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Optimisation of submerged culture conditions for the production of mycelial biomass and exopolysaccharide by *Pleurotus nebrodensis*

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Abstract - The optimisation of submerged culture conditions and nutritional requirements was studied for the production of exopolysaccharide (EPS) from *Pleurotus nebrodensis.* The optimal temperature and initial pH for both mycelial growth and EPS production in shake flask cultures were 25 °C and 8.0, respectively. Maltose was found the most suitable carbon source for both mycelial biomass and EPS production. Yeast extract was favourable nitrogen source for both mycelial biomass and EPS production. Optimum concentration of each medium component was determined using the orthogonal matrix method. The optimal combination of the media constituents for mycelial growth and EPS production was as follows: 200 g l^{-1} bran, 25 g l^{-1} maltose, 3 g l^{-1} yeast extract, 1 g l^{-1} KH_2PO_4, 1 g l^{-1} $MgSO_4$ $7H_2O$. Under the optimal conditions, the mycelial biomass (4.13 g l^{-1}) and EPS content (2.40 g l^{-1}) of *Pleurotus nebrodensis* was 2.3 and 3.6 times compared to the control with basal medium respectively.

Key words: *Pleurotus nebrodensis*, biomass, exopolysaccharide, optimisation.

INTRODUCTION

The wild species of *Pleurotus nebrodensis* is a precious, edible and medicinal mushroom, it only distributes in the arid desert of Xinjiang area in China. In 1983, wild *Pleurotus nebrodensis* was cultivated successfully on wood chips of spruce, cottonseed hulls and brans by Chinese scientists for the first time (Chen, 1986).

Pleurotus nebrodensis is a tasting crisp and delicious with pleasant aroma functional mushroom; it is rich in protein, amino acids, polysaccharide, edible fibre and mineral elements. Various experiments have demonstrated that *Pleurotus nebrodensis* had physiological effects, such as preventing cardiovascular disease and rachitis (Lin, 2000). A number of pharmacologic studies have shown that polysaccharide of *Pleurotus nebrodensis* had immunological activities (Gan and Lv, 2001; Deng and Lv, 2002), antioxidative and antitumor effects (Zheng *et al*., 2005). In fact, the importance of the medicinal properties of *Pleurotus nebrodensis* polysaccharides had been realised all over the world by now.

Polysaccharide of *Pleurotus nebrodensis* was normally extracted from its fruiting bodies (Dong *et al*., 2004b). However, solid-cultivation would not only take a long period of time, but also get low products. Therefore, researchers had tried to obtain polysaccharide of *Pleurotus nebrodensis* from its mycelia (Yang *et al*., 2004; Li *et al*., 2005). Actually, liquid culture had a lot of advantages for gaining mass production, such as it is relatively easy to harvest mycelia and extract useful material from fermentation broth (Zhang *et al*., 2002), especially, it facilitated the release of polysaccharide. Therefore, liquid culture of *Pleurotus nebrodensis* is worthy of investigating greatly.

In recent years, submerged cultivation for the production of mycelial biomass from *Pleurotus nebrodensis* has been investigated widely (Li *et al*., 2003; Dong *et al*., 2004a, 2004c), while submerged cultivation for production of exopolysaccharide (EPS) has not been reported yet up to now. In this study, *Pleurotus nebrodensis* was cultivated in liquid media, and then the optimum nutritional requirements of media and environmental conditions of culture for producing EPS were investigated.

MATERIALS AND METHODS

Microorganism and culture conditions. J3 (conserved in our laboratory), a strain of *Pleurotus nebrodensis*, was used in this experiment. This strain was incubated on PDA slant (20% w/v potato, 2% w/v dextrose, 2% w/v agar) at 25 °C for about 12 days.

Inoculum preparation and flask cultures. *Pleurotus nebrodensis* was initially grown on PDA medium in a Petri dish for about 12 days, and then transferred to liquid media by punching out approximately 0.5-cm^2 mycelial block from the agar plate culture. The fermentation culture was grown in a 250-ml Erlenmeyer flask containing 100-ml tested medium and shaken continuously at 160 rpm, 25 °C for 6 days after being laid still for 24 h.

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The tested medium used for all flask culture experiments was derived from the basal medium with natural initial pH. The composition of the basal medium was 10% w/v bran, 1% w/v sucrose, 0.3% w/v peptone, 0.1% w/v KH_2PO_4, 0.05% w/v $MgSO_4$ $7H_2O$ (Jiang *et al*., 2005).

