilis* Bs-07 resulted in the highest conversion of the *A. auricula* polysaccharide and p-hydroxybenzoic acid was found to be the best donor group. For obtaining the best conditions of the medium, we made a response surface optimized test by adopting the Box-Behnken central composite method through Design-Expert 8.05 software to optimize the microbial conversion medium. The best conditions for microbial conversion were a polysaccharide concentration of 4.0 mg/mL, a pH of 6, and a ratio of substrate to donor of 40∶1. In addition, an orthogonal experiment using the Minitab 16 software, which was designed to optimize the transformation conditions, showed that the optimum transformation conditions were a temperature of 35 ℃, a rotational speed of 120 r/min, and a conversion time of 48 h.

Results of the in vitro experiments indicated that the transformed *A. auricula* polysaccharide had a stronger anticoagulant activity than the untransformed polysaccharide. What is more, the result also showed that it had a better anticoagulant effect, and acylated polysaccharide had a large potential value on developing functional food or drugs.

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枯草芽孢杆菌 Bs-07 液态发酵提高黑木耳多糖抗凝血功能

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摘要: 为了提高黑木耳多糖的抗凝血功能,应用微生物发酵技术转化黑木耳多糖得到抗凝血活性更强的黑木耳多糖衍生物. 研究了黑木耳多糖转化的最佳发酵条件,比较了未转化和转化后的黑木耳多糖的体外抗凝血功能. 通过响应面和正交试验得出最佳转化条件:将浓度 4.0 mg/mL 的黑木耳酸性多糖 AAP_3 溶液与对羟基苯甲酸充分混合(体积比为40∶1),调 pH 至6.0,接种枯草芽孢杆菌 Bs-07,在温度为 35 ℃,旋转摇床转速为 120 r/min 的条件下,发酵 48 h,转化率达 40% 以上. 检测转化后黑木耳多糖衍生物 APTT,PT 和 TT 的数据结果显示,转化后黑木耳多糖衍生物体外抗凝血功能得到了明显提高.

关键词: 黑木耳多糖;微生物发酵;抗凝血
中图分类号: TS202.3