Optimisation of the composition of fermentation medium. The selection of carbon and nitrogen sources is the first step in the course of media optimisation. Sucrose (1.500 g), dextrose (1.737 g), maltose (1.579 g), lactose (1.579 g) and fructose (1.579 g) were provided with the proportion equal to the carbon content of 1.5 g sucrose (0.632 g) in the basal medium separately. During in the course of screening for the nitrogen sources, peptone (0.300 g), yeast extract (0.623 g), beef extract (0.335 g), ammonium nitrate (0.125 g), ammonium sulphate (0.206 g), sodium nitrate (0.265 g) and ammonium chloride (0.166 g) were tested with the proportion equivalent to the nitrogen content of 0.3 g peptone (0.044 g). The orthogonal test was applied to determine the optimum combination of carbon and nitrogen sources after finding out the optimal kind of carbon and nitrogen sources.

Optimisation of environmental conditions. The single-factor test was used to determine the optimal environmental conditions respectively. Inoculation volume, incubation temperature, revolution speed, initial pH value, culture time and the volume of liquid media in 250 ml-sized flask were tested in this experiment which was conducted in the optimum fermentation medium obtained above.

Measurement of the mycelial biomass and EPS. At the end of liquid culture course, the original inoculated mycelial block was discarded and then the fermentation broth was centrifuged (4000 rpm, 20 min. Dry weight of mycelia was measured after rinsing the mycelial pellet with distilled water for three times and then drying at 60 °C to a constant weight. Five millilitres of supernatant fluid was mixed with triplicate volume of 95% ethanol, stirred vigorously and kept at 4 °C for 18 h. After centrifugation (4000 rpm, 10 min), the supernatant was discarded, and the precipitate, i.e. the crude EPS, was dissolved with distilled water heated to 60 °C. The EPS content was determined by phenol-sulphuric acid method with dextrose as the standard (Chaplin and Kennedy, 1994).

Statistical analysis. In shake-flask experiments, all treatments were run in triplicate and all experiments were repeated at least twice. Data from each treatment were subjected to DPS for ANOVA (analysis of variance), and Duncan's multiple range test was used to determine significant differences ($p < 0.05$) among the treatments.

RESULTS AND DISCUSSION

Effects of carbon and nitrogen sources on biomass and EPS content

Although some researchers have reported the effect of liquid media component on the content of EPS from *Pleurotus ostreatus* (Rosado *et al*., 2003), *Boletus edulis* (Deng and Chen, 2005) and *Ganoderma lucidum* (Wang *et al*., 2005), the optimal carbon and nitrogen sources and environmental conditions of producing EPS from *Pleurotus nebrodensis* have not been reported. The biomass and content of EPS were observed in liquid media containing five different carbon sources (Table 1). The optimum carbon source of producing maximum biomass (1.9 g l^{-1}) and EPS (1.7 g l^{-1}) was maltose. In the liquid medium containing maltose, the yield of biomass and EPS was obviously higher than sucrose (47 and 42%), dextrose (38 and 45%), lactose (6 and 30%), fructose (62 and 35%) respectively. By ANOVA, the analysis of variance showed the different effect of the five carbon sources on biomass ($p < 0.01$) and on EPS ($p < 0.05$).

TABLE 1 - Effect of carbon and nitrogen sources on production of biomass and EPS by *Pleurotus nebrodensis*

	Biomass (g l^{-1})	Content of EPS (g l^{-1})
Carbon sources		
Sucrose	1.01	0.992
Dextrose	1.19	0.947
Maltose	1.91	1.710
Lactose	1.79	1.200
Fructose	0.73	1.120
	**	*
Nitrogen sources		
Peptone	1.65	0.686
Yeast extract	1.93	1.040
Beef extract	1.97	0.792
Ammonium nitrate	0.49	0.798
Ammonium sulfate	1.23	0.864
Sodium nitrate	1.08	0.771
Ammonium chloride	1.21	0.867
	**	*

*: $p < 0.05$, **: $p < 0.01$.

The EPS content (Table 1), in the medium containing yeast extract, was the highest (1.04 g l^{-1}), while the biomass was 1.93 g l^{-1}. The biomass was 1.97 g l^{-1} in the medium containing beef extract, a little higher than that in the medium containing yeast extract, but the EPS content was only 0.792 g l^{-1}, significantly lower than that in the medium containing yeast extract. The yield of biomass and EPS in medium containing yeast extract was higher than that in medium containing peptone (14.5 and 34.0%), ammonium nitrate (74.6 and 23.3%), ammonium sulphate (36.3 and 16.9%), sodium nitrate (44.0 and 25.9%), ammonium chloride (37.3 and 16.6%) respectively. On account of EPS content being the main examined object, yeast extract was then determined as the optimal nitrogen source. Similar to the experiment screening for the optimal carbon sources, ANOVA revealed the diverse effect of seven nitrogen sources on biomass ($p < 0.01$) and on EPS ($p < 0.05$).

TABLE 2 - Effect of carbon and nitrogen sources on production of biomass and EPS by *Pleurotus nebrodensis*

Levels	Factors (g l^{-1})				
	A	B	C	D	E
1	100	15	2	1	0.5
2	150	20	3	2	1.0
3	200	25	4	3	1.5

A: bran, B: maltose, C: yeast extract, D: KH_2PO_4, E: $MgSO_4 \cdot 7H_2O$.

Optimisation of culture medium by orthogonal test

A five-factor-three-level orthogonal design was adopted as shown in Table 2 and the results of the orthogonal test were summarised in Table 3. By ANOVA, only maltose was found to have a significant influence on the content of EPS ($p < 0.01$). The degree of the influence of all the factors on biomass and on EPS came in this turn: B > C > E > D > A and B > A > E > C > D, respectively. Finally, we determined the optimal composition of culture medium: 20% w/v bran, 2.5% w/v maltose, 0.3% w/v yeast extract, 0.1% w/v KH_2PO_4, 0.1% w/v $MgSO_4 \cdot 7H_2O$.

Effects of environmental conditions on biomass and EPS content

Pleurotus nebrodensis could grow at initial pH value ranging from 5 to 10 and its mycelial biomass came to a head at initial pH 6 (Table 4), in accordance with the results reported by some researchers (Dong *et al*., 2004c). However, the EPS content reached its maximum (1.84 g l^{-1}) at initial pH 8, significantly higher than that at the others (Table 5). Initial pH value had a remarkable influence not only on mycelial biomass, but also on EPS by ANOVA analysis ($p < 0.01$). So we chose initial pH 8 as the optimum in view of EPS content.

TABLE 3 - Results of the orthogonal test

No.	1	2	3	4	5	Biomass	EPS content
	A	B	C	D	E	(g l^{-1})	(g l^{-1})
1	1	1	1	1	1	2.30	0.638
2	1	1	1	1	2	2.15	0.683
3	1	1	1	1	3	2.75	0.552
4	1	2	2	2	1	3.10	0.846
5	1	2	2	2	2	5.10	0.950
6	1	2	2	2	3	2.30	0.944
7	1	3	3	3	1	4.90	1.230
8	1	3	3	3	2	2.10	1.440
9	1	3	3	3	3	6.70	1.020
10	2	1	2	3	1	3.80	0.670
11	2	1	2	3	2	2.45	0.683
12	2	1	2	3	3	2.20	0.795
13	2	2	3	1	1	3.45	0.859
14	2	2	3	1	2	3.90	0.832
15	2	2	3	1	3	3.10	0.910
16	2	3	1	2	1	3.70	0.766
17	2	3	1	2	2	3.15	1.670
18	2	3	1	2	3	4.55	1.590
19	3	1	3	2	1	3.50	0.824
20	3	1	3	2	2	2.50	1.380
21	3	1	3	2	3	3.65	0.936
22	3	2	1	3	1	2.10	0.800
23	3	2	1	3	2	3.05	0.790
24	3	2	1	3	3	3.10	0.859
25	3	3	2	1	1	2.30	0.872
26	3	3	2	1	2	3.40	3.410
27	3	3	2	1	3	4.50	3.250
K_1	3.49	2.81	2.98	3.09	3.24		
K_2	3.37	3.24	3.24	3.51	3.09		
K_3	3.12	3.92	3.76	3.38	3.65	∑=89.8	
R	0.37	1.11	0.78	0.42	0.56		
Significance test							
K_1'	0.922	0.796	0.928	1.330	0.834		
K_2'	0.975	0.866	1.380	1.100	1.320		
K_3'	1.460	1.690	1.05	0.921	1.210	∑=30.2	
R'	0.538	0.894	0.452	0.413	0.481		
Significance test	**						

A: bran, B: maltose, C: yeast extract, D: KH_2PO_4, E: $MgSO_4 \cdot 7H_2O$. **: $p < 0.01$.

TABLE 4 - Effect of environmental conditions on production of biomass by *Pleurotus nebrodensis*

Initial pH	Biomass (g l^{-1})	Temp. (°C)	Biomass (g l^{-1})	Time (d)	Biomass (g l^{-1})	Mv[a] (ml)	Biomass (g l^{-1})	Iv[b] (cm^2)	Biomass (g l^{-1})	Rs[c] (rpm)	Biomass (g l^{-1})
5.0	4.37	20	4.43	2	0.32	50	2.74	0.25	1.07	130	1.70
6.0	4.83	25	5.13	3	0.57	100	1.55	0.5	1.43	160	2.33
7.0	4.43	30	4.17	4	0.89	150	0.87	1.0	2.73	190	3.13
8.0	4.13			5	0.98	200	0.59	1.5	3.03		
9.0	3.93			6	1.13						
10.0	3.60			7	1.17						
				8	2.90						
	**				**		**		**		**

[a] Mv: medium volume, [b] Iv: inoculation volume, [c] Rs: revolution speed. **: $p < 0.01$.

The maximal biomass and EPS yield were both obtained highly when the culture temperature was 25 °C (Tables 4, 5), consistent with some reports in 25 °C optimum for fungi growth (Li *et al*., 2003; Dong *et al*., 2004c). 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 biomass remarkably increased until day 8 ($p < 0.01$) and the maximal EPS content (2.38 g l^{-1}) occurred at day 5 ($p < 0.01$), similar to the value reported by Yang *et al.* (2006) in *Pleurotus ferulae*. The EPS yield (2.36 g l^{-1}) of day 4 was only a little lower than that of day 5, but we still chose day 4 as the optimal culture time for the sake of shortening total fermentation period.

The capacity of liquid media in a 250-ml Erlenmeyer flask had a significant effect on both biomass and EPS content ($p < 0.01$) (Tables 4, 5). Despite of the highest biomass in 50-ml liquid media, accordant with the report by Li *et al*. (2003), the EPS yield reached the maximum when the capacity of liquid media was 100 ml. The optimal inoculation volume of producing EPS was 0.5-cm^2 mycelial block (Table 5). With the revolution speed increasing, the biomass of *Pleurotus nebrodensis* increased gradually (Table 4), but the EPS yield came to a head at 160 rpm (Table 5). To our surprise, the effect of revolution speed on biomass and EPS content also displayed significantly ($p < 0.01$). Revolution speed of 160 rpm, due to the lower wastage of electricity, the smaller damage to shaker and the higher security in large scale production, was finally decided.

Validity experiment

The control group was cultured in basal media under the conditions of natural initial pH, 25 °C incubation temperature, 160 rpm revolution speed, 100-ml liquid media in a 250 ml-sized flask, 0.50-cm^2 mycelial block of inoculation volume and 7 days culture time. The tested group was conducted under the optimised conditions by orthogonal test and single-factor test. The mycelial biomass (4.13 g l^{-1}) and EPS content (2.40 g l^{-1}) of *Pleurotus nebrodensis* in tested group were 2.3 and 3.6 times compared to the control, respectively.

The optimal conditions of producing biomass and EPS by *Pleurotus nebrodensis* in submerged culture could offer references to large-scale cultivation, but further research of industrial fermentation and exploitation should be done continuously.

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TABLE 5 - Effect of environmental conditions on production of EPS by *Pleurotus nebrodensis*

Initial pH	EPS (g l^{-1})	Temp. (°C)	EPS (g l^{-1})	Time (d)	EPS (g l^{-1})	Mv[a] (ml)	EPS (g l^{-1})	Iv[b] (cm^2)	EPS (g l^{-1})	Rs[c] (rpm)	EPS (g l^{-1})
5.0	1.21	20	2.55	2	2.13	50	1.35	0.25	1.53	130	1.89
6.0	1.21	25	2.62	3	2.15	100	2.77	0.50	1.72	160	2.88
7.0	1.53	30	2.53	4	2.36	150	1.58	1.00	1.35	190	2.41
8.0	1.84			5	2.38	200	1.56	1.50	1.30		
9.0	1.43			6	2.08						
10.0	1.23			7	1.97						
				8	1.66						
	**				**		**		**		**

[a] Mv: medium volume, [b] Iv: inoculation volume, [c] Rs: revolution speed. **: $p < 0.01$.

